MBBR Ammonia Removal: An Investigation of Nitrification Kinetics, Biofilm and Biomass Response, and Bacterial Population Shifts During Long-Term Cold Temperature Exposure

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A thesis submitted under the supervision of Dr. Robert Delatolla in partial fulfillment of the requirements for the degree of Masters of Applied Science in Environmental Engineering.

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ABSTRACT

New federal regulations with regards to ammonia in wastewater effluent discharge will require over 1000 existing wastewater treatment facilities to be upgraded. Although biological treatment is the most common and economical means of wastewater ammonia removal, nitrification rates can be completely impeded at cold temperatures. Moving bed biofilm reactors (MBBR) have shown promise as an upgrade nitrifying unit at pilot-scale and full-scale applications with respect to low temperature nitrification. MBBR technologies offer the advantages of less space requirement, utilizing the whole tank volume, no sludge recycling, and no backwashing, over other attached growth systems.

Two laboratory MBBRs were used in this study to investigate MBBR nitrification rates at 20^{o}C, after long-term exposure to 1^{o}C, and at the kinetic threshold temperature of 5^{o}C. Furthermore, the biologically produced solids from the MBBR system 20^{o}C and after long-term exposure to 1^{o}C, and the Arrhenius temperature correction models used to predict nitrification rates after long-term exposure to 1^{o}C. The nitrification rates at 1^{o}C over a four month exposure period as compared to the rate at 20^{o}C were 18.7 ± 5.5% and 15.7 ± 4.7% for the two reactors. The nitrification rate at 5^{o}C was 66.2 ± 3.9% and 64.4 ± 3.7% compared to the rate measured at 20^{o}C for reactors 1 and 2, respectively, and as such was identified as the kinetic temperature threshold. The quantity of solids detached from the nitrifying MBBR biocarriers was low and did not vary significantly at 20^{o}C and after long-term exposure to 1^{o}C. Lastly, a temperature correction model based on exposure time to cold temperatures, developed by Delatolla et al. (2009) showed a strong

correlation to the calculated ammonia removal rates relative to $20^{o}C$ following a gradual acclimatization period to cold temperatures.

Biofilm morphology along with biomass viability at various depths in the biofilm were investigated using variable pressure electron scanning microscope imaging (VPSEM) and confocal laser scanning microscope (CLSM) imaging in combination with viability live/dead staining. The biofilm thickness along with the number of viable cells showed significant increases after long-term exposure to $1^{o}C$ while the dead cell coverage did not show significant changes. Hence, this study observed higher cell activities at warm temperatures and a slightly greater quantity of biomass with lower activities at cold temperatures in nitrifying MBBR biofilms. Using DNA sequencing analysis, *Nitrosomonas* and *Nitrosospira* (ammonia oxidizers) as well as *Nitrospira* (nitrite oxidizer) were identified in which no population shift was observed during $20^{o}C$ and after long-term exposure to $1^{o}C$. Furthermore, a number of non-nitrifiers were identified in the biofilm during warm and cold temperatures presenting the possibility that their presence may have provided some form of protection to the nitrifiers during long-term temperature exposure.

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Lastly, I'd like to thank my closest friends and family members who never really understood or knew what my research work was about, but was always 100% supportive and proud. I'd like to dedicate this thesis to my mom, who I'm sure has guided me through life for the last 15 years.

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LIST OF ABBREVIATIONS

AOB	Ammonia Oxidizing Bacteria
BAF	Biological Aerated Filter
BOD	Biochemical Oxygen Demand
$BVRR_A$	Biofilm Volume Ammonia Removal Rate
CLSM	Confocal Laser Scanning Microscope
C/N	Carbon to Nitrogen Ratio
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EPS	Extracellular Polymeric Substances
FBBR	Fluidized-Bed Bioreactor
HRT	Hydraulic Retention Time
MBBR	Moving Bed Biofilm Reactor
MTBL	Mass Transfer Boundary Layer
NOB	Nitrite Oxidizing Bacteria
OTU	Operational Taxonomic Unit
R1	Reactor 1
R2	Reactor 2
RBC	Rotating Biological Contactor
rRNA	Ribosomal Ribonucleic Acid
SARR	Surface Area Removal Rate

SRT	Solids Retention Time
SWW	Synthetic Wastewater
T	Temperature
TCAG	The Centre for Applied Genomics
TKN	Total Kjehldahl Nitrogen
TSS	Total Suspended Solids
$VCRR_A$	Viable Cell Ammonia Removal Rate
VPSEM	Variable Pressure Scanning Electron Microscope
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Ammonia is one of four deleterious constituents that has recently been regulated by the Federal Government of Canada (Canada Gazette, 2012). Ammonia removal in conventional rural Canadian wastewater treatment plants (WWTPs) is currently limited or non-existent in winter months. Biological nitrification is often preferred and used as a means to remove ammonia in wastewater (Metcalf and Eddy, 2003); however, numerous studies and practical experiences have identified the effect of low temperatures on microbial populations as a major cause for loss of nitrification (Sharma and Ahler, 1977; Zhu and Chen, 2002; Env. Canada, 2003; Salvetti et al., 2006).

A large number of wastewater facilities in northern countries, where land is available, are treatment lagoons and as such are the conventional method of wastewater treatment in Canadian rural communities. Treatment lagoons in Canada are subject to very low temperatures in the winter months and although this treatment technology is successful at removing organics year round, it lacks the ability to perform nitrification at cold temperatures (Env. of Canada, 2003; Metcalf and Eddy, 2003). Due to new federal regulations in regards to ammonia discharge in wastewater effluent, which limit the discharge of the toxic ammonia (NH_3) to less than 1.25 mg-NH_3-N/L at 15 ± 1^{o}C in medium to high flow treatment plants, many existing treatment lagoons will be upgraded in the near future (Canada Gazette, 2012).

With a lack of sufficient ammonia removal at cold temperatures in conventional rural wastewater treatment facilities, there has been an increased interest in nitrifying biofilm systems as upgrade units for existing systems. Attached growth processes have shown to achieve higher nitrification rates as compared to suspended growth processes under warm temperatures (Aravinthan et al., 1998). Microorganisms within a biofilm establish symbiotic relationships in their environment and demonstrate an increased inclination for survival compared to microorganisms in suspension (Dunne, 2002). In addition, due to mass transfer limitations, nitrifying bacteria within a biofilm are less susceptible to temperature effects (Wijffels et al., 1995).

The autotrophic bacteria responsible for nitrification at warm temperatures are believed to be responsible for nitrification at cold temperatures (Wijffels et al., 1995). However, recent studies have observed bacterial population shifts in ammonia oxidizing bacteria (AOB) with changes in temperature (Hallin et al., 2005; Layton et al., 2005; Siripong and Rittmann, 2007). Nitrite oxidizing bacteria (NOB), on the other hand, have not been shown to experience a population shift with changes in temperature but rather during substrate scarcity or temporary substrate elevations (Gieseke et al., 2003; Haseborg et al., 2010; Huang et al., 2010). *Nitrosomonas* are considered to be the dominant AOB population in wastewaters (Park et al., 2008; Ducey et al., 2009; Rodriguez-Caballero et al., 2012). *Nitrosospira*, another terrestrial AOB genera, has been found to co-exist with *Nitrosomonas* in small amounts but *Nitrosospira* is much more temperature sensitive (Park et al., 2008); therefore, *Nitrosomonas* becomes the dominant AOB genera at low temperatures. The two common genera of NOB observed in wastewater at all temperatures are *Nitrobacter* and *Nitrospira* (Wagner et al., 2001; Daims et al., 2001; Siripong and Rittmann, 2007).

Moving bed biofilm reactors (MBBRs) have shown promise as an upgrade or replacement technology for low temperature nitrification (Andreottola et al., 2000; Wessman and Johnson, 2006; Delatolla et al., 2011). The performance of MBBR upgrade systems to treat ammonia has been well demonstrated at numerous operating lagoons; however, MBBR units have been installed to treat wastewater exiting from the first or middle pond of multiple pond lagoon systems where the temperature drop is limited (Wessman and Johnson, 2006; Houweling et al., 2007; Delatolla et al., 2011).

Nitrifying MBBR upgrades installed after the first pond are fed wastewater with a limited temperature drop relative to the influent temperature. As shown in Figure 1.1, the effluent of pond 1 will not only have the highest temperature (T), relative to the effluent of the other ponds, but also the highest carbon to nitrogen (C/N) ratio; therefore, the upgrade MBBR unit's biofilm (option 1 in Figure 1.1) will be composed of both heterotrophic (carbon oxidizers) and autotrophic (nitrifiers) growth. The heterotrophic bacteria will ultimately outcompete the autotrophic bacteria for nutrients and oxygen within the biofilm matrix due to their high metabolic growth rate, resulting in a suppression of autotrophic activity. The generation time of nitrifiers is extremely slow compared to carbon (C) oxidizers. In fact, the yield of nitrifiers is minute compared to that of C oxidizers. Based on this phenomenon, previous studies show that organic loading, represented as biochemical oxygen demand (BOD), should be kept as low as possible to promote and maintain nitrification in MBBR units. Tertiary nitrification requires the BOD to Total Kjehldahl Nitrogen (BOD/TKN) ratio to be less than 1.0 (WEF, 2011), where nitrification has shown to be completely subsided at organic loads exceeding 5 g-BOD/m^3 (Hem et al., 1994).

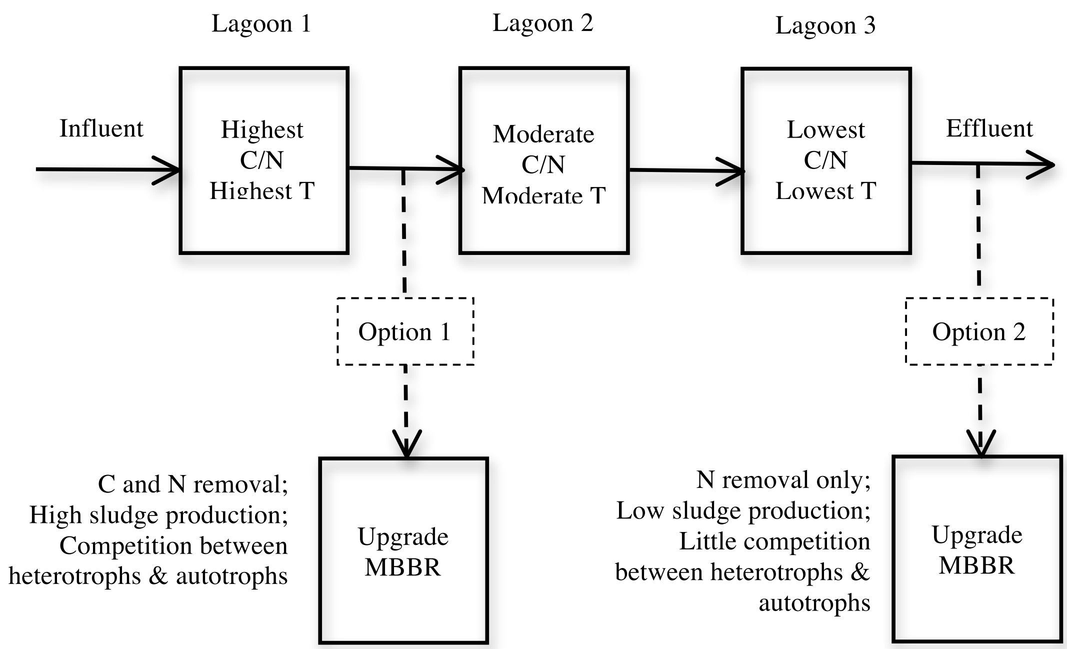

Figure 1.1: Schematic of installation options of an upgrade nitrifying MBBR system at a three pond, lagoon treatment system

An MBBR performing carbon removal and nitrification (option 1 in Figure 1.1) will produce biofilm with a thicker C oxidizing layer on the outer surface and a thin nitrifying layer closer to the substratum (Figure 1.2 where MTBL is the mass transfer boundary layer) (Metcalf and Eddy, 2003; Lee et al., 2004; WEF, 2011). The outer heterotrophic layer has greater access to higher concentrations of substrate and oxygen in the bulk liquid and subsequently may grow thick enough to smother the underlying nitrifying layer. Although C oxidizers and nitrifiers do not share the same substrate they both require oxygen; hence in nitrifying biofilms that sustain significant heterotrophic growth, the oxygen concentrations decrease significantly through the upper C oxidizing layer, resulting in less oxygen availability to the nitrifiers and ultimately limiting autotrophic activity (Figure 1.2b). Furthermore, the high growth rate of heterotrophs will also create high sludge production in the MBBR upgrade unit and produce a redundancy in the treatment system as the upgrade unit will remove BOD that can be readily removed in the previous ponds.

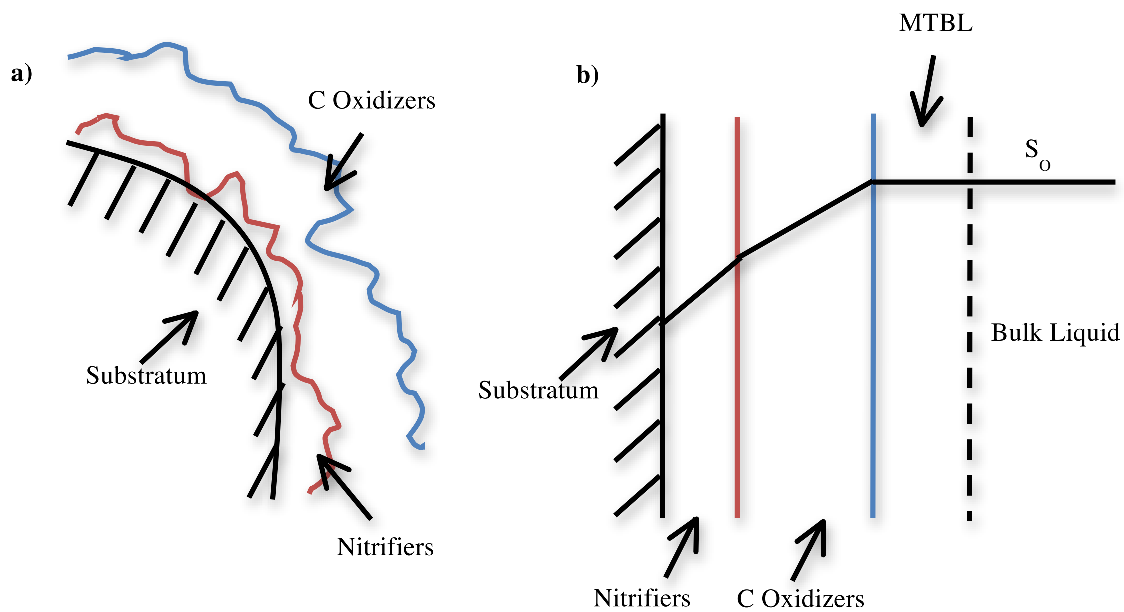

Figure 1.2: High C/N ratio biofilm: a) overgrowth of C oxidizers smothering nitrifiers b) oxygen concentration, S_o, through the biofilm

Although the middle pond will have a lower C/N ratio than the first pond, the optimum C/N ratio for nitrification actually exists in or after the last pond. The last pond of a lagoon system will have the lowest C/N ratio with BOD/TKN values of approximately 1.0 or less. Thus, a nitrifying MBBR unit treating effluent from the last lagoon (option 2 in Figure 1.1) will theoretically perform nitrification with limited C removal since the majority of the carbon will have already been removed in previous ponds. The low C/N ratio will promote autotrophic bacteria without promoting a large population of heterotrophic bacteria and thus will reduce the competition between heterotrophs and autotrophs. However, the installation of the MBBR upgrade unit after the last pond will result in very low temperature wastewater being fed to the MBBR unit in the cold season. The last lagoon in a Canadian multiple lagoon treatment system often reaches and is maintained at temperatures as low as 1^{o}C in the winter months. It is this capacity of a nitrifying MBBR unit to function at temperatures of 1^{o}C for the span of a Canadian winter that is the inspiration for this study.

As shown in Figure 1.3, an MBBR designated for nitrification only (option 2 in Figure 1.1) will theoretically produce a very small heterotrophic biofilm layer on the outer surface of the nitrifying layer. A low C/N ratio will reduce competition between C oxidizers and nitrifiers, facilitating access to high concentrations of oxygen from the bulk liquid to the nitrifying biofilm layer (Figure 1.3b). Therefore, as a nitrifying upgrade technology, the installation of the MBBR unit should be considered after the last treatment pond in lagoon systems if the system can be shown to perform nitrification at 1^{o}C for the span of a Canadian winter (Delatolla et al., 2011). Furthermore, although the slow growth rate of autotrophs and the limited presence of heterotrophs will theoretically result in low sludge production in the MBBR upgrade unit, the placement of the MBBR unit after the last pond will require an investigation of the sludge production by the MBBR upgrade unit. As the proposed installation of the MBBR unit will become the final unit prior to discharge, the generation of solids must be limited to prevent the discharge of total suspended solids (TSS) concentrations that exceed regulations or subsequently necessitate the addition of a solids separation unit.

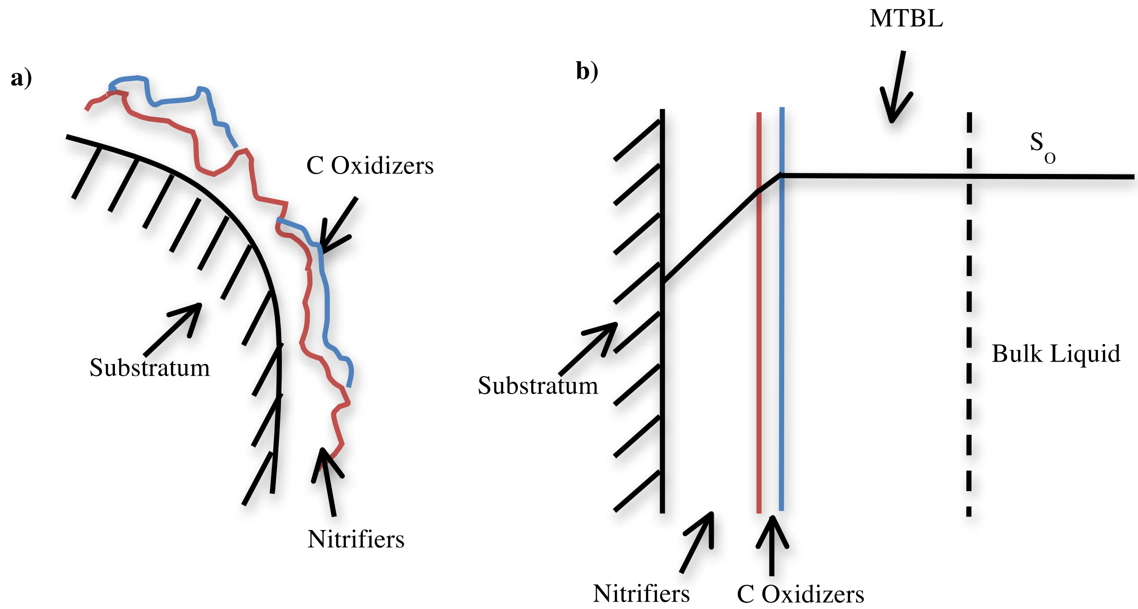

Figure 1.3: Low C/N ratio biofilm: a) minimal growth of C oxidizers on outer layer of nitrifiers b) oxygen concentration, S_o, through the biofilm

Although the performance of MBBRs has been well demonstrated for short-term low temperature nitrification, the literature lacks fundamental information regarding long-term nitrification at very cold temperatures. Furthermore, current knowledge on the response of nitrifying biofilm and bacterial population shifts during long-term exposure to temperatures of 1^{o}C is limited and is non-existent for MBBR treatment systems. Research on long-term MBBR nitrification at 1^{o}C will provide valuable new knowledge on the potential design and installation of nitrifying MBBR upgrade units for hundreds of existing lagoon treatment systems in Canada.

1.2. AIM OF STUDY

This research will provide a new fundamental understanding of long-term cold temperature nitrification using MBBR technologies. Beyond studying the nitrification kinetics, the production of biologically detached solids will be investigated in addition to the biofilm and biomass response and the identification of bacterial populations during long-term exposure to cold temperatures. This research will provide new and critical knowledge on the performance of MBBRs as a nitrifying upgrade system to treat very cold effluent exiting the last pond of a multiple lagoon treatment system. The specific objectives of this research are as follows:

- quantify MBBR nitrifying kinetic rates at 20^{o}C as baseline kinetics and at 1^{o}C for four months, the duration of time representative of a Canadian winter;
- identify a nitrifying kinetic temperature threshold;
- investigate the correlation of the Arrhenius temperature correction coefficient to predict nitrification rates under long-term low temperature exposure;
- characterize the biologically produced solids of the nitrifying MBBR system at 20^{o}C and after four months exposure to 1^{o}C;
- characterize the effects of long-term exposure to 1^{o}C on the thickness and morphology of MBBR nitrifying biofilms;
- characterize the viability of the nitrifying organisms, investigate AOB and NOB populations responsible for nitrification at 1^{o}C and identify population shifts in AOB and NOB populations with long exposure to 1^{o}C.

1.3. THESIS ORGANIZATION

Chapter 1 presents background information on the significance of this research as well as the objectives of this study. Chapter 2 presents a literature review on nitrification microbiology, nitrification kinetics, biofilms, current technologies used for ammonia removal in wastewater treatment plants, and the design and operational considerations for MBBR systems. Chapter 3 describes the experimental overview, a description of the laboratory reactors, and methodologies used.

The ability of MBBR technologies to perform nitrification at low temperatures for the duration representative of a Canadian winter is investigated in Chapter 4. This work has been accepted for publication in the journal of Water Environment Research (WER) under the following title: *MBBR nitrification during long-term exposure to cold temperatures* by V. Hoang, R. Delatolla, A. Gadbois, and E. Laflamme. The nitrification rate within two laboratory MBBRs exposed to a temperature of 1^{o}C for four months is investigated to replicate cold temperature conditions in WWTPs located in Northern Canada. A nitrification kinetic threshold temperature is also identified during these experiments. Furthermore, the biologically produced solids of the MBBRs are analyzed at 20^{o}C and after four months exposure to 1^{o}C. Finally, temperature correction coefficient (θ) models, with respect to the exposure time to cold temperatures, are investigated to predict nitrification rates of MBBR systems operating at low temperatures for extendeds period of time.

In Chapter 5, the analysis of biofilm morphology and viability as well as the bacterial population shifts of the MBBR biofilm during long-term cold temperature exposure are investigated. This work will be submitted to the journal of Environmental

Science & Technology under the following title: *MBBR nitrifying biofilm and biomass response to long-term exposure to 1^{o}C* by V. Hoang, R. Delatolla, T. Abujamel, W. Mottawea A. Stinzi, A. Gadbois, and E. Laflamme. Variable pressure scanning electron microscopy is used to examine the biofilm thickness and morphology and confocal laser scanning microscopy in combination with fluorescence staining is used to quantify the viability of the cells embedded in the biofilm as the MBBR system is exposed to cold temperatures for four months. Moreover, the microbial community and bacterial populations of the biofilm are identified at 20^{o}C and 1^{o}C using DNA sequencing analysis. The objective of this work is to expand on the findings discussed in Chapter 4 and aims to validate that cells embedded in the biofilm exist at a higher level of activity in warm temperatures and at a lower level of activity in cold temperatures.

Finally, Chapter 6 presents all conclusions drawn from this research in regards to nitrification kinetics, biofilm morphology, biomass viability, and bacterial population in response to long-term exposure to 1^{o}C as well as future recommendations.

1.4. CONTRIBUTIONS OF AUTHORS

During the course of this work two scientific papers have been developed and are listed below along with an overview of the authors' contributions.

Article 1:

Hoang, V., Delatolla, R., Gadbois, A., Laflamme, E. *MBBR nitrification during long-term exposure to cold temperatures.* Accepted for publication in Water Environment Research (WER).

V. Hoang: Conducted literature review, developed and conducted experimental procedure, analyzed results, and wrote manuscript.

R. Delatolla: Provided supervision in the development experimental procedure, analysis of results, and reviewed manuscript.

A. Gadbois: Provided technical assistance in experimental procedure, analysis of results and reviewed manuscript.

E. Laflamme: Provided technical assistance in experimental procedure, analysis of results and reviewed manuscript.

Article 2:

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Stinzi, A. Gadbois, A., Laflamme, E., Abujamel, T. *MBBR nitrifying biofilm and biomass response long-term exposure to 1^{o}C.* In preparation for submission to Environmental Science & Technology.

V. Hoang: Conducted literature review, developed and conducted experimental procedure, analyzed results, and wrote manuscript.

R. Delatolla: Provided supervision in the development experimental procedure, analysis of results, and reviewed manuscript.

T. Abujamel: Provided technical assistance in experimental procedure of DNA extraction and amplification, performed sequencing analysis, and provided expertise in sequencing analysis interpretation.

W. Mottawea: Performed sequencing analysis.

A. Stinzi: Provided technical assistance and expertise in DNA sequencing analysis and interpretation.

A. Gadbois: Provided technical assistance in experimental procedure, analysis of results and reviewed manuscript.

E. Laflamme: Provided technical assistance in experimental procedure, analysis of results and reviewed manuscript.

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CHAPTER 2: LITERATURE REVIEW

2.1. BIOLOGICAL WASTEWATER TREATMENT

Wastewater treatment is classified into four general levels of treatment. Preliminary and primary treatment uses physical screening and sedimentation to remove course material, grit, and solids from the wastewater. Secondary treatment units promote biological growth to remove carbonaceous material from the wastewater and either incorporate or are followed by a solids separation unit. Tertiary treatment units use either biological or physical/chemical methods of treatment to removal nutrients from the wastewater and/or polish the effluent prior to leaving the system. Finally, wastewater treatment systems may also include disinfection treatment prior to discharge.

The main objectives of biological wastewater treatment are to promote microbes and microbial activity that transform biodegradable constituents into acceptable end products, while promoting microbial conditions that are easily separated from the wastewater or readily settle out of solution as suspended solids and capture colloidal solids. Biological treatment units are designed to oxidize carbon and remove nutrients such as nitrogen and phosphorus.

2.1.1. Microbial Metabolism

Microbial growth is cell replication in response to the physio-chemical environment. Microorganisms require specific substrates, pH, temperatures, and dissolved oxygen (DO) concentrations for growth. Four main components are required for microbial growth: a carbon source, an energy source, a terminal electron acceptor, and nutrients. Figure 2.1 below shows a general schematic of bacteria metabolic processes (Metcalf and Eddy, 2003).

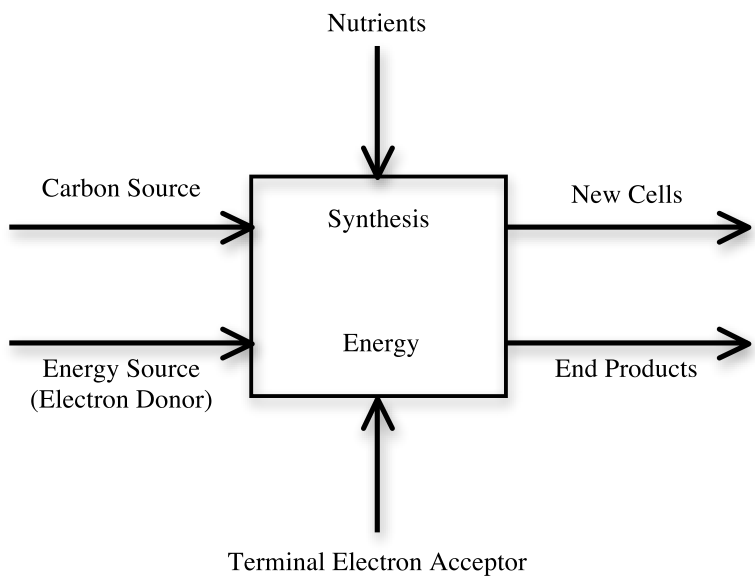


Figure 2.1: General bacteria metabolism

Metabolism is a series of redox (reduction-oxidation) reactions that regulate the energy required for cell synthesis, maintenance, and endogenous decay. Enzymes released by microorganisms act as a catalyst for many metabolic processes since these reactions are naturally slow processes unless a catalyst is present. Metabolism involves two key processes: anabolism, in which cells build molecules from small units, and catabolism, in which cells break down molecules into smaller subunits. Both of these processes are redox reactions and thus involve an electron donor and an electron acceptor. The electron donor is the reducing agent that donates electrons, whereas the electron acceptor is the oxidizing agent that gains electrons. Microorganism classification based on electron acceptor is shown in Table 2.1.

Table 2.1: Classification of organisms based on electron acceptor

Classification	Electron Acceptor
Aerobic	O_2
Obligate Aerobic	Only O_2
Anoxic	NO_2^-, NO_3^-
Facultative Aerobic	O_2, NO_2^-, NO_3^-
Anaerobic	Electron acceptors other than O_2

Metabolism is the relationship between the energy transference of catabolic and anabolic reactions. In other words, anabolic metabolism uses the energy produced by catabolic metabolism for cell synthesis. Referring to Figure 2.1, catabolic metabolism is the reaction between the electron donor and electron acceptor to produce energy and end products, while anabolic metabolism is the reaction between the carbon source, nutrients, and energy produced from catabolism to maintain existing cells and synthesize new cells.

Energy is obtained from either photosynthesis or chemical oxidation. In biological wastewater processes, carbon is predominantly oxidized by organoheterotrophic bacteria in an aerated basin and nitrification is performed by lithoautotrophic bacteria in the same basin or a downstream aerated unit. Heterotrophic bacteria utilize organic carbon sources, whereas autotrophic bacteria utilize inorganic carbon sources for cell synthesis. Organic carbon concentrations are generally measured as the biochemical oxygen demand (BOD) or the chemical oxygen demand (COD) of the wastewater. Table 2.2 represents the classification of organisms based on their energy source (electron donor) and carbon source. Furthermore, microorganisms will also thrive in a specific temperature range. Table 2.3 classifies organisms based on their preferred temperature range.

Table 2.2: Classification of organisms based on energy source and carbon source

Classification	Energy Source	Carbon Source
Organotrophs	Organic	-
Lithotrophs	Inorganic	-
Heterotroph	-	Organic
Autotroph	-	Inorganic

Table 2.3: Classification of organisms based on optimal growth temperatures

Classification	Temperature Range (°C)
Psychrophilic	0 - 15
Mesophilic	15 – 45
Thermophilic	> 45

2.1.2. Bacterial Growth & Energetics

Bacterial growth in a batch reactor can be characterized by four distinct phases and represented by the Monod growth curve (Monod, 1949). Batch growth is a process in which a culture is grown in a vessel of fixed volume and the contents are removed after a measured amount of time. The growth conditions within the vessel are constantly changing. Batch growth undergoes four distinct stages: lag phase, exponential growth phase, stationary phase, and death phase. These stages are described in Table 2.4 and Figure 2.2.

Table 2.4: Description of the four phases of bacterial growth in a batch reactor

Phase	Description
Lag	Acclimatization stage where cells adapt to their new environment; could be very long depending on inoculum age
Exponential	Nutrients and substrates are in excess; cells have adapted to their environment and grow rapidly; no inhibitions
Stationary	Net biomass growth is zero; nutrients and substrates become limited; growth rate is offset by death rate; cell lysing may be occurring; inhibition may be occurring
Death	No or little growth is occurring; biomass concentration declines at a first order rate

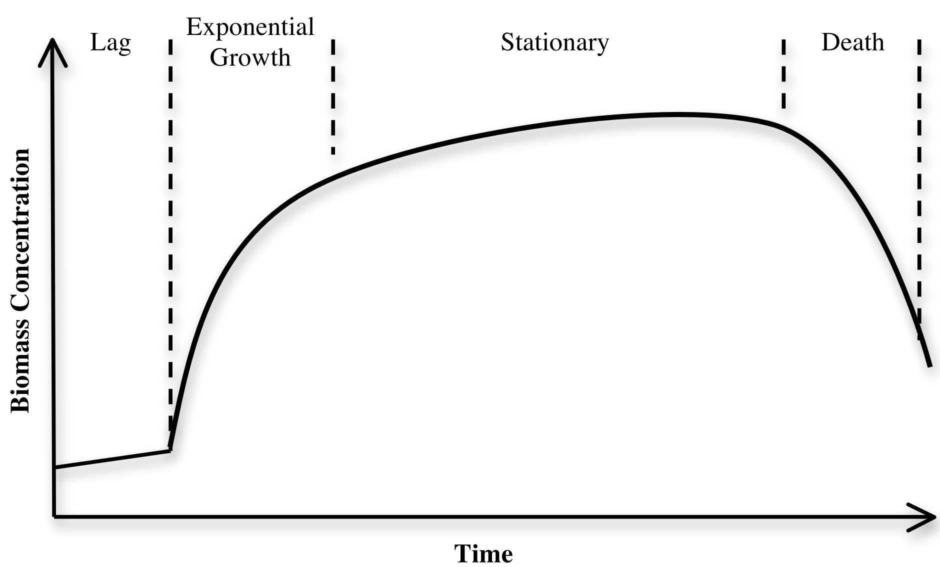


Figure 2.2: Monod growth curve characterized by four phases

Kinetics are expressions that represent the growth rate of cells and the removal rate of constituents within a system. A reaction rate, *r*, may be represented by the following equation:

$$r = k[C]^n \qquad 2.1$$

where k is the kinetic rate coefficient, C is the constituent concentration (g/m^3), and n is the order of reaction. The units of the kinetic rate coefficient will depend on the order of reaction. Kinetic rates are commonly expressed as zero, first, or second order as represented in Table 2.5 but can also be expressed as mixed order. The reaction rate is dependent on the constituent concentration unless the reaction is zero order.

Table 2.5: Expressions that represent the rate of reaction

Order	**Reaction rate**
Zero	$r = k[C]^0 = k$
First	$r = k[C]^1$
Second	$r = k[C]^2$
n^{th}	$r = k[C]^n$

The Monod equation assumes Michaelis-Menten kinetics in which a single enzyme system is responsible for substrate uptake and substrate uptake by enzymes is low and therefore, not limiting for growth. The term *specific* refers to rates normalized per existing mass of biomass. The specific growth rate, μ (g-new cells/g-cells·d), is given in Equation 2.2 and growth rate, μ_g (g/m^{3}d), is given in Equation 2.3.

$$\mu = \frac{\mu_{max} S}{K_s + S} - b \qquad 2.2$$

$$\mu_g = \frac{\mu_{max} SX}{K_s + S} - bX \qquad 2.3$$

where μ_{max} is the maximum specific growth rate (g-new cells/g-cells·d), K_S is the half velocity constant (g/m^3), S is the substrate concentration (g/m^3), b is the endogenous decay coefficient (g-cells/g-cells·d), and X is the biomass (microorganisms) concentration (g-cells/m^3). The specific growth rate is zero order when $S >> K_S$, first order when $S << K_S$, and mixed order if otherwise. Graphically, as seen in Figure 2.3, the K_S value represents the substrate concentration corresponding to the half value of μ_{max}.The K_S value will dictate how well a microorganism will grow and survive given the available amount of substrate. That is, microorganisms that have a small K_S value will thrive at a low substrate concentrations compared to those with higher K_S values.

In biological wastewater treatment, a key principle of concern is substrate removal and is characterized by substrate utilization, r_{su} (g-substrate/m^{3}d), which is expressed by the following equation:

$$r_{su} = -\frac{kSX}{K_s + S} \qquad 2.4$$

where k is the maximum specific substrate utilization rate (g-substrate/g-cells·d). Zero order substrate utilization is strictly dependent on K_S, whereas first order substrate utilization is dependent on both K_S and S. Furthermore, the specific substrate utilization rate, q (g-substrate/g-cells·d), is represented by Equation 2.5. Figure 2.3 is a graphical representation of maximum specific growth rate and maximum substrate utilization (Benefield and Randall, 1980).

$$q = -\frac{kS}{K_s + S} \qquad 2.5$$

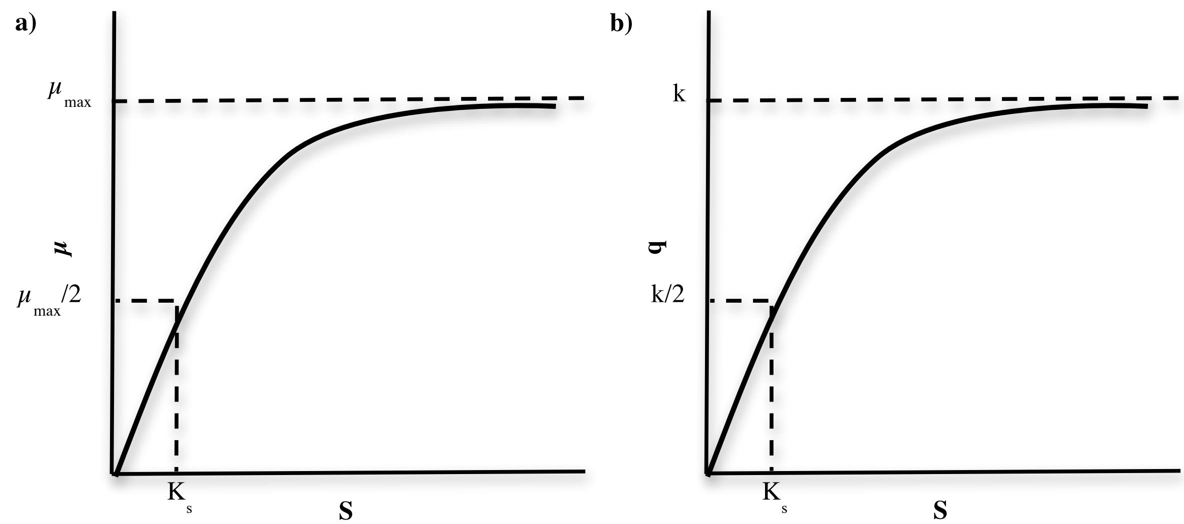


Figure 2.3: Graphical representation of specific growth rate and substrate utilization

Microorganisms consume substrate and produce new cells, in other words, biomass is continuously produced as compounds in the wastewater are consumed and degraded. Since biomass is mostly organic matter it is often measured as volatile suspended solids (VSS) in biological wastewater treatment. The biomass produced relative to the substrate consumed is defined as biomass yield, Y (g-biomass produced/g-substrate utilized), and can be represented by the following equation (Metcalf and Eddy, 2003):

$$Y = g \text{ of biomass produced/ } g \text{ of substrate utilized} \qquad 2.6$$

Furthermore, the exponential microbial growth in which all catabolic energy is used for synthesis is referred to as true cell yield, Y_T (g-biomass produced/g-substrate utilized), and can be expressed as Equation 2.7 using the relationship between maximum specific growth rate and maximum specific substrate utilization rate:

$$Y_T = \frac{\mu_{max}}{k} \tag{2.7}$$

2.2. NITRIFICATION

Biological treatment is often used as an economical means of ammonia removal. Nitrification is defined as the biological oxidation of ammonia (NH_3/NH_4^+), to nitrite (NO_2^-), followed by the oxidation of nitrite to nitrate (NO_3^-). In this manuscript, ammonia will refer to the sum of ionized ammonia or ammonium, NH_4^+, and un-ionized ammonia or free phase ammonia, NH_3. The two-step nitrification process can be described by the following chemical equations:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \tag{2.8}$$

$$NO_2^- + 1.5\ O_2 \rightarrow NO_3^- \tag{2.9}$$

NH_4^+ and NH_3 concentrations in water exist in equilibrium with their relative concentrations being directly related to the pH and temperature of the solution. The conversion of ionized to un-ionized ammonia increases as the pH increases. At pH levels of 9.4 or greater, NH_3 is strongly favored. Speciation of ammonia can be expressed in the form of the Henderson-Hasselbalch equation, where the acid dissociation constant, *pKa*, is 9.25 for ammonia (Petrucci et al., 2007):

$$pH = pKa + log\frac{[NH_3]}{[NH_4^+]} \tag{2.10}$$

Conversely, since the percentage of NH_3 is directly related to pH and temperature of the solution, the following equations can be used to determine *pKa* and the percent of NH_3, respectively (Canada Gazette, 2010):

$$pKa = 0.09 + \left(\frac{2730}{273 + T}\right) \tag{2.11}$$

$$\%NH_3 = \left(\frac{1}{1 + 10^{pKa - pH}}\right) \times 100\% \tag{2.12}$$

where *pKa* will change according to the temperature, *T* (^{o}C). As pH and temperature increase, the percent NH_3 will increase. In contrast, when the pH and temperature decreases, the percent NH_3 will decrease.

Ammonia concentration in released wastewater effluent can cause acute and/or chronic toxicity to aquatic life (Canada Gazette, 2004). Acute toxicity of ammonia is the concentration of ammonia in an effluent that kills more that 50% of rainbow trout over a 96-hour period of exposure. Environment Canada has recognized that ammonia concentrations as low as 1.25 mg-NH_3-N/L can be acutely lethal to receiving waters (Canada Gazette, 2012). Chronic toxicity of ammonia is its capacity, as a single substance or in combination with other substances, to cause harmful effects on the exposed organism throughout or during the life of the organism. This exposure may be on a repeated or continuous basis over an extended period of time. Fish exposed to chronic toxicity levels of ammonia will have reduced reproductive capacity as well as growth retardation in young fish and benthic invertebrate populations. To prevent chronic toxicity of ammonia to fish in discharge streams, wastewater effluent concentrations should not exceed 0.019 mg-NH_3-N/L (Canada Gazette, 2004).

2.2.1. Microbial Metabolism of Nitrifiers

Figure 2.4 represents the metabolism of nitrifying bacteria, where NH_4^+ is the electron donor, carbon dioxide (CO_2) is the inorganic carbon source, and oxygen (O_2) is the terminal electron acceptor.

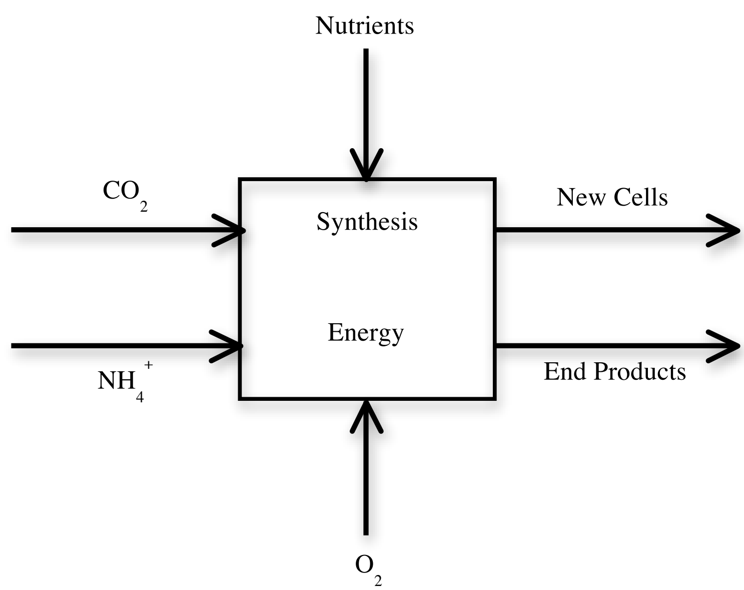


Figure 2.4: Metabolism of nitrifying bacteria

According to "textbook" knowledge, the two common bacteria genera responsible for the ammonia oxidation and nitrite oxidation are *Nitrosomonas* and *Nitrobacter*, respectively (Metcalf and Eddy, 2003). These two bacteria are lithoautotrophic and are distinctively different. There are a number of other autotrophic bacteria genera capable of ammonia oxidation and nitrite oxidation (Painter, 1970). Additional ammonia oxidizing bacteria (AOB) include: *Nitrosococcus, Nitrosospira, Nitrosolobus,* and *Nitrosorobrio.* Other nitrite oxidizing bacteria (NOB) include: *Nitrococcus, Nitrospira, Nitrospina, and Nitroeystis.*

2.2.2. Bacterial Energetics of Nitrifiers

The growth rate of nitrifying bacteria can be represented by the Monod equation where the specific growth rate of nitrifiers, μ_N (g-cells/g-cells·d), can be expressed in the following equations with dissolved oxygen (DO) in excess (Equation 2.13) and with DO being limiting (Equation 2.14) (Metcalf and Eddy, 2003):

$$\mu_N = \frac{\mu_{N\,max} S_N}{K_N + S_N} - b_N \qquad 2.13$$

$$\mu_N = \left(\frac{\mu_{N\,max} S_N}{K_N + S_N}\right)\left(\frac{DO}{K_O + DO}\right) - b_N \qquad 2.14$$

where μ_{Nmax} is the maximum specific growth rate of nitrifiers (g-new cells/g-cells·d), S_N is the nitrogen substrate concentration (g/m^3), K_N is the half velocity constant in which ammonia-nitrogen (substrate) concentration is at half of the specific substrate utilization rate (g/m^3), b_N is the endogenous decay coefficient of the nitrifiers (g-VSS/g-VSS·d), DO is the dissolved oxygen concentration (g/m^3), and K_o is the saturation constant for DO (g/m^3).

Typical values of half velocity constants (K), biomass yields (Y), and maximum specific growth rates (μ_{max}) for the most common AOB genera, *Nitrosomonas*, and NOB genera, *Nitrobacter*, as well as heterotrophs from an activated sludge fed with glucose substrate are represented in Table 2.6 (Sharma and Ahlert, 1976; Metcalf and Eddy, 2003).

Table 2.6: Half velocity constants, biomass yields, and maximum specific growth rate values for *Nitrosomonas*, *Nitrobacter*, and activated sludge heterotrophs

	K (g/m^3)		**Y** (g-cells/g-substrate utilized)		μ_{max} ($time^{-1}$)
	Energy Substrate	Electron Acceptor	Theoretical	Experimental	
Nitrosomonas	0.06 – 5.6	0.3 – 1.3	0.29	0.03 – 0.13	0.46 – 2.2
Nitrobacter	0.06 – 8.4	0.25 – 1.3	0.084	0.02 – 0.08	0.28 – 1.4
Heterotrophs	<1 - 181	0.0007 – 0.1	0.40	0.37 – 0.79	7.2 – 17.0

The energy substrate half velocity constant range for nitrifiers is much lower than that of heterotrophs (Table 2.6). Therefore, nitrifiers exhibit significantly lower kinetics of substrate oxidation. Furthermore, the electron acceptor half velocity constant range for nitrifiers is higher than that of heterotrophs, equating to nitrifiers requiring more oxygen to oxidize ammonia and nitrite than heterotrophs require to oxidize carbon.

The biomass yield and maximum specific growth rate of heterotrophs are significantly higher than that of the autotrophic *Nitrosomonas* and *Nitrobacter* (Table 2.6). The conversion of carbon dioxide into cellular carbon compounds that is performed by autotrophs requires a reduction step and a high input of energy relative to heterotrophic carbon assimilation, resulting in significantly lower autotrophic energy yields, lower biomass yields, and lower growth rates (Metcalf and Eddy, 2003). Heterotrophic carbon oxidizers in wastewater treatment plants have a biomass yield of approximately 0.4 g-VSS/g-COD while autotrophic nitrifiers (AOB and NOB as a community of organisms) have a significantly lower reported biomass yield of approximately 0.12 g-VSS/g-NH_4^+-N (Metcalf and Eddy, 2003). Furthermore, the rate of exponential growth (Figure 2.2) of a bacteria culture is defined as the generation time or doubling time of a bacterial population; heterotrophs have a generation time of approximately 30 minutes whereas autotrophs have a generation time between 8 – 60 hours (Sharma and Alhert, 1976; Metcalf and Eddy, 2003). These parameters are of key importance in biological wastewater treatment design since carbon and ammonia oxidation often takes place in one basin (single-stage) when suspended growth treatment is used.

2.2.3. Dissolved Oxygen Requirements

Theoretically, the quantity of DO required for nitrification is 4.57 g-O_2/g-NH_4^+-N oxidized. This can be determined directly from the stoichiometric relationship in Equation 2.15 (Metcalf and Eddy, 2003):

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O \qquad 2.15$$

More specifically, from the two-step nitrification stoichiometric relationship in Equation 2.8 and 2.9, it is observed that more oxygen is required for ammonia oxidation than nitrite oxidation with 3.43 g-O_2/g-NH_4^+-N oxidized and 1.14 g-O_2/g-NO_2^--N oxidized, respectively. However, in an activated sludge system, nitrifiers are distributed within flocs that contain heterotrophic bacteria and various solids; therefore, the effect of DO is affected by the floc size and density, where nitrifiers within the depths of the floc will have limited access to DO due to mass transfer effects (Metcalf and Eddy, 2003).

Similarly, DO mass transfer limitations will also affect nitrifying biofilm that is embedded at depths in attached growth systems. Partial nitrification has been observed at low DO concentrations due to low DO inhibition effects being greater for NOB as compared to AOB (Metcalf and Eddy, 2003).

2.2.4. pH & Alkalinity Requirements

Nitrification rates decrease significantly at pH levels decrease below 6.8. Optimal nitrification rates will occur at pH levels in the range of 7.0 – 8.0. Specifically, optimum pH levels for *Nitrosomonas* and *Nitrobacter* are in the range of 7.9 – 8.2 and 7.2 – 7.6, respectively (Alleman, 1984).

In addition, the equilibrium between un-ionized and ionized ammonia (NH_3/NH_4^+) along with nitrite and nitrous acid (NO_2^-/HNO_2) is also controlled by the pH of the wastewater. Un-ionized ammonia, NH_3, and nitrous acid, HNO_2, have been shown to inhibit both *Nitrosomonas* and *Nitrobacter*. Similar to ionized and un-ionized ammonia, nitrite and nitrous oxide will exist in equilibrium and their relative concentrations are directly related to the pH of the solution. Table 2.7 below shows NH_3 and HNO_2 concentration ranges that initiate inhibition of nitrifying bacteria (Anthonisen et al., 1976).

Nitrification releases hydrogen (H^+) and thus decreases the pH of the solution. Particularly, the oxidation of NH_4^+ releases hydrogen ions and will decrease the pH of a non-buffered wastewater. This in turn will shift the NH_3/NH_4^+ equilibrium and will decrease the inhibitory NH_3 concentration. However, a decrease in pH in a non-buffered wastewater will also affect the NO_2^-/HNO_2 equilibrium and will increase the inhibitory HNO_2 concentrations in the wastewater. Hence, a buffered wastewater is required to ensure appropriate pH levels for nitrification. The necessary alkalinity required for nitrification of 7.14 g-$CaCO_3$/g-NH_4^+-N oxidized is determined using the stoichiometric relationship shown in Equation 2.16 (Metcalf and Eddy, 2003).

$$NH_4^+ + 2HCO_3^- + 2\ O_2 \rightarrow NO_3^- + 2\ CO_2 + 2\ H_2O \qquad 2.16$$

Table 2.7: Un-ionized ammonia and nitrous acid concentrations that initiate inhibition to nitrifiers

	NH_3 (mg-N/L)	**HNO_2 (mg-N/L)**
Nitrosomonas	10 – 150	0.22 – 2.8
Nitrobacter	0.1 – 1.0	0.22 – 2.8

2.2.5. Temperature

Nitrifying bacteria have been found to be extremely sensitive to low temperatures; nitrification rates have been reported to increase 12 - 50% with a 4ºC increase and decrease 8 - 30% with a 1ºC drop when compared to nitrification rates at 21.3ºC (Barritt, 1933; Srna and Baggaley, 1975). The minimum generation time of nitrifiers at 30ºC (optimal temperature) is approximately 15 hours, whereas the generation time at 5ºC is approximately 200 hours (Wijffels et al., 1991). The optimum temperature for nitrification is within the range of 28 – 36ºC, thus classifying nitrifers as mesophilic bacteria (Sharma and Ahlert, 1976).

The Arrhenius equation can be used to express the effects of temperature on nitrification growth rates in which a temperature correction factor, θ, is applied (Metcalf and Eddy, 2003):

$$\mu_T = \mu_{max,20°C}\, \theta^{\,T\text{-}20°C} \qquad 2.17$$

where μ_T is the specific growth rate (g-cells/g-cells·d) at the temperature of interest, $\mu_{max,20°C}$ is the maximum specific growth rate (g-cells/g-cells·d) at 20°C and T is the temperature of interest (°C). The ammonia removal rate can thus be expressed using the temperature correction factor as follows (Metcalf and Eddy, 2003):

$$k_2 = k_1 \theta^{\,(T2\text{-}T1)} \qquad 2.18$$

where k_1 and k_2 are ammonia removal rates at temperatures $T1$ and $T2$ (°C), respectively. This assumes that the nitrification reaction rate is zero order, which has been observed at ammonia concentrations above 3 – 5 mg/L (USEPA, 1993; Hem et al., 1994).

The θ factor has been determined in previous studies to range from 1.076 to 1.165 (Downing and Hopwood, 1964; Painter and Loveless, 1983; Oleszkiewicz and Berquist, 1988). Average values of 1.098 and 1.058 were determined under ammonia limiting conditions and oxygen limiting conditions, respectively (Salvetti et al., 2006). In addition, θ values have shown to differ for suspended and attached growth processes (Aravinthan et al., 1998) with a θ value of 1.09 was established for moving bed biofilm reactor (MBBR) systems (Rusten et al., 1995). Nonetheless, a reported value of 1.072 has been accepted for wastewater treatment plant (WWTP) design (WERF, 2003).

A large variation in reported θ values is a concern in the design of treatment systems operating under various conditions. Factors that can greatly affect the temperature correction factor include the acclimatization period to new temperatures as compared to shock temperature changes to the system as well as the duration in which the system is exposed to the new temperature (Hwang and Oleszkiewicz, 2007; Delatolla et al., 2009). Hwang and Oleszkiewicz (2007) demonstrated that a gradual decrease in temperature from 20^{o}C to 10^{o}C was accurately modeled by a θ value of 1.072; however, the same change in temperature imposed as an immediate decrease in temperature produced a significantly higher θ value of 1.116. These findings are subsequently supported by similar results observed by Delatolla et al. (2009). Furthermore, the duration that the system is exposed to the new low temperatures has also shown to affect the θ value. Exposure time to low temperatures of 4^{o}C following an acclimatization period of 20 days resulted in increasing θ values from 1.002 to 1.216 as the exposure time increased from 5 days to 115 days (Delatolla et al., 2009). The following equation was therefore formulated to model the increase in θ values during prolonged exposure to cold temperatures (Delatolla et al., 2009):

$$\theta = 3.81\times 10^{-2}\cdot ln(t)+9.83\times 10^{-1} \qquad 2.19$$

where t is the number of days of low temperature exposure. Consideration as to whether the system experiences gradual or abrupt exposure to low temperatures and the duration of exposure time to low temperatures have thus been shown to more accurately estimate the θ values and ultimately long-term low temperature nitrification rates.

2.3. SUSPENDED GROWTH BIOLOGICAL TREATMENT SYSTEM

Suspended growth processes depend on free-floating microorganisms for heterotrophic carbon oxidation and autotrophic nitrification. The two conventional wastewater treatment systems in Canada are lagoon systems (facultative and aerated) and activated sludge systems.

2.3.1. Lagoons

Conventional facultative lagoons and aerated lagoons represent the majority of WWTPs in Canada, as these systems are conventional methods of treatment in rural communities in Canada. In fact, there are over 1000 lagoons systems currently operating in Canada as wastewater treatment facilities. Aerated lagoons and aerated facultative lagoons are low rate suspended growth processes and are often popular when vast space is available. Operating and maintenance cost are generally minimal and require a low level of skill to operate. Aerated lagoons utilize aeration to increase DO values and maintain microbial growth in suspension and typically operate at continuous discharge. Aerated lagoons offer the potential for effective nitrification under ideal conditions (warm temperatures) and a long solids retention time (SRT); however, lagoon systems that are susceptible to cold climates and seasonal changes demonstrate low nitrification rates (USEPA, 1993). SRTs for aerated lagoons with sludge recycling are typically increased up to 30 days during the cold season from an SRT of 10 – 20 days during the warm season (Metcalf and Eddy, 2003). As compared to a conventional activated sludge process, lagoons do not always include the provision of sludge recycle and are inherent to less process control.

2.3.2. Activated Sludge

The activated sludge process is the conventional method of wastewater treatment in largely populated areas in Canada. Nitrification in these systems can be achieved by either a single stage or two-stage activated sludge process. A common approach for BOD removal and nitrification is a single stage activated sludge process, which includes an aeration tank, clarifier, and sludge recycle. External secondary clarification is employed downstream of the aerated tank for the separation of total suspended solids (TSS) from the mixed liquor. The separated and thickened sludge stream is recycled back into the aerated tank to maintain a high concentration of active biomass. Nitrifying activated sludge processes require an increase in SRT to allow for the development of slow growing nitrifying bacteria. The minimum SRT required to achieve complete nitrification is 3 – 18 hours, whereas the minimum SRT required for BOD removal is 1 – 2 hours in domestic wastewater (Grady et al., 1999).

Two-stage activated sludge processes separate BOD removal and ammonia removal: an initial high rate carbon oxidation followed by a separate low rate nitrification process. Process separation can reduce the competition between heterotrophic bacteria (carbon oxidizers) and autotrophic bacteria (nitrifiers) as well as remove or reduce any toxic or inhibitory compounds that may affect nitrification. However, capital and maintenance costs associated with two-stage activated sludge systems are often higher than single stage activated sludge systems (Env. Canada, 2003).

2.4. BIOFILM

In natural aquatic ecosystems, microorganisms that colonize on surfaces outnumber suspended microorganisms (ZoBell, 1943). Bacteria that grow on surfaces are advantageous as they establish specific locations and symbiotic relationships in their environment. Attachment to a surface can provide a non-hostile environment, which in turn will provide some protection from predation and harmful substances in the environment. In addition, nutrients in an aqueous environment are concentrated near the surface or the surface itself can act as the nutrient source. The inclination of bacterial adhesion to surfaces suggests a strong survival instinct and advantage over suspended bacteria (Dunne, 2002).

Biofilm can be defined as a "structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" (Costerton et al., 1999). Extracellular polymeric substances (EPS) or exopolysaccharides produced by bacteria and other cells hold the biofilm together. The release of EPS will form glycocalyx, or "glue", through interactions of organic and inorganic molecules in the environment. When fully hydrated, glycocalyx is predominantly water and when partially hydrated glycocalyx is predominantly anionic and efficient for trapping nutrients and minerals from the surroundings. Biofilms consist of heterogeneous species that form symbiotic relationships with one another; byproducts produced by one organism can act as a substrate for another organism. The complex biofilm matrix formed by EPS contains polysaccharides, proteins, glycoproteins, phospholipids, nucleic acid, and humic acid (McSwain et al., 2005).

Biofilm layers are not a planar surface but are extremely non-uniform structures that contain protrusions, bumps, and voids (Costerton et al., 1995; Metcalf and Eddy, 2003). The biofilm density will vary amongst different types of biofilm and will also vary with biofilm depth. Furthermore, uniform growth does not occur across the substratum surface since hydrodynamic and design configurations of biofilm systems can often cause the periodic loss of large patches of biofilm (Hinton and Stensel, 1991).

2.4.1. Bacterial Attachment

Bacterial adhesion to a surface is initiated only when there is an initial attraction between the microbes and the surface. Surfaces can be abiotic (non-living) or biotic (living). The first stage of attachment is an irreversible process referred to as primary adhesion. Microorganisms must be in close proximity to the surface to promote attraction or repulsion forces; the net sum of these forces will determine adhesion (Dunne, 2002). The surface must be conditioned to overcome net repulsion forces through the use of molecular interactions. The attachment of organisms is mostly found on hydrophobic surfaces (Bryers, 2000). The second stage of attachment is referred to as secondary adhesion in which the primarily attached microorganisms will consolidate their adhesion to the surface by excreting EPS. Consequently, the presence of one species can promote adhesion of another species.

Planktonic cells, active or dead, along with inert material can be recruited to attach to the biofilm from the bulk liquid phase. Recruitment can be random or specific. Specific recruitment is referred to as coaggregation in which genetically distinct bacteria become attached to one another (Rickard et al. 2003).

2.4.2. Biofilm Maturation

The overall density and complexity of the biofilm increases as the organisms begin to actively replicate and die. Growth potential is affected by the availability of nutrients in the immediate environment, bulk phase conditions (temperature, pH, oxygen, carbon source, and osmolarity), and bulk liquid dynamics (Dunne, 2002). A maturing biofilm is defined as a film that has reached a critical mass. Subsequently, dynamic equilibrium is initiated as bacteria located in the outmost layer detach or die to maintain a quasi equilibrium state. A healthy and actively growing biofilm may thus shed daughter cells that have the potential of colonizing downstream surfaces (Characklis, 1990).

2.4.3. Bacterial Detachment

Bacterial detachment will occur due to one or more of the following processes: abrasion, erosion, sloughing, or grazing (Dunne, 2002; Metcalf and Eddy, 2003). Erosion is the loss of small pieces of biofilm due to liquid shear forces near the biofilm surface. Abrasion, similar to erosion, is the loss of small pieces of biofilm but is caused by particle collision. Sloughing is the loss of large patches of biofilm due to a depletion of nutrients or sudden changes in hydrodynamic stress. Lastly, grazing occurs when protozoa or other predators consume the outer layer of the biofilm.

2.4.4. Mass Transfer Effects

Mass transfer effects govern substrate removal within many biofilms and specifically in biofilm wastewater treatment processes. Suspended growth systems are bio-kinetically limited, whereas attached growth systems are mass transfer limited. The pathway of nutrients and oxygen into a biofilm is a complex process. As described in Figure 2.5, the substrate particle must first be transported to the biofilm surface and adhere to the surface before being broken down in the extracellular intrabiofilm and degraded within the cell (WEF, 2011).

Microbial growth reactions are thus heterogeneous reactions and involve more than one phase; both inter- and intra-phase transport of the substrate takes place. Mass transfer to the biofilm is controlled by the hydraulic regime of the bulk liquid, whereas mass transfer in the biofilm is controlled by molecular diffusivity of the substrate and biofilm structure (WEF, 2011).

The rates of various mechanisms of the overall biodegradation process comprise the overall observed rate, which is also referred to as the global rate. The intrinsic rate of biodegradation is defined as the rate of biodegradation by the cells independent of mass transfer effects. One of the individual mechanistic reactions is rate limiting and this rate will control the overall observable rate and specifically is equal to the global rate of biodegradation. Therefore, the rate of each individual reaction step in the biofilm biochemical transformation is calculated to determine the rate limiting step.

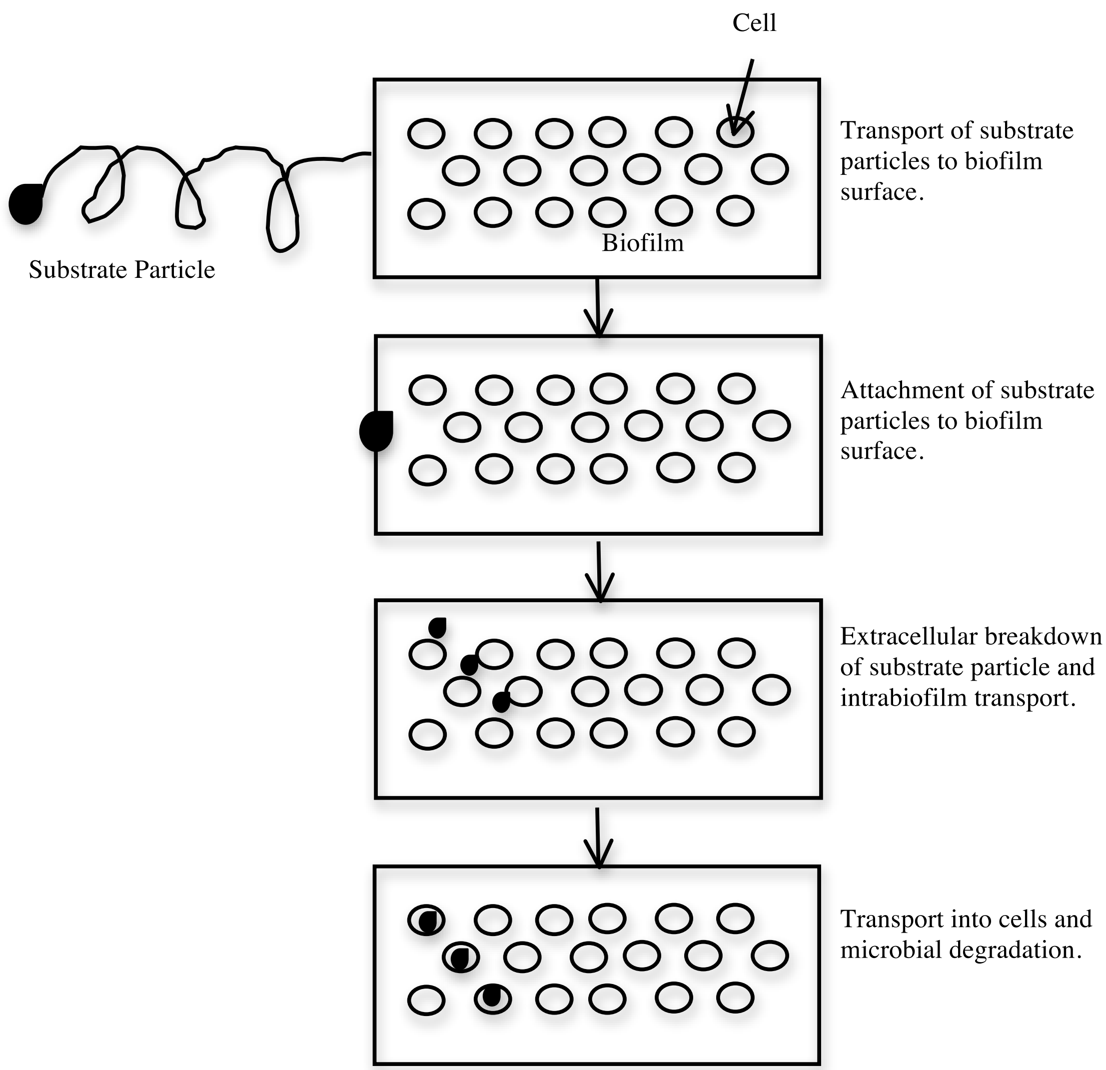


Figure 2.5: Pathway of substrate particles for biochemical transformation in biofilm

The rate of reaction for biofilm reactors is often expressed relative to the surface area or volume of the media (catalyst) in which the biofilm attaches as opposed to the liquid volume. Figure 2.6 shows the boundary layers of a biofilm as substrate transports to the biofilm surface and into the biofilm, where S_b is the substrate concentration in the bulk liquid (g/m^3), Ss is the substrate concentration at the biofilm surface (g/m^3), and L is the biofilm thickness (m) (Metcalf and Eddy, 2003).

Transport of nutrients, substrates, and other constituents is first resisted as the constituents travel from the bulk liquid phase to the biofilm surface through the mass transfer boundary layer (MTBL), also referred to as the stagnant fluid layer (Figure 2.6).

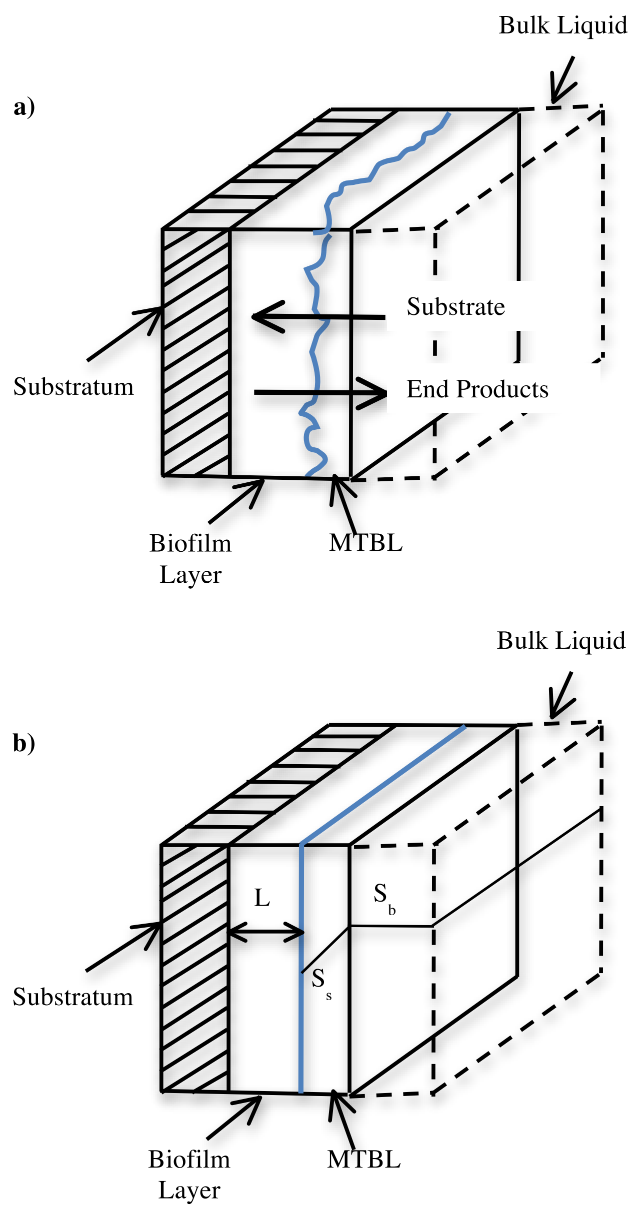


Figure 2.6: Mass transport: a) biofilm boundary layers b) substrate concentration through biofilm

The MTBL thickness varies with fluid properties and particularly with the velocity of the bulk liquid (Metcalf and Eddy, 2003; WEF, 2011). Substrate and oxygen concentrations being consumed by cells embedded in the biofilm are often lower with depth in the biofilm as compared to concentrations in the bulk liquid. As a result, the rate of mass transfer into and through the film is often rate limiting.

Mass transport to the biofilm, r_{sf} (g/m^{2}d), is represented as a surface flux across the MTBL while the mass transport inside the biofilm, r_{bf},(g/m^{2}d) is represented as a flux through the biofilm. Fick's Law of Diffusion is used to define r_{sf} and r_{bf} in Equations 2.20 and 2.21, respectively (Metcalf and Eddy, 2003; WEF, 2011):

$$r_{sf} = -D_w\left(\frac{dS}{dx}\right) = -D_w\left(\frac{S_b - S_s}{L}\right) \qquad 2.20$$

$$r_{bf} = -D_e\left(\frac{dS}{dx}\right) \qquad 2.21$$

where D_w is the diffusion coefficient in water (m^2/d), S_b is the bulk liquid concentration (g/m^3), S_s is the substrate concentration at the outer biofilm (g/m^3), L is the effective thickness of the biofilm (m), D_e is the diffusion coefficient in the biofilm (m^2/d), and dS_f/dx is the substrate concentration gradient (g/m^3·m). The rate of substrate utilization, r_{su}, at any point in the biofilm is governed by Equation 2.4 (Section 2.1.2).

Either the electron donor or electron acceptor will often be the diffusion rate limiting processes into and across the biofilm. Using the relationship between the electron donor and electron acceptor in the bulk liquid, Williamson and McCarty (1976) proposed a mechanistic model to describe substrate flux limitations. The effect of substrate flux limitations is described by the following relationship, which is true if the election acceptor is rate limiting:

$$S_{ba} < \frac{D_{wd} v_a mw_a}{D_{wa} v_d mw_d} S_{bd} \qquad 2.22$$

where S_{ba} is the bulk liquid electron acceptor substrate concentration (mg/L), D_{wd} is the diffusivity coefficient of the electron donor in water (cm^2/d), mw_a is the molecular weight of the electron acceptor (g), v_a is molar stoichiometric reaction coefficient for the electron acceptor (mol), D_{wa} is the diffusivity coefficient of the electron acceptor in water (cm^2/d), mw_d is the molecular weight of the electron donor (g), v_d is molar stoichiometric reaction coefficient for the electron donor (mol), and S_{bd} is the bulk liquid electron donor substrate concentration (mg/L). This equation is used to determine the quantity of relative S_{ba} needed to sustain electron donor utilization in the biofilm. It should be noted that nitrification rates in biofilm systems are often rate limited by DO concentrations (electron acceptor) in the bulk liquid as oppose to ammonia concentrations (electron donor).

2.4.5. Nitrifying Biofilm

Since nitrifiers are slow growing organisms and nitrifying biofilm can take an extensive time to reach full potential in a biofilm system, nitrification rates may still be increasing after the first year of operation (Boller and Gujer, 1986; Wessman and Johnson, 2006). Therefore, the first year of operation will generally be regarded as an acclimitization period where the biofilm is in a young state. Ammonia loading rates of a nitrifying biofilm system during a start-up phase should be kept low to promote attachment and growth; moreover, ammonia loading should be increased slowly to prevent instability of the system (Rusten et al., 2006; Bassin et al., 2012).

A low carbon/nitrogen (C/N) ratio in the system will reduce competition between heterotrophs and autotrophs and is essential for the development of nitrifying biofilm. However, it has been shown that the time required to grow stable nitrifying biofilm is significantly shortened when there is a heterotrophic growth start-up phase where organic compounds are fed to the biofilm system (Bassin et al., 2012). Heterotrophs are fast-growing organisms and will result in higher biomass production and consequently, higher EPS concentrations. EPS acts as a fixation matrix that supports the adhesion of biomass to the substratum and as a result will facilitate initial attachment of bacteria (Tijhuis et al., 1994; Cammarota and Santa'anna, 1998). Furthermore, when organic matter and/or particulate matter increases in the system, nitrifying biofilm concentrations become diluted and this, in turn, can decrease the nitrification reaction rate. A low alkalinity can also decrease the reaction rate due to a pH reduction inside the biofilm; a higher residual alkalinity is required for a thicker biofilm.

2.5. ATTACHED GROWTH BIOLOGICAL TREATMENT SYSTEMS

Attached growth processes are another form of biological treatment that use microorganisms attached to substrata for carbon oxidation and nitrification. Attached growth processes rely on microorganisms that are attached to inert support material. Bacteria in wastewater attach themselves to a substratum to form a biofilm layer. Suspended and colloidal solids may attach and decompose into soluble products on the surface of the biofilm, as they often cannot penetrate through the void spaces of the film.

Dissolved constituents however, pass through the film via diffusion. Substrate removal is thus governed by mass transfer effects through the biofilm and bulk liquid. Therefore, attached growth processes put forward a higher level of design complexity compared to suspended growth processes. Similar to suspended growth processes, BOD removal and nitrification can be achieved in a single-stage process or can be separated into two-stage processes. Common attached growth technologies capable of ammonia removal include (Env. Canada, 2003; Metcalf and Eddy, 2003):

- trickling filters;
- rotating biological contactors (RBC);
- biological aerated filtration (BAF);
- moving-bed biofilm reactors (MBBR).

These attached growth processes can be used for BOD removal only, combined BOD removal and nitrification, or tertiary nitrification. Trickling filters, RBCs, BAFs, and MBBRs are all capable of producing an effluent with total ammonia-nitrogen concentration lower than 5 mg/L at various flow rates and under conventional treatment temperatures (Env. Canada, 2003). BAFs and MBBRs represent newer and more efficient technologies of biofilm systems compared to trickling filters and RBCs and offer the advantage of requiring less space (Oleszkiewicz and Barnard, 2006).

2.5.1. Trickling Filter

Trickling filters include a solid substratum to support the growth of an active biofilm. The filter contains a bed of rocks, gravel or polyvinyl chloride (PVC).

Wastewater is pumped to the top of a rotating arm where the wastewater is sprayed downwards through the media bed. Figure 2.7 illustrates a trickling filter with its rotating arm spraying wastewater into the bed rock filter. The oxygen needed for treatment is provided by a natural draft or forced air. The bed rock located in the upper portion of the filter will contain thicker biofilm with heterotrophic bacteria whereas the bed rock located in the lower portion of the filter will contain a thinner biofilm with autotrophic bacteria (Figure 2.7) in a single stage filter (Metcalf and Eddy, 2003). This process can operate at various hydraulic and feed loads, where the wastewater flow approaches plug flow conditions in the system.

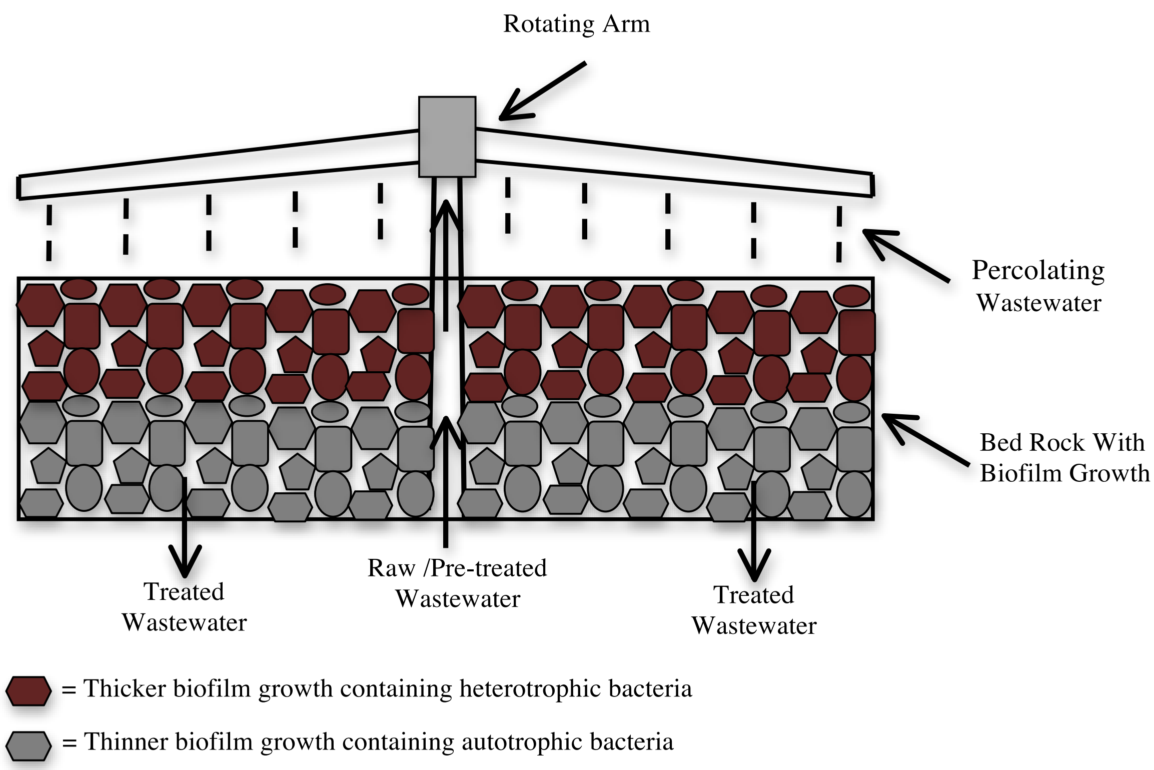


Figure 2.7: Schematic of a trickling filter

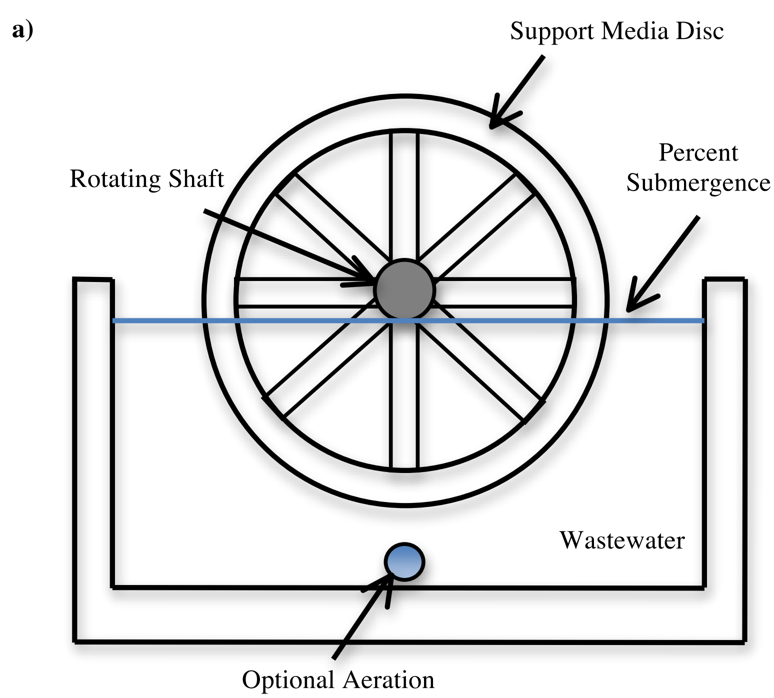
Both BOD removal and nitrification can be achieved using a single stage trickling filter with rock or plastic packing material at low organic loads (Stenquist et al., 1974; Parker and Richards, 1986). To initiate nitrification, BOD loading should be less than 30 mg/L; for complete nitrification, BOD loading should be less than 15 mg/L (Bruce et al., 1975).

Operating costs are low to moderate and the level of skill required to operate the system is moderate. Although trickling filters require less energy compared to the conventional activated sludge process, there are a few disadvantages to consider. Trickling filters are suitable for low flow rates or moderate to high flow rates (Env. Canada, 2003; Metcalf and Eddy, 2003). Furthermore, since biofilm growth is not uniformly distributed and the liquid does not flow evenly over the packed media, biofilm overgrowth on some portions of the packed media can cause clogging. Specifically, high loading rates (particularly under carbon removal conditions) can cause excess growth of biofilm, which in turn clog the pores of the media and directly affect the actual retention time of the system. Trickling filters may also require additional pre-treatment and can have the potential for odour problems if proper ventilation is not provided.

2.5.2. Rotating Biological Contactors (RBC)

RBC technologies consist of a cylindrical synthetic substratum bundle mounted on a horizontal rotating shaft, which is partially submerged in a holding tank where biofilm develops on the substratum. The number of RBC units per pass depends on the flow rate and influent ammonia concentration. Similar to the trickling filter, RBCs require a low BOD loading to initiate and perform nitrification and are sensitive to the diffusion efficiency of nutrients into the biofilm (Env. Canada, 2003). Conventional RBCs are 35 – 40% submerged but can be modified into submerged biological contactors (SBR), which are submerged up to 70 – 90% (WEF, 2000). Figure 2.8 shows the front, side, and top view of an RBC system.

The RBC system has minimal space requirements, easy process control and is capable of achieving a high degree of wastewater purification including nitrification (Env. Canada, 2003). RBCs do however, require frequent shaft and mechanical drive maintenance as well as additional protection from cold climates. Excess biofilm growth can cause clogging in void spaces in the media and weigh down the RBC causing mechanical failures.



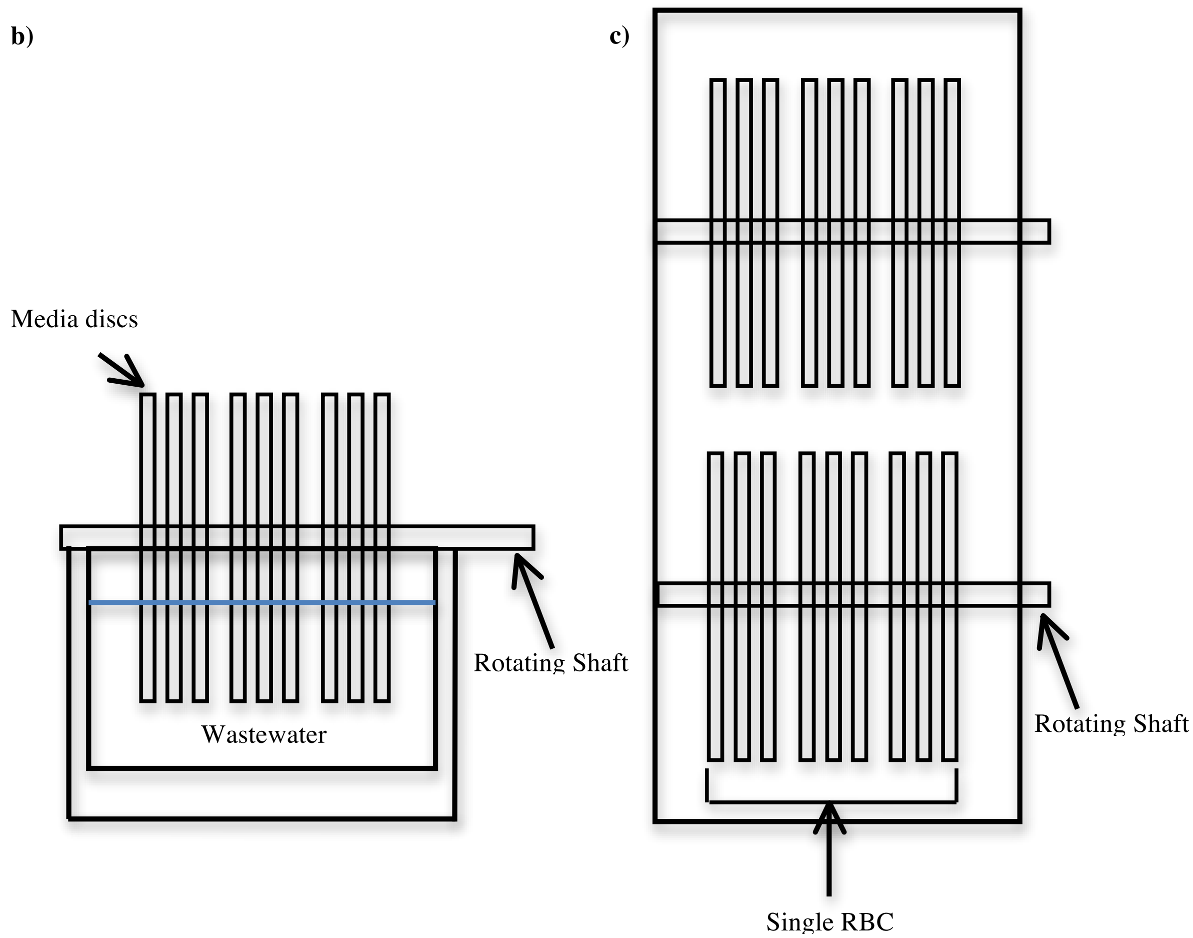


Figure 2.8: Schematic of an RBC system: a) front view of one RBC b) side view of one RBC c) top view of multiple RBC trains

2.5.3. Biological Aerated Filter (BAF)

BAF systems, also referred to as aerobic submerged fixed-bed systems, can be designed to operate under various configurations. BAFs contain comprise of packing material that is submerged in a vertical cell containing media of sand, gravel, activated carbon, or polystyrene beads. The media offers a high surface area for biofilm growth and the fixed-bed provides simultaneous biological treatment and suspended solids filtration (Env. Canada, 2003). Designs differ according to packing configuration, inlet and outlet flow distribution, and upstream collection and storage systems (Metcalf and Eddy, 2003).

The Biocarbone process uses high-density packing material and is a downflow process, whereas the Biofor and the Biostyr are upflow processes that use high-density and low-density packing material, respectively. Similar to a BAF, the fluidized-bed bioreactor (FBBR) is an aerobic submerged mobile bed reactor. FBBRs use lightweight media that are maintained in suspension by air or water flow (WEF, 2000).

Downflow configurations often generate fast clogging of the media and require a higher backwashing frequency. From these observations, upflow configurations are preferred over downflow configurations due to lower head loss and lower backwashing frequency (Env. Canada, 2003). The HRT for BAFs are generally 1 – 1.5 hours (Metcalf and Eddy, 2003) and backwashing cycles are required to maintain the biofilm and wash out suspended solids trapped in the packed bed. BAFs can be placed in series of treatment trains and switched to parallel treatment when hydraulic flows are high. Thus the design and operation of BAF systems require a considerably high level of complexity.

BAF systems can be used for both BOD removal and nitrification. To maintain a high rate of nitrification in a combined BOD removal and nitrification system, organic loading should be low based on the volume of media in the treatment cell (WEF, 2000). BAF technologies offer the advantage of being compact and do not require secondary or intermediate settling but do however require frequent backwashing and are susceptible to clogging.

2.5.4. Moving Bed Biofilm Reactors (MBBR)

MBBR technologies use biocarriers to support and maintain the growth of active biofilm. Carriers are produced by various suppliers and thus vary in shape and size. The specific surface area available for biofilm growth varies from 500 – 1200 m^2/m^3 depending on the carrier type and manufacturer (Ødegaard, 2006). The carriers are maintained in constant suspension and movement within the treatment system to allow for optimal contact of the biofilm with oxygen and substrate concentrations in the bulk liquid. Biofilm growth occurs on the inner portion of the carrier to protect the biofilm, to a certain extent, from external forces. These carriers are self-cleaning as collision of the carriers with one another and hydrodynamic forces in the system maintain the biofilm thickness. The available surface area for biofilm growth can vary depending on the carrier type. The percentage fill, by volume, of the media bed relative to the total reactor volume is recommended to be between 25 – 70% (WEF, 2000; Metcalf and Eddy, 2003; Rusten et al., 2006). Therefore, to accommodate for future increases in treatment capacity, the percent fill can simply be augmented through the addition of more carriers. Furthermore, carriers have been found to last as long as 15 years with minimum wear and tear (Rusten et al., 2006).

Similar to the previously mentioned attached growth processes, MBBRs can be used for both BOD removal and nitrification. MBBRs do not occupy a large area, they do not require sludge recycling, or backwashing and solids produced through the detachment of biofilm can be settled using a final clarifier (Metcalf and Eddy, 2003; Ødegaard, 2006). The operating and maintenance costs associated with MBBR technologies are generally low compared to conventional suspended growth processes and other attached growth processes but depending on aeration rates, costs can also be high.

2.6. LOW TEMPERATURE NITRIFICATION IN WASTEWATER TREATMENT SYSTEMS

2.6.1. Recent Canadian Federal Regulation of Ammonia Discharge

Although a medium to highly concentrated municipal Canadian WWTP influent only contains approximately 25 mg/L of total ammonia (Metcalf and Eddy, 1991), in which the fraction of toxic un-ionized ammonia is small, a significant amount of ammonia is generated during anaerobic digestion and anaerobic respiration. The degradation of amino acids produce NH_4^+/NH_3 as a means of maintaining alkalinity in the digester. The produced ammonia react with CO_2 and water to form alkalinity as ammonium bicarbonate ($NH_4(HCO_3)$). Total ammonia concentrations between 500 – 2000 mg/L are often found in a typical anaerobic digester (WEF, 1996; Metcalf and Eddy, 2003). Furthermore, a lagoon system with a long SRT can produce an anaerobic zone at the bottom of the pond and generate ammonia through ammonification. Therefore, in addition to ammonia in the wastewater influent, the ammonia generated during anaerobic digestion requires treatment before effluent discharge.

Ammonia has been recognized as a deleterious substance and has recently been regulated by the Federal Government of Canada. New federal wastewater effluent regulations aim to directly reduce the mass of pollutants discharged in surface waters from WWTPs. The regulations apply to any WWTP (both intermittent and continuous wastewater systems) that discharge a daily volume of 100 m^3 or more effluent. The ammonia discharge in wastewater effluent has been federally regulated to be less than 1.25 mg-NH_3-N/L at 15 ± 1^{o}C. The discharge of total ammonia is expected to be reduced by 16 000 metric tonnes annually in Canada and consequently will reduce the release of un-ionized ammonia and its toxic effects to receiving waters (Canada Gazette, 2012).

2.6.2. Potential of Biological Nitrication of Wastewater

The effect of cold temperature on biological treatment systems has been shown to decrease biological activity and overall treatment efficiency (Metcalf and Eddy, 2003). Understanding nitrification at low temperatures is of key importance for cold climate countries. As mentioned in the previous section, lagoon facilities represent over 1000 municipal WWTPs in Canada with aerated treatment ponds reaching temperatures as low as 1^{o}C during winter months. Existing WWTPs will require upgrading in the near future in order to meet the newly implemented ammonia discharge regulations (Canada Gazette, 2012). Although a number of various biological treatment technologies are capable of sufficient ammonia removal in wastewater, only a few are effective at low temperatures due to the sensitive nature of nitrifying bacteria.

Nitrification in an activated sludge process has been shown to decrease 50% at 12^{o}C and 100% at 5^{o}C, where a sharp decrease in nitrification was observed at 15^{o}C (Borchardt, 1966). For sufficient ammonia removal, nitrifying systems require long retention times when the concentration of nitrifiers in the system is low, when the sludge age is low, or when the temperature of the system is low (Sharma and Ahlert, 1976). Suspended growth systems usually require a longer retention time compared to attached growth systems (Sharma and Ahlert, 1976), as attached growth processes have the ability to maintain a higher biomass concentration and higher biomass age compared to suspended growth processes (Bryers, 2000).

Although previous studies have shown that suspended growth processes are capable of low temperature nitrification, these studies did not investigate long-term nitrification (Oleszkiewicz and Berquist, 1988; Head and Oleszkiewicz, 2004; Hwang and Oleszkiewicz, 2007). To accommodate for a low concentration of nitrifiers, low sludge age, and/or low temperatures, these studies seed low temperature systems with nitrifying biomass from high temperature systems to achieve nitrification. In this manner, nitrifying biomass from a high temperature system must be continuously supplied to the low temperature system in order to achieve consistent and sufficient ammonia removal over short periods of time. However, this process has never been shown to be effective over long exposure times and would be very costly over long winter periods since nitrifying biomass from a high temperature system must be continuously purchased or a high temperature nitrifying system must be operated in conjunction with the low temperature system to provide the active biomass.

In contrast, nitrifying biofilm systems show great promise of consistent nitrification at low temperatures for extended periods of time (WEF, 1998; Andersson et al., 2001; Delatolla et al., 2009). Compared to suspended nitrifiers, higher nitrification rates have been demonstrated by attached nitrifiers at warm temperatures (Aravinthan et al., 1998). In addition, attached nitrifiers have been shown to be less sensitive to low temperatures than suspended nitrifiers due to diffusion limitations of the biofilm matrix and an increase substrate affinity (Wijffels et al., 1995). Therefore, attached growth processes should be considered as a potential upgrade technology for long-term cold temperature nitrification in existing Canadian WWTPs.

2.7. MBBR AS A POTENTIAL NITRIFYING UPGRADE TECHNOLOGY FOR COLD TEMPERATURE TREATMENT

A number of different attached growth technologies capable of sufficient ammonia removal are described in Section 2.5. The MBBR system is an attractive upgrade technology for low temperature nitrification over other attached growth technologies because MBBRs offer the advantages of less space requirement, utilizing the whole tank volume, no sludge recycling, and no backwashing. The MBBR is also designed such that the attached biofilm is self-maintaining and the carriers are highly durable (Rusten et al., 2006). In addition, MBBR systems can be designed to be easily upgraded to accomidate for future projected population growth as the percent fill of media can simply be increased through the addition of more carriers into the basins as needed.

The performance of MBBRs as an upgrade nitrifying technology has been demonstrated for pilot-scale and full-scale systems (Wessman and Johnson, 2006; Ødegaard, 2006; Houweling et al., 2007; Delatolla et al., 2011). These systems are capable of ammonia removal at temperatures as low as 4^{o}C and below. However, these MBBR upgrades were installed to treat effluent exiting the first or middle lagoon where temperature drops were limited or cold temperatures were only experienced on a short-term basis. As mentioned in Chapter 1, the placement of an upgrade nitrifying MBBR system in a multiple lagoon treatment system should be considered after the last lagoon, where temperatures can drop to 1^{o}C for an extended period of time, for optimum C/N ratios and to prevent carbon removal redundancy. Current research and literature, however, lack information regarding long-term nitrification at 1^{o}C.

2.7.1. Nitrifying MBBR Operational Conditions

Major design and operational factors that influence nitrifying biofilm development and nitrification rates are ammonia loading rates, organic loading rates and DO concentrations (Hem et al., 1994; Rusten et al., 2006; Bassin et al., 2012). The intrinsic rate of nitrification is expressed by Michaelis-Menten kinetics (Equation 2.18) and is often assumed to be zero order to simplify the analytical solution in MBBR reactors as elevated DO values are maintained in the system. Although several assumptions are made under certain operating conditions, it is acceptable for biofilm reactor design (Salvetti et al., 2006). To compensate, the overall reaction rate, often equal to the substrate flux into the biofilm, is used as a kinetic design parameter. The empirical equation of ammonia flux, *J*, for MBBR nitrification design, with temperature effects, is represented by the following equation (WEF, 2011):

$$J = k\theta^{(T2-T1)}S^{n} \tag{2.24}$$

where k is the reaction rate constant, θ is the Arrhenius temperature correction factor, $T1$ is the low temperature of interest (^{o}C), $T2$ is the reference temperature (^{o}C), S is the ammonia (substrate) concentration (g/m^3), and n is the order of reaction. Diffusional resistance and the increase in DO and substrate saturation can mask the effects of temperature on nitrification rates, where the effect of temperature on the nitrification has been observed to be less under oxygen limiting conditions compared to ammonia limiting conditions (Salvetti et al., 2006).

Ammonia concentration limits nitrification rates only at low concentrations of approximately 1 – 3 mg-NH_4^+-N/L, whereas when ammonia concentrations are above 3 mg-NH_4^+-N/L, nitrification rates are governed by organic loading and DO concentration. It has been found that, in the absence of organic matter, the transition from ammonia limiting to oxygen limiting conditions occur when the DO to ammonia concentration ratio is about 2.7 g-O_2/g-NH_4^+-N at a DO concentration of 9 – 10 mg-O_2/L and 3.2 g-O_2/g-NH_4-N at a DO concentration of 6 mg-O_2/L (Gönenc and Harrermoës, 1985; Szwerinski et al., 1986).

The intrinsic reaction rate in an MBBR biofilm is influenced by both present and past loading concentrations; biofilm acclimatized with a high ammonia loading is found to have a reaction rate almost twice that of a biofilm acclimatized with a low ammonia loading (Hem et al., 1994). Rusten et al. (1995) observed k to be 0.6 – 0.7 m/hr for tertiary MBBR nitrification. Furthermore, when nitrification is ammomia limited or partly ammonia limited, the rate of reaction is first or mixed order; when nitrification is oxygen limited, the rate of reacton is zero order. Therefore, a value of n = 0.7 is used to design a nitrifying MBBR when ammonia is limited and a value of n = 0 is used when oxygen is limited. Although a DO concentration of 2 – 3 mg-O_2/L has shown to be substantial for carbon removal, a high DO concentration (above 6 mg-O_2/L) is required to initiate and maintain nitrification when the organic loading is greater than approximately 4 g-BOD_7/m^2d, (Ødegaard, 2006). Therefore, organic loading should be kept as low as possible to initiate and maintain nitrification. Table 2.8 shows nitrification rates with respect to organic loading (Hem et al., 1994) and Table 2.9 shows the effect of organic load on the requirement of DO for MBBR ammonia removal (Rusten et al., 2006).

Table 2.8: Nitrification rates according to organic loading rates

Organic Loading Rate (g-BOD_7/m^2d)	**Nitrification Rate (g-NH_4^+-N/m^2d)**
1 – 2	0.7 – 1.2
2 – 3	0.3 – 0.8
> 5	Very close to zero

Table 2.9: Effects of organic loading on DO requirements for ammonia removal

Organic Loading ($g\ BOD_7/m^2d$)	**DO Concentration ($mg\ O_2/L$)**	**Removal Rate ($g\ NH_4^+$-N/m^2d)**
1	5	1
3	8	1

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CHAPTER 3: METHODS AND MATERIALS

3.1. EXPERIMENTAL DESIGN

Two identical laboratory moving bed biofilm reactors (MBBRs) were operated under identical and continuous conditions during the entire experimental phase. A temperature-controlled room was used to house the reactors for temperatures below 20^{o}C. The temperature of the feed and the reactors were measured using a standard thermometer. The MBBRs were operated at 20^{o}C for six weeks and subsequently acclimatized over a four week period to 1^{o}C and maintained at 1^{o}C for a period of four months. Following the experiments at 1^{o}C, the MBBRs were acclimatized and operated at 5^{o}C for two weeks to investigate this potential kinetic threshold temperature. Nitrification kinetic rates were measured at 20^{o}C, 1^{o}C, and 5^{o}C under steady state conditions. Particularly, nitrification kinetic rates were measured after six weeks at 20^{o}C and after one month, three months, and four months at 1^{o}C. To analyze the kinetic temperature threshold, nitrification kinetics were then measured after two weeks at 5^{o}C. Nitrogen mass balances based on removed ammonia (NH_4^+-N) and produced nitrite (NO_2^--N) and nitrate (NO_3^--N) were performed for each kinetic data set measured to monitor the pathway of ammonia oxidation and nitrite oxidation.

The hydraulic retention time (HRT) of the two reactors during experiments at 20^{o}C was 4 hours and was increased to 6 hours for experiments at 1^{o}C and 5^{o}C. The HRT was adjusted at low temperatures to conserve synthetic wastewater (SWW) during lower kinetic rates while maintaining a conventional HRT value between 2 and 6 hours for nitrification (WEF, 2011). The pH was maintained between 7 – 8 and the dissolved oxygen (DO) concentration was maintained between 7 – 8 mg-O_2/L throughout the entire experimental phase. These values again correspond to conventional operation conditions (Metcalf and Eddy, 2003; Ødegaard, 2006).

3.2. REACTORS

The reactors were designed as completely stirred tank reactors (CSTRs) with a total volume of 2 L. K3 Anoxkaldnes carriers were used in both reactors at a 40% media fill value (Figure 3.1) and this fill percentage was below the recommended maximum fill percentage of 70% and above the minimum value of 25% (Rusten et al., 2006; WEF, 2011). The K3 carriers are high-density polyethene and are cylindrical shaped with an inside crosspiece and outside longitude fins. These carriers are 7 mm in depth, 10 mm in diameter, and have a surface area available for biofilm attachment of 500 m^2/m^3 (Rusten et al., 2006). Adequate aeration was provided to ensure movement and suspension of carriers and to prevent denitrification.

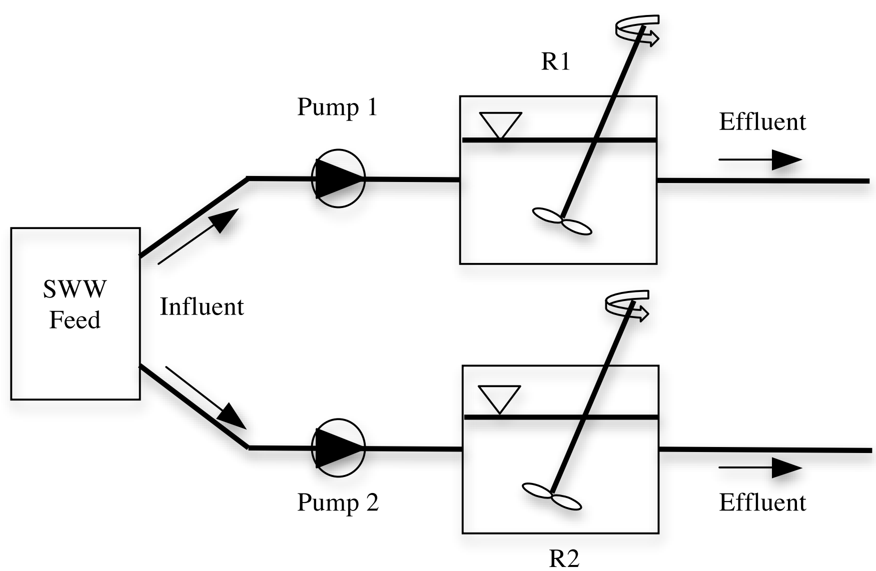

Figure 3.1: Schematic of the laboratory setup of reactors

All carriers were inoculated in an MBBR nitrifying pilot plant that was operated at the Vaudreuil, QC wastewater treatment plant. Reactor 1 was denoted as R1 and reactor 2 was denoted as R2. A slightly older and more developed biofilm was used in R1 and a younger biofilm was used in R2 since R1 was operated for approximately six months longer than R2 before the start of the experiments.

3.3. SYNTHETIC WASTEWATER RECIPE

SWW was used for all experiments in this study. The SWW recipe was formulated using a variety of sources (De Beer et al., 1993; Hem et al., 1994; Gieseke et al., 2001; Lee et al., 2004; Rochex et al., 2008; Xia et al., 2010) to ensure sufficient amounts of nutrients and ammonia to the nitrifying biofilm. No carbon sources were provided to in the continuous feed to allow for the investigation of nitrification with minimal heterotrophic growth affects.

The SWW recipe is as follows: $(NH_4)_2SO_4$ (104 mg/L), $NaHCO_3$ (286 mg/L), $FeSO_4 \cdot 7H_2O$ (5 mg/L), KH_2PO_4 (80 mg/L), $CaCl_2 \cdot 2H_2O$ (29 mg/L), and $MgSO_4 \cdot 7H_2O$ (71 mg/L). Trace nutrients included $MnCl_2 \cdot 4H_2O$ (0.2 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.05 mg/L), $CuSO_4 \cdot 5H_2O$ (0.2 mg/L), $CoCl_2 \cdot 6H_2O$ (0.002 mg/L), and $ZnSO_4 \cdot 7H_2O$ (0.06 mg/L).

3.4. CONSTITUENTS: NITROGEN, PH, DO, TSS AND VSS

To quantify nitrification kinetic rates, NH_4^+/NH_3-N, NO_2^--N, and NO_3^--N concentrations were measured using standard methods (APHA, 1995). Method 4500C-

NH_3, method 4500B-NO_2^-, and method 4500B-NO_3^- was used to measure ammonia, nitrite, and nitrate, respectively once both reactors reached steady state conditions at 20°C, 1°C, and 5°C. DO and pH were measured using an Orion 5-Star pH/RDO Multiparameter Meter (Thermo Scientific). The pH of the reactors was controlled using sodium hydroxide (NaOH) or sulfuric acid (H_2SO_4) to increase or decrease the pH, respectively. Solids analysis of the effluent was measured using standard methods for total suspended solids (TSS) and volatile suspended solids (VSS) (APHA, 1995). Method 2540D and method 2540E was used to measure TSS and VSS, respectively.

Three samples (triplicates) were measured to determine NH_4^+-N, NO_2^--N, and NO_3^--N concentrations as well as pH and DO concentrations throughout the entire experimental phase. Steady state conditions were verified by a 10% change, or less, in nitrogen mass balances of triplicates from five consecutive samples. Hence 15 samples were used to measure NH_4^+-N, NO_2^--N, NO_3^--N, pH and DO. A minimum of 15 samples was also used to measure the effluent TSS and VSS concentrations during steady state conditions for the 20°C experiments and after four months at 1°C.

3.5. BIOFILM THICKNESS AND MORPHOLOGY

Biofilm thickness and morphology was investigated using a Tescan Vega II-XMU variable pressure scanning electron microscope (VPSEM). A variable pressure chamber in a VPSEM replaces the high-pressure chamber found in a traditional SEM. This variable pressure chamber eliminates the need for destructive preparation associated with traditional SEM imaging (Little et al., 1991; Priester et al., 2007). Biofilm samples analyzed using VPSEM imaging do not require pre-treatment. Kimwipes™ were used, however, to carefully remove excess liquid in the pore space of the carriers before being placed directly into the low-pressure chamber of the microscope for imaging. Samples were analyzed at a pressure of 40 Pa and magnifications between ×20 and ×2000. AxioVision LE (Carl Zeiss Microscopy Software) was used to directly measure biofilm thickness on the acquired images.

Biofilm thickness and morphology was investigated at 20^{o}C and after one month and four months exposure to 1^{o}C. One carrier from each reactor was used for analysis at 20^{o}C and one carrier from each reactor was used for analysis at 1^{o}C. Images were captured at random locations on the carrier and a minimum of 20 thickness measurements was used to complete a data set for analysis.

3.6. BIOMASS VIABILITY

The viability of embedded cells in the biofilm samples were analyzed using confocal laser scanning microscope (CLSM) imaging in combination with a FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen) as fluorescent stains. A CLSM uses pinpoint illumination, unlike a conventional fluorescence microscope, to eliminate unfocused background details and thus has the ability to capture images at selective depths of a specimen without physically sectioning a specimen. The viability kit includes SYTO9, which stains all cells green, and propidium iodide, which stains only cells that have a damaged cell membrane. Therefore, live cells are presumed as those with intact cell membranes. Live cells will hence fluoresce green (SYTO9) and dead cells will fluoresce red (combination of SYTO9 and propidium iodide). The stain may penetrate depths of approximately 100 µm into the biofilm (Ødegaard, 2006). The samples in this study were stained for approximately 40 minutes while covered to prevent overexposure of light to the fluorescent stain. The stained samples were then rinsed off with sterile water to remove excess stain before imaging.

Biomass viability was investigated at 20^{o}C, following one month at 1^{o}C, and following four months at 1^{o}C. One carrier from each reactor was used for analysis at 20^{o}C and one carrier from each reactor was used for analysis at 1^{o}C. Random sections of the carrier were cut, without detaching the biofilm from the carrier itself, to enable visualization of all sections of the carriers and the respective attached biofilm.

Images were acquired using a Zeiss LSM 5 Pascal confocal microscope equipped with an Argon laser (488 nm, 514 nm) and a HeNe laser (543 nm). Sample images with a thickness of 1.0 µm and an area of 214 × 143 µm were captured using a 36× oil immersion lens for viewing. A total of four stacks (where each stack is comprised of five images), or 20 images total, per reactor were acquired and analyzed for each experimental phase. Stack images were taken at random locations on the carrier where each stack had a maximum vertical depth of 80 µm. This allowed for analysis of the upper portion of the biofilm where substrate and nutrients were most exposed without penetrating beyond the limit of the stain penetration. Full substrate penetration has been observed to reach depths of approximately 100 µm in wastewater biofilms (Ødegaard, 2006).

Vision Assistant 7.1 (National Instruments, LabView 8.0) was used to quantify the percentage of live (stained green) and dead (stained red) cells. This was performed by a calibration method where the size of a single cell on the CLSM image was verified to be in the range of a real cell. Single cells were identified as stained cells that did not appear in as a cluster in the CLSM image (Figure 3.2). A total of 20 verifications were performed for each image. The colour threshold function on NI Vision Assistant was used to segment the illuminated portion of the image that represents the cells of interest. Portions of the image that represent pore space or unfocused areas were excluded in the quantification of percent cell coverage. This process was performed for illuminated green cells and subsequently for illuminated red cells.

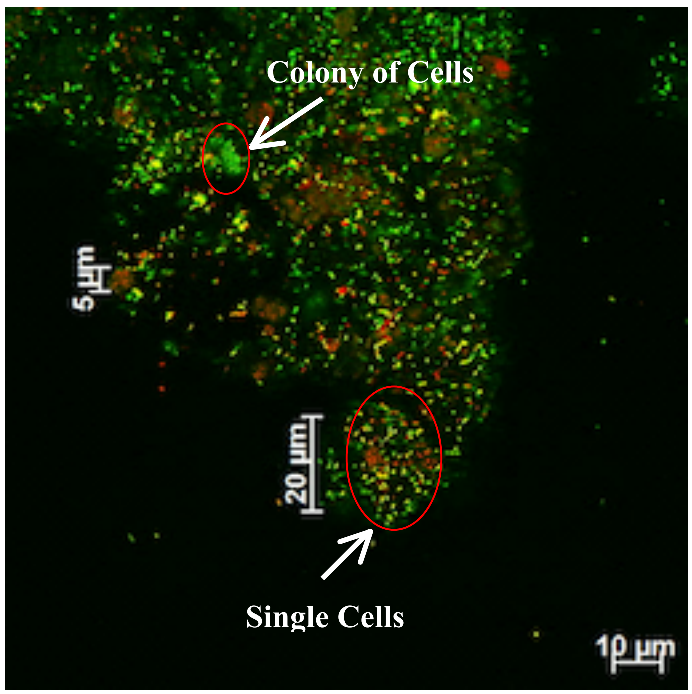

Figure 3.2: Identification of clustered cells and single cells on a CLSM image

3.7. CHARACTERIZATION OF MICROBIAL POPULATIONS

Microbial populations of the MBBR biofilm were characterized using a protocol in which the DNA is extracted and then amplified for analysis. DNA was extracted with two mechanical lysis cycles in a FastPrep® Instrument (MP Biomedicals) set at speed 6.0 for 40 seconds using a FastDNA® Spin Kit (MP Biomedicals) and following the manufacturer's instructions. Extracted DNA was then stored at -20^{o}C until it was used for library construction.

The extracted DNA was amplified through a two-step polymerase chain reaction (PCR). The PCR primers and protocol was based upon Sundquist et al. (2007) was used to allow for a unique barcode combination for each sample. The extracted DNA was then inspected on a 2% agarose gel and purified using a Montage PCR96 Cleanup Kit (Millipore). The purified amplicons (amplified pieces of DNA) concentration was quantified using a Quant-iT™ dsDNA BR Assay Kit (Invitrogen). 50 ng of each amplicon was pooled and then sequenced at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto.

Upon receiving sequence reads of the amplicons from the TCAG, quality filtering of the merged reads was then performed using the Galaxy tool (Goecks et al., 2010) in which only sequences with an exact match to the amplification primers were kept for analysis. The average reads per sample was 901 070 ± 331 232.

Clustering the reads at 97% sequence similarity resulted in an average operational taxonomic units (OTUs) number of 1289 ± 60.64 per sample. OTUs clustering was performed at 97% sequence similarity to bacterial taxa assignment against the Greengenes database (released February 4, 2011) and relative abundance of assigned bacterial taxa was then computed using the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010).

3.8. STATISTICAL ANALYSIS

Due to the small size of the laboratory MBBR (total volume of 2 L) and the limited amount of active K3 carriers in each reactor (40% media fill), the amount of carriers used for biofilm thickness and morphology, and biomass viability was designated systematically to prevent a significant decrease in media fill percentage. Although only one carrier per reactor was used for each VPSEM and CLSM imaging during each experimental phase, the microscopic images captured represent a very small section of the carrier and thus examining various random sections on the carrier allowed for a statistically viable analysis. Furthermore, all carriers were inoculated under the same conditions and again operated under the same conditions in their corresponding reactors. Therefore, this work employs the assumption that a single carrier in R1 is believed to be representative of all carriers in R1, and likewise, a single carrier in R2 is representative of all carriers in R2. T-tests and p-values (0.05, signifying statistical relevance) were used to compare statistically significant differences in values between the two reactors and between experiments at 20^oC, 1^oC and 5^oC. A combined error was used for errors that include more than one variable (Appendex A).

For the sequencing analysis, only a composite sample of the biofilm from each reactor, under steady state operating conditions, at 20^oC and 1^oC was analyzed due to complexities of the method and time limitations. As such, statistically relevant changes are not possible to be observed. Future recommendations will include the analysis of replicates for sequencing analysis.

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CHAPTER 4: MBBR NITRIFICATION DURING LONG-TERM EXPOSURE TO COLD TEMPERATURES

4.1. SETTING THE CONTEXT

The article presented in Chapter 4 is entitled *MBBR nitrification during long-term exposure to cold temperatures* by V. Hoang, R. Delatolla, A. Gadbois, and E. Laflamme. This article has been accepted for publication in Water Environment Research (WER). This paper describes the nitrification relative kinetic rates of a laboratory scale MBBR treatment system operating at $1^{o}C$ for approximately four months to replicate conditions at a wastewater treatment facility in Northern Canada. This chapter addresses the objectives of quantifying the relative kinetic rates of nitrification at $20^{o}C$ and $1^{o}C$, identifying the kinetic threshold, investigating the correlation of the temperature correction coefficient, and characterizing the biologically produced solids of the MBBR system.

4.2. INTRODUCTION

Ammonia is a deleterious constituent released from wastewater treatment plants (WWTPs) and is responsible for acute and chronic toxicities in lakes and rivers where treatment is not applied (Chambers et al., 1997). Ammonia removal in conventional northern WWTPs is currently at risk of being limited or non-existent in winter months due to severely cold temperatures. Therefore, ammonia discharge in wastewater effluent has recently been regulated by northern countries such as Canada to reduce risk to fisheries resources, ecosystem health, and human health (Canada Gazette, 2012). Numerous studies and practical experience have identified the effect of low temperatures on nitrifying bacteria as the cause for loss of nitrification (Sharma and Ahler, 1976; Painter and Loveless, 1983; Zhu and Chen, 2002; Salvetti et al., 2006). Attached growth processes and specifically the moving bed biofilm reactor (MBBR) have shown promise in achieving short-term ammonia removal at cold temperatures below $4^{o}C$ (Andreottola et al., 2000; Wessman and Johnson, 2006; Delatolla et al., 2011).

MBBR technologies have become an attractive process compared to conventional treatment processes. MBBRs are compact units that utilize the whole tank volume and offer the advantage of no sludge recycling and no backwashing (Ødegaard, 2006). The MBBR process uses biocarriers, which vary in size and shape, to support and maintain the growth of active biofilm within the treatment systems. An aeration system is incorporated in the system to keep the carriers in constant suspension and movement to provide the attached biofilm with adequate oxygen and access to the bulk liquid substrate. The active biofilm grows on the inner portion of the carrier where it is protected, to a certain degree, from external collisions and forces. The operating and maintenance costs associated with MBBR technologies are generally low and MBBRs are capable of producing an effluent with total ammonia-nitrogen concentration lower than 5 mg/L at various flow rates and under conventional treatment temperatures (Env. Canada, 2003).

With over 1000 treatment lagoons in operation, this treatment system represents the majority of wastewater treatment facilities currently operating in Canada (Env. Canada, 2003). The performance of nitrifying MBBR upgrade systems in northern countries has been demonstrated at a selection of operating lagoons with the MBBR unit being installed to treat wastewater exiting the first pond of multiple treatment lagoon ponds, where the temperature drop is limited (Wessman and Johnson, 2006; Houweling et al., 2007; Delatolla et al., 2011). However, the optimum carbon/nitrogen (C/N) ratio for nitrification exists at the end of the last pond, where the temperature may drop to 1ºC during the winter months. The nitrification kinetics of the MBBR system over long exposure periods to a temperature of 1ºC has yet to be investigated and quantified.

The installation of an upgrade system at the end of functioning lagoons where the C/N ratio is low will eliminate the redundant carbon removal that occurs in upgrade nitrifying systems applied after the first pond. As such, post solids separation after an upgrade nitrifying MBBR installed following the last pond is an important consideration; biological solids generated by the MBBR unit may compromise the effluent TSS concentration with respect to effluent solids regulations. The objectives of this research are to quantify the nitrification rate of MBBR nitrification at 20^{o}C and at 1^{o}C for a time period representative of a northern winter, identify the kinetic threshold temperature, quantify biofilm detachment of the MBBR system at 20^{o}C and 1^{o}C, and investigate the correlation of a temperature correction factor to predict the kinetics of a nitrifying MBBR system operating at 1^{o}C for an extended period of time.

4.3. METHODS

4.3.1. Reactors

The experimental design of this study includes two laboratory MBBRs operated under continuous conditions at 20ºC for six weeks, at 1ºC for four months, the extent of an average Canadian winter, and at 5ºC (kinetic threshold temperature) for two weeks. Figure 4.1 shows a schematic of the laboratory reactors. A temperature-controlled chamber was used to house the reactors during experiments below room temperature.

Synthetic wastewater (SWW) and K3 Kaldnes carriers were used for this study. The K3 carriers are small cylindrical shaped high-density polyethene with an inside crosspiece and outside longitude fins. These carriers have a surface area available for biofilm attachment of 500 m^2/m^3 (Rusten et al., 2006). The K3 Kaldnes carriers occupied approximately 40% by volume of both 2-litre completely mixed reactors, reactor 1 (R1) and reactor 2 (R2), respectively. This fill percentage was below the recommended maximum fill percentage of 70% (Rusten et al., 2006). All carriers were inoculated in an MBBR nitrifying pilot plant that was operated at the Vaudreuil, Quebec, Canada wastewater treatment plant.

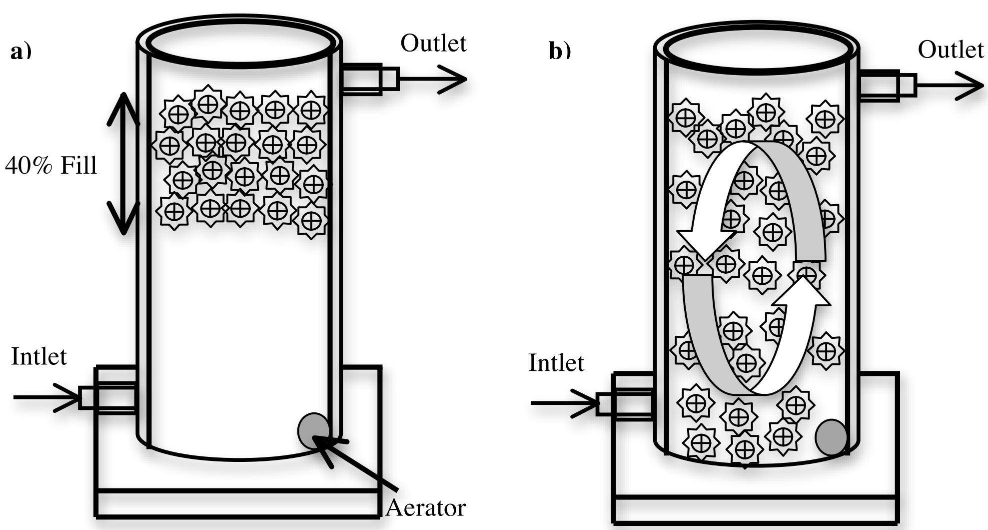


Figure 4.1: Schematic of MBBR reactors showing a) carrier percent fill and b) aerated reactor in operation

4.3.2. Experiments

Synthetic wastewater was prepared according to the following recipe: $(NH_4)_2SO_4$ (104 mg/L), $NaHCO_3$ (286 mg/L), $FeSO_4{\cdot}7H_2O$ (5 mg/L), KH_2PO_4 (80 mg/L),

$CaCl_2 \cdot 2H_2O$ (29 mg/L), and $MgSO_4 \cdot 7H_2O$ (71 mg/L). Trace nutrients included molybdenum, copper, cobalt and zinc. No organic carbon sources were added in order to investigate attached growth kinetics of nitrification in MBBR systems with minimal interferences due to heterotrophic growth.

Both reactors were first operated at 20^{o}C for six weeks, where steady state conditions were observed and then acclimatized over a four week period to 1^{o}C and maintained at 1^{o}C for four months. Following the 1^{o}C experiments, the temperatures of both reactors was increased to 5^{o}C for a period of two weeks, where steady state conditions were again observed.

The reactors were operated under the same conditions of temperature, pH, and dissolved oxygen (DO) throughout the entire experimental phase at all temperatures. The DO in both reactors was maintained between 7 and 8 mg-O_2/L and the pH was maintained between 7 and 8. High aeration was provided to both reactors to maintain sufficient movement of the carriers within the reactors and to prevent denitrification. The reactors were operated at a hydraulic retention time (HRT) of 4 hours during the experiments at 20^{o}C and the first month of the experiments at 1^{o}C. Subsequently, the HRT of the two reactors were increased to 6 hours for the remainder of the experiments at 1^{o}C. The HRT was adjusted to conserve SWW during lower kinetic rates while maintaining conventional HRT values between 2 and 6 hours. Nitrogen mass balances at 20^{o}C, 1^{o}C and 5^{o}C were calculated to validate that nitrification was the pathway of ammonia removal. Mass balances were quantified at 1^{o}C numerous times over the four month exposure period to ensure nitrification, with mass balances validated as soon as both reactors reached steady state conditions (at day 40) and then again at day 69, day 136, and day 160 during the experimental phase.

4.3.3. Analysis

Ammonia (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations of the reactors were measured using standard methods (APHA, 1995). Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations of the effluent from both reactors were also measured using standard methods (APHA, 1995).

4.4. RESULTS

4.4.1. Nitrification Kinetics

MBBR ammonia removal kinetics at cold temperatures is calculated relative to the surface area of the carriers. Furthermore, in order to provide a direct comparison to the 20^{o}C rates of removal, the ammonia removal kinetics of R1 and R2 are presented relative to the removal rates at 20^{o}C during the 39-day acclimatization period from 20^{o}C to 1^{o}C and throughout the 121-day exposure period to 1^{o}C (Figure 4.2).

The average removal rate at 20^{o}C (day zero) in both reactors was comparable to those reported in the literature (Hem et al., 1994; Rusten et al., 1995; Andreottola et al., 2000) and did not show any significant changes during the acclimatization stage. In fact, the removal rates increased slightly as compared to the removal rate measured at 20^{o}C within the first 37 days of acclimatization. A sharp decrease in ammonia removal was then observed once the temperature in both reactors was below 5^{o}C (after day 37). The ammonia removal rates at 5°C were thus measured following the 1°C experimental phase to validate this potential kinetic threshold. Table 4.1 shows the ammonia removal rates of both reactors at 5°C and 1°C relative to the removal rates at 20°C. A significant decrease in nitrification rates is observed at temperatures above and equal to 5°C and between 5°C and 1°C; thus indicating that 5°C is a kinetic threshold for nitrification at cold temperature in MBBR systems.

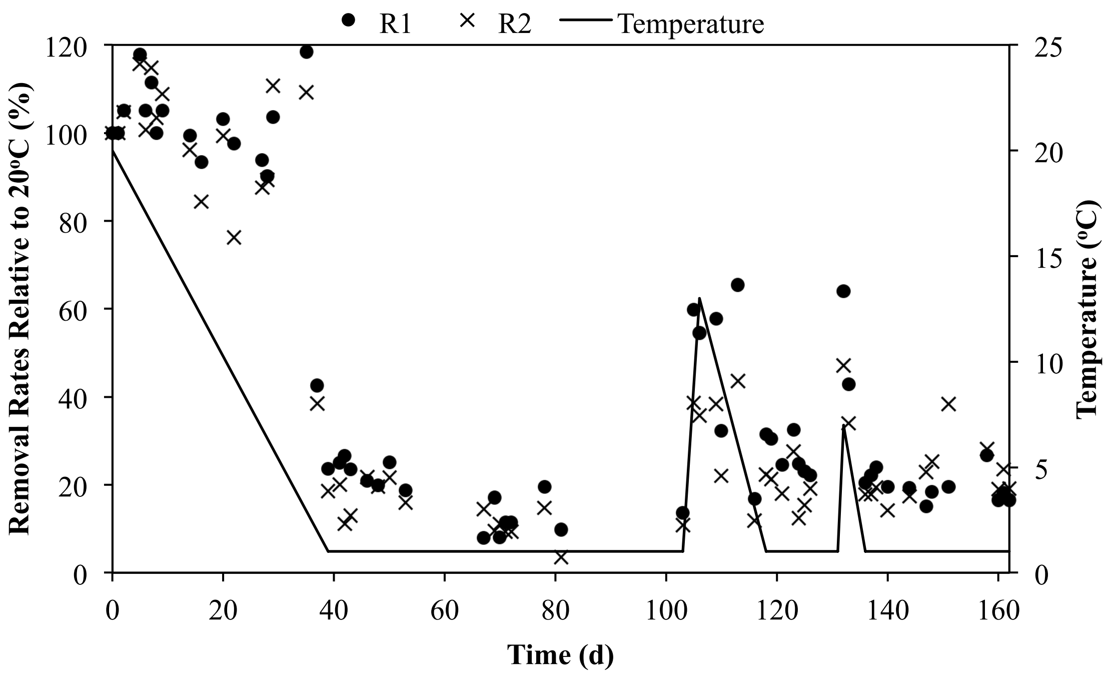


Figure 4.2: Ammonia removal rate of R1 and R2 relative to 20°C during acclimatization and exposure to 1°C

Compared to the removal rate at 20°C, the average ammonia removal rate at 1°C over 121 days of exposure were measured to be 18.7 ± 5.5% and 15.7 ± 4.7%, for reactors R1 and R2, respectively. These results demonstrate that even after a long exposure period to 1°C, MBBR systems loaded with SWW and no organic carbon are able to maintain moderate ammonia removal rates. Thus, the findings support the hypothesis that MBBRs are a promising nitrifying upgrade technology for ammonia treatment in northern countries under very cold temperatures.

Table 4.1: Rate of ammonia removal of R1 and R2 at 5°C and after 121 days of exposure to 1°C relative to rate of removal at 20°C (n = 15)

Temperature (°C)	**Rate of Ammonia Removal Relative to 20°C (%)**	
	R1	**R2**
5	66.2 ± 3.9	64.4 ± 3.7
1	18.7 ± 5.5	15.7 ± 4.7

4.4.2. Biological Solids Detachment

The effluent of both reactors was collected for analysis to compare TSS concentrations at 20°C and after 121 days at 1°C. SWW without particulate matter was used to feed both reactors; hence the TSS measured in the effluent was directly produced by biofilm detachment from the carriers and subsequently the TSS production in this study is considered as the biofilm detachment rate. The following equation was used to calculate the detachment rate:

$$\text{Detachment Rate} = TSS \cdot Q / (V_b \cdot a) \qquad 4.1$$

where TSS (g/L) is the effluent TSS concentration, Q (L/d) is flow rate in and out of the reactor, V_b (m^3) media bed volume, and a (m^2/m^3) is the specific surface area of the biocarrier. The average effluent TSS concentrations for the two reactors were found to be 2.2 mg/L at 20°C and 3.0 mg/L at 1°C, where both reactors exhibited very similar effluent TSS concentrations. The average detachment rates of the two reactors were calculated to be 0.044 ± 0.027 g-TSS/m^{2}d and 0.059 ± 0.019 g-TSS/m^{2}d at 20°C and 121 days at 1°C, again where the detachment rates in the two reactors were similar. A slightly higher, but not statistically significant, TSS concentration and detachment rate was observed at 1°C even though the observed mass of biofilm on the carriers appeared similar at 20°C and 1°C.

4.5. DISCUSSION

4.5.1. Low Temperature Effects on Nitrification Kinetics

Although the removal rate at 1°C in this study could not be compared to those in the literature due to lack of available information, nitrification rates measured at 4°C during the acclimatization phase were in accordance with those found in a past study (Delatolla et al, 2011). The slight discrepancy between the ammonia removal rates of R1 and R2 observed in Figure 4.2 is statistically insignificant and most likely due to natural variances inherent to the systems and the analytical methods.

Although both reactors were consistently maintained at 1°C for the first two months of operation, two short temperature spikes occurred during the experimental phase due to malfunctions of the temperature-controlled chamber (Figure 4.2). The removal rates for both reactors rapidly increased as the temperature increased from 1°C to 13°C on day 106 and from 1°C to 7°C on day 132. During the first temperature spike, from 1°C to 13°C, the ammonia removal rates increased by 340% and 244% for R1 and R2, respectively. To prevent additional shock to the system, the cold room temperature was decreased steadily over a two week time period to slowly re-acclimatize the systems back to low temperatures. The second spike, from 1°C to 7°C, showed an ammonia removal rate increase of 149% and 152% for R1 and R2, respectively. The reactors were decreased back to 1^{o}C in two days. With a rapid temperature decrease from 7^{o}C to 1^{o}C, the removal rates decreased approximately 66% in R1 and 63% in R2.

Following these shock temperature changes, both reactors demonstrated the ability to quickly recover and perform consistently for the last month of operation at 1^{o}C. Delatolla et al. (2009) performed temperature shock experiments using a biological aerated filtration (BAF) system for ammonia removal and observed increases of 200% to 300% in removal rate when the temperature was rapidly increased from 4^{o}C to 20^{o}C and a decrease of 50% in removal rate when the temperature was rapidly decreased from 20^{o}C to 4^{o}C. Although the temperature change of the reactors in the current study did not reach 20^{o}C during the shock events, a similar response of the MBBR systems was observed as compared to the BAF systems investigated in Delatolla et al. Thus it appears that nitrifying biofilm systems, regardless of reactor design, are capable of quickly adjusting ammonia removal rates to temperature changes without losing the ability to nitrify.

Nitrogen mass balances based on the removed NH_4^+-N and produced NO_2^--N and NO_3^--N were used to monitor the pathway of nitritification (oxidation of ammonia to nitrite) and nitratification (oxidation of nitrite to nitrate). Nitrite concentrations in both reactors during the first two months of experiments at 1^{o}C did not increase above 0.5 mg-N/L. During the last month of experiments at 1^{o}C, after shock temperature events had passed, nitrite concentrations in R1 again did not increase above 0.5 mg-N/L, whereas a slight increase to 1.1 mg-N/L was observed in R2 and remained consistent until the end of the experiments at 1^{o}C. This indicates that nitratification proceeded to approximate completion. However, during the shock temperature events when the temperature was rapidly increased, the nitrite concentration increased to maximum values of 2.0 mg-N/L and 4.9 mg-N/L in R1 and 2.5 mg-N/L and 4.4 mg-N/L in R2 during temperature spikes to 13^{o}C and 7^{o}C, respectively. These nitrite concentrations subsided in approximately three days following the sudden increase in temperature; indicating that the rate of nitratification cannot respond as quickly as the rate of nitritification during a temperature shock event. These findings are supported by the results of Delatolla et al. (2009) who also observed increased nitrite concentrations during shock temperature events in BAF treatment systems.

Since nitrite did not accumulate during the acclimatization phase, where temperature was decreased slowly in the reactors, a slow temperature change enables the slower rate of nitratification to adjust to the temperature change in accordance with the faster rate of nitritification. It should also be noted that both reactors showed higher nitrite concentrations during the second temperature spike even though the first temperature spike had a higher increase in temperature. This suggests that the nitrite-oxidizers may be more sensitive to successive shock temperature changes. Nitrogen mass balances of removed NH_4^+-N, and produced NO_2^--N and NO_3^--N were within the range of +/- 10%. NH_4^+-N removed was slightly higher than NO_2^--N and NO_3^--N produced, which is expected since NH_4^+-N will be preferentially used as a source of nitrogen for cell synthesis (Rusten et al., 1995).

The sharp decrease in ammonia removal rates at 1^{o}C observed in this study could be due to a significant loss or death of cells in the biofilm caused by exposure to cold temperatures. However, the immediate increase in kinetics during the temperature spikes indicates that this may not be the case. The attached nitrifying bacteria demonstrate the ability to adjust and perform at high removal rates quickly following sudden increases in temperature. Taking into account that the minimum generation time of nitrifiers at 30°C (optimal temperature) is approximately 15 hours and the generation time at 5°C is approximately 200 hours (Wijffels et al., 1991), these findings suggest that the nitrifying bacteria simply exist at a higher activity level at higher temperatures and at a lower activity level as temperature decreases. However, this inference requires additional insight into the survival of the organisms embedded in the biofilm to be validated; work that will be addressed in Chapter 5 of this manuscript.

4.5.2. Low Temperature Effects on Detached Biological Solids

Full-scale nitrifying MBBR systems have low HRTs and are not designed to promote hydrolysis, thus a significant portion of the TSS that enter these systems will exit in the effluent. The effluent TSS concentrations measured in this study indicate that the detached TSS from the nitrifying carriers will increase the effluent TSS concentration by less than 4 mg/L. Although the study was performed at a C/N of approximately zero, the very low TSS concentrations measured support the promise of applying an MBBR system at the end of the last pond of a lagoon treatment system without additional solids separation.

4.5.3. Temperature Correction Coefficient Analysis

Nitrifying bacteria have been known to be extremely sensitive to low temperatures, where nitrification rates have been reported to increase between 12 and 50% with a 4ºC increase and decrease between 8 and 30% with a 1°C drop when compared to nitrification rates at 21.3°C (Barritt, 1933; Srna and Baggaley, 1975). A temperature correction factor is commonly used to express the effects of temperature on nitrification growth rates (Metcalf and Eddy, 2003):

$$\mu_T = \mu_{max,20°C}\theta^{T\text{-}20°C} \tag{4.2}$$

where θ is the temperature correction factor, μ_T is the specific growth rate at the temperature of interest (time^{-1}), $\mu_{max,20°C}$ is the maximum specific growth rate at 20°C (time^{-1}) and T is the temperature of interest (°C). Numerous studies over the years on suspended growth nitrifiers have shown that the temperature correction factor can range from 1.076 to 1.165 (Downing and Hopwood, 1964; Painter and Loveless, 1983; Oleszkiewicz and Berquist, 1988). Furthermore, an average value of 1.098 and 1.058 was found (Salvetti et al., 2006) for ammonia limiting conditions and oxygen limiting conditions, respectively. Although this range of values is seen in the literature, an Arrhenius temperature correction factor of 1.072 has been reported and commonly used for WWTP design (WERF, 2003). θ values have also shown to differ between suspended and attached growth processes (Aravinthan et al., 1998), with a θ value of 1.09 being suggested for MBBR systems (Rusten et al., 1995). The ammonia removal rate for attached growth treatment MBBR systems is expressed using the temperature correction factor as follows:

$$SARR_{A2} = SARR_{A1}\theta^{(T2\text{-}T1)} \tag{4.3}$$

where $SARR_{A1}$ and $SARR_{A2}$ are surface area ammonia removal rates (g NH_4^+-N/m^2·d) at temperatures $T1$ and $T2$ (°C), respectively. Equation 4.3 assumes that the nitrification reaction rate is zero order, which is often the case at ammonia concentrations equal to or above 3 – 5 mg/L (USEPA, 1993; Hem et al.,1994). A large variation and lack of consistent θ values in the literature is a concern when designing treatment systems operating at various conditions. Factors that can greatly affect the temperature correction factor include whether the nitrifiers are planktonic or sessile, an acclimatization temperature change compared to a shock temperature change to the system as well as the duration at which the system is exposed to very low temperatures. Compared to a gradual decrease in temperature using a θ value of 1.072, Hwang and Oleszkiewicz (2007) demonstrated that a rapid decrease in temperature increased the θ value to 1.116, resulting in a much smaller nitrification rate as predicted with a θ value of 1.072. Furthermore, exposure time to temperatures below 5°C following the acclimatization period has shown to result in increasing θ values as the exposure time progresses (Delatolla et al., 2009).

Delatolla et al. (2009) showed that θ values for an attached growth BAF system exposed to 4°C ranged between 1.002 at initial exposure to cold conditions and increased to 1.216 after 115 days of exposure. Thus the following equation was formulated to correlate to the change in kinetics with exposure time to cold conditions:

$$\theta = 3.81\times10^{-2} \ln(t) + 9.83\times10^{-1} \qquad 4.4$$

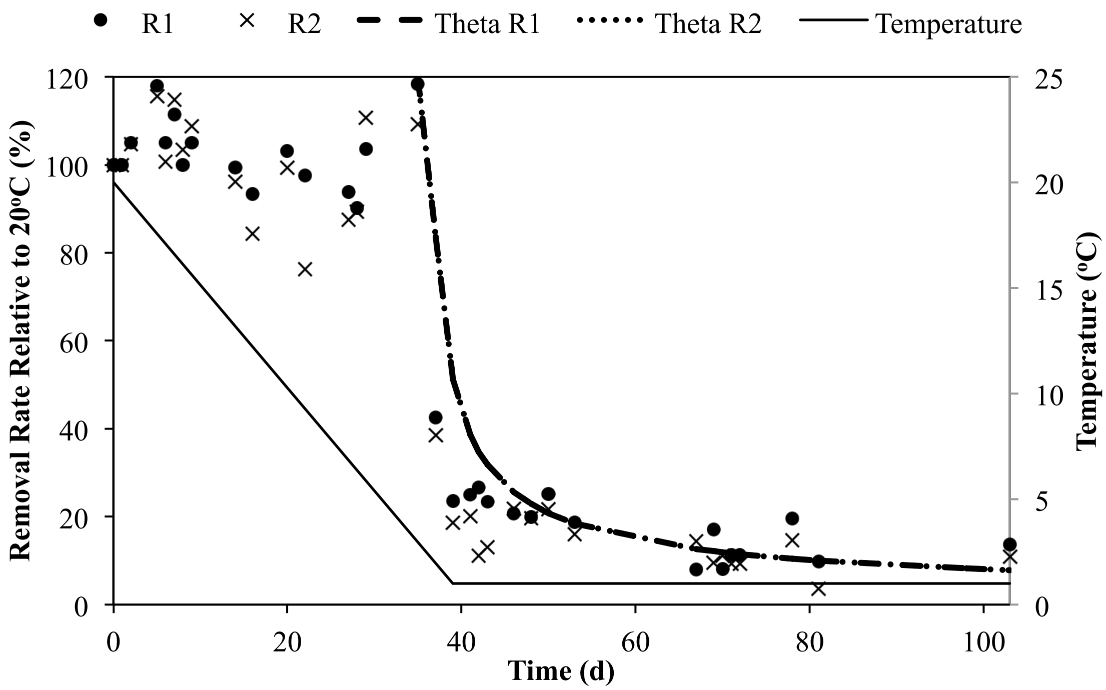
where t is the time exposed (d) to 4°C. The Delatolla et al. θ model was evaluated in this study for measured ammonia removal rates at 1°C during 121 days of exposure time relative to rates at 20°C. This model, which takes into consideration the low temperature exposure time, provided a strong correlation to the ammonia removal rates of the MBBR reactors and thus was evaluated as part of this study.

The ammonia removal rate at 1°C predicted with Equation 4.3, using a temperature correction factor of 1.072, was calculated to be much larger than the measured removal rates. The removal rate at 1°C relative to 20°C was predicted to be approximately 26%, while an average removal rate for both reactors of 17.2 ± 5.1% was measured.

Another approach to predicting removal rates is taking into account the exposure time in which the system is exposed to cold temperatures. The Delatolla et al. θ model demonstrates changing θ values as the time of exposure changes (Equation 4.4). As such, the Delatolla et al. θ model was applied to Equations 4.4 to predict θ values and then applied to Equation 4.3 to predict the corresponding relative removal rates. The results demonstrate a strong correlation where the R^2 values for the Delatolla et al. θ model following the acclimatization period were 0.86 for R1 and 0.84 for R2 while the R^2 values decreased to 0.68 and 0.82 for R1 and 0.63 and 0.41 for R2 during the first and second temperature shock events, respectively (Table 4.2).

The relative removal rates predicted using θ values calculated with Equation 4.4 were very similar for R1 and R2 before the temperature spikes (day 35 to day 103); in fact, the two models overlap one another (Figure 4.3a). However, the relative removal rates of the two reactors show a slightly larger variance after the two temperature shock events (day 103 to day 162), where the relative removal rates for R1 was slightly higher than that of R2 (Figure 4.3b). As such, the Delatolla et al. model performed best after an acclimatization period as compared to shock temperature changes.

a)



b)

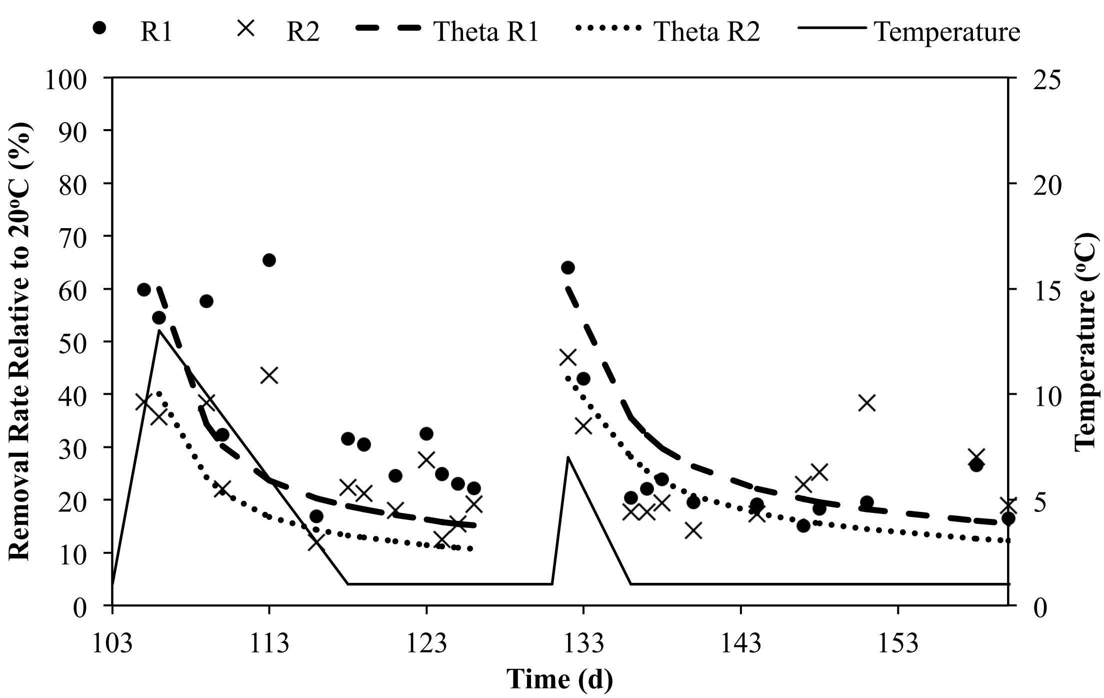


Figure 4.3: a) Delatolla et al. θ model applied to removal rates of R1 and R2: a) before temperature spikes b) after temperature spikes

Table 4.2: R^2 values of Delatolla et al. θ model correlation to experimental ammonia removal rates of R1 and R2 (n = 15)

Experimental Phase	Time (d)	R1	R2
Before temperature spike	35 - 103	0.86	0.84
After first temperature spike	103 - 126	0.68	0.63
After second temperature spike	132 - 162	0.82	0.41

4.6. CONCLUSION

The results of this study reveal that MBBRs are a promising technology for long-term ammonia removal at cold temperatures. A kinetic rate threshold temperature was observed at 5°C, which ammonia removal rates decrease rapidly. Consistent removal rates were observed for two months after exposure to 1°C. Despite temperatures spikes during the exposure to 1°C, the system was able to recover and perform consistently for the remainder of the experimental phase, with nitrite accumulations in the reactors subsiding within a few days. The removal rate at 1°C was 18.7 ± 5.5% and 15.7 ± 4.7%, for reactors R1 and R2, respectively as compared to the removal rate at 20°C. Effluent TSS concentrations measured were used to calculate biofilm detachment rates and were determined to be very low at both 20°C and after 121 days at 1°C. Therefore, this study shows the promise of installing a nitrifying MBBR upgrade system at the end of a treatment train without additional solids separation. With respect to predicting ammonia removal rates based on a temperature correction factor, the exposure time in which the system is exposed to cold temperatures should be taken into consideration. The traditional θ value resulted in high predicted rates and as such, the Delatolla et al. (2009) θ model was investigated and showed a strong correlation to the measured relative ammonia removal rates throughout various stages of the experiments where the θ value changes with exposure time. Furthermore, it should be noted that the Delatolla et al. θ model showed a stronger correlation relative rates of removal after an acclimatization period as compared to after rapid temperature changes.

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CHAPTER 5: MBBR NITRIFYING BIOFILM AND BIOMASS RESPONSE TO LONG TERM EXPOSURE TO 1^{o}C

5.1. SETTING THE CONTEXT

The article presented in Chapter 5 is entitled *MBBR nitrifying biofilm and biomass response to long-term exposure to 1^{o}C* by V. Hoang, R. Delatolla, T. Abujamel, W. Mottawea, A. Stinzi, A. Gadbois, and E. Laflamme. This article has been prepared for the submission to Environmental Science & Technology. This paper uses a combination of two microscopic techniques to examine the biofilm morphology and biomass viability as well as investigate the microbial population shifts of a laboratory scale MBBR treatment systems operating at 1^{o}C for four months. This chapter addresses the objectives of investigating biofilm thickness and morphology, quantifying the biomass live and dead cell coverage at cold temperatures, characterizing the viability of the nitrifying organisms, and investigating population shifts in the AOB and NOB communities during long-term exposure to 1^{o}C.

5.2. INTRODUCTION

Ammonia discharged from wastewater effluent is responsible for the highest rate of acute and chronic toxicity in Canadian waters (Chambers et al., 1997) and therefore has recently been regulated by the Federal Government of Canada (Canada Gazette, 2012). Although biological treatment is the most economical means of ammonia removal (Metcalf and Eddy, 2003), nitrification in cold climates can become extensively limited or non-existent due to the temperature sensitivity of nitrifiers. Previous studies and practical experiences have shown a sharp decrease in nitrification rates at low temperatures (Sharma and Ahler, 1976; Painter and Loveless, 1983; Zhu and Chen, 2002; Salvetti et al., 2006). However, attached growth processes and specifically the moving bed biofilm reactor (MBBR) have shown promise for low temperature nitrification. MBBR systems have demonstrated success as an upgrade or replacement technology for low temperature ammonia removal (Wessman and Johnson, 2006; Houweling et al., 2007; Delatolla et al., 2011).

Currently there are over 1000 wastewater treatment lagoon systems operating in Canada and due to new federal regulations in regards to ammonia discharge in wastewater effluent, many of these existing treatment lagoons will be upgraded in the near future (Canada Gazette, 2012). Although MBBR upgrade units have demonstrated the ability to perform nitrification at low temperatures, these nitrifying MBBR units have been tested to treat effluent exiting the first pond of multiple lagoon treatment systems where temperature drops are limited and the carbon/nitrogen (C/N) is higher than the subsequent ponds (Delatolla et al., 2011). The optimum carbon/nitrogen (C/N) ratio for nitrification occurss at the last lagoon where temperatures drop to 1^{o}C for extended periods of time. Installing MBBR upgrade units after the last pond will allow the system to benefit from low C/N ratios, which will reduce the size and operational costs of the upgrade unit and prevent carbon removal redundancy in the downstream ponds. Long-term MBBR nitrification at 1^{o}C, however, has yet to be investigated or quantified. Beyond quantifying the nitrification kinetics of MBBR units at 1^{o}C, an investigation of the nitrifying biofilm and biomass response to long-term cold temperature exposure is necessary to evaluate MBBRs as a feasible nitrifying upgrade technology for cold climate lagoons and moreover, build a fundamental understanding of the ability of wastewater biofilms to nitrify at cold temperatures.

Autotrophic bacteria responsible for nitrification were not believed to experience a population shift as temperatures decrease since the same bacterial population was believed to be responsible for nitrification at warm and cold temperatures (Wijffels et al., 1995). More recently however, it has been observed that the ammonia oxidizing bacteria (AOB) populations, which co-exist in wastewater, demonstrate shifts in their community populations with a change in temperature (Hallin et al., 2005; Layton et al., 2005; Siripong and Rittmann, 2007). Nitrite oxidizing bacteria (NOB), however, have not been shown to experience a population shift during temporal changes but rather during substrate scarcity or temporary elevations (Gieseke et al., 2003; Haseborg et al., 2010; Huang et al., 2010). *Nitrosomonas* are considered to be the dominant AOB population in wastewaters and are believed to co-exist with *Nitrosospira* (Park et al., 2008; Ducey et al., 2009; Rodriguez-Caballero et al., 2012) and two common genera of NOB in wastewater are *Nitrobacter* and *Nitrospira* (Wagner et al., 1996; Daims et al., 2001; Siripong and Rittmann, 2007).

Thus, this work will investigate the response of the MBBR nitrifying biofilm, the embedded biomass and population shifts of the embedded bacterial community as treatment systems are exposed to cold temperatures. Particularly, this study aims to characterize the effects of long-term exposure to 1^{o}C on the thickness and morphology of MBBR nitrifying biofilms, the viability of cells in the biofilm and the bacterial community shifts. This study utilized a variable pressure scanning election microscope (VPSEM) and a confocal laser scanning microscope (CLSM) with protocols that minimize the destructive effects on the MBBR biofilm and DNA sequencing to monitor effects on the bacterial community. 16S rRNA gene analysis was used to classify the AOB and NOB communities of the MBBR system at 20^{o}C and after four months exposure to 1^{o}C. Furthermore, the ammonia removal rate with respect to the total cell coverage and biofilm volume throughout long-term exposure to 1^{o}C was compared to traditional biocarrier surface area rates of removal.

5.3. METHODS & MATERIALS

5.3.1. Biofilm Samples

The nitrifying biofilm analyzed in this study was taken from two laboratory scale MBBRs operating under continuous conditions with a hydraulic retention time (HRT) of 4 hours during 20^{o}C experiments and during the first month of operation at 1^{o}C. The HRT was subsequently increased to 6 hours for the remainder of experiments at 1^{o}C to conserve the feed while maintaining a traditional HRT during lower kinetics rates. The pH of both reactors was maintained between 7 and 8 and the dissolved oxygen (DO) concentration was maintained between 7 and 8 mg-O_2/L. Both reactors were operated at 20^{o}C for six weeks and then acclimatized to 1^{o}C and operated at 1^{o}C for four months. Synthetic wastewater (SWW) was used to provide the nitrifying bacteria with nutrients and substrate. The SWW was prepared according to the following recipe: $(NH_4)_2SO_4$ (104 mg/L), $NaHCO_3$ (286 mg/L), $FeSO_4 \cdot 7H_2O$ (5 mg/L), KH_2PO_4 (80 mg/L), $CaCl_2 \cdot 2H_2O$ (29 mg/L), and $MgSO_4 \cdot 7H_2O$ (71 mg/L). Trace nutrients included molybdenum, copper, cobalt and zinc. No organic carbon sources were added in order to investigate attached growth kinetics of nitrification in the MBBRs with limited effects due to heterotrophic growth.

K3 AnoxKaldnes carriers were used as substratum for biofilm growth and all carriers were inoculated in an MBBR nitrifying pilot plant that was operated at the Vaudreuil, QC, Canada wastewater treatment plant. K3 carriers have a specific surface area of 500 m^2/m^3 (Rusten et al., 2006) and the carrier percent fill of both reactors was 40% throughout the study. The biofilm in reactor 1 (R1) was slightly more developed than reactor 2 (R2) at the start of the study due to difference in operation times in the inoculation reactors. R1 was operated for approximately six months longer than R2 at the start of the study. The biofilm in R1 was thus considered more mature as the biofilm was already developed at the start of the experimental phase as compared to R2, which was slightly less developed at the start of the study and thus developed throughout the study. The biofilm on the R1 carriers at the start of the study was darker in colour and covered more carrier surface area than the R2 carriers (Figure 5.1).

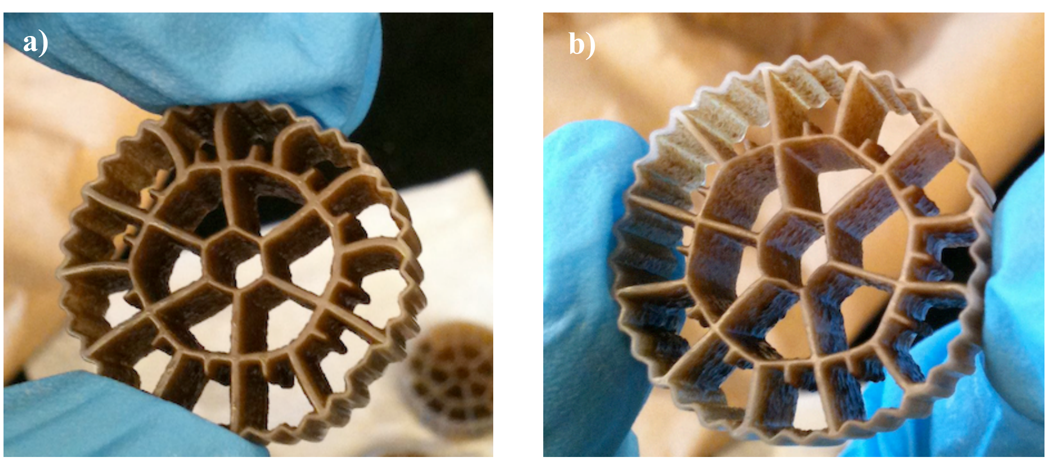


Figure 5.1: Photos of initial biofilm coverage on carriers a) mature biofilm in R1 and b) young biofilm in R2

5.3.2. Nitrification Kinetics

Nitrification rates were measured at 20^oC, once both reactors reached 1^oC and again after one month, three months, and four months of operation at 1^oC. Ammonia (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations were measured using standard methods (APHA, 1995) throughout the experimental phase. In this study, ammonia removal rates of the laboratory MBBRs are relative to the surface area of the carriers and the average removal rates at 1^oC are represented as the percent removal relative to the removal rate at 20^oC. The nitrogen constituents were measured in triplicate.

5.3.3. VPSEM Image Acquisition & Analysis

VPSEM imaging was used for direct imaging of biofilm specimens without pre-treatment; thus eliminating the destructive effects of traditional scanning electron microscopy (SEM) pre-treatment. Samples were analyzed at a pressure of 40 Pa on a Tescan Vega II-XMU. Images were captured at magnifications between 20× and 2000× and biofilm thicknesses were directly measured using AxioVision LE (Carl Zeiss Microscopy Software). Twenty thickness measurements along with morphological observation images were acquired at random locations on the carrier for each experimental phase.

5.3.4. CLSM Sample Preparation

CLSM imaging was used to capture high-resolution optical images of the biomass at selective depths in the biofilm. The CLSM configuration did not require the biofilm to be detached from the substratum, hence minimizing loss of biomass due to traditional biofilm detachment protocols. The FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen) was used to analyze the viability of the samples and specifically quantify the live and dead cells in the biofilm. This kit provides a two-colour fluorescent assay for bacterial viability. SYTO9 stains all cells and propidium iodide stains cells with a damaged cell membrane. Hence, live cells (green) are labeled as those that contained intact cell membranes and dead cells (red) were those that contained damaged cell membranes.

The K3 carriers were cut at various sections to enable attached biofilm to be visualized using the CLSM. Random locations of the biofilm attached to the carrier were analyzed with the biofilm remaining attached to the substratum. After fluorescent staining, biofilm samples were covered and protected from light for approximately 40 minutes to allow for stain penetration to depths of approximately 100 μm in the biofilm. Biofilm samples were subsequently rinsed prior to microscopy.

5.3.5. CLSM Image Acquisition & Analysis

Fluorescent stained biofilm samples were analyzed using a Zeiss LSM 5 Pascal confocal microscope equipped with an Argon laser (488 nm, 514 nm) and a HeNe laser (543 nm). A 36× magnification oil immersion lens was used to view and capture 1.0 μm thick, 214 × 143 μm optical sections. A total of four stacks of five images, or 20 images total, per reactor was acquired and analyzed during each experimental phase. Stack images were taken at random locations on the biofilm with maximum vertical depths of 80 μm to ensure that stain penetration did not limit the identification of the cells and to capture the upper portion of the biofilm that is most exposed to substrate and nutrients. Full substrate penetration has been observed to be less than 100 μm in wastewater MBBR biofilms (Ødegaard, 2006).

Vision Assistant 7.1 (National Instruments, LabView 8.0) was used to quantify the percent coverage of live and dead cells in the nitrifying biofilm. The colour threshold function in NI Vision Assistant was used to segment the illuminated cells of interest from the rest of the CLSM image. The CLSM images captured slices at various depths in the biofilm and as such, pore spaces often compromised a portion of the acquired image. Pore areas appeared as black areas in the CLSM images and were excluded from cell coverage calculations in the study. The biofilm area of each image was quantified as the portion of the image excluding the pore spaces and the percent coverage of the illuminated cells in the biofilm relative to the biofilm area of each image. This procedure was first performed for the live cells, stained in green, and subsequently for the dead cells, stained in red.

5.3.6. DNA Extraction, Amplification, & Analysis

A composite biofilm sample from each reactor during each experimental phase was used for sequencing analysis. DNA was extracted from the composite sample using a FastDNA® Spin Kit (MP Biomedicals) with a FastPrep® Instrument (MP Biomedicals) set at speed 6.0 for 40 seconds. Extracted DNA was stored at -20^{o}C until it was used for library construction.

DNA amplification was achieved by polymerase chain reaction (PCR) using a Phusion® High-Fidelity PCR Master Mix (Finnzyme). The extracted DNA is then inspected with 2% agarose and purified with a Montage PCR96 Cleanup Kit (Millipore). A Quant-iT™ dsDNA BR Assay Kit (Invitrogen) was then used to quantify the purified amplicons (amplified pieces of DNA). 50 ng of each amplicon was sequenced at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto.

The sequence ends of the amplicons sequenced by the TCAG were merged with pair-end sequences and the Galaxy tool (Goecks et al., 2010) was then used for quality filtering in which only sequences with exact matches to the primers were analyzed. The average reads per sample was 901 070 ± 331 232. Operational taxonomic units (OTUs) clustering at 97% sequence similarity, bacterial taxa assignment against Greengenes database (released February 4, 2011), and relative abundance of assigned bacterial taxa was computed using the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). This analysis was used to simply identify the microbial community present in the biofilm at 20^{o}C and 1^{o}C.

5.3.7. Statistical Analysis

For nitrification kinetics, biofilm morphology, and biomass viability analysis, statistically significant differences in values obtained at 20^{o}C and 1^{o}C and between the two reactors were tested by comparing 95% confidence intervals and confirmed using the t-test and p-values (0.05 signifying statistical relevance). Sequencing analysis was examined using a composite biofilm sample in which statistical differences cannot be compared between samples. A combined error was used to errors that contained more than one variable (Appendix A).

5.4. RESULTS

5.4.1. Nitrification Kinetics

The relative nitrification kinetics of the laboratory MBBRs during long-term exposure to 1°C is expressed as the average removal rates compared to 20°C in this study (Figure 5.2). The average removal rates of the two reactors at 1°C after four months of exposure relative to the removal rates at 20°C were measured to be approximately 17.2 ± 5.1%.

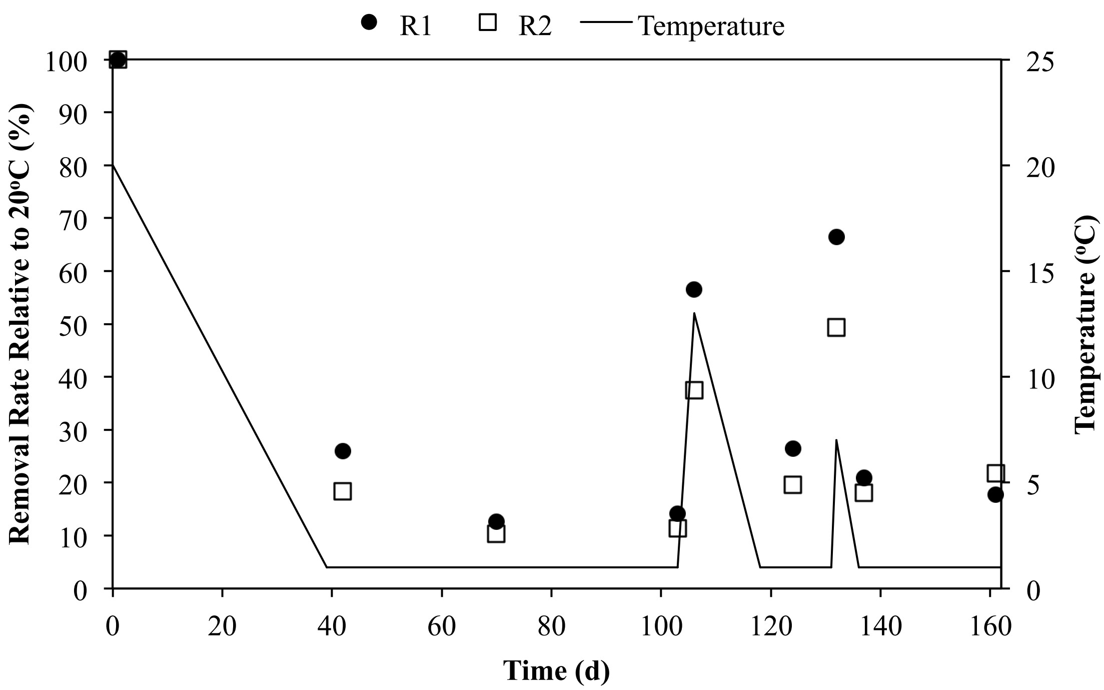


Figure 5.2: Average removal rate of R1 and R2 during long-term 1°C exposure and during rapid temperature spikes

The removal rates in R1 were slightly higher than those in R2 most likely due to natural variances inherent to the systems, errors inherent to the analytical methods, and/or the biofilm in R1 being more mature than the biofilm in R2. Two temperature shocks were observed due to malfunctions of the temperature controlled chamber that housed the

MBBR reactors (Figure 5.2). An increase in removal rate in both reactors was observed with a rapid increase in temperature.

5.4.2. Biofilm Morphology

VPSEM was used to directly measure the thickness of the biofilm and characterize the morphology of the biofilm in this study without pretreatment of the samples (Figure 5.3). Since conventional MBBR design is based upon the assumption that the inner surface area of the carrier supports the attachment of biofilm, only the inner surface of the carriers was investigated for thickness and used to characterize the biofilm in this study. Images acquired at 90× magnification clearly showed attached biofilm along the inner surfaces of the carriers while the biofilm attached to the outer surfaces of the carrier was not visible at this same magnificent due to the very thin nature of the outer carrier biofilm.

The VPSEM images demonstrated that the biofilm at the corners and ridges of the inner surface of the carriers, where the biofilm was best protected from higher intensities of abrasion and erosion, was thicker than the biofilm along the straight inner surfaces of the carrier (Figure 5.3). The nitrifying biofilm was thus not uniformly thick and nor was the surface of the biofilm smooth. Throughout the experiments at 20^{o}C and the long exposure to 1^{o}C, the morphology of the biofilm qualified from the VPSEM images did not display any notable changes.

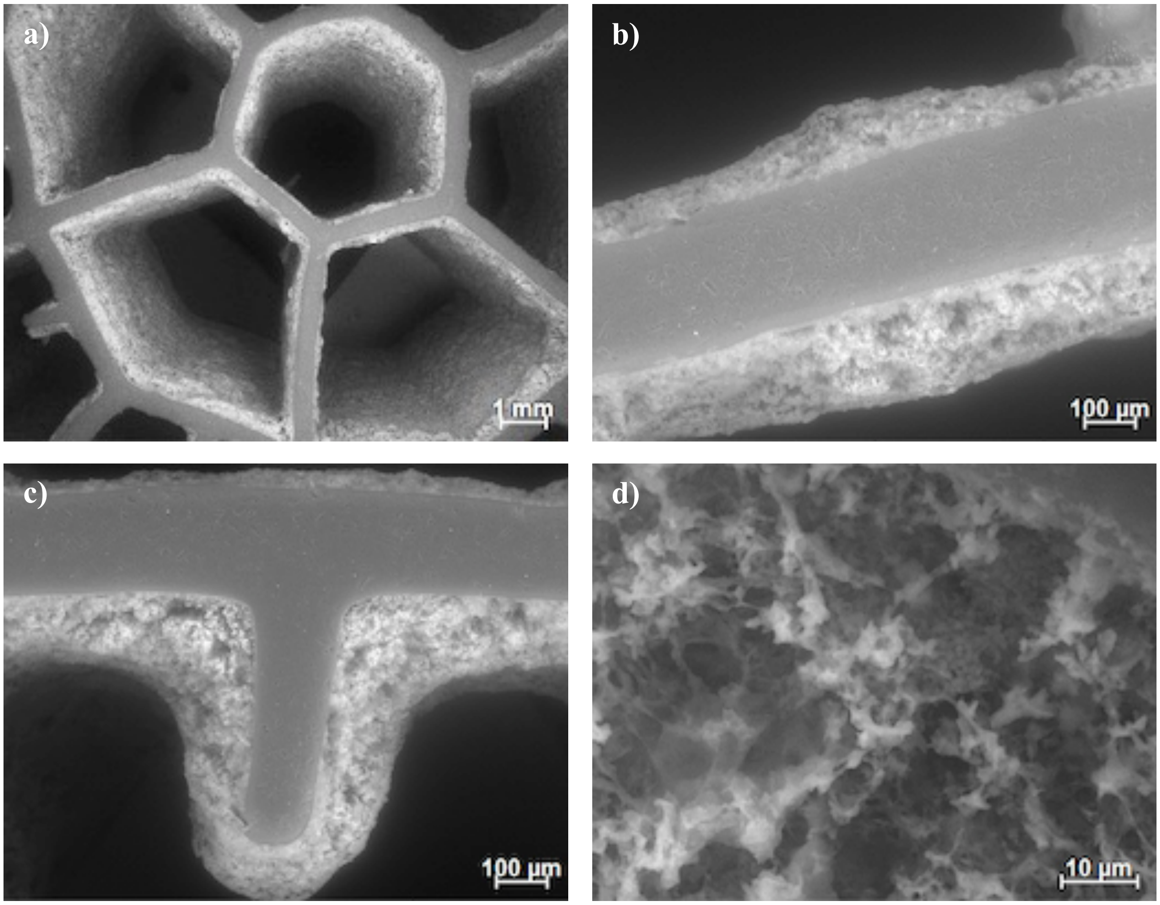


Figure 5.3: VPSEM images of nitrifying biofilm attached to a single K3 carrier sampled from the laboratory MBBR: a) image of biofilm coverage on the inner portion of the K3 at 16× magnification, b) and c) images displaying biofilm thicknesses on an edge and on a ridge of the K3 carrier at 90× magnification, respectively, and d) image displaying the morphology of the biofilm at 2000× magnification

Biofilm thicknesses in R1 were significantly greater than the biofilm thicknesses in R2 (Figure 5.4). Again, this was expected since the biofilm in R1 was more mature at the start of the study as compared to R2. Furthermore, a significant increase in biofilm thickness in both reactors was observed after four months of exposure to 1^{o}C as compared to 20^{o}C.

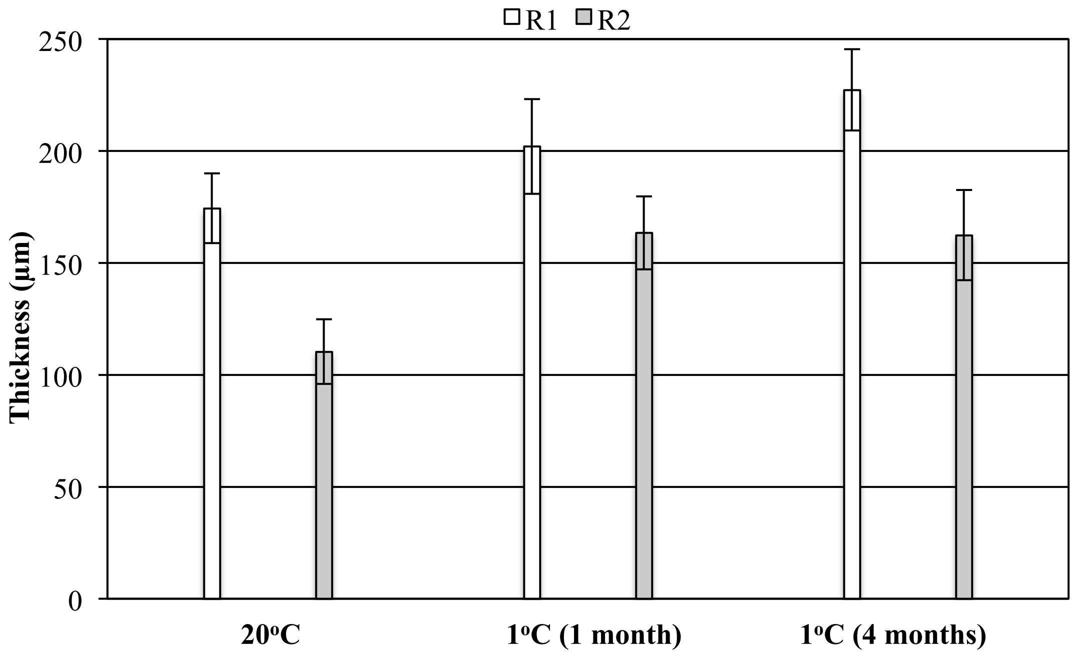
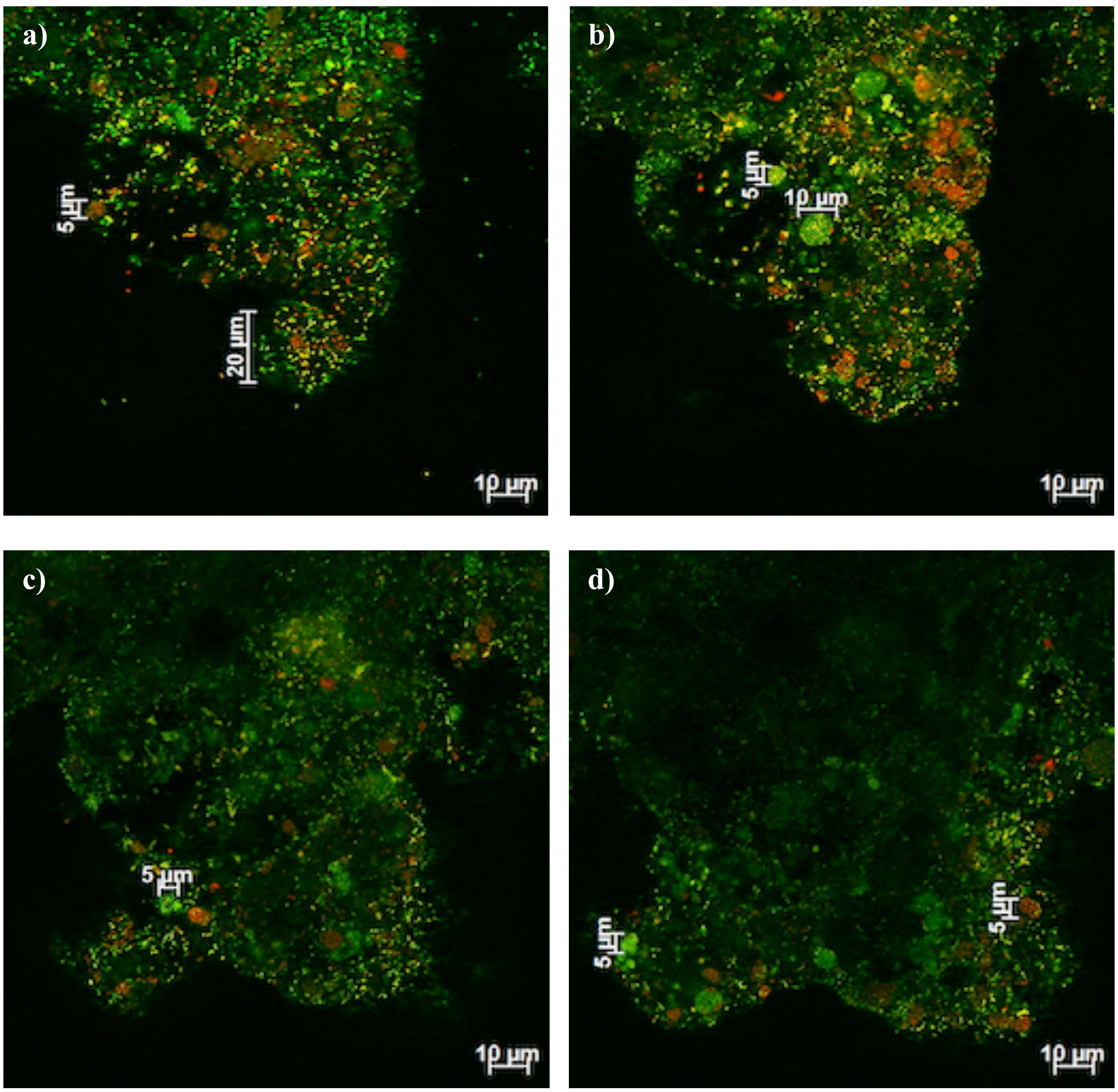


Figure 5.4: Average biofilm thickness with 95% confidence intervals for R1 and R2 throughout the experimental phase (n=10)

5.4.3. Viability of Biomass

CLSM in combination with viability staining was used to quantify the live and dead cell coverage of the biofilm at depths within the biofilm, where live cells were illuminated green and dead cells were illuminated red (Figure 5.5).

No significant change in biofilm live cell coverage was observed between experiments at 20°C and after one month of exposure to 1°C. However, there was a significant increase in live cell coverage after four months at 1°C compared to the experiments at 20°C (Figure 5.6). Particularly, the live cell coverage was observed to increase by a magnitude of 2.5 ± 1.5% in R1 and 3.9 ± 1.9% in R2 after four months of exposure to 1°C. This is an increase of 1.7 times and 2.1 times in live cell coverage as compared to 20°C in R1 and R2, respectively. Dead cell coverage did not show significant changes throughout the experimental phase, where the average dead cell coverage was observed to be 1.4 ± 0.7% at 20°C and 1.0 ± 0.6% at 1°C for the two reactors.



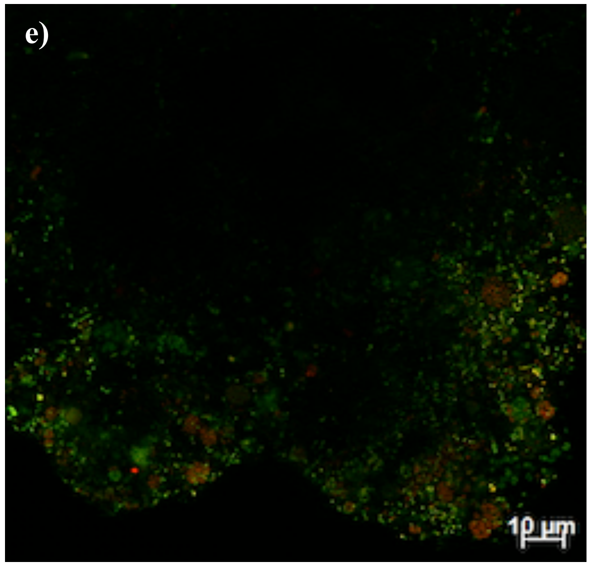


Figure 5.5: CLSM stack of images of nitrifying biofilm attached to the K3 carrier; live cells are illuminated green and dead cells are illuminated red. Images are 143 × 143 µm in lateral area: a) biofilm layer farthest from carrier surface (top) b) 5 µm depth c) 10 µm depth d) 15 µm depth and e) biofilm layer closest to carrier

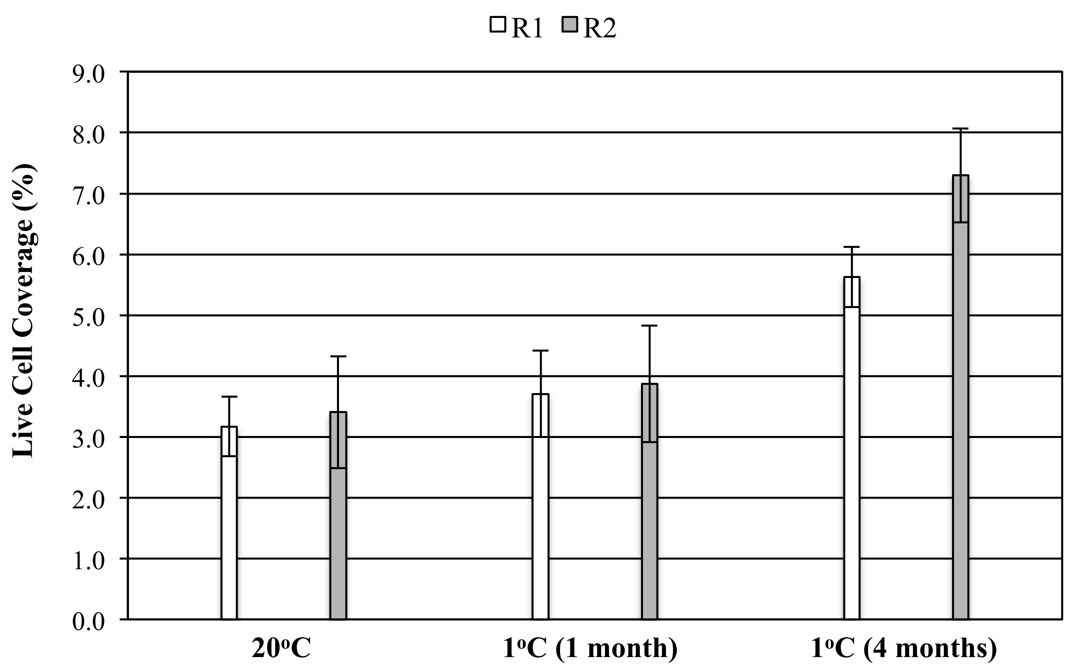


Figure 5.6: Average live cell coverage with 95% confidence intervals for R1 and R2 at each experimental phase (n=20)

5.4.4. Bacterial Population

OTUs from all samples were identified 25 bacterial phyla. The three most abundant phyla identified in the two reactors were *Proteobacteria, Nitrospirae,* and *Bacteroidetes* at both 20^{o}C and after four months of exposure to 1^{o}C. The majority of sequences (42.99–50.60%) in the samples belong to the *Proteobacteria* phylum. This phylum includes the class *β-Proteobacteria* that is comprised of organic matter decomposing bacteria, AOB, NOB, and denitrifying bacteria. Interestingly, the phylum *Nitrospirae*, which is the phylum of major NOBs, was found to be the second major bacterial phylum in the young (R2) biofilm at both 20°C and 1°C; in the mature (R1) biofilm, *Nitrospirae* was the fourth major bacterial phylum at 20°C and 1°C with *Acidobacteria* being the third most abundant phylum. In both mature and young biofilms, the percent abundance of *Nitrospirae* increased after long-term exposure to 1^{o}C (Table 5.1).

Table 5.1: Percent abundance of the major bacterial phyla identified

Bacterial Phyla	**Abundance (%)**			
	20^{o}C		**1^{o}C**	
	R1	**R2**	**R1**	**R2**
Proteobacteria	50.60	47.99	47.04	42.99
Nitrospirae	3.82	19.83	7.88	30.40
Bacteroidetes	24.83	10.09	8.48	14.99

At the genus level, *Nitrosomonas* and *Nitrosospira* (*Proteobacteria* phylum) were identified as the two AOB genera at 20^{o}C and after long-term exposure at 1^{o}C where both reactors showed a higher relative abundance of *Nitrosomonas* than *Nitrosospira* at both temperatures. Other major AOB genera, such as *Nitrosococcus*, were not detected in either reactor. Furthermore, *Nitrospira* (*Nitrospirae* phylum) was identified to be the main NOB genus at 20°C and after long-term exposure at 1°C in both of reactors. The relative abundance of *Nitrospira* in the young biofilm was higher than the mature biofilm at both warm and cold temperatures. At 20°C, *Nitrospira* accounts for 19.83% of total bacteria in R1 where the abundance increased 1.5 times at 1°C to reach 30.40%. In the mature biofilm, *Nitrospira* abundance was 3.82% at 20°C and doubled after four months at 1°C to reach 7.88% (Table 5.2).

Table 5.2: Percent abundance of identified AOB and NOB genera

Type	Genus	Abundance (%)			
		20°C		1°C	
		R1	R2	R1	R2
AOB	*Nitrosomonas*	3.68	2.45	0.002	9.35
AOB	*Nitrosospira*	0.56	1.84	0.0002	0.37
NOB	*Nitrospira*	3.82	19.83	7.88	30.40

Denitrifiers were detected in all samples where 90% of the genera identified in this group were *Nitrosomonas*, *Flavobacterium*, *Pseudomonas*, and *Rhodoplanes*, with *Nitrosomonas* being a potential AOB and denitrifier that will act as an AOB in the presence of ammonia (Bock et al., 1995). The denitrifiers accounted for 5.02% of bacteria in the young biofilm at 20°C and 10.73% at 1°C. Their relative abundance was lower in the mature biofilm where they show 0.09% and 6.16% of total bacteria at 20°C and 1°C, respectively.

ANaerobic AMMonium Oxidation (anammox) bacteria, mainly represented by the phylum *Planctomycetes*, were also among the detected bacteria. Major bacterial genera belong to the *Planctomycetes* phylum include *Planctomyces*, *Isosphaera*, *Rhodopirellula*, and *Pirellula*. Anammox bacteria represented 0.11–3.78% of the total bacteria. *Planctomycetes* was detected in highest relative abundance in the mature biofilm at 1°C (3.78%) and was lower at 20°C (0.83%). The young biofilm demonstrated lower relative abundances of *Planctomycetes* at both 1°C (0.11%) and 20°C (1.04%).

5.5. DISCUSSION

5.5.1. Effect of Low Temperature on Nitrification Kinetic Rate

Previous studies and practical experiences have shown a sharp decrease in nitrification rates at low temperatures. The average MBBR nitrification rate in this study was measured to be 17.2 ± 5.1% at 1^{o}C compared to the rate at 20^{o}C. The decrease of ammonia removal rates as temperatures decreased could be due to the loss or death of nitrifying bacteria during cold temperature exposure. However, both reactors were able to quickly recover from the rapid temperature changes return to the pre-change removal rates for the remainder of the experiment at 1^{o}C. The ability of the MBBRs to quickly adapt and recover from two temperature spikes suggests that the nitrifiers were not lost nor lysed but rather maintained in the biofilm. Both laboratory MBBRs showed an immediate increase in ammonia removal rates while experiencing two rapid temperature increases. A similar occurrence was observed during temperature shock experiments of a nitrifying biological aerated filtration (BAF) system (Delatolla et al., 2009). These findings suggest that the nitrifying bacteria exist at a higher level of activity at higher temperatures and at a lower level of activity at lower temperatures. These results demonstrate the potential of the MBBR system to perform consistent nitrification at $1^{o}C$ over an extended period of time representative of a Canadian winter.

5.5.2. Effect of Low Temperature on Nitrifying Biofilm

A significant increase in biofilm thickness was observed in both reactors after four months of exposure to $1^{o}C$ compared to $20^{o}C$. This observation is consistent with previous studies that showed increased nitrifying biofilm thicknesses with exposure to cold temperatures (Bjornber et al., 2009; Delatola et al., 2012). Particularly, Bjornberg et al. (2009) observed a relatively thin biofilm of approximately 57 µm in a full-scale MBBR system during the summer months and an average increase in biofilm thickness of 40 µm after the winter months. This observed increase in biofilm thickness at colder temperatures may be due to increased DO concentration in the reactors at cold temperatures and resulting in an increase of penetration depth into the biofilm. The concentration of ammonia in the two reactors was above 5 mg-N/L during the experiments, which suggests that DO is the mass transfer limited constituent for bacterial growth (Gujer and Boller, 1986). Hence, higher DO concentrations will promote penetration of oxygen to deeper depths in the biofilm where oxygen was previously limited and subsequently may stimulate the growth of thicker biofilms.

5.5.3. Effect of Low Temperature on Viability of the Nitrifying Biomass

It should be reiterated that the increase in viable cells observed in this study occurred in a biofilm that showed a significant increase in thickness while the kinetic rates dropped considerably to an average of 17.2 ± 5.1% of the rates observed at 20°C. Therefore, the observed thicker biofilm and the increase cell coverage of viable cells after long exposure to cold temperatures demonstrate that a larger quantity of viable cells are promoted at 1°C as compared to 20°C; however, these cells are significantly less active with respect to the oxidation of ammonia and nitrite.

Furthermore, the live cell coverage in R2 was similar to that in R1 at 20°C and one month exposure at 1°C but was significantly greater than R1 after four months of exposure to 1°C (Figure 5.6). It should be noted that throughout the study and specifically after four months of exposure to 1°C, the more mature biofilm in R1 showed significantly greater biofilm thicknesses than the thicknesses measured in R2. Moreover, both reactors demonstrated similar kinetics at all temperatures. Hence, the younger and thinner biofilm in R2 maintained comparable rates of nitrification with respect to a more mature and thicker biofilm at all temperatures in the study by maintaining a larger quantity of viable biofilm cells per area of biofilm.

Although viable cell quantities were shown to increase with exposure to cold temperature and differences were observed between younger and more mature biofilms, no significant change in dead cell coverage was observed between the two reactors or at the different temperatures of the study. The average ratio of dead cells to viable cells was approximately 0.4 ± 0.3 at 20°C and decreased slightly to 0.2 ± 0.1 at 1°C for both reactors. The increased biofilm volume and quantity of viable cells with stagnant percentage coverage of dead cells suggests evidence of biofilm growth as opposed to cell death due to cold temperature exposure.

5.5.4. Nitrification Kinetics Normalized to Biofilm Volume and Viable Biomass at 1^{o}C

The application of VPSEM and CLSM imaging in combination with viability staining provided valuable information with respect to the response of the attached MBBR biofilm and biomass to exposure to cold temperature. The results were integrated with measured MBBR nitrification rates to normalize nitrification kinetics to the biofilm volume and viable biomass at 1^{o}C. Based on the biofilm thickness measurements acquired in this study, a biofilm volume ammonia removal rate (BVRR$_A$) was calculated according to the following equation:

$$BVRR_A = (C_{OUT} - C_{IN}) \cdot Q / (L_{AVG} \cdot V_b \cdot a) \qquad 5.2$$

where C_{OUT} and C_{IN} are ammonia concentrations of the outlet and inlet (mg-N/L), respectively, Q is the flow rate in and out of the reactors (L/d), L_{AVG} is the average biofilm thickness (m), V_b is the media bed volume (m^3), and a is the specific surface area of the carrier (m^2/m^3) . Furthermore, using the results obtained from the CLSM analysis, ammonia removal rates were also established relative to the viable cells (VCRR$_A$) according to the following equation:

$$VCRR_A = (C_{OUT} - C_{IN}) \cdot Q / (LC \cdot V_b \cdot a) \qquad 5.3$$

where LC is the live cell coverage. The BVRR$_A$ and VCRR$_A$ of both laboratory MBBRs after four months exposure to 1^{o}C relative to the BVRR$_A$ and VCRR$_A$ at 20^{o}C are compared to the surface area removal rates measured (Table 5.4). A sharp decrease in BVRR$_A$ and VCRR$_A$ is observed when both reactors are exposed to cold temperatures;

the $BVRR_A$ and $VCRR_A$ at 1°C was approximately 10 times less than the $BVRR_A$ and $VCRR_A$ at 20°C.

Table 5.3: Removal rates, $BVRR_A$ and $VCRR_A$, with standard deviation values of R1 and R2 at 1°C relative to 20°C after four months exposure time to 1°C

	Removal Rates at 1°C relative to 20°C (%)	**$BVRR_A$ at 1°C relative to 20°C (%)**	**$VCRR_A$ at 1°C relative to 20°C (%)**
R1	18.7 ± 5.5	13.3 ± 6.4	9.7 ± 8.2
R2	15.7 ± 4.7	14.5 ± 7.7	9.9 ± 7.6

Both the $BVRR_A$ and $VCRR_A$ at 1°C relative to 20°C were significantly less than the removal rates at 1°C relative to surface area removal rates at 20°C. The $BVRR_A$ at 1°C corresponds to a thicker biofilm and thus higher biofilm volume along with a much lower removal rate as compared to the $BVRR_A$ calculated at 20°C that corresponds to a thinner biofilm and larger removal rate. Thus the larger biofilm thickness during cold temperatures increased the difference between the removal rates relative to the biofilm volume as compared to the carrier surface area. Similarly, the $VCRR_A$ at 1°C corresponds to a higher percentage of live cells but a much lower removal rate as compared to those at 20°C. Hence the $BVRR_A$ and $VCRR_A$ confirm that thinner nitrifying biofilm with fewer cells and higher activity levels exist at warmer temperatures and thicker nitrifying biofilm with more cells and lower activity levels exist at cold temperatures in MBBR systems investigated in this study.

5.5.5. Effect of Low Temperature on Bacterial Population

The AOB and NOB genera identified in this study are in general agreement with those found in past studies investigating microbial communities of nitrifying biofilms (Okabe et al., 2002; Gieseke et al., 2003; Park, 2008). Specifically, *Nitrosospira* has been found to not be an influential AOB genus in wastewaters (Schramm et al., 1999; Daims et al., 2001; Gieseke et al., 2001). Particularly, *Nitrosospira* has been observed to co-exist in lower numbers relative to *Nitrosomonas*, the dominant AOB population in wastewaters, with *Nitrosospira* exhibiting higher temperature sensitivity (Park et al., 2008). This study supports previous observations and shows that *Nitrosomonas* and *Nitrosospira* co-existed at both 20^{o}C and 1^{o}C. *Nitrosomonas* dominated the AOB group with at least a 1.3 times higher abundance than *Nitrosospira* and *Nitrosospira* showed a low relative abundance throughout the entire experimental phase. Although *Nitrosospira* has been previously shown to be more sensitive to temperature than *Nitrosomonas*, an increase in relative abundance shift was observed in both mature (R1) and young (R2) biofilms after long-term exposure to 1^{o}C The observed increase in relative abundance of *Nitrosomonas* in the young biofilm and the decrease in relative abundance in the mature biofilm during exposure to cold temperatures may be related to the lower live cell counts observed in the mature biofilm at cold temperatures relative to the young biofilm.

Nitrobacter and *Nitrospira* have been shown to be the two common genera of NOBs in wastewater (Wagner et al., 1996; Daims et al., 2001; Siripong and Rittmann, 2007). Recent studies however, have shown that *Nitrospira*, not *Nitrobacter*, are the dominant nitrite oxidizers in wastewater systems (Daims et al., 2001; Zeng et al., 2009). In particular, studies have shown that *Nitrobacter* was dominant when the available substrate was abundant and *Nitrospira* thrived when substrate concentrations were scarce or when the system experienced temporary nitrite elevations (Schramm et al., 1999; Gieseke et al., 2003; Blackburne et al., 2007; Haseborg et al., 2010; Huang et al., 2010). No NOB population shift was observed in this study with *Nitrospira* being identified as the genera responsible for nitrite oxidation at both 20^{o}C and 1^{o}C, while *Nitrobacter* was not detected in either reactor throughout the entire experimental phase. *Nitrospira* was not only shown to be the only identified NOB genera in both young and mature biofilms at 20^{o}C and during long exposures to 1^{o}C, but its relative abundance was also observed to increase in both reactors during exposure to cold temperatures. Hence, these observations support the kinetic and microscopic findings that acclimatized nitrifying MBBRs are capable of maintaining or increasing the NOB population during long exposure to very cold temperatures.

The non-nitrifying bacteria identified in this study coincide with those identified in past studies investigating activated sludge and biofilm systems treating wastewater (Benedict and Carlson, 1971; Okabe et al., 2002; Wagner and Loy, 2002). Numerous heterotrophic species belonging to the *Bacteroidetes, Chloroflexi, Proteobacteria,* and *Acidibacteria* phyla were identified in this study along with the nitrifying bacteria belonging to the *Proteobacteria* (AOB) and *Nitrospirae* (NOB) phyla. It has been reported that species of the *Proteobacteria* phylum form dense biofilm layers close to the surface of substrata and provide a growth matrix for nitrifiers (Wagner and Loy, 2002; Ducey et al., 2010). Numerous bacterial strands have been identified as cryoprotective exopolysaccharides (EPS) producers that when mixed with *E.Coli* were shown to increase the survival rate during freeze and thaw cycles (Sung and Joung 2007). As such, it has been previously hypothesized that the presence of these protective non-nitrifiers can actually improve nitrification rates at cold temperatures (Ducey et al., 2010). Thus, a symbiotic relationship between nitrifiers and the identified non-nitrifiers in this study could potentially offer cold temperature resistance in biofilms and be a contributing factor to the maintenance of nitrification during long exposure to cold temperatures observed in this study.

5.6. CONCLUSION

The application of VPSEM and CLSM in combination with viability staining provided valuable information with respect to the response of the attached MBBR biofilm and biomass to long-term cold temperature exposure. The results were integrated with measured MBBR nitrification rates and demonstrated an increase in biofilm thickness and live cell coverage in both reactors after four months exposure to 1^oC while kinetic rates of nitrification decreased significantly. The calculated dead cell coverage during experiments at 20^oC and 1^oC, on the other hand, did not show significant differences and thus supports evidence of biofilm growth as opposed to cell death due to cold temperature exposure.

A high percentage of live cell coverage corresponds to a low removal rate after four months of exposure to 1^oC. To further analyze the ammonia removal rates with respect to the nitrifying biomass, biofilm volume removal rates ($BVRR_A$) and viable cell coverage removal rates ($VCRR_A$) were determined. The calculated $BVRR_A$ and $VCRR_A$ for R1 and R2 at 1^oC were both approximately 10 times less than the removal rates calculated for experiments at 20^oC but corresponded to higher biofilm thickness and higher percent live cell coverage. These findings suggest a strong correlation between the nitrifying bacteria activity level and the change in temperature, where a lower quantity of nitrifying bacteria was shown to exist at a higher level of activity at warm temperatures and a greater quantity of nitrifiers existed at a lower level of activity after long exposure to cold temperatures. Lastly, no nitrifying microbial population shift was observed after four months exposure to cold temperatures in which AOBs were identified to be *Nitrosomonas* and *Nitrosospira* and the NOB was identified as *Nitrospira* at both 20^{o}C and 1^{o}C. A number of non-nitrifiers were also identified in the biofilms at both temperatures suggesting nitrification rates at 1^{o}C may have been maintained partially due to the protection of nitrifiers by non-nitrifiers.

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CHAPTER 6: CONCLUSION

Moving bed biofilm reactor (MBBR) nitrification kinetics, biofilm mass, volume, and morphology, biologically produced solids, biomass viability, and nitrifier population shifts in response to long-term exposure to 1^{o}C was investigated during the course of this research. The main conclusions that were drawn from this work are as follows:

- the nitrification rate at 1^{o}C over a four month exposure period was maintained at a moderate rate throughout the entire period of exposure;
- a kinetic temperature threshold was observed at 5^{o}C in which the nitrification rate at 5^{o}C increased to approximately 3.6 times the rates observed at 1C;
- the MBBR system was able to quickly recover and perform at high ammonia removal rates after experiencing two rapid temperature increases;
- the duration of which a biofilm system is exposed to cold temperatures and whether this system is acclimatized to cold temperatures or experiences a rapid temperature decrease are important factors when applying a temperature correction factor (θ) to predict ammonia removal rates;
- the Delatolla et al. (2009) temperature correction model, developed for a biological aerated filter (BAF) and based on the duration of exposure time to cold temperatures, showed a strong correlation to the observed MBBR ammonia removal rates following an acclimatization period from 20^{o}C to 1^{o}C;
- biological solids produced by the laboratory MBBRs were measured to be less than 5 mg-TSS/L at 20^{o}C and after long-term exposure to 1^{o}C signifying that a nitrifying MBBR unit placed after the last lagoon pond would not compromise the effluent in terms of solids regulations;

- biofilm thickness increased significantly with long exposure to cold temperatures as nitrification rates decreased, indicating growth of biofilm that could be stimulated by an increase in dissolved oxygen (DO) saturation at low temperatures;
- live cell coverage of the upper layer of the biofilm (100 μm) showed an increase of approximately 1.9 times after four months of exposure to 1^{o}C as compared to 20^{o}C, indicating that larger quantity of viable cells are promoted at 1^{o}C than 20^{o}C but the cells at 1^{o}C are significantly less active;
- dead cell coverage of the upper layer of the biofilm (100 μm) did not show significant changes after four months of exposure to 1^{o}C as compared to 20^{o}C;
- the biofilm volume removal rates ($BVRR_A$) and viable cell coverage removal rates ($VCRR_A$) were calculated and was determined to be approximately 10 times less than the measured surface area removal rates suggesting a thinner biofilm with fewer cells exist at warm temperatures and a thicker biofilm with more cells exist at cold temperatures;
- a microbial population shift was not observed in the biofilm during long-term exposure to 1^{o}C, where ammonia oxidizing bacteria (AOB) were identified as *Nitrosomonas* and *Nitrosospira* and the nitrite oxidizing bacteria (NOB) was identified as *Nitrospira* at both 20^{o}C and 1^{o}C;
- non-nitrifying bacteria were identified at 20^{o}C and after long-term exposure to 1^{o}C and their presence may have provided some form of protection to the nitrifiers during cold temperature exposure;

- the ability of the MBBR system to consistently perform nitrification over a duration of four months at 1^oC, being able to recover from two rapid temperature changes, not showing changes in dead cell coverage while showing significant changes in biofilm thickness and live cell coverage after four months of exposure to 1^oC, suggests that nitrifying biofilm performs at a higher level of cell activity during warm temperatures and a lower level of cell activity at cold temperatures.

6.1. FUTURE RECOMMENDATIONS

Future recommendations are herein proposed to further demonstrate the feasibility of nitrifying MBBRs as a cold temperature upgrade technology installed after the last treatment lagoon pond.

1. To minimize the effects of heterotrophic bacteria on nitrification, a zero carbon source synthetic wastewater was fed to the lab-scale MBBR system during this study. As a result, near ideal conditions were provided for the investigation of cold temperature effects on nitrification kinetic rates, nitrifying biofilm and biomass response, and nitrifiying population shifts. Thus, future studies using a carbon feed or real wastewater is recommended.

2. To further this research, it is recommended that the next step is to perform a pilot-scale study.

3. Due to the time constraints of this study, only CSLM analysis was used to examine biomass viability and was thus limited to the quantification of live and dead cells as appose to AOBs and NOBs. It is recommended that fluorescence in-situ hybridization (FISH) analysis in combination with CSLM can provide a means to quantitatively analyze the size, distribution, and percent coverage of nitrifying bacteria.

4. Beyond the scope and time constraints of this research, the live/dead cell coverage ratio with respect to biofilm depth was not investigated. This information, however, can provide great insight to mass transfer limitations on nitrification rates during long-term exposure cold temperatures.

5. Due to the complexity of the sequencing methods and the time constraints of this project, a composite biofilm sample per reactor during each experimental phase was used for sequencing analysis. As such, for future experiments, at least four samples (n = 4) per reactor during each experimental phase are needed to calculate p-values for statistical analysis.

6. This work suggests the upgrade nitrifying MBBR be installed to treat effluent exiting the last lagoon of multiple treatment lagoons where the C/N ratio is low. Although the competition between heterotrophs and autotrophs are reduced in this manner, the presence of carbon will still produce a substantial amount of heterotrophic biofilm in the nitrifying MBBR system. Consequently, the effect of

carbon oxidizers on nitrifying biofilm is of key importance for full-scale applications. The next step in furthering this research is to investigate MBBR cold temperature nitrification at a laboratory-scale level using synthetic wastewater with carbon sources and subsequently at a pilot-scale level using real municipal wastewater.

APPENDIX A: COMBINED ERROR CALCULATIONS

If a variable, Z, depends on one or more variables, A and/or B, that have independent errors, ΔA and/or ΔB, then the combined error, ΔZ, is calculations based on the following relationships represented in Table A.1 below.

Table A.1: Rules for calculating combined errors

Relation Between Z, A, and B	**Combined Error Calculation for Z**
Z = A + B	$(\Delta Z)^2 = (\Delta A)^2 + (\Delta B)^2$
Z = A – B	$(\Delta Z)^2 = (\Delta A)^2 + (\Delta B)^2$
Z = A × B	$\left(\frac{\Delta Z}{Z}\right)^2 = \left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2$
Z = A/B	$\left(\frac{\Delta Z}{Z}\right)^2 = \left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2$
$Z = A^n$	$\left(\frac{\Delta Z}{Z}\right) = n\left(\frac{\Delta A}{A}\right)$