

# **Nitrifying Moving Bed Biofilm Reactors at Low Temperatures and Cold Shock Conditions: A Kinetic, Biofilm and Microbiome Study**

Warsama Ahmed



uOttawa

Thesis submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy in Civil Engineering

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

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## Preface

This thesis is an original work by Warsama Ahmed. This research was conducted at the University of Ottawa under the supervision of Dr. Robert Delatolla. Four manuscripts were prepared for publications in peer-reviewed journals as part of this research, with versions of these manuscripts appearing in Chapters 3 to 6 of this dissertation.

Chapter 3 includes a version of Publication 1:

Ahmed, W., Tian, X., Delatolla, R., 2019. *Nitrifying moving bed biofilm reactor: Performance at low temperatures and response to cold shock*. *Chemosphere* 229, 295–302. (Published)

Chapter 4 includes a version of Publication 2:

Ahmed W., Delatolla R. *Biofilm and Microbiome Response of Attached Growth Nitrification Systems Across Incremental Decreases in Temperatures*. (Journal of Water Process Engineering – Submitted)

Chapter 5 includes a version of Publication 3:

Ahmed, W., Delatolla, R. *Microbial Response of Nitrifying Biofilms to Cold shock*. (Journal of Environmental Science: Water Research & Technology – Submitted)

Chapter 6 includes a version of Publication 4:

Ahmed, W., Delatolla, R. *Biofilm Technologies as Upgrades to Low Temperature Passive Treatment Systems*. (Journal of Biotechnology – In preparation)

The work in this dissertation contributed to the design of the first three low carbon lagoon upgrade nitrifying MBBR systems in Canada. The kinetic findings of this study crucial for the temperature dependent rate modeling of this biofilm technology will also be published in the WEF Manual of Practices No. 29: Biological Nutrient Removal.

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

## Abstract

The nitrification process, the biologically mediated process of ammonia treatment in water resources recovery facilities (WRRF), remains the most common treatment process to mitigate the adverse effects of effluent ammonia discharges in surface water. However, it is well established that the temperature-sensitive process of nitrification remains hindered at low temperatures in conventional suspended growth technologies; specifically, passive treatment systems such as the lagoons, representing over 50% of Canadian treatment facilities in operation. As such, nitrification in lagoon facilities remains unreliable during the cold seasons with no nitrification occurring at 1°C. In contrast to suspended growth systems, attached growth technologies such as the moving bed biofilm reactors (MBBR) have recently been proven capable of achieving significant nitrification rates at temperatures as low as 1°C and are proposed as suitable upgrade systems to the common lagoon facility to reach year-long ammonia treatment targets. As such, the main objective of this research is to investigate and expand the current knowledge by investigating the key research questions lacking in the current literature on post-carbon, low temperature nitrifying MBBR systems.

With this aim, a temperature-controlled study of attached growth nitrification kinetics was conducted to isolate the effects of low temperatures on nitrifying MBBR system performance down to 1°C. A removal rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d is identified as the 1°C intrinsic removal rate and the design removal rate for nitrifying MBBR systems at low temperatures. Considering this intrinsic rate at 1°C, an assessment of reactor efficiency at elevated TAN concentrations typical of non-combined sewer systems indicates that a two reactor in-series MBBR system configuration is recommended for retrofitting lagoon facilities connected to sanitary sewers.

The study of the reactor performance to temperatures as low as 1°C demonstrates a non-linear decline in removal efficiency between 10°C and 1°C, with the existence of a kinetic threshold temperature delineated between 4°C and 2°C. As such, this delineated temperature range accounts for a significant decline in the performance of low carbon nitrifying MBBR systems during the onset of the cold seasons. This research identifies new recommended Arrhenius correction coefficient values taking into account this kinetic threshold temperature, with a coefficient of 1.049 being recommended above the kinetic threshold ( $\geq 4^\circ\text{C}$ ) and 1.149 below the threshold temperature at 1°C. Moreover, since the elapsed time to low temperature was identified as a key factor of attached growth nitrification kinetics, a modified theta model accounting for temperature and time is proposed in this research to accurately model the rate of nitrifying MBBR systems between 4°C and 1°C.

Finally, with the severe adverse effects of sudden decreases in temperature, or cold shocks, on nitrification kinetics being previously demonstrated but not well understood, this research compares acclimatized and cold shocked MBBR reactors down to 1°C. The findings indicate 21% lower kinetics in the cold shocked reactor with reactor efficiencies never reaching those of the acclimatized reactor despite extended operation at 1°C. Thus, the research delineates the potentially lasting effects of extreme weather events such as cold air outbreaks and snowmelt periods on nitrifying MBBR system performance. On the other hand, these same findings demonstrate the resiliency of nitrifying MBBR reactors as nitrification was maintained within these systems despite being cold-shocked down from 10°C and 1°C.

This study of attached growth kinetics was coupled with an investigation of the nitrifying biofilms, biomass, and microbiome responses to low temperatures and cold shock down to 1°C to provide an understanding of the changes occurring in these systems down to the cellular level.

Comparisons of acclimatized and cold shocked nitrifying biofilms responses down to 1°C were characterized by increases in biofilm thickness, increases in biomass viability; and, greater shifts in microbiome communities occurring above 4°C in the acclimatized biofilm. Considering these observations, results also indicated a significant increase in nitrifiers per carrier above 4°C. As such, these findings suggested that the bulk of nitrifying biofilm adaptation to cold temperatures occurs above 4°C, a crucial adaptation phase in acclimatized systems. This adaptation phase is shown to be lacking in cold-shocked systems, with the cold shocked biofilm and microbiome demonstrating significant differences with the acclimatized systems' biofilm and microbiome.

This research was performed to answer the critical research questions relating to the design and operation of the post-carbon, low temperature nitrifying MBBR systems, with the first low temperature MBBR systems being scheduled to begin operation in the fall of 2020. This research expands the current knowledge on low temperature attached growth nitrification kinetics as well as cold shocked attached growth nitrification kinetics in MBBR systems down to 1°C. In addition, this research delineates the effects of low temperatures and cold shocks on the nitrifying MBBR system's biofilms and their embedded cells.

## **Acknowledgements**

I would like to express my sincere gratitude to Dr. Robert Delatolla for his guidance and support during my research. I would like to thank Dr. Robert Delatolla for sharing his passion for research and instilling in me the importance of combining different fields of research as I embarked on this journey. I want to give my thanks to Professor Robert Delatolla in patiently teaching us the art of writing, for which I am grateful. Finally, I thank him and his wife, Melanie Buteau-Delatolla, for their kindness and encouragement during my graduate studies.

I also thank all the members of my research group for their support, contributions and for creating a fun and welcoming environment in which to conduct research.

I acknowledge and thank Veolia Water Technologies and Associate Engineering for their contribution to this research. I would also like to extend my sincere gratitude to the Department of Civil Engineering faculty members and support staff at uOttawa.

Finally, thank you to friends and family for their support, especially my parents, without whom this thesis would not have been possible.

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## List of Acronyms

AOB	Ammonia oxidizing bacteria
ATP	Adenosine triphosphate
BAF	Biologically active filters
BOD	Biological oxygen demand
cBOD <sub>5</sub>	Carbonaceous biochemical oxygen demand
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
DO	Dissolved oxygen
E	Removal Efficiency
EPS	Extracellular polymeric substances
FLASH	Fast length adjustment of short reads
HRT	Hydraulic retention time
IFAS	Integrated fixed film activated sludge
KEGG	Kyoto Encyclopedia of Genes and Genomes
MBBR	Moving bed biofilm reactors
MTBL	Mass transfer boundary layer
NOB	Nitrite oxidizing bacteria
OTU	Operational taxonomical unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PI	Propidium iodide
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative insights into microbial ecology
RBC	Rotating Biological Contactors
SALR	Surface area loading rate
SARR	Surface area removal rate
sCOD	Soluble chemical oxygen demand
TAN	Total ammonia nitrogen
TAN <sub>eff</sub>	Effluent total ammonia nitrogen
TF	Trickling Filters
TN	Total nitrogen
TP	Total phosphorus
TSS	Total suspended solids
USEPA	United States Environmental Protection Agency
VLR	Volumetric loading rate
VPSEM	Variable pressure electron microscopy
VRR	Volumetric Removal Rate
VSS	Volatile suspended solids
WRRF	Water resources recovery facility
WSER	Wastewater systems effluent regulations

# Chapter 1. Introduction

## 1.1. Background

The nutrient-rich effluents discharged by water resources recovery facilities (WRRF) have been identified as critical factors for surface water toxicity and eutrophication (Murdoch et al., 2000; Driscoll et al., 2003; Preston et al., 2011). Elevated concentrations of effluent total ammonia nitrogen (TAN), comprising unionized ammonia ( $\text{NH}_3$ ) and ammonia ion ( $\text{NH}_4^+$ ), can lead to severe adverse effects on the receiving water bodies, from changes in colour in otherwise clear surface waters to the direct negative effects on aquatic life due to toxicity (Llyod and Herbert, 1960; Wright et al., 1986; Playle and Wood, 1989). Moreover, TAN along with phosphorus can also lead to eutrophication, defined as excessive plant growth and algae blooms, eventually resulting in odour problems from decomposing plants and low dissolved oxygen (DO) levels which again adversely affect aquatic life (Shammas, 1986; USEPA, 1993; Gerardi, 2002).

This reality has led to increasingly stringent regulations around the world, including Canada (EEC, 1991; Canada Gazette, 2012; USEPA, 1983; USEPA, 2016). The Canadian Fisheries Act's Wastewater Systems Effluent Regulations (WSER) regulates the discharge of four deleterious substances. The effluent concentration limits defined in this regulation are with respect to carbonaceous biochemical oxygen demand (cBOD) and the total suspended solids (TSS) to not exceed 25 mg/L, for the residual chlorine ( $\text{Cl}^-$ ) to be equal or less than 0.02 mg/L and for unionized ammonia as nitrogen ( $\text{NH}_3\text{-N}$ ) to be lower than 1.25 mg/L at  $15 \pm 1^\circ\text{C}$ . The regulation also indicates that effluent wastewater passes an acute lethality test (LC50), defined as less than 50% mortality of rainbow trout after 96 hours in 100% wastewater effluent. For the latter toxicity test, TAN concentrations of 15 mg/L to 20 mg/L have been reported to fail the LC50 test (Di Giulio and Hinton, 2008; CCME, 2010). However, in such cases, the facilities may seek temporary

authorization to discharge as long as the concentrations of unionized ammonia nitrogen in the receiving surface water is less than or equal to 0.016 mg/L at any point that is at a distance of 100 m from the effluent discharge point. In other regions, such as the United States and the European Union, state or region-specific guidelines also currently set out to regulate total nitrogen point source pollution on sensitive receiving water bodies (USEPA 1993; USEPA 2016; EEC, 2017).

Conventional suspended growth systems have been proven capable of reducing cBOD, TSS as well as achieving nitrification; however, low temperatures have a well-reported adverse effect on nitrification with a positive correlation between decreasing nitrification kinetics and decreasing temperatures in these systems (Painter and Loveless, 1983; van Dyke et al., 2003; Ducey et al., 2010; Champagne et al., 2017; Chen et al., 2018). Moreover, nitrification in passive treatment systems such as the lagoon, representing 54% of Canadian WRRFs currently in operation or a total number of 1244 facilities in Canada along with 8000 facilities in the United States, remains unreliable during the cold seasons (Houweling et al., 2007; ECCO, 2016; Statistics Canada, 2018). The temperature-sensitive process of nitrification in lagoons declines as temperatures decrease below the optimal growth temperatures (25°C-30°C) of the mesophilic nitrifying organisms, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), responsible for the two-step process of nitrification (Sharma and Ahlert, 1977; Painter and Loveless, 1983; Zhu and Chen, 2002; Salvetti et al., 2006). Moreover, with their long hydraulic retention times (HRT) and large surface areas, lagoons are prone to heat loss during cold season operation which leads to lower temperatures, ice covering of the surface and subsequently low oxygen concentrations that exacerbate the adverse effects of low temperatures on nitrification and eventually ceases the nitrification process in these systems (Houweling et al., 2007; Racys et al. 2018).

On the other hand, nitrifying attached growth systems, although impeded by low temperature as well, have been shown to be affected to a significantly lesser extent compared to suspended growth systems. For decades, biofilm technologies such as the rotating biological contactors (RBC), the trickling filters (TF), the biologically active filters (BAF) and the moving bed biofilm reactors (MBBR) have been proven to attain significant nitrification rates below 15°C (Parker et al., 1989; Boller et al., 1990; Anderson et al., 1994; Ødegaard et al., 1994; Rusten et al., 1995; Ødegaard, 2000; Kapoor et al., 2003; Delatolla et al., 2009; Delatolla et al., 2010; Hoang et al., 2014a; Young et al., 2016). The latter biofilm technology, the MBBR system, has been investigated for over two decades as an add-on nitrifying unit to passive treatment systems to achieve desired TAN discharge objectives (Rusten et al., 1995; Houweling et al., 2007; Delatolla et al., 2010; Leyva-Díaz et al., 2017). These systems benefit from the advantages innate to biofilm technologies; such as small land footprints, low emission of effluent solids, resilience to environmental changes such as temperature, and the capacity to maintain active slow growing organisms such as nitrifiers necessary for TAN removal (Wang et al., 2005; Barwal and Chaudhary, 2014; Dezotti et al., 2018). In addition to the many inherent advantages of attached growth systems, the MBBR system possesses unique benefits such as their low operational intensity which renders them potential candidates as upgrade units for passive treatment systems (Ødegaard, 2006; Forrest et al., 2016). Furthermore, nitrifying MBBR systems have demonstrated the capacity to achieve significant nitrification rates at temperatures as low as 1°C at both the laboratory and pilot scale (Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017).

## 1.2. Statement of Problem

The nitrifying MBBR systems have been reported capable of maintaining significant nitrification rates at temperatures as low as 1°C in several studies (Almomani et al., 2014; Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017). Although these studies on low temperature nitrifying MBBR systems have been proven capable of conducting nitrification at both the laboratory and pilot scale down to 1°C, these studies were either conducted with synthetic wastewater or with real wastewater without significant temperature-control precision. Thus, the intrinsic 1°C removal rate for nitrifying MBBR systems using a temperature-controlled investigation and real wastewater has yet to be precisely quantified.

Furthermore, previous studies on low temperature nitrifying MBBR systems were conducted with TAN concentrations of approximately 20 mg/L, which are typical of lagoon effluent concentrations of systems fed by combined sewers (Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017). Current engineering standards in North America and Europe dictate the separation of new sewer systems into sanitary and stormwater sewers and present combined sewer systems to be transitioned into two distinct collectors to provide overflow control and mitigate the effects of large storm events (Diogo et al., 2018). Nonetheless, these standards towards separate sewer systems result in raw wastewater no longer being diluted by stormwater runoff with higher nutrient concentrations entering WRRFs which can impact the design and operation of post-carbon removal, nitrifying lagoon upgrade MBBR systems (Delatolla and Babarutsi, 2005). As no studies exist in the current literature on low temperature nitrifying MBBR systems treating sanitary sewers municipal wastewater down to 1°C, a gap of knowledge remains on the design and performance of low temperature nitrifying MBBR systems treating elevated TAN concentrations typical of sanitary sewers.

To optimize the design and operation of low temperature nitrifying MBBR systems, the kinetic threshold temperature, or the temperature below which a significant decrease in nitrification kinetics occurs, must be precisely delineated. In a study conducted at 20°C, 5°C and 1°C on nitrifying MBBR systems, a potential temperature threshold at 5°C was suggested (Hoang et al., 2014a). However, due to the limited number of temperatures investigated in the above-mentioned investigation, a study investigating incremental decreases in temperatures on attached growth nitrification kinetics has yet to be conducted to precisely identify the kinetic threshold temperature for the attached growth nitrification.

Furthermore, in the aim to optimize the design and operation of these low temperature nitrifying MBBR systems, the Arrhenius temperature correction coefficients are practical to predict nitrification kinetics in MBBR systems with respect to temperatures (Rusten et al., 1995; Zhang et al., 2014; Young et al., 2017). The conventional correction coefficients of 1.098 and 1.058 reported by Salvetti et al. (2006) for ammonia limited and oxygen limited conditions respectively are the commonly used coefficients for nitrifying MBBR systems (WEF, 2011). However, these correction coefficients are only reported for temperatures above 10°C. Young et al. (2017) in their pilot study with limited temperature control on 1°C nitrifying MBBR systems proposed correction coefficients of 1.086 and 1.09 for temperatures below 12°C. Moreover, Young et al. (2017) have reported, between 5°C and 1°C, a strong correlation ( $R^2 = 0.77$ ) between their measured nitrification rates and modeled rates obtained with the Delatolla et al. (2009) theta model, a temperature-dependent Equation to model attached growth nitrification kinetics as a function elapsed time at 4°C. As such, Arrhenius temperature correction coefficients and theta model investigations down to 1°C for nitrifying MBBR systems have yet to be conducted with enhanced temperature control.

As the adverse effects of low temperatures on the performance of nitrifying MBBR systems kinetics have been reported in several studies as mentioned above, there are limited studies on the impact of rapid decreases in temperature, or cold shocks, on both suspended growth and attached growth nitrification kinetics (Hwang and Oleszkiewicz, 2007; Delatolla et al., 2010). Cold shock events in WRRFs may occur during periods of snowmelt and during extreme weather events such as cold air outbreaks predicted to increase in frequency in the upcoming decades in several regions around the world, including North America (Kanno et al., 2016; Champagne et al., 2017; Smith and Sheridan, 2018). Hwang and Oleszkiewicz (2007) reported in a comparison study between a suspended growth nitrifying system acclimatized to low temperatures by incremental decreases in temperature and cold shocked nitrifying system that the latter system displayed 20% lower nitrification rates with no recovery of the kinetics to match the acclimatized system. However, this cold shock study was conducted with temperatures above 10°C and with suspended growth systems. As for studies on cold shocked nitrifying attached growth systems, the lowest temperature investigated to date is 4°C by Delatolla et al. (2010). This work is signifying a need to investigate the adverse effects of cold shocks on attached growth nitrification kinetics in MBBR systems down to 1°C.

While the current knowledge on low temperature and cold shocked attached growth nitrification kinetics is expanded, it is also essential to investigate the effects of low temperatures and rapid decreases in temperatures down to the cellular level to understand their impacts on nitrifying MBBR systems and their kinetics. For this reason, the nitrifying biofilms, biomass, and microbiome responses in low temperature nitrifying MBBR systems were investigated down to 1°C (Hoang et al., 2014b; Young et al., 2017). Still, the current available studies either lacked precise temperature-controlled experimentations or the use of real wastewater to properly

delineated the adverse effects of low temperatures on the nitrifying biofilms and their embedded cells; moreover, currently no studies have been conducted on cold shocked nitrifying MBBR systems down to the cellular level. Thus, the necessity for temperature-controlled investigations of low temperatures and cold shocks adverse effects on nitrifying biofilms, biomass, and microbiome in nitrifying MBBR systems down to 1°C as to date such studies have yet to be conducted.

Finally, the above-mentioned investigations on low temperature nitrifying MBBR systems provide key knowledge on this type of attached growth system intended as low carbon upgrade units to passive treatment systems. As the remaining gaps of knowledge listed above are addressed as well; a synthesis of the current findings of low temperature nitrifying MBBR systems down to 1°C remains to be available to stakeholders. Such a review and synthesis of the current state of the art will provide a crucial understanding for the optimal design and operation of low carbon low temperature nitrifying MBBR systems and aims to summarize the answers to the remaining research questions as the first biofilm technologies are being scheduled to begin operation in Canada in the fall of 2020.

### **1.3. Research Objectives**

The main objective of this research is to investigate and expand the current knowledge on post-carbon, low temperature nitrifying MBBR systems and cold shocked nitrifying MBBR systems. As such, the scope of this work is to study low temperature and cold shock effects on attached growth nitrification kinetics down to 1°C as well as the nitrifying biofilms, biomass, and microbiome responses to low temperatures and cold shocks in nitrifying MBBR systems. Explicitly, the specific objectives of this thesis are to determine:

- I. The 1°C intrinsic ammonia removal rate of nitrifying MBBR systems.

- II. The design configuration of low temperature nitrifying MBBR systems configured for the treatment of elevated TAN concentrations typical of sanitary sewers.
- III. The kinetic threshold temperature below which attached growth nitrification kinetics significantly shift due to the adverse effects of low temperatures.
- IV. The Arrhenius temperature correction coefficients and the theta model accounting for exposure time to low temperatures for attached growth nitrification kinetics down to 1°C.
- V. The extent of cold shock adverse effects down to 1°C on attached growth nitrification kinetics.
- VI. The nitrifying biofilm responses to low temperatures down to 1°C with respect to biofilm morphology (thickness, mass, and density), biomass viability, and the nitrifying microbiome responses to low temperatures down to 1°C.
- VII. The nitrifying biofilm responses to cold shocks down to 1°C with respect to biofilm morphology (thickness, mass, and density), biomass viability, and the nitrifying microbiome responses to cold shocks down to 1°C.

## 1.4. Thesis Organization

The background information of this research, the statement of the problem, the rational and objectives of this research, and the list of publications developed in the scope of this research are described in Chapter 1. Chapter 2 presents the review of the current literature relevant to the objectives of this research and the work presented in the subsequent chapters.

Chapter 3 is a version of the study published in the Journal of Chemosphere under the title: *Nitrifying moving bed biofilm reactor: Performance at Low Temperatures and Response to Cold-shock* by W. Ahmed, X. Tian and R. Delatolla. The low temperature and cold shocked attached growth nitrification kinetics down to 1°C in the MBBR system are investigated in this publication.

Chapter 4 is a version of the manuscript submitted to the journal of Water Process Engineering titled: *Biofilm and Microbiome Response of attached growth Nitrification Systems Across Incremental Decreases in Temperatures* by W. Ahmed and R. Delatolla. This study aims to investigate the changes occurring in nitrifying biofilms during their acclimatization to low temperatures down to the microbiome level. This study includes an assessment of attached growth nitrification kinetics, an analysis of the nitrifying biofilm's adaptation to decreasing temperatures with respect to biofilm thickness, mass, and density; an assessment of the biomass availability, and an investigation nitrifying biofilm microbial communities at different temperatures down to 1°C.

Chapter 5 is a version of the study submitted to the journal of Environmental Science: Water Research & Technology titled: *Microbial Response of Nitrifying Biofilms to Cold Shock* by W. Ahmed and R. Delatolla. This study includes an investigation of cold shocked attached growth nitrification kinetics and an analysis of the effects of cold shocks in nitrifying MBBR systems down to the microbiome level. This study delineates cold shock responses down to 1°C with an assessment of the biofilm morphology (thickness, mass, and density), the biomass viability, and the embedded microbial communities.

Chapter 6 is a version of the manuscript in preparation for submission to the journal of Biotechnology titled: *Review of Biofilm Technologies as Upgrade Systems to Low Temperature Passive Treatment Systems* by W. Ahmed and R. Delatolla. This publication aims to review currently available nitrifying biofilm technologies as add-on solutions to passive treatment systems to attain nitrification in low temperatures. In addition, this study synthesizes current knowledge with respect to low temperature nitrifying MBBR systems for their optimal design and operations as upgrade post-carbon removal units.

Finally, Chapter 7 presents the discussion and conclusion of this dissertation. Also presented in this chapter are future directions and recommendations with respect to this research project.

## 1.5. Novelty and Key Contributions

With the findings of this research, four scientific journal manuscripts have been written and collated into the chapters of this manuscript-based thesis as described in the above Section 1.4. The novelty of the research in each chapter of this dissertation is listed below.

### **Publication 1 (Presented in Chapter 3):**

Ahmed, W., Tian, X., Delatolla, R., 2019. Nitrifying moving bed biofilm reactor: Performance at low temperatures and response to cold shock. *Chemosphere* 229, 295–302. (Published)

Author Contributions:

1. W. Ahmed: Contributed to the experimental design, performed the data collection, analysis, and wrote the manuscript.
2. X. Tian: Reviewed the data and the manuscript.
3. R. Delatolla: Developed the research question, the experimental design, directed the research, and revised the manuscript.

Chapter 3 presents the only temperature-controlled investigation of low temperature nitrifying MBBR systems using real wastewater and elevated TAN concentrations typical of sanitary sewer systems. This investigation is also the only study of cold shocks on attached growth nitrification kinetics down to 1°C. The findings of this study further improve the design and operation of low carbon nitrifying MBBR systems destined for low temperature operation. The key findings include the intrinsic removal rate of nitrifying MBBR systems at 1°C, the optimal design of these systems for the treatment of elevated TAN concentrations typical of non-combined sewers, the Arrhenius correction coefficients and a Theta model for low temperature modeling of

nitrifying MBBR systems performance down to 1°C, and the extent of cold shocks adverse effects on reactor performance during periods of snowmelt and periods of extreme weather events.

**Publication 2 (Presented in Chapter 4):**

Ahmed, W., Delatolla, R. Biofilm and microbiome response of attached growth nitrification systems across incremental decreases in temperatures (Manuscript submitted to the Journal of Water Process Engineering)

Author Contributions:

1. W. Ahmed: Contributed to the experimental design, performed the data collection, analysis, and wrote the manuscript.
2. R. Delatolla: Developed the research question, the experimental design, directed the research and revised the manuscript.

Chapter 4 is comprised of the first investigation on nitrifying biofilm and microbiome adaptations to low temperatures using both temperature-controlled experimentation and real wastewater to isolate the effects of decreasing temperatures on nitrifying MBBR systems down to the microbiome level. The findings of this study provide an in-depth understanding of the adaptation of nitrifying biofilms and nitrifying microbiome to low temperatures; thus, providing insights on the changes occurring in nitrifying MBBR systems during the onset of low temperatures.

**Publication 3 (Presented in Chapter 5):**

Ahmed, W., Delatolla, R. *Microbial response of nitrifying biofilms to cold shock*. (Manuscript submitted to the Journal of Environmental Science: Water Research & Technology)

Author Contributions:

1. W. Ahmed: Contributed to the experimental design, performed the data collection, analysis, and wrote the manuscript.

2. R. Delatolla: Developed the research question, the experimental design, directed the research and revised the manuscript.

Chapter 5 includes is the first and only study using modern molecular techniques to investigate the adverse effects of cold shocks on nitrifying biofilms but also the first study assessing cold shocked attached growth nitrification kinetics with extended exposures to 1°C. The findings in the chapter aim to provide additional knowledge on nitrifying MBBR systems' performance and microbiome response. The findings of this chapter include new insights on cold shocks' adverse effects to understand the effects on attached growth nitrification kinetics.

#### **Publication 4 (Presented in Chapter 6):**

Ahmed, W., Delatolla, R. *Review of biofilm technologies as upgrade systems to low temperature passive treatment systems*. (Manuscript in preparation for submission to the journal of Biotechnology)

Author Contributions:

1. W. Ahmed: Performed the data collection and analysis, the literature review and wrote the manuscript.
2. R. Delatolla: Developed the research question, the experimental design, directed the research and revised the manuscript.

Finally, Chapter 6 presents a review on the design of nitrifying attached growth systems available as add-on solutions to passive treatment systems to attain nitrification at low temperatures. More specifically, this review is focused on nitrifying biofilm technologies reported to have reached significant nitrification rates at temperatures below 15°C. For this aim, the performance, and the operational parameters of low temperature nitrifying RBC, TF, BAF; and, MBBR systems, as well as less common and emerging biofilm technologies, are summarized. In addition, a synthesis of key design parameters and the microbial response of 1°C nitrifying MBBR

systems is presented to guide the optimal design and operation of this new application of this technology.

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## Chapter 2. Literature Review

### 2.1. Wastewater Effluent Regulations

In Canada, wastewater effluent regulations for water resources recovery facilities (WRRF) are provided under the Fisheries Act. These wastewater systems effluent regulations (WSER) define limits for total suspended solids (TSS), carbonaceous biological oxygen demand (cBOD), total residual chlorine (Cl<sup>-</sup>); and, unionized ammonia nitrogen (NH<sub>3</sub>) as listed in Table 2.1.

Table 2.1. Federal effluent regulations

Constituent	Concentration (mg/L)
TSS	≤ 25
cBOD	≤ 25
Cl <sup>-</sup>	≤ 0.02
NH <sub>3</sub> -N	<1.25*

\*At 15 ± 1°C

The speciation of TAN is related to the pH and temperature where the fraction of unionized ammonia is determined with Equations 2.1 and 2.2, where  $T$  represents the temperature (°C), and  $pKa$  is the acid dissociation (Metcalf & Eddy, 2014).

$$pKa = 0.09 + \frac{2730}{273 + T} \quad 2.1$$

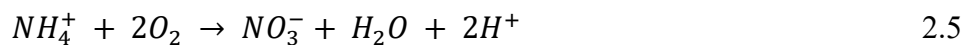
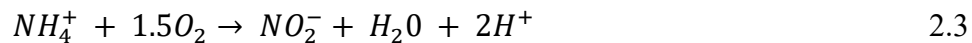
$$\%NH_3 = \frac{1}{1 + 10^{pKa - pH}} \times 100\% \quad 2.2$$

The other crucial WSER regulation is the acute lethality test, LC50 test, which is defined as 50% mortality of rainbow trout after 96 hours in 100% wastewater effluent (Canada Gazette, 2012). This regulation represents a challenge for Canadian WRRFs with their average municipal

TAN concentration of 20-30 mg/L when it is reported that concentrations of 15-20 mg/L fail the acute lethality test (Di Giulio and Hinton, 2008).

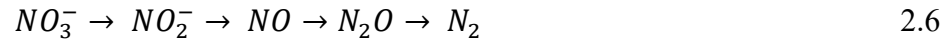
## 2.2. Nitrification Process

The most widely used and cost-effective means of treating ammonia in wastewater remains the biologically mediated process of nitrification (Balakrishnan and Eckenfelder, 1969; Metcalf & Eddy, 2014; Zhou et al., 2016). Nitrification is a two-step process with the overall reaction represented by Equation 2.5 which encompasses Equation 2.3, the oxidation of ammonia to nitrite reaction; or nitritation, as well as the Equation 2.4 the nitratation, the oxidation of nitrite to nitrate (Kindaichi et al., 2004; Monteiro et al., 2014). Dissolved oxygen (DO) requirements for the process of nitrification is 4.57 gO<sub>2</sub>/NH<sub>4</sub><sup>+</sup>-N oxidized as nitritation and nitratation requiring ratios of 3.43 gO<sub>2</sub>/g NH<sub>4</sub>-N and 1.14 gO<sub>2</sub>/g NO<sub>2</sub><sup>-</sup>-N respectively while the nitrification's alkalinity ratio is 7.14 gCaCO<sub>3</sub>/g<sup>1</sup>NH<sub>4</sub><sup>+</sup>-N (Metcalf & Eddy, 2014).



The nitrification end product, NO<sub>3</sub><sup>-</sup>, has also been reportedly capable of worsening eutrophication in rivers; moreover, nitrate tends to migrate to groundwater with its high aqueous solubility and negative charge where it has been identified as causing severe adverse health effects, including cancers (Gibert et al., 2008; Nuhoglu et al., 2002). There are currently no effluent regulations on effluent nitrate; however, the World Health Organization has set a NO<sub>3</sub><sup>-</sup>-N limit of 11.3 mg/L in drinking water while the United States Environmental Protection Agency (USEPA) has set the limit at 10 mg/L same as in Canada (WHO, 1984; USEPA, 1987; Health Canada, 2003). In WRRF, the biological process of denitrification is the most economical means of reducing NO<sub>3</sub><sup>-</sup>

concentration in wastewater (Schipper et al., 2010). Denitrification, or  $\text{NO}_3^-$  reduction to  $\text{N}_2$  gas, occurs through a series of intermediate products, as shown by Equation 2.6. It is estimated that a ratio of biological oxygen demand (BOD) to  $\text{NO}_3^-$  of 4 is needed for denitrification to occur while the alkalinity ratio is  $3.57 \text{ gCaCO}_3/\text{gNO}_3^-$ -N (Barth et al., 1968).



### 2.3. Nitrifying Bacteria

The process of nitrification in WRRFs is regulated by bacteria defined as nitrifiers with the first step mediated by ammonia-oxidizing bacteria (AOB) and the second step by nitrite oxidizing bacteria (NOB) (Kindaichi et al., 2004; Monteiro et al., 2014). The prominent AOBs observed in wastewater are *Nitrosomonas*, *Nitrospira* and *Nitrosococcus* (Rowan et al., 2003; Monteiro et al., 2014). *Nitrobacter* and *Nitrospira* have been identified as the main NOB with the latter identified as the more prominent organism in WRRFs (Wagner et al., 1996; Daims et al., 2001). Contrary to the bacteria regulating the nitrification process, a larger set of phylogenetically diverse bacteria have been identified for the denitrification process (Knowles, 1982; Knowles, 1996; Verbaendert et al., 2011). Bacteria such as *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Flavobacterium*, *Halobacterium*, *Hypomicrobium*, *Methanomonas*, *Moraxella*, *Neisseria*, *Paracoccus*, *Propionibacterium*, *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Spirillum*, and *Vibrio* amongst other genera were identified as denitrifiers in full-scale WRRF (Payne, 1976; Gayle, 1989; Lu et al., 2014; Metcalf & Eddy, 2014).

### 2.4. Bacterial Growth and Energetics

The sum of enzyme-mediated oxidation-reduction reactions in bacteria defined as the metabolism is comprised of anabolic processes (maintenance and synthesis) and catabolic processes with the latter providing the cellular energy in the stored as adenosine triphosphate (ATP) and required by the former (Wessmann et al., 2006; Metcalf & Eddy, 2014). Nitrifiers are classified as chemolithoautotrophs and use inorganic chemical compounds as electron donors and an inorganic carbon source in their metabolic processes. AOBs are aerobes that degrade  $\text{NH}_4^+$  as their electron donor and oxygen as their electron acceptor; similarly, NOBs are also aerobes by using oxygen as their electron acceptor; however, they use  $\text{NO}_2^-$  as the electron donor (Figure 2.1).

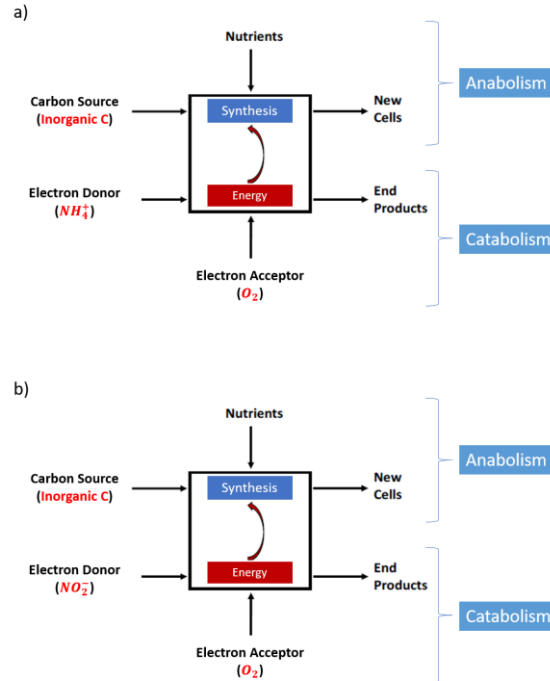


Figure 2.1. Metabolism of a) AOB and b) NOB adapted from Metcalf & Eddy (2014).

The growth of these organisms is characterized by four phases, which are the lag phase, the growth phase, the stationary phase and the death phase, as demonstrated in the Monod Growth curve shown in Figure 2.2 (Monod, 1949). The lag phase is the period needed by cells to adapt to a new environment, which is accompanied by increased activity in cellular metabolism; the log (or

exponential) growth phase is characterized by cellular division; the stationary phase is characterized in a cessation of growth; the death phase is characterized by decay and decrease in the number of cells.

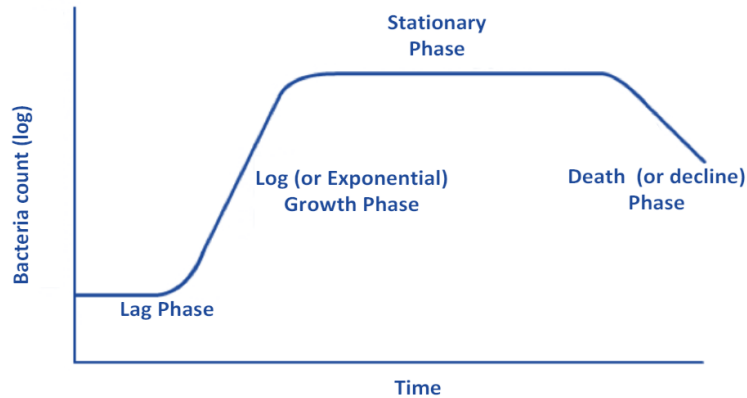


Figure 2.2. Bacterial growth curve modified from Metcalf & Eddy (2004).

## 2.5. Biofilms

A biofilm is the aggregation of organisms embedded in extracellular polymeric substances (EPS) (Costerton et al., 1999; Flemming and Wingender 2010). As bacteria aggregate into biofilms, they transition from their planktonic state to a sessile state in which they can conduct new metabolic processes beneficial to the survival, maintenance and proliferation of the biofilm community (Davey and O'toole, 2000; Donlan, 2002; Dunne, 2002).

## 2.6. Mass Transfer Limited Kinetics

In the attached growth systems, substrate utilization occurs within the biofilms growing on support media. As displayed in Figure 2.3, a stagnant liquid layer, or mass transfer boundary layer (MTBL), separates the biofilm from the bulk liquid through which substrates transition through in order to be degraded within the biofilm. Substrate consumption is diffusion limited in attached growth systems as substrate concentration is reduced with biofilm depth. The rate of mass transfer

through the MTBL is expressed as the mass per area per unit of time ( $\text{gN}/\text{m}^2\text{d}$ ) as in Equation 2.7, while the rate of mass transfer and substrate utilization rates within the biofilm are given by Equations 2.8 and 2.9.

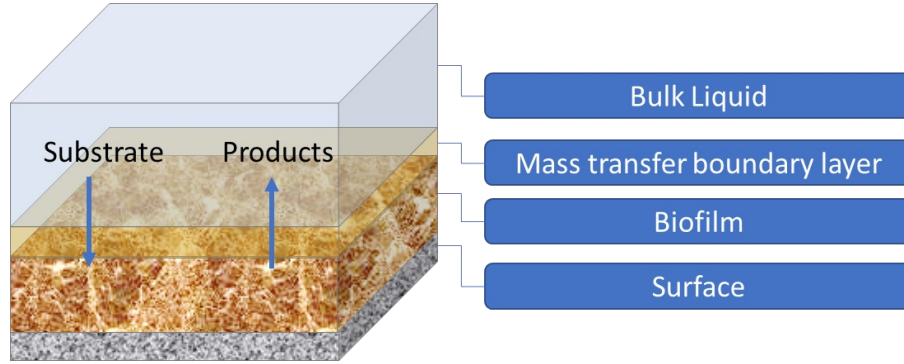


Figure 2.3. Cross-sectional representation of a biofilm and support media.

$$r_{sf} = -D_w \frac{dS}{dx} = -D_w \frac{(S_b - S_s)}{L} \quad 2.7$$

Where  $r_{sf}$  = rate of substrate surface flux,  $\text{gN}/\text{m}^2\text{d}$

$D_w$  = diffusion coefficient of substrate in water,  $\text{m}^2/\text{d}$

$dS/dx$  = substrate concentration gradient,  $\text{g}/\text{m}^3 \cdot \text{m}$

$S_b$  = bulk liquid substrate concentration,  $\text{g}/\text{m}^3$

$S_s$  = substrate concentration at outer layer of biofilm,  $\text{g}/\text{m}^3$

$L$  = effective thickness of stagnant film,  $\text{m}$

$$r_{bf} = -D_e \frac{dS_f}{dx} \quad 2.8$$

Where  $r_{bf}$  = rate of substrate flux in biofilm due to mass transfer,  $\text{gN}/\text{m}^2\text{d}$

$D_e$  = effective diffusivity coefficient in biofilm,  $\text{m}^2/\text{d}$

$dS_f/dx$  = substrate concentration gradient,  $\text{g}/\text{m}^3\text{m}$

$$r_{su} = \frac{kS_f X}{K_s + S_f} \quad 2.9$$

where  $r_{su}$  = rate of substrate utilization in biofilm, gN/m<sup>2</sup>d

$S_f$  = substrate concentration at a point in the biofilm, g/m<sup>3</sup>

## 2.7. Biofilm Technologies

### 2.7.1. Rotating Biological Contactors

RBC systems are one of the oldest attached growth systems currently employed for biological ammonia removal. Conventionally, these bioreactors consist of rotating discs made from various materials mounted on a rotating horizontal shaft partially submerged in wastewater; typically, a submergence of 40% (Dutta et al., 2007; Ravi et al., 2013). RBC parameters such as media types, rotation speed, staging configuration, organic loading rate, recycling regime, hydraulic retention time and submergence all affect nitrification rates in this type of biofilm system (Bagchi et al., 2012; Feng et al., 2017; Hassard et al., 2015; Ishiguro, 1983; Pal et al., 2016; Patwardhan, 2003; Peters and Wu, 1984; Torretta et al., 2017). For low temperature applications of RBC systems employed in municipal treatment plants have been reported capable of reaching significant nitrification rates at an average temperature of 13°C (Miller et al., 1981). At temperatures below 13°C, a significant decline in nitrification rates are observed as approximately 2.5 times more surface area will be required in order to attain the same removal rates recorded at 13°C (Rodgers and Zhan, 2003). Moreover, in these types of systems, it is estimated that a performance loss of 4.5%/°C occurs as temperature declines (Nowak, 2000). Nonetheless, Kapoor et al. (2003) have reported that with proper design and operation of nitrifying RBC systems, significant removal efficiencies of 95% can be attained at temperatures as low as 8°C.

Pano and Middlebrooks (1983) proposed the Equation 2.10 to establish the relationship between nitrification, temperature and the organic loads as chemical oxygen demand (COD) in RBC systems with  $f_i$  representing the fraction of maximum nitrification rate and  $M$  the organic load as gCOD/m<sup>2</sup>d when it is between 4.3 and 14.3 gCOD/m<sup>2</sup>d. The fraction of the maximum removal rate can then be substituted in Equation 2.11 to determine the surface area removal rate (SARR) with respect to temperature and organic load in RBC treatment trains. The modeled nitrification rate at a given temperature is represented by  $k_n$  while  $A$  and  $K_N$  represent the total surface area and the half saturation constant. The terms  $C_0$ ,  $C_f$ ,  $C_{1M}$ ,  $C_2$ ,  $C_3$ , and  $C_4$  represent the influent concentration, the effluent concentration and the influent concentrations in the stages 1, 2, 3 and 4 respectively.

$$f_i = 1.43 - 0.1M \quad 2.10$$

$$\frac{Q(C_0 - C_f)}{\sum_{i=1} A} = k_N \left[ \frac{f_i C_{1M}}{K_N + C_{1M}} + \frac{C_2}{K_N + C_2} + \frac{C_3}{K_N + C_3} + \frac{C_4}{K_N + C_4} \right] \quad 2.11$$

### 2.7.2. Trickling Filters

Another biofilm technology utilized for ammonia treatment in wastewater is the trickling filter (TF), which is a non-submerged fixed film bioreactor. These biofilm systems have a fixed media, a wastewater distribution system which distributes the influent on the media and a drainage system to collect the effluent as main components (Wik, 2004; Vianna et al., 2012; Ali et al., 2017). Three types of configuration could be used to attain nitrification in TF systems; a single pass configuration with a single reactor in which nitrification occurs at lower segments of the bioreactor once the organic matter has been consumed in the upper segments; a recirculation configuration with a single reactor in which the effluent is recirculated to the top of the reactor to perform nitrification; and finally an in-series configuration in which nitrification is performed in the latter bioreactors in the treatment train once the organic load has been reduced in the first bioreactors

(Brooks et al., 1984; Tekerlekopoulou and Vayenas, 2003; Muñoz-Sánchez et al., 2013; Zhang et al., 2015; Ali et al., 2017; Bornemann et al., 2018).

Studies have demonstrated that single pass TF systems attain lower and inconsistent performance to reach effluent targets compared to other TF configurations as nitrification rates are hindered by the availability of organic matter untreated in the upper levels of the media (Anderson et al., 1994; Daigger et al., 1994; Parker et al., 1995; Surampalli et al., 1995; Pearce, 2004). A conventional design surface area loading rates (SALR) for these systems are between 0.2 and 1.0 gN/m<sup>2</sup>d for mixed biomass reactors destined for cBOD<sub>5</sub> and ammonia removal, while loading rates are 0.5 to 2.4 gN/m<sup>2</sup>d for systems destined for nitrification only (Daigger and Boltz, 2011).

Gujer and Boller (1986) proposed the Equation 2.12 to determine SALR with respect to temperature where  $SARR_{max,T}$  is the maximum attainable removal rate at a given temperature that could be modeled using an Arrhenius temperature correction.

$$SALR = SARR_{max,T} \cdot \frac{C_f}{K_N + C_f} \quad 2.12$$

To determine the SARR in relation to the temperature and depth within the bioreactor Parker et al. (1989) proposed Equation 2.13 when the increase in depth influences nitrification rate and the Equation 2.14 for when no change in nitrification rate is assumed with the increase in depth. In these Equations,  $\alpha$  represents the specific surface area of the media (m<sup>2</sup>/m<sup>3</sup>),  $k$  the empirical nitrification rate decline with depth (m<sup>-1</sup>) between 0 and 0.16 (m<sup>-1</sup>),  $v_h$  represents the hydraulic loading rate, including recirculation if present (m<sup>3</sup>/m<sup>2</sup>d) and finally  $z$  represents the depth (m).

$$\frac{\alpha \cdot SARR_{max,T}}{k \cdot v_h} \cdot (1 - e^{-k \cdot z}) = C_0 - C_f + K_N \cdot \ln\left(\frac{C_0}{C_f}\right) \quad 2.13$$

$$\frac{\alpha \cdot SARR_{max,T}}{v_h} = C_0 - C_f + K_N \cdot \ln\left(\frac{C_0}{C_f}\right) \quad 2.14$$

The Equation 2.15 provides the relationship of the oxygen transfer efficiency and SARR at a given temperature between 5 and 25°C where  $E_{O_2}$  is the dimensionless effectiveness factor of the media ranging from 0.93 to 0.96 (Gujer and Boller, 1986) and  $SARR_{O_2, max, T}$  the maximum oxygen flux a given temperature.

$$SARR_{max,T} = E_{O_2} \cdot \frac{SARR_{O_2, max, T}}{4.3} \cdot \frac{C_f}{K_N + C_f} \quad 2.15$$

### 2.7.3. Biological Aerated Filters

Biologically active filters (BAF) are fixed film submerged bioreactors employed for the process of nitrification in the last several decades in WRRFs (Payraudeau et al., 2001; Chang et al., 2009). This type of reactor can be characterized by the direction of the flow, and further the fluid velocity, the type of media, the backwashing regime. For low-temperature applications, nitrifying BAF systems have been reported to perform better than non-submerged and partially submerged attached growth systems reviewed above and activated sludge reactors, as reported by Hansen et al. (2007) in their long-term study. Paffoni et al. (1990) demonstrated that BAF systems are robust add-on units as tertiary treatment solutions for existing WRRF with rates of 500 gN/m<sup>3</sup>d at temperatures between 12°C and 13°C.

Delatolla et al. (2009) demonstrated that these bioreactors are capable of maintaining nitrification despite long exposures to low temperatures down to 4°C, with removal rates as high as 16% of the rates recorded at 20°C. The exposure time to low temperatures is an additional crucial factor of nitrification as demonstrated by the Delatolla et al. (2009) Theta model characterized by Equation 2.16 in which the correction coefficient increases with the elapsed time  $t$ .

$$\theta = 391 \times 10^2 \cdot \ln(t) + 9.83 \times 10^{-1} \quad 2.16$$

These same authors have demonstrated the severe adverse effects of cold shocks on nitrification kinetics with a drastic decrease in the nitrifying BAF systems' efficiency of 56%. At the low temperature of 4°C, these nitrifying BAF systems are capable of reaching removal rates of 180 gN/m<sup>3</sup>d (Delatolla et al., 2010).

#### **2.7.4. Moving Bed Biofilm Reactors**

MBBR systems are recent attached growth systems developed four decades ago with free-floating carriers serving as support media for the biofilm; mixing is provided through aeration, which also provides the DO required for processes such as nitrification (Oleszkiewicz and Barnard, 2006). The search for a compact and economical treatment solution has led to the design of this type of bioreactor which, in addition benefits inherent to biofilm technologies, present their own advantages such as a small footprint and a simplistic operation (Ødegaard et al., 1994; Ødegaard, 1999; Ødegaard, 2006).

The capability of MBBR systems to perform nitrification at low temperatures has been demonstrated for several decades, as listed in Table 2.2. In a study between 5°C and 15°C, Andreotolla et al. (2000) compared the performance of MBBR and RBC systems and determined that MBBR systems maintained stable rates of 0.9 gN/m<sup>2</sup>d below 8°C as opposed to the RBC system. At lower temperatures, MBBR systems are capable of reaching significant removal rates of 140 gN/m<sup>3</sup>d at 4°C, rates comparable to the more operational intensive BAF system (Delatolla et al., 2010). The same authors have demonstrated that a theta model accounting for the exposure time at low temperatures in nitrifying BAF has accurately predicted the adverse effects of exposure time on the nitrifying MBBR systems kinetics as well.

The low operational intensity of MBBR systems makes them good candidates as add-on technologies for lagoon facilities to attain low temperature nitrification as demonstrated at the pilot scale (Delatolla et al., 2010; Houweling et al., 2007; Ødegaard, 2006). These studies on add-on nitrifying MBBR systems determined the importance of not placing these systems between the lagoons of a multipond facility but at the end to conduct nitrification under low carbon conditions.

Following these findings, post-carbon removal low temperature nitrifying MBBR systems have been investigated down to 1°C. This type of biofilm technology has been shown capable of attaining nitrification rates as elevated as 0.35 gN/m<sup>2</sup>d at temperatures as low as 1°C and removal efficiencies on average 20% of the efficiencies attainable at 20°C during extended operations at both the pilot and laboratory scale (Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017).

Table 2.2. Overview of low temperature nitrifying MBBR systems studies.

<b>Study scale &amp; Media</b>	<b>T (°C)</b>	<b>Key Findings</b>	<b>References</b>
Pilot & Lab scale Polyethylene carriers; SA = 200 m <sup>2</sup> /m <sup>3</sup>	15°C	<ul style="list-style-type: none"> <li>• Nitrification oxygen limited at 2 gO<sub>2</sub>/gTAN and ammonia limited above 5 gO<sub>2</sub>/gTAN.</li> <li>• Transition from ammonia limited to oxygen limited conditions at 2.7 gO<sub>2</sub>/gTAN for 9-10 gO<sub>2</sub>/m<sup>3</sup> and 3.2 gO<sub>2</sub>/gTAN for 6 gO<sub>2</sub>/m<sup>3</sup>.</li> <li>• Nitrification severely impeded at organic loads higher than 5 gBOD<sub>7</sub>/m<sup>2</sup>d.</li> </ul>	(Hem et al., 1995)
Full-scale Hydroxyl-Pac; SA =568 m <sup>2</sup> /m <sup>3</sup>	8 - 21	<ul style="list-style-type: none"> <li>• Removal efficiencies of 91.4% below 12°C and 92.4% below 15°C.</li> <li>• Inner interim biofilm thinner than the inner edge biofilm in the summer while they display the same thickness in the winter.</li> </ul>	(Bjornberg et al., 2009)
Pilot-scale Plastic Carriers; SA =310 m <sup>2</sup> /m <sup>3</sup>	7 - 18	<ul style="list-style-type: none"> <li>• Nitrification rate post-denitrification stage of 1.24 gN/m<sup>2</sup>d.</li> <li>• Nitrification rate pre-denitrification stage of 1.01 gN/m<sup>2</sup>d.</li> </ul>	(Rusten et al., 1995)

Full & Pilot-scale Plastic carriers. SA = 300 m <sup>2</sup> /m <sup>3</sup>	6 - 15	<ul style="list-style-type: none"> <li>• 20-25% higher nitrification rates post denitrification stage.</li> <li>• Nitrification inhibited at organic loads greater than 4.5 gbsCOD/m<sup>2</sup>d.</li> <li>• Increasing DO concentrations mitigate the negative effects of decreasing temperatures.</li> <li>• Correction coefficient of <math>\theta = 1.09</math> between the temperature of 7 and 18°C.</li> <li>• Nitrification rates at 15°C of 0.35 and 0.15 gN/m<sup>2</sup>d under ammonia and oxygen-limiting conditions.</li> <li>• Average removal efficiencies of 76% at 13.7°C.</li> <li>• An increase in DO limits the negative effects of decreasing temperatures on nitrification rates.</li> <li>• Nitrification is oxygen limited at 2-3 mgTAN/L.</li> </ul>	(Ødegaard et al., 1999)
Pilot-scale Plastic carriers; SA = 300 m <sup>2</sup> /m <sup>3</sup>	3 - 14	<ul style="list-style-type: none"> <li>• Nitrification rates of: 1.2 -1.5 gN/m<sup>2</sup>d at 15°C 1.2 gN/m<sup>2</sup>d at 11°C 1.17 gN/m<sup>2</sup>d at 10°C.</li> <li>• Primary factors influencing nitrification rates are the organic load, TAN and DO concentrations.</li> <li>• The nitrification rate linearly dependent on the oxygen concentration at 10 mgO<sub>2</sub>/L and above.</li> </ul>	(Ødegaard, 2006)
Pilot-scale Kaldnes media	3 -15	<ul style="list-style-type: none"> <li>• Nitrification rate of 210 gN/m<sup>3</sup>d at 8°C 140 gN/m<sup>3</sup>d at 4°C</li> <li>• Significant nitrification rates MBBR attained under extended operation (3.5 months) at 6-8°C.</li> <li>• Exposure time to low temperatures identified as an important factor of nitrification kinetics.</li> <li>• Nitrification rates accurately predicted by Delatolla et al. (2009) Theta model accounting for exposure times at 4°C.</li> </ul>	(Delatolla et al., 2010)
Full-scale AnoxKaldnes K1 carriers; SA =350 m <sup>2</sup> /m <sup>3</sup>	2 - 8	<ul style="list-style-type: none"> <li>• Removal efficiencies of 40% at 4.2°C.</li> </ul>	(Wessman and Johnson 2006)

Pilot-scale AnoxKaldnes K1 carriers; SA = 500 m <sup>2</sup> /m <sup>3</sup>	1 - 20	<ul style="list-style-type: none"> <li>• Nitrification rates of 110 and 120 gN/m<sup>3</sup>d at 1°C.</li> <li>• Nitrification rates at 1°C are 40% of the rate attainable at 20°C.</li> <li>• The transition from 1 to 20°C is well predicted by the Delatolla et al. (2009) theta model with a strong correlation (R<sup>2</sup> = 0.79 and 0.9).</li> </ul>	(Almomani et al., 2014)
Lab-scale AnoxKaldnes K3 carriers; SA = 500 m <sup>2</sup> /m <sup>3</sup>	1 - 20	<ul style="list-style-type: none"> <li>• The relative removal efficiency of 40% at 1°C compared to the recorded efficiency 20°C.</li> <li>• Nitrification rate threshold temperature at 5°C, below which a significant decrease in the removal rate is observed.</li> <li>• Nitrification rates at 1°C between 18.7± 5.5% and 15.7 ± 4.7% of the rates attainable at 20°C.</li> <li>• No significant changes in the mass of biofilm between 20 and 1°C.</li> <li>• Temperature correction coefficient <math>\theta = 1.072</math> and <math>1.09</math> resulted in modeled rates 30% higher than the measured rates for <math>T_1 = 17.5^\circ\text{C}</math> and <math>T_2 = 1^\circ\text{C}</math>.</li> <li>• Measured rates at 1°C were well predicted by the Delatolla et al. (2009) theta model (R<sup>2</sup> = 0.86).</li> </ul>	(Hoang et al., 2014a)
Lab-scale AnoxKaldnes K5 carrier; SA 800 m <sup>2</sup> /m <sup>3</sup>	1 - 20	<ul style="list-style-type: none"> <li>• Increase in biofilm thickness after 4 months at 1°C.</li> <li>• -Increase in viable cell coverage after 4 months at 1°C.</li> <li>• Nitrosomonas and Nitrospira identified as main AOB and NOB.</li> </ul>	(Hoang et al., 2014b)
Pilot-scale AnoxKaldnes K5 carriers; SA = 800 m <sup>2</sup> /m <sup>3</sup>	1 - 20	<ul style="list-style-type: none"> <li>• Nitrification occurs 22 hours after reactor startup with seeded carriers from a carbon-rich environment.</li> <li>• 18 days to attain steady state nitrification kinetics at 1°C.</li> <li>• The transition from a carbon-rich environment to low carbon environment led to a sloughing event and a microbial community shift characterized by an increase in AOB, NOB and extracellular polymeric substance.</li> </ul>	(Young et al., 2016)
Pilot-scale AnoxKaldnes K5 carrier;	1 - 20	<ul style="list-style-type: none"> <li>• Nitrification rate of 0.35 gN/m<sup>2</sup>d at 1°C.</li> </ul>	(Young et al., 2017)

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## **Chapter 3. Nitrifying Moving Bed Biofilm Reactor: Performance at Low Temperatures and Response to Cold Shock Operation**

### **3.1. Context**

Chapter 3 presents the study published in the journal of Chemosphere under the title: *Nitrifying moving bed biofilm reactor: Performance at low temperatures and response to cold shock* by W. Ahmed, X. Tian and R. Delatolla, (Chemosphere, 2019. 229, 295–302). The low temperature and cold shocked attached growth nitrification kinetics down to 1°C in the MBBR system are investigated in this study which is the only temperature-controlled investigation of low temperature nitrifying MBBR systems using real wastewater and elevated TAN concentrations typical of sanitary sewer systems. This investigation is also the only study of cold shocks on attached growth nitrification kinetics down to 1°C.

### **3.2. Abstract**

In contrast with suspended growth systems, attached growth technologies such as the moving bed biofilm reactors (MBBR) have recently been proven capable of achieving significant nitrification rates at temperatures as low as 1°C. The purpose of this study was to investigate the performance of the nitrifying MBBR system at elevated municipal concentrations with exposures to low temperatures and cold shock conditions down to 1°C with an enhanced temperature-controlled room. A removal rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d was identified as the intrinsic rate of nitrifying MBBR systems at 1°C and thus also proposed as the conservative rate for low temperature design of these systems. A temperature threshold between 2°C and 4°C was identified at which attached growth nitrification displays a significant decrease in kinetics. Arrhenius correction coefficients of 1.086 and 1.09 previously applied for low temperature nitrifying MBBR systems resulted in conservative modeled removal rates on average 21% lower than the measured

rates. Thus, an Arrhenius correction coefficient of 1.049 is proposed between the temperatures of 10°C and 4°C and another correction coefficient of 1.149 to model rates at 1°C. For the transition from 4°C to 1°C, the adjustment of a previously reported Theta model is proposed in this study to account for exposure time at low temperatures; with the modified model showing strong correlation with measured rates ( $R^2 = 0.88$ ). Finally, a comparison of nitrification kinetics between MBBR systems acclimatized to 1°C and systems that are cold shocked to 1°C demonstrated that shocked removal rates are 21% lower.

### **3.3. Introduction**

Eutrophication and water toxicity in surface waters are caused by nutrient discharge of water resource recovery facilities (WRRF) (Murdoch et al., 2000; Driscoll et al., 2003; Preston et al., 2011). To mitigate the adverse effects of nutrients such as ammonia in surface waters, various countries are introducing increasingly stringent regulations for point source polluters (EEC, 1991; Canada Gazette, 2012; USEPA, 2016). The biologically mediated ammonia removal process of nitrification is one of the most economical means of treating ammonia in wastewater and meeting discharge regulations (Balakrishnan and Eckenfelder, 1969). Nitrification is a two-step process regulated by two phylogenetically different autotrophic nitrifiers, with the first step, nitritation, being regulated by ammonia oxidizing bacteria (AOB) and the second step, nitratation, being regulated by nitrite oxidizing bacteria (NOB) (Kindaichi et al., 2004; Monteiro et al., 2014).

Many conventional suspended growth systems are capable of effectively reducing carbonaceous biochemical oxygen demand, total suspended solids as well as achieving nitrification; however, low temperatures have shown a well-reported adverse effect on nitrification with a positive correlation between decreasing kinetics and decreasing temperatures in these systems (Painter and Loveless, 1983; Van Dyke et al., 2003; Ducey et al., 2010; Champagne et al.,

2017; Chen et al., 2018). On the other hand, attached growth systems have been reported capable of attaining significant nitrification rates at temperatures as low as 1°C (Almomani et al., 2014; Hoang et al., 2014a; Young et al., 2016). For over two decades, attached growth systems, such as the MBBR technology, have been investigated as add-on nitrifying units to achieve desired discharge objectives (Rusten et al., 1995a; Houweling et al., 2007; Delatolla et al., 2010; Leyva-Díaz et al., 2017). The many inherent advantages of MBBR systems, such as their low operational intensity, renders these systems potential candidates as add-on units for passive treatment systems (Ødegaard, 2006; Forrest et al., 2016). Moreover, these systems benefit from the advantages innate to biofilm technologies; such as small land footprints, low emission of effluent solids, resilience to environmental changes such as temperature, and the capacity to maintain active slow growing organisms such as nitrifiers (Wang et al., 2005; Barwal and Chaudhary, 2014; Dezotti et al., 2018).

Although attached growth systems have demonstrated the capacity to achieve significant nitrification rates at temperatures as low as 1°C at both the laboratory and pilot scale, these studies were either conducted with synthetic wastewater or with real wastewater but when using real wastewater were performed with limited temperature control (Hoang et al., 2014a; Young et al., 2016; Young et al., 2017a). Thus, the intrinsic removal rate at a controlled temperature of 1°C using real wastewater, expressly the conservative rate at low temperatures, has yet to be precisely quantified. Furthermore, the above-mentioned field and laboratory scale studies on low temperature nitrification were conducted with an average influent total ammonia nitrogen (TAN) concentration of 20 mg/L, representative of effluents from lagoon treatment systems fed by combined sewer systems. However, current engineering standards in numerous countries dictate that sewer systems be divided into sanitary sewers and stormwater sewers (Diogo et al., 2018). Moreover, combined sewer systems are to be transitioned into two distinct collectors to

mitigate the effects of large storm events and provide overflow control. This results in the raw wastewater in collectors no longer being diluted by stormwater runoff, thus resulting in higher influent nutrient concentrations entering WRRF, which can impact the design and operation of post-carbon removal, lagoon upgrade nitrifying MBBR systems (Delatolla and Babarutsi, 2005). With no studies in the current literature having been performed on nitrifying MBBR systems treating non-combined sewer municipal wastewater at 1°C, a gap of knowledge exists with respect to the performance of nitrifying MBBR systems treating elevated ammonia concentrations from non-combined sewers at very low temperatures.

The temperature threshold below which a significant decrease in MBBR nitrifying kinetics occurs due to the adverse effects of low temperatures also has yet to be precisely identified. Hoang et al. (2014a) have suggested a potential threshold temperature of 5°C or below in their investigation conducted at 20°C, 5°C and 1°C. Due to the limited number of temperatures investigated in the above-mentioned study, an investigation identifying the temperature threshold of attached growth nitrification kinetics by means of incremental decreases in temperatures is required to precisely anticipate the kinetic response to low temperature operation of nitrifying MBBR systems.

The Arrhenius temperature correction coefficient has been used in previous studies to model removal rates that account for temperature changes in nitrifying MBBR systems (Rusten et al., 1995b; Salvetti et al., 2006; Zhang et al., 2014). The change in removal rate due to changes in temperature is determined by Equation 3.1, where  $k_1$  and  $k_2$  represent the removal rates (gN/m<sup>3</sup>d),  $T_1$  and  $T_2$  the temperatures in degree Celsius (°C) and  $\theta$  the Arrhenius temperature correction coefficient.

$$k_2 = k_1 \cdot \theta^{(T_2 - T_1)} \quad 3.1$$

Salveti et al. (2006) have reported the commonly used correction coefficient values of 1.098 and 1.058 for ammonia limited and oxygen limited attached growth nitrification (Water Environment Federation, 2011). However, these correction coefficients have been reported for temperature values above 10°C. Previous studies on MBBR systems acclimatized to 1°C have demonstrated the potential of this technology to perform low temperatures nitrification (Almomani et al., 2014; Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016, Young et al., 2017a, Young et al., 2017b). In a recently published study on 1°C nitrifying MBBR, Arrhenius correction coefficients of 1.086 and 1.09 were applied to temperatures below 12°C and demonstrated strong correlations ( $R^2 = 0.89$ ) between the measured and modeled removal rates (Young et al., 2017a). Although Equation 3.1 can be used to model the temperature effects on the nitrification rates, it does not take into account another important factor; the exposure time. Delatolla et al. (2009) proposed Equation 3.2, a temperature dependent Equation to model the attached growth nitrification rates as a function of elapsed time at 4°C; where  $\theta$  represents the Arrhenius temperature correction coefficient and  $t$  the elapsed time in days.

$$\theta = 3.81 \times 10^{-2} \cdot \ln(t) + 9.83 \times 10^{-1} \quad 3.2$$

Young et al. (2017a) have reported, in their application of the Delatolla et al. (2009) Theta model, a strong correlation ( $R^2 = 0.77$ ) between measured and modeled attached growth nitrification rates during the temperature transition from 5°C to 1°C. Nevertheless, Arrhenius temperature correction coefficients and a theta model factoring the exposure time have yet to be investigated as low as 1°C with enhanced temperature control.

To achieve efficient operation of nitrifying MBBR systems, it is essential to evaluate the effects of cold shocks on nitrification kinetics not only to account for periods of snowmelt, but also the projected increase in frequency of extreme weather events and cold air outbreaks (Gao et

al., 2015; Kanno et al., 2016; Champagne et al., 2017; Smith and Sheridan, 2018). Hwang and Oleszkiewicz (2007) in a comparison study between a shocked nitrifying reactor and a reactor acclimatized by incremental decreases in temperature, reported 20% lower nitrification rates for the shocked system. However, in the above study the cold shock experiment was conducted with suspended growth systems and at temperatures above 10°C. For MBBR systems, the lowest temperature reached in a previous study on cold shock was only 4°C (Delatolla et al., 2010). Thus, cold shock treatment studies down to 1°C for nitrifying MBBR systems have yet to be conducted and hence require investigation to further our understanding of the potential of MBBR units as lagoon upgrade systems.

The overall aim of this work is to optimize the design of low carbon, nitrifying MBBR systems as upgrade units to lagoon treatment systems by identifying their performance at influent TAN concentrations typical of non-combined sanitary sewers during the exposure to low temperatures down to 1°C and cold shock operation to 1°C. The specific objectives of this study are to determine the intrinsic ammonia removal rate of nitrifying MBBR systems at 1°C; to determine the temperature threshold below which nitrification kinetics decrease significantly; to identify the Arrhenius temperature correction coefficients for attached growth nitrification at low temperatures; and to delineate the extent of cold-shock adverse effects on attached growth nitrification kinetics at low temperatures during long operation. These objectives are to be investigated using enhanced temperature-controlled experiments and real wastewater while operating MBBR systems to meet an effluent TAN ( $TAN_{eff}$ ) concentration of 10 mg/L.

## **3.4. Materials and Methods**

### **3.4.1. Experimental Setup**

This study was conducted in an enhanced temperature-controlled room to ensure operational temperature precision for the kinetics testing of three laboratory scale MBBR reactors (Figure 3.1). The three MBBR reactors; reactor 1 (R1), reactor 2 (R2) and reactor 3 (R3) with volumes of 2.2 L, 2.2 L and 0.75 L respectively were utilized in this study. The smaller volume of R3 relative to R1 and R2 was selected to accommodate the limited volume of feed wastewater transported to the testing laboratory. The reactors housed AnoxKaldnes K5 carriers (Lund, Sweden) with diameters of 25 mm, heights of 4 mm and surface area to volume ratios of  $800 \text{ m}^2/\text{m}^3$ . The K5 carriers were seeded carriers collected from a partially nitrifying, carbon removal integrated fixed film activated sludge facility located in Hawkesbury, Ontario, Canada, and were operated within benchtop nitrifying MBBR. The fill percentages of the reactors were set below the 67% maximum recommended for MBBR systems (Rusten et al., 2006). Aeration was supplied to the reactors with an air pump and air diffusers to provide dissolved oxygen (DO) and allow adequate mixing of the carriers in the reactors. MBBR systems for a period of seven months before the start of this study (Young et al., 2017).

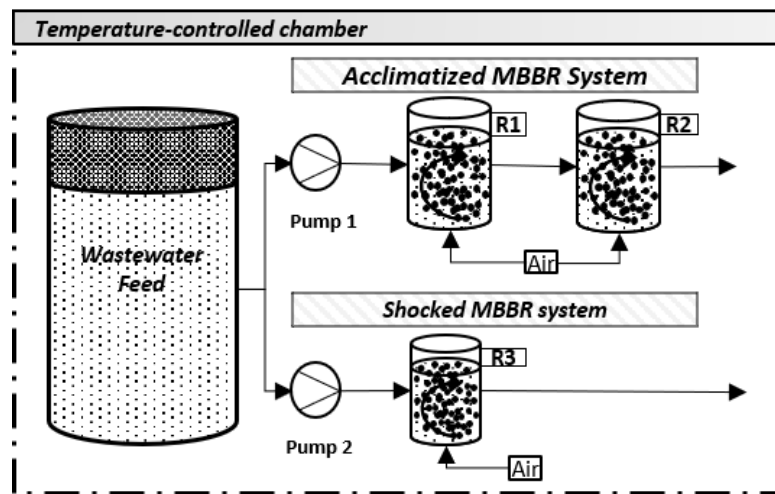


Figure 3.1. Experimental setup of the acclimatized and shocked MBBR systems in the temperature-controlled room.

### 3.4.2. Wastewater Feed

All three reactors were fed with an effluent collected from the outlet of a continuous flow; partially aerated multipond lagoon facility fed by a non-combined sewer system. The characteristics of the lagoon facility's effluent, which was also used as the feed to the MBBR reactors in this study, are shown in Table 3.1.

Table 3.1. Feed characteristics for the acclimatized and shocked MBBR systems.

<b>Constituent</b>	<b>Concentration (mg/L)</b>
TAN (NH <sub>4</sub> <sup>+</sup> /NH <sub>3</sub> -N)	39.97 ± 0.36
Nitrite (NO <sub>2</sub> <sup>-</sup> -N)	0.08 ± 0.02
Nitrate (NO <sub>3</sub> <sup>-</sup> -N)	3.22 ± 0.03
Biochemical oxygen demand (BOD)	13.13 ± 0.7
Alkalinity (CaCO <sub>3</sub> )	303.41 ± 2.94
Total suspended solids (TSS)	7.09 ± 0.62
Volatile suspended solids (VSS)	5.87 ± 0.61

### 3.4.3. Reactor Operation

The fully inoculated R1 system was operated at decreasing temperatures from 10°C to 1°C in a temperature-controlled room in decrements of 2°C to ensure a slow acclimatization to each temperature. Specifically, R1 was operated at 10°C, 8°C, 6°C, 4°C, 2°C and 1°C. R1 was operated at each incremental decrease in temperature until steady state kinetics were achieved, with steady state operation being defined as removal rate fluctuations within ± 10%. R1 was operated for a minimum of 20 hydraulic retention times at each temperature value in addition to demonstrating steady operation at each temperature investigated in this study. The operation of R1 was used to identify a potential kinetic threshold temperature of nitrifying MBBR systems operating at low

temperatures, to quantify the intrinsic Arrhenius correction coefficient of nitrifying MBBR systems and to determine the intrinsic removal rate of nitrifying MBBR systems at 1°C.

To determine the optimal design of nitrifying MBBR at 1°C treating TAN concentrations typical of non-combined sanitary sewers, R1 was operated for 73 days at 1°C with reactor R2 placed in-series to R1. R2 was again fully inoculated and was subjected to the same temperature acclimatization regime as R1 prior to being placed in-series to R1. The operation of R2 in-series to R1 enabled the design of a single MBBR reactor (R1) to be compared to the operation of two MBBR reactors in-series (R1 and R2) at 1°C. R1 and R2 were operated for 73 days at 1°C to study the performance of the reactors across extended operational time at the very low temperature of 1°C.

To assess the effects of cold shock on nitrifying MBBR systems reactor R3 was operated at steady state at 10°C for longer than 20 hydraulic retention times prior to being cold-shocked to 1°C. The temperature in R3 was shocked from 10°C directly to 1°C. Subsequent to being shocked down to 1°C, R3 was operated for 50 days at 1°C to compare the performance of the shocked R3 reactor to the acclimatized R1 reactor across an extended operational time.

#### **3.4.4. Chemical Analyses**

The following parameters were analyzed using triplicate testing during the study: the concentrations of TAN, NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, sCOD, CaCO<sub>3</sub>, TSS, VSS, as well as DO, pH and temperatures. As the reactors were operated with constant flow rates and consistent influent concentrations, grab samples were collected from within each of the reactors for analyses. The samples were harvested at approximately the center of the reactor basins and at half of the depth of the reactors. All samples were analysed within 4 hours of collection. The following standard methods were used to measure nitrogen constituents and solids: 4500 NH<sub>3</sub>; 4500 NO<sub>2</sub><sup>-</sup>; 4500 NO<sub>3</sub><sup>-</sup>

; 2540D-TSS (TSS dried at 103°C-105°C) and 2540E-VSS (fixed and volatile suspended solids ignited at 550°C) (APHA, 2005). sCOD and CaCO<sub>3</sub> were determined using HACH methods 8000 and 10239 with a DR 5000 Spectrophotometer (HACH, CO, US). DO, pH and temperature were measured using DO and pH VWR probes (ON, Canada) and Lascar Electronics temperature probes (PA, US).

### **3.4.5. Statistical Analyses**

The student *t-test* was used to validate significant statistical differences for TAN concentrations, removal rates, removal efficiencies, and detachment rates with a *p*-value less than 0.05 indicating significance. Pearson's correlation was used for correlation analysis between modeled and measured removal rates with *p*-value less than 0.05 indicating significance. Error bars in figures signify 95% confidence intervals.

## **3.5. Results and Discussion**

### **3.5.1. Intrinsic Removal Rates of Nitrifying MBBR Systems at 1°C**

Sanitary sewage systems (or non-combined sewage systems) separate the municipal wastewater containing blackwater and greywater from surface runoffs and hence prevent sewage overflows in periods of heavy rains or ice melts (Brombach et al., 2005; Ahm et al., 2016). However, this may result in nutrient concentrations from black and grey waters no longer diluted by surface runoffs. The lagoon effluent used in this study originated from a multipond facility receiving wastewater from non-combined sewers with an average TAN concentration of  $39.97 \pm 0.36$  mg/L. This concentration is approximately double the concentrations investigated in previous studies where nitrifying MBBR systems were naturally acclimatized to 1°C (Hoang et al., 2014a; Young et al., 2016).

The loading rate for R1 at 1°C was set to 186.89 gN/m<sup>3</sup>d, above the previously reported attainable removal rate of 167.24 gN/m<sup>3</sup>d for nitrifying MBBR systems at 1°C in a pilot study (Young et al., 2017). In contrast, the average 1°C removal rate recorded at steady state in this study for R1 is only 98.44 ± 4.69 gN/m<sup>3</sup>d; 40% lower than the previously reported rate of 167.24 gN/m<sup>3</sup>d at 1°C (Young et al., 2017). The discrepancy in rates is likely due to the limited temperature control in the above-mentioned study as pilot nitrifying reactors benefit from natural and slight temperature fluctuations, observed in full-scale systems, thus displaying higher rates than reactors strictly operating at 1°C. Hence, the enhanced temperature-controlled environment used in this study indicates that 98.44 gN/m<sup>3</sup>d is the intrinsic removal rate of nitrifying MBBR at 1°C. Moreover, this rate of 98.44 gN/m<sup>3</sup>d is also representative of the most conservative rate to consider for the design of nitrifying MBBR systems destined for low temperature operation. Nonetheless, the steady state removal rate of 98.44 ± 4.69 gN/m<sup>3</sup>d corresponds to a removal efficiency of 55.80 ± 1.64% and an average effluent concentration of 17.43 ± 0.6 mgTAN/L; thus, an effluent concentration exceeding the set limit of 10 mgTAN/L common to many WRRF.

To attain effluent concentrations below 10 mgTAN/L, a second reactor, R2, was introduced in-series following R1. In a two MBBR in-series configuration, the second reactor benefits from lower loading rates with the potential of operating under ammonia mass transfer limited conditions; thus, operating at higher efficiencies than the first reactor and improving the overall treatment capability of the MBBR system. Moreover, due to its higher treatment efficiency and lower loading rates, a smaller second reactor in-series can be utilized to reduce the footprint of the MBBR systems.

Increasing the volume of a single MBBR reactor or increasing the carrier percent fill of the reactor would be possible to attain the targeted effluent concentration; however, in the context of

a full-scale design a single MBBR with a large basin would require a larger footprint than two MBBR in-series whereas an MBBR with an initial high carrier percent fill would have a lower upgradability potential of two reactors in-series with lower percent carrier fills.

Adding the second reactor, R2, in-series following R1 resulted in TAN effluent concentrations below the set threshold of 10 mg/L (Figure 3.2). The average TAN effluent concentration for the MBBR system in-series (R1-R2) is  $5.68 \pm 0.74$  mg/L with a removal efficiency of  $85.62 \pm 1.67\%$ . R2 attains a removal efficiency of  $68.43 \pm 3.58\%$  and thus is 12.63% more efficient than R1. Adding R2 as a polishing reactor decreased TAN effluent concentration by 67%, supporting the proposed solution of a two MBBR in-series configuration to treat TAN concentrations from non-combined sewer systems at temperatures as low as 1°C.

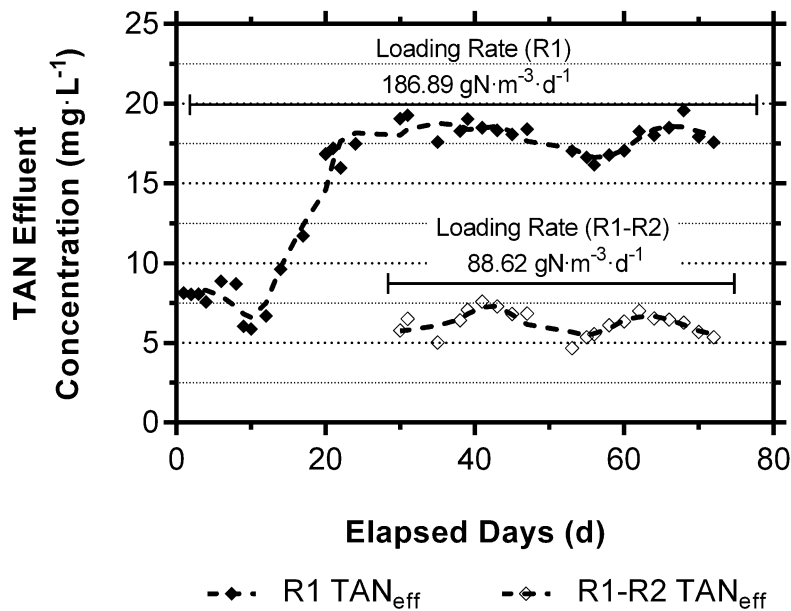


Figure 3.2. TAN effluent concentration across time for R1 and R1-R2 in-series at 1°C.

### 3.5.2. Temperature Threshold of MBBR Nitrification Kinetics

As previously mentioned, it is well established that low temperatures have a negative effect on nitrification kinetics in attached growth systems. Consequently, for the operation of MBBR

systems in cold climates it is essential to establish the decrease in nitrification kinetics that occurs at low temperatures and to identify any temperature threshold values associated with significant decreases in kinetics. For this aim, the removal rates of R1 were measured and the removal efficiencies calculated at steady state operation at 10°C, 8°C, 6°C, 4°C, 2°C and 1°C (Figure 3.3). No statistical difference was found between 10°C and 8°C operation in terms of removal rates with averages of  $363.79 \pm 4.85$  gN/m<sup>3</sup>d and  $368.70 \pm 4.85$  gN/m<sup>3</sup>d respectively as the nitrification rates were mass transfer limited. A decrease in removal rates is observed at 6°C with an average rate of 344.13 gN/m<sup>3</sup>d signifying an 8% decline in rates compared to the 8°C operation. The average removal rate for the subsequent decrements in temperature at 4°C, 2°C and 1°C were 270.43 gN/m<sup>3</sup>d,  $221.29 \pm 28.86$  gN/m<sup>3</sup>d and  $98.44 \pm 4.69$  gN/m<sup>3</sup>d respectively.

At 10°C, TAN effluent concentration for R1 was reduced to an average of  $1.49 \pm 0.19$  mgTAN/L from an average influent concentration of  $39.97 \pm 0.36$  mg/L. This represents a removal efficiency of 96.31%. As the temperature was decreased in decrements of 2°C from 10°C to 4°C, the removal efficiency remained steady with efficiencies above 91% at 8°C, 6°C and 4°C operation with the highest TAN<sub>eff</sub> recorded at 3.53 mg/L. The first significant drop in removal efficiency is recorded below 4°C, when the temperature was set to 2°C, at which the removal efficiency decreases to 74.82% and the TAN<sub>eff</sub> increases to  $10.14 \pm 2.50$  mg/L. Compared to 4°C operation with a removal efficiency of  $93 \pm 0.23\%$  and TAN<sub>eff</sub> of  $2.85 \pm 0.09$  mg/L, a 19.55% decrease in efficiency and an increase of 7.29 mg/L in TAN<sub>eff</sub> is recorded at the temperature of 2°C. Consequently, the temperature range between 2°C and 4°C is the threshold temperature below which nitrification becomes significantly hindered by low temperatures.

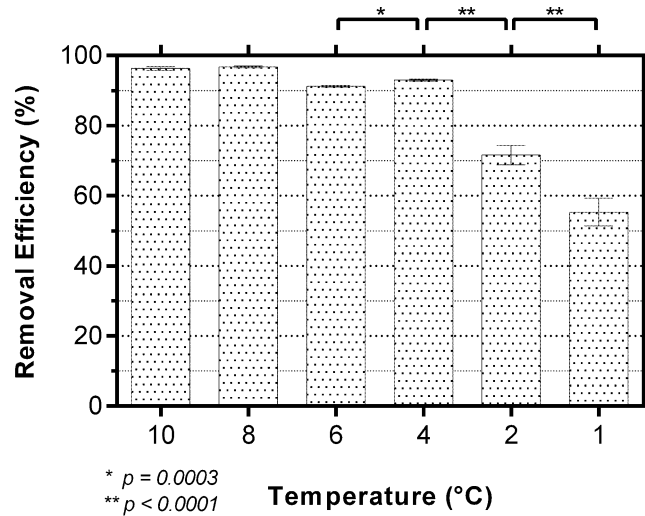


Figure 3.3. Average and 95% confidence interval of TAN removal efficiencies for R1 across temperature.

As previously stated, nitrification is a two-step process regulated by two phylogenetically distinct bacteria; the ammonia oxidizing bacteria and the nitrite oxidizing bacteria (Kindaichi et al., 2004). Evaluating nitrite accumulation across temperatures in the reactor should indicate which one of the two steps becomes hindered at the threshold between 2°C and 4°C delineated above. Measured nitrite concentrations across temperatures suggest that the significant decrease in nitrification observed below 4°C is likely not due to a suppressed nitrite oxidation. Nitrite as percent  $\text{NO}_x$  remains below 10% across temperatures with  $9.4 \pm 0.9\%$  being the highest measured percent  $\text{NO}_x$  at 6°C. At 2°C, the temperature at which the measured nitrification rates significantly declined, the percent  $\text{NO}_x$  was  $5.03 \pm 0.57\%$ . Hence, these results indicate that it is the nitrification step, rather than nitrification that is adversely affected by the operation below 4°C in systems that are acclimatized to low temperatures. This observation is supported by previous findings that demonstrated that NOB have a higher growth rate than AOB at temperatures below 15°C (Paredes et al., 2007; Gilbert et al., 2014; Ge et al., 2015). Potentially, an investigation of the microbial

communities in the biofilm across the different temperatures may clarify the changes occurring within the biofilm between the temperatures of 2°C and 4°C.

### 3.5.3. Temperature Correction Coefficients for MBBR Nitrification

To study the effects of temperatures on nitrification, an Arrhenius temperature correction coefficient can be used to model removal rates for MBBR systems (Rusten et al., 1995b; Salvetti et al., 2006; Zhang et al., 2014). As previously mentioned, Arrhenius correction coefficients of 1.086 and 1.09 were recently applied to model removal rates at temperatures below 12°C for nitrifying MBBR (Young et al., 2017a). Applying these same correction coefficients of 1.086 and 1.09 resulted in modeled removal rates lower than the measured rates of R1 at each of the temperatures investigated in this study except for 1°C (Figure 3.4). For instance, modeling the removal rate from 10°C to 6°C with  $\theta = 1.09$  resulted in a rate of 255.69 gN/m<sup>3</sup>d; predicting a rate decrease of 30% compared to the rate measured at 10°C. However, the measured removal rate of R1 at 6°C is 344.13 gN/m<sup>3</sup>d which is only 5.3% lower than the rate at 10°C. In other words, applying a correction coefficient of  $\theta = 1.09$  underestimated R1's kinetics by 25.35% at the temperature of 6°C. As shown in Figure 3.4, the same observation is made at the other temperatures as the application of  $\theta = 1.086$  and  $\theta = 1.09$  models conservative rates on average 21% lower than the measured rates except at 1°C. As reported above, there is a distinct temperature threshold between 2°C and 4°C with respect to a significant decline in attached growth nitrification kinetics. Therefore, an Arrhenius temperature correction coefficient of 1.049 to predict, with a stronger correlation ( $R^2 = 0.93$ ), R1's measured rates between 10°C and 4°C is proposed while a second coefficient of 1.149 is proposed to model the rates at 1°C. Discrepancies in Arrhenius temperature correction coefficients are to be expected as they are dependent on several factors such as the temperature range investigated; the magnitude of the temperature shift; the rate at which the

temperature is decreased; the precision in temperature control; whether the nitrification reaction is oxygen or ammonia rate limited; the operational conditions (pH, alkalinity, etc.); and the structure and ecology of the microbial community in the biofilm (Beales, 2004; Salvetti et al., 2006; Hwang and Oleszkiewicz, 2007; Mannucci et al., 2015).

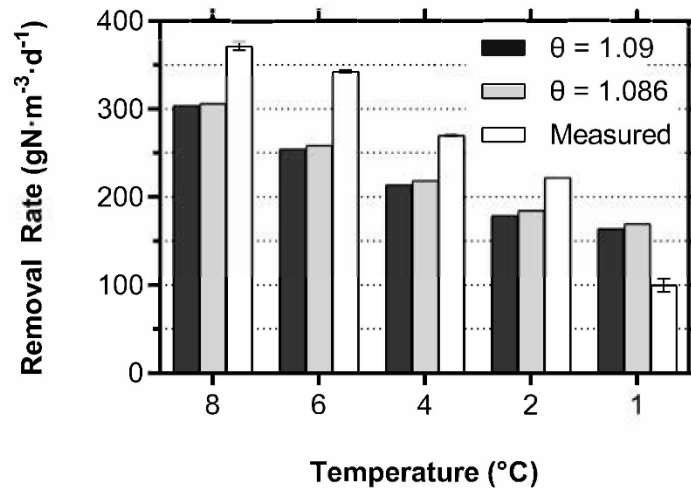


Figure 3.4. Average and 95% confidence interval of measured and modeled removal rates for R1 across temperature. Modeled removal rates were obtained by applying Arrhenius correction coefficients of 1.09 and 1.086.

Although the two proposed correction coefficients of 1.049 and 1.149 can be applied to model the temperature effect on the nitrification rates, they do not take into account another important factor; the exposure time. Delatolla et al. (2009) proposed for attached growth systems a temperature dependent Equation to model the nitrification rate as a function of elapsed time. Equations 3.1 and 3.2 can be used in series to one another to estimate the removal rates as cold temperature exposure conventionally progress at cold climate treatment facilities. For MBBR systems, the Delatolla et al. (2009) Theta model was capable of predicting the transition from 8°C to 4°C (Delatolla et al., 2010). More recently, Young et al. (2017a) reported that this model can also be applied for the transition from 5°C to 1°C with a strong correlation ( $R^2 = 0.89$ ) between modeled and measured rates. For this study, the Theta model displays significantly higher rates

than the measured rates for the transition from 4°C to the 25<sup>th</sup> day of operation at 1°C at which steady state kinetics were reached in R1 (Figure 3.5). Once more, the limited temperature control in the Young et al. (2017a) pilot study could possibly explain the higher measured rates closer to the rates displayed by the Theta model. With the accurate temperature control measures of this experiment, the lower measured removal rates indicate that for the transition from 4°C to 1°C, a modified Theta model is required. Hence, Equation 3.3 is proposed to obtain a modified Theta model to predict the rates below 4°C until 1°C steady state kinetics are reached. With the strong correlation ( $R^2 = 0.88$ ) obtained between modeled and measured rates, the modified Delatolla et al. (2009) Theta model can be applied to factor in the exposure time below 4°C.

$$\theta = 1.17 \times 10^{-1} \cdot \ln(t) + 9.19 \times 10^{-1} \quad 3.3$$

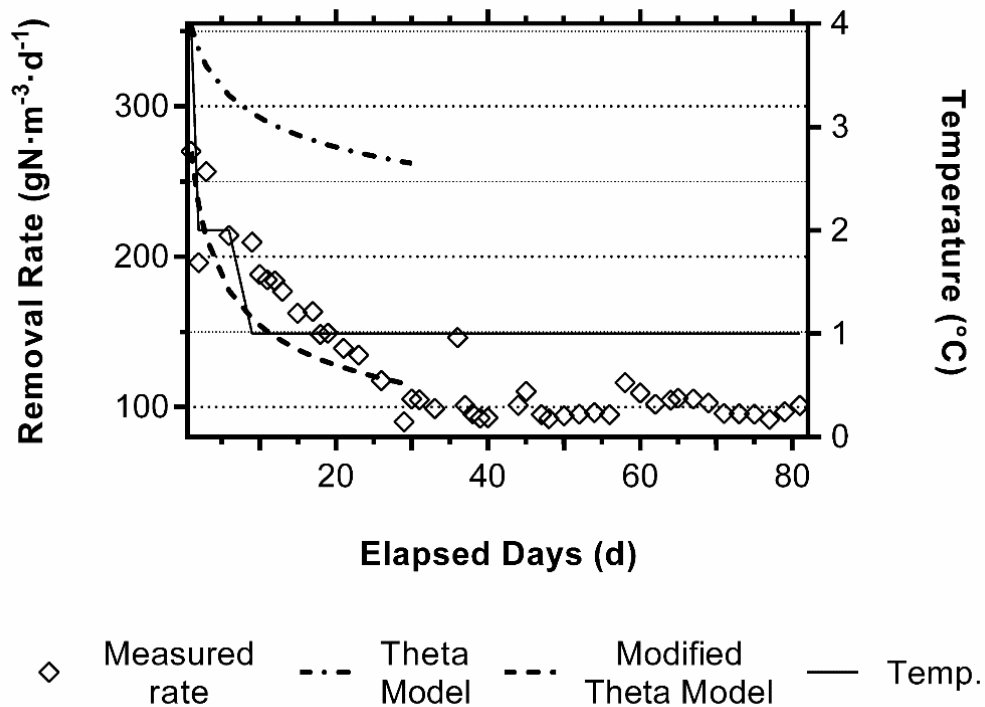


Figure 3.5. Removal rates, Theta model and the modified Theta model across time for R1 between the temperatures of 4°C and 1°C. Delatolla et al. (2009) Theta model and the modified Theta model were applied for the transition from 4°C to the first 25 days at 1°C.

### 3.5.4. Cold-Shock Kinetics of Nitrifying MBBR Systems

A rapid decrease in temperature, or cold-shock treatment, has been shown to have a significant adverse effect on nitrifying MBBR systems (Delatolla et al., 2010). A reactor, R3, cold-shocked from 10°C to 1°C and set at the same loading rate as the acclimatized reactor, R1, was studied in this work to research the effects of cold shock on nitrifying MBBR systems' performance. In a comparison between the shocked reactor and the acclimatized reactor at 1°C, statistically different removal rates were obtained (Figure 3.6). The left box plot in Figure 3.6 represents the steady state removal rates of R1 while the right box plot represents the steady state removal rates of R3 and demonstrates that the shocked reactor underperformed compared to the acclimatized reactor at 1°C. The nitrification rates of the shocked reactor are on average 21% lower than the rates of the acclimatized reactor; with an average shocked removal rate of  $78.01 \pm 5.73$  gN/m<sup>3</sup>d compared to  $98.44 \pm 4.69$  gN/m<sup>3</sup>d at 1°C. Moreover, the lowest removal rate observed for the shocked reactor is 56.34 gN/m<sup>3</sup>d while the lowest ever reached by the acclimatized reactor is 84.19 gN/m<sup>3</sup>d. During the operation at 1°C, the shocked reactor never recovered to reach the rates attained by the acclimatized reactor as this lack of performance observed in R3 was supported by previous investigations performed on cold-shocked attached growth and suspended growth nitrifying systems (Hwang and Oleszkiewicz, 2007; Delatolla et al., 2010).

An assessment of solids production in both the acclimatized and shocked reactors indicates that the acclimatized reactor has an average detachment rate of  $26 \pm 6.5$  gTSS/m<sup>3</sup>d and an average effluent TSS concentration of  $23.19 \pm 5.06$  mg/L; higher rates than the shocked reactor with an average detachment rate of  $1.68 \pm 0.67$  gTSS/m<sup>3</sup>d and average effluent TSS concentration of  $10.40 \pm 1.4$  mg/L (Supplemental Figure 3.7). The detachment process in the biofilm may occur through

various mechanisms such as the grazing by predators, by erosion and abrasion characterized as the loss of small sized particles measurable as  $\mu\text{m}$  range solids, and by sloughing, the loss of larger particles measurable as mm solids (Bryers, 1987; Bester et al., 2013; Butler and Boltz, 2014). It is also widely understood that the detachment process is dependent on growth rates and thus substrate utilization rates (Speitel and DiGiano, 1987; Peyton and Characklis, 1993; Stewart, 1993). This growth rate dependent model of the detachment process supports the observation of higher detachment rates in the acclimatized reactor which is achieving higher nitrification rates than the shocked reactor.

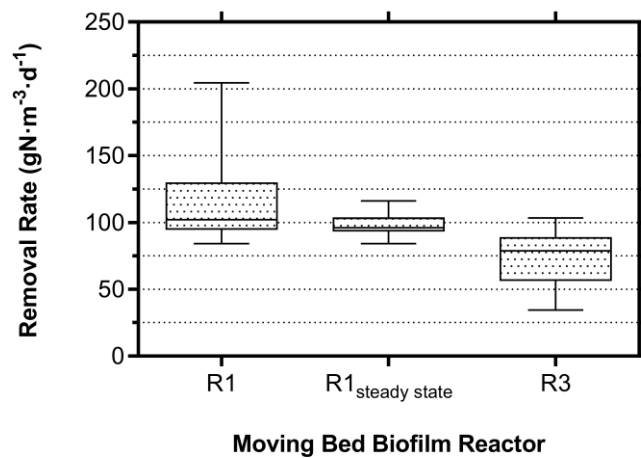


Figure 3.6. Removal rates for R1 and R3 at 1°C. The range of measured removal rates for R1 and R3 are characterized by the span between the minimum and maximum, the first and third quartiles and the median: R1 (n = 29; median = 95.99  $\text{gN}/\text{m}^3\text{d}$ ), R3 (n = 20; median = 84.98  $\text{gN}/\text{m}^3\text{d}$ ).

However, to understand the discrepancies in detachment and nitrification rates between the acclimatized and shocked reactors it is necessary to consider the severe impacts of cold shocks on microorganisms embedded in the biofilm. Sudden decrease in temperature may lower nitrification rates by causing direct adverse effects in cells such as impeded transcription and translation; reduced enzyme activities due to conformational instability; and impeded substrate

uptake due to membrane rigidity increase (Horn et al., 2007; Phadtare and Severinov, 2010; Barria et al., 2013; Eshwar et al., 2017). On the other hand, in the detachment process, cold shocks seem to have an indirect impact through the bacteria's cold-shock response mechanisms mitigating the adverse effects of temperature decreases (Phadtare et al., 1999; Wouters et al., 2001; Caruso et al., 2018). For instance, biofilm formation regulators such as cyclic diguanylate (ci-di-GMP) are down regulated in a cellular response to cold shocks triggering a shift from the sessile state to the planktonic state which eventually leads to biofilm dispersal (Bester et al., 2013; Toyofuku et al., 2016; Díaz-Salazar et al., 2017; Lin Chua et al., 2017; Wang et al., 2017). This contradicts the observations in detachment rates between the shocked and acclimatized reactors; indeed, no sloughing event was recorded during the operation of the shocked reactor (Supplemental Figure 3.8). In addition to the lack of nitrification recovery in the shocked reactor, it is unclear whether it is due to a late onset of the cold-shock response mechanisms of the nitrifiers in the reactor or whether their adaptation mechanisms are incapable of mitigating rapid decreases in temperature. Extensive research on cold-shock response mechanisms in AOB and NOB is necessary to fully understand the effects of cold shocks on nitrifying MBBR systems during periods of snowmelt and extreme weather events.

### **3.6. Conclusions**

The current literature lacks studies on 1°C nitrifying MBBR systems using real wastewater with temperature control precision to determine the intrinsic removal rate of these systems at 1°C; the optimal reactor configuration for the treatment of TAN concentrations typical of sanitary sewer systems; the Arrhenius corrections coefficients down to 1°C; and, the extent of cold-shocks adverse effects on attached growth nitrification down to 1°C. This study addresses these gaps in knowledge and provides information that is necessary to expand the application of the MBBR

technology to lagoon treatment systems as nitrifying add-on, upgrade units. With the enhanced temperature control applied in this study a rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d is reported as the intrinsic removal rate at 1°C for nitrifying MBBR systems. A two reactor in-series is proposed as the optimal configuration for nitrifying MBBR upgrade systems for passive treatment systems fed by sanitary sewer systems. A temperature threshold between 2°C and 4°C was delineated at which a significant drop in the nitrification rate occurs with results indicating that the decline in nitrification kinetics below 4°C is due to the decline in nitritation rates rather than nitrification rates.

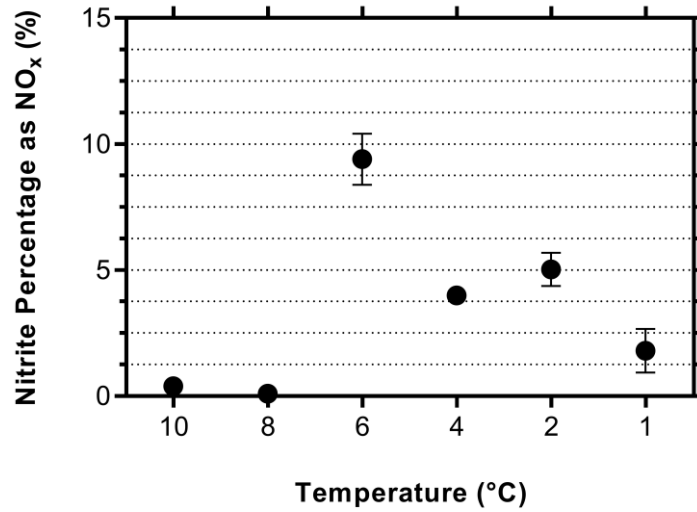
An Arrhenius correction coefficient of 1.049 between the temperatures of 10°C and 4°C and a second coefficient of 1.149 for 1°C are proposed for nitrifying MBBR systems. For the transition from 4°C to 1°C, the application of the Delatolla et al. (2009) Theta model accounting for the exposure time at low temperatures displayed higher rates compared to the measured rates; thus, a modified Theta model that strongly correlates ( $R^2 = 0.88$ ) with the measured removal rate is proposed for the transition from low temperatures (4°C) to very low temperatures (1°C) for nitrifying MBBR systems.

Cold-shock experiments demonstrated that cold-shocked reactor R3 is less effective in reducing TAN concentrations with on average 21% lower removal rates compared to R1, the acclimatized reactor. In addition to lower nitrification rates, R3 displayed lower detachment rates and a lack of nitrification recovery throughout its operation at 1°C.

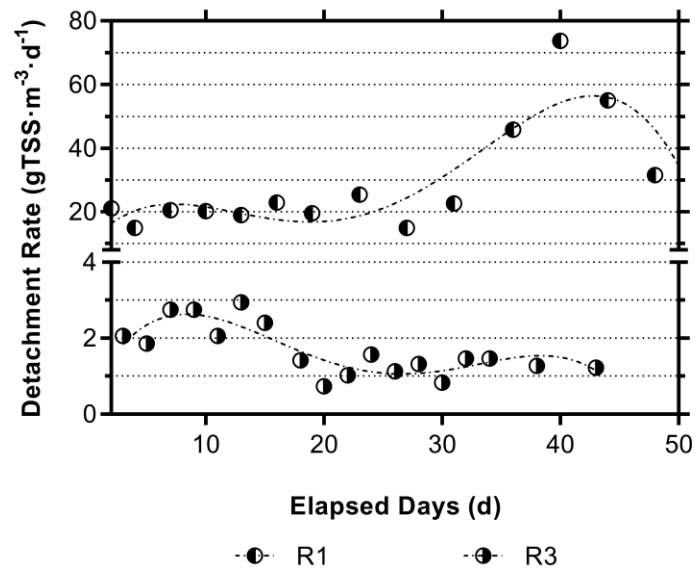
### **3.7. Acknowledgements**

The authors would like to thank Associated Engineering and Veolia Water Technologies Canada for their technical support.

### 3.8. Supplemental Materials



Supplemental Figure 3.7. Average and 95% confidence interval of nitrite percentages as NO<sub>x</sub> for R1 across temperature.



Supplemental Figure 3.8. Detachment rates for R1 and R3 at 1°C across time.

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# **Chapter 4. Biofilm and Microbiome Response of Attached Growth Nitrification Systems Across Incremental Decreases to Low Temperatures**

## **4.1. Context**

Chapter 4 presents the study submitted to the journal of Water Process Engineering titled: *Biofilm and microbiome response of attached growth nitrification systems across incremental decreases in temperatures* by W. Ahmed and R. Delatolla. This study aims to investigate the changes occurring in nitrifying biofilms during their acclimatization to low temperatures down to the microbiome level. This study includes an assessment of attached growth nitrification kinetics, an analysis of the nitrifying biofilm's adaptation to decreasing temperatures with respect to biofilm thickness, mass, and density; an assessment of the biomass viability, and an investigation nitrifying biofilm microbial communities at different temperatures down to 1°C. As such, this study is the first investigation on nitrifying biofilm and microbiome adaptations to low temperatures using both temperature-controlled experimentation and real wastewater to isolate the effects of decreasing temperatures on nitrifying MBBR systems.

## **4.2. Abstract**

The study investigates low temperature attached growth rates and nitrifying biofilm, biomass, and microbiome responses down to 1°C. Two moving bed biofilm reactors (MBBR) were acclimatized from 10°C to 1°C by incremental decreases in temperatures. A non-linear decline in nitrification with decreasing temperatures was recorded with a kinetic threshold temperature delineated between 4°C and 2°C, at which a significant decline in efficiency of 22.47% occurred. Assessment of the nitrifying biofilm thickness shows a greater increase in thickness occurring above the kinetic threshold with an increase of 25.47% in biofilm thickness between 10°C and 4°C, followed by lesser increases of 6.55% and 9.60% at 2°C and 1°C. The biofilm density

decreases between 10°C and 1°C as no statistically relevant changes in biofilm mass is measured along with the measured increases in biofilm thickness. Similarly, the percentage of viable cells of the nitrifying biofilm occurs above the kinetic threshold temperature with an increase of 24.01% in viable cells between 10°C and 4°C, followed by lesser non-statistically relevant increases at 2°C and 1°C. No statistically significant change in the number of estimated species is observed; however,  $\beta$ -diversity analysis indicates greater microbial community shifts above the kinetic threshold followed by lesser shifts between 2°C and 1°C. *Nitrosomanadacea* and *Nitrosopira* are the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) families in the biofilm with relative abundances of  $3.43 \pm 0.51\%$  and  $0.94 \pm 0.44\%$  respectively at 10°C. A change in NOB relative abundance occurred between the temperatures of 10°C and 1°C, decreasing to  $0.19 \pm 0.10\%$  above the kinetic threshold. The estimation of nitrifiers per carrier in the bioreactors, based on biofilm thickness, biomass viability and relative abundances, shows an increase of 30.2% in AOB/carrier above the kinetic threshold temperature ( $> 4^\circ\text{C}$ ) followed by lesser increases down to 1°C.

### **4.3. Introduction**

The elevated ammonia concentrations recorded in surface waters in North America and around the world are mainly due to the anthropogenic release from point source polluters such as water resources recovery facilities (WRRF) (Murdoch et al., 2000; Driscoll et al., 2003; Preston et al., 2011). The severe environmental and economic impacts of water toxicity and eutrophication caused by the ammonia discharged into receiving water bodies are well understood, which has led to a trend towards stringent effluent regulations in numerous countries to mitigate the adverse effects of deleterious substances from WRRF (USEPA, 1983; EEC, 1991; Canada Gazette, 2012; Yadu et al., 2018). The biologically mediated two-step process of nitrification remains the most

common solution for ammonia removal from wastewater (Balakrishnan and Eckenfelder, 1969a; Zhou et al., 2016b). That being said, the temperature-sensitive process of nitrification remains hindered at low temperatures in conventional suspended growth technologies to eventually cease below 4°C with no nitrification recorded at 1°C. However, nitrifying attached growth systems such as the moving bed biofilm reactors (MBBR) have been shown capable of maintaining nitrification down to 1°C (van Dyke et al., 2003; Almomani et al., 2014; Hoang et al., 2014b; Hoang et al., 2014a; Young et al., 2016; Young et al., 2017a; Chen et al., 2018; Ahmed et al., 2019). In addition, nitrifying biofilm technologies have been presented as a potential upgrade solution for ammonia removal for the numerous passive treatment systems in operation in northern countries due to their efficiency and their low operational intensity (Delatolla and Babarutsi, 2005; Ødegaard, 2016; Zhou et al., 2018).

Nitrifying attached growth systems possess advantages inherent to biofilm technologies such as the resiliency to environmental stressors and the ability to retain slow growing organisms such as the nitrifiers responsible for the nitrification process (Barwal and Chaudhary, 2014; Wang et al., 2015; Dezotti et al., 2018). The nitrifying biofilm in these bioreactors has been shown to be composed of phylogenetically diverse organisms in addition to the autotrophic communities responsible for the two-step process of nitrification commonly referred to as ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Kindaichi et al., 2004; Monteiro et al., 2014; Correa et al., 2018; Saidu et al., 2018). Studies have identified the dominant nitrifying genera in WRRFs as *Nitrosomonas* as the dominant AOB and *Nitrospira* as the dominant NOB (Almstrand et al., 2013; Leyva-Díaz et al., 2017; Zhang et al., 2018).

The effects of low temperatures on nitrifying biofilm, biomass and microbiome in attached growth systems were investigated at temperatures as low as 1°C (Hem et al., 1994; Rusten et al.,

1995; Salvetti et al., 2006; Delatolla et al., 2010; Almomani et al., 2014; Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017a). Previous findings indicate that increases in nitrifying biofilms' thickness are observed at low temperatures down to 1°C along with decreases in biofilm densities linked to limited increases in biofilm mass despite the observed increased thickness (Hoang et al., 2014a; Young et al., 2017a).

A recent study on 1°C nitrifying attached growth systems was unable to achieve incremental, precise and stable decreases in temperatures while using real wastewater to accurately isolate the kinetic change of attached growth nitrification across incremental temperature change (Ahmed et al., 2019). In particular, this study confirmed the non-linear relationship between declining temperatures and attached growth nitrification kinetics with a first significant decrease in kinetics delineated between the temperatures of 2°C and 4°C. However, no characterization of the changes occurring within the nitrifying biofilm, biomass and microbiome at this kinetic threshold temperature was reported. Therefore, signifying that the effects of low temperature on nitrifying biofilms down to the cellular level have yet to be accurately defined by incremental decreases in temperatures using a temperature-controlled setting with the system being fed with real wastewater.

This study aims to delineate the response of nitrifying biofilms and the embedded microbiome of low temperature attached growth systems during the transition to lower temperatures and the onset of low temperatures. In particular, this study investigates the nitrifying biofilm response with respect to thickness, mass, density, biomass viability and as well as the response of the embedded microbial communities during the transition and onset of low temperatures.

#### **4.4. Materials and Methods**

#### 4.4.1. Experimental Setup and Operation

The experimental setup of this study consists of two laboratory scale MBBR reactors that are identical in design and were operated in parallel to ensure repeatability of reactor performance in a temperature-controlled chamber throughout the experiment (Figure 4.1). The two attached growth reactors, MBBR1 and MBBR2, have the same volume of 2.2 L and housed AnoxKaldnes K5 carriers (Lund, Sweden) with diameters of 25 mm, heights of 4 mm and surface area to volume ratios of  $800 \text{ m}^2/\text{m}^3$ , collected from a partially nitrifying Integrated Fixed Active Sludge (IFAS) system located in Hawkesbury, Ontario, Canada. The MBBR reactors were operated at fill percentages below the maximum recommended 67% fill for MBBR systems (Rusten et al., 1995). Aeration was supplied to the reactors with an air pump and air diffusers to provide dissolved oxygen (DO) for the aerobic reaction of nitrification and allow adequate mixing of the carriers in the reactors.

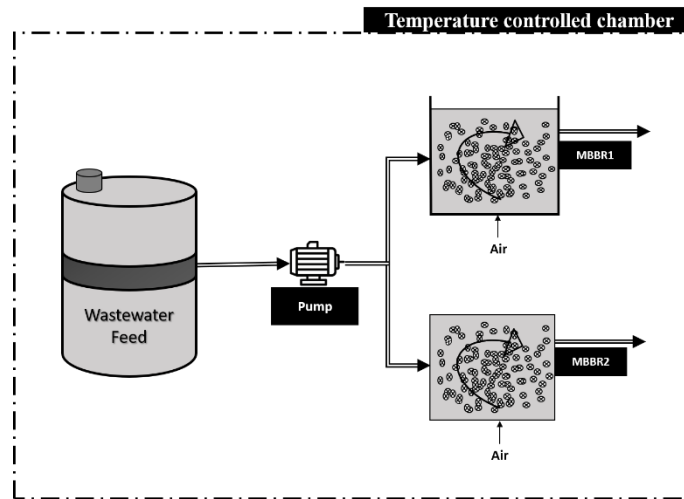


Figure 4.1. Schematic of laboratory MBBR system comprised of two identical MBBR reactors denoted MBBR1 and MBBR2.

The biofilm system, comprised of two identical MBBR reactors, was operated in parallel with temperatures decreasing from  $10^{\circ}\text{C}$  to  $1^{\circ}\text{C}$  in the temperature-controlled chamber in decrements of  $2^{\circ}\text{C}$  to ensure a slow acclimatization to each temperature. Specifically, the

operational temperatures were 10°C, 8°C, 6°C, 4°C, 2°C and 1°C. The MBBR reactors were operated at each incremental decrease in temperature until steady-state removal efficiency was achieved, with the steady-state operation being defined as removal efficiency fluctuations below  $\pm 10\%$ . The reactors were operated for a minimum of 20 hydraulic retention times at each temperature value in addition to demonstrating steady operation at each temperature investigated in this study. The MBBR reactors were fed with real wastewater collected from a full-scale, partially aerated, continuous flow multi-pond lagoon facility with a hydraulic retention time of 20.3 days. The wastewater constituents of the full-scale facility effluent used to feed the MBBR reactors are listed in Table 4.1.

Table 4.1. Constituents of the lagoon effluent used as feed for the MBBR system.

Constituent	Concentration (mg/L)
TAN (NH <sub>4</sub> <sup>+</sup> /NH <sub>3</sub> -N)	39.97 $\pm$ 0.36
Nitrite (NO <sub>2</sub> <sup>-</sup> -N)	0.08 $\pm$ 0.02
Nitrate (NO <sub>3</sub> <sup>-</sup> -N)	3.22 $\pm$ 0.03
Biochemical oxygen demand (BOD)	13.13 $\pm$ 0.7
Alkalinity (CaCO <sub>3</sub> )	303.41 $\pm$ 2.94
Total suspended solids (TSS)	7.09 $\pm$ 0.62
Volatile suspended solids (VSS)	5.87 $\pm$ 0.61

#### 4.4.2. Wastewater Constituent Analysis

Standard methods 4500 NH<sub>3</sub>, 4500 NO<sub>2</sub><sup>-</sup>, 4500 NO<sub>3</sub><sup>-</sup>, 2540D-TSS (TSS dried at 103 °C - 105°C) and 2540E-VSS (fixed and volatile suspended solids ignited at 550°C) were used to measure water constituents (APHA, 2005). The soluble chemical oxygen demand (sCOD) and alkalinity (CaCO<sub>3</sub>) concentrations were measured using HACH methods 8000 and 10239 with a DR 5000 Spectrophotometer (HACH, CO, USA). DO, pH and temperatures were measured using

DO and pH probes (VWR, ON, Canada) and a temperature data logger (Lascar Electronics, PA, USA).

#### **4.4.3. Biofilm Morphology**

Images used to assess the biofilm thickness were acquired using the Vega II-XMU SEM (Tescan USA Inc., PA, US) variable pressure scanning electron microscopy (VPSEM) by acquiring 20 images from triplicate carriers (Delatolla et al., 2009a; Tian and Delatolla, 2019). 100 measurements were taken per image using NI Vision Assistant's Machine Vision Advanced Edge Detection tool (National Instruments, LabView 14, TX, US). Biofilm mass was determined using a modified Delatolla et al. (2010) method on triplicate carriers. Harvested carriers were dried at 105°C overnight then cooled down in a desiccator for 20 min before measuring the weight of each carrier. Next, the carriers were thoroughly abraded with a brush to remove the biofilm; the clean carriers were then dried at 105°C overnight and cooled down in a desiccator for 20 min before measuring their weight. Biofilm mass was calculated by subtracting the initial weight of the carrier and its embedded biofilm by the weight of the clean carrier.

#### **4.4.4. Cell Viability**

Cell viability was assessed using a confocal laser scanning microscope (CLSM) (Zeiss, VA, US), FilmTracer LIVE/DEAD Biofilm viability kit (Life Technologies, CA, US) and Calcofluor White Stain (Sigma-Aldrich, MO, US), by acquiring 25 images from triplicate carriers with a  $\times 63$  water objective. Viable cells were identified with the green stain, SYTO9, a membrane-permeant and non-viable cells with propidium iodide (PI), a cell membrane-impermeable only visible in cells with compromised cell membranes. The extracellular polymeric substance (EPS) was identified with Calcofluor White Stain to delineate the biofilm. Viable and non-viable cells were quantified using NI Vision (National Instruments, LabView 14, TX, US) (Young et al.,

2017a; Tian and Delatolla, 2019). The percent of viable biomass cells embedded in the biofilm is defined in this study as the quantified viable cells divided by the total cells (viable and non-viable) multiplied by 100.

#### **4.4.5. Microbiome Analysis**

The microbiome was analyzed following the method by Young et al. (Young et al., 2017a). DNA was extracted from five carriers using FastDNA Spin Kit (MP Biomedicals, CA, US) and amplified targeting the 16s rRNA gene with two polymerase chain reactions (PCR) amplification; first PCR amplifying the V6 region and adding the barcodes and Illumina sequencing adapters, and the second PCR amplifying the product of the first PCR and adding Illumina flow cell adapters. A 2% agarose gel was used to inspect the amplicons before purifying them with Montage PCR95 cleanup kit (EMD Millipore, Millipore Sigma, MA, US). The amplicons were quantified using Qant-iT dsDNA HS Assay Kit (Life Technologies, ON, Canada) then a pooled sample containing 50 ng of DNA from each sample was sent for processing with a Hiseq2500 at the Center for Applied Genomics (TCAG, Toronto, ON, Canada).

Sequenced reads assembly and quality filtering were performed using Fast length Adjustment of Short reads (FLASH) software with a minimum quality score of 20 over 90% of sequences (Magoč and Salzberg, 2011). Then using Novobarcode, the reads were demultiplexed and trimmed of their barcodes before analysis (Goecks et al., 2010). Operational taxonomical units (OTU) clustering at 97% sequence similarity was performed with the Quantitative Insights into Microbial Ecology (QIIME v.1.9.1) software (Caporaso et al., 2010a). Taxonomy was assigned using UCLUST algorithm and Greengenes 13.5 as a reference database before performing alpha diversity (Chao1 index) and beta diversity analyses. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed based

on the 16S rRNA sequencing data (Langille et al., 2013). The gene counts for each predicted pathways were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa and Goto, 2000; Kanehisa et al., 2019; Kanehisa, 2019).

#### **4.4.6. Statistical Analysis**

Statistical significance was determined using a one-way ANOVA for the removal efficiency, biofilm thickness, biofilm mass and biomass viability with a *p*-value less than 0.05 indicating significance. Kruskal Wallis sum-ranked was used to determine statistical significance for relative microbial abundance and analysis of similarities (ANOSIM) for beta diversity's significance with *p*-value less than 0.05 indicating significance. Statistical significance for PICRUST analysis through KEGG pathways identification was accepted at a *p*-value of less than 0.05.

### **4.5. . Results and Discussion**

#### **4.5.1. Low Temperature Attached Growth Nitrification Kinetics**

To determine the effects of low temperatures down to 1°C on attached growth nitrification efficiency, two nitrifying MBBR systems performance were measured at 10°C, 8°C, 6°C, 4°C, 2°C and 1°C (Figure 4.2). The measured total ammonia nitrogen (TAN) removal efficiencies remained above 85% with efficiencies of  $96.13 \pm 0.44\%$ ,  $97.23 \pm 0.67\%$ ,  $88.74 \pm 2.73\%$ ,  $95.98 \pm 3.21\%$  at 10°C, 8°C, 6°C and 4°C, respectively. Although there was no statistically relevant change in TAN removal efficiency between 10°C and 4°C, a significant decrease in efficiency is observed between 10°C and 6°C. At the temperature of 6°C, not only does the TAN removal efficiency decreased, but also the rates of nitrification and denitrification diverged. Nitrification at 6°C showed lower rates compared to denitrification and hence a slight accumulation of nitrite is observed at this temperature. This decline in TAN removal efficiency and nitrification are shown to be a temporary

decrease in the system at 6°C, as both the TAN removal and nitrification rates increase at 4°C. This temporary observed decrease likely shows the first effects of temperature on the performance of the system, with these effects being overcome with extended time of operation.

A significant decrease in attached growth nitrification performance is also recorded below 4°C, more specifically between the temperatures of 4°C and 2°C as the removal efficiencies decreased from  $95.98 \pm 3.21\%$  at 4°C to  $73.51 \pm 3.51\%$  at 2°C; representing a decline in efficiency of 22.47%. This pattern of change in efficiency demonstrates a non-linear relationship between decreasing temperatures and declining attached growth nitrification efficiency. As such, the data indicates the existence of a kinetic threshold temperature for attached growth nitrification between the temperatures of 4°C and 2°C. This threshold temperature previously delineated to be below the temperature of 5°C in low temperature nitrifying biofilm systems by Hoang et al. (2014a) and below 4°C by Ahmed et al. (2019) in temperature-controlled studies. This first threshold is followed by a second significant decline in efficiency, between the temperatures of 2°C and 1°C, characterized by a decrease in removal efficiency of 14.63%.

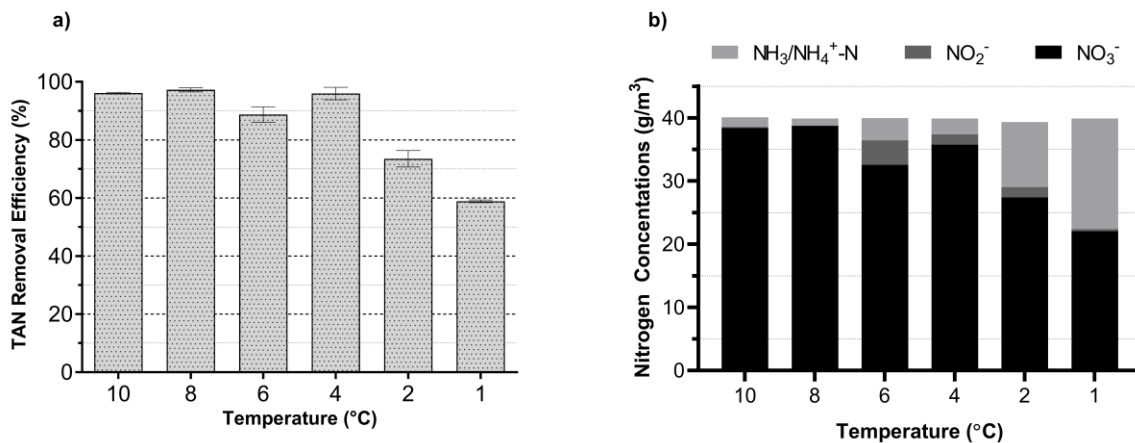


Figure 4.2. Nitrifying MBBR performance across temperatures, a) average and 95% confidence intervals of TAN removal efficiencies and b) nitrogen constituent concentrations.

Measurements of TAN, nitrite, and nitrate concentrations in the reactors show a nitrogen mass balance with an error of less than 4% (Figure 4.2b). This nitrogen mass balance confirms nitrification as the pathway for ammonia removal and also confirms a lack of significant denitrification in the reactors. Nitrite percentage as NO<sub>x</sub> (%NO<sub>x</sub>) above the kinetic threshold temperature was measured at  $0.40 \pm 0.02\%$ ,  $0.08 \pm 0.02\%$ ,  $9.42 \pm 0.96\%$ , and  $3.98 \pm 0.01\%$  at 10°C, 8°C, 6°C and 4°C; while nitrite as %NO<sub>x</sub> below the threshold temperature was  $5.04 \pm 0.51\%$  and  $1.45 \pm 0.15\%$  at 2°C and 1°C. Therefore, the first increase in nitrite is recorded at 6°C or above the kinetic threshold temperature. As such, nitrite accumulation across temperatures in the reactors indicates that the significant decline in removal efficiency occurring between 4°C and 2°C is preceded by a decline in nitrification, the first reaction and rate-limiting step of nitrification.

#### **4.5.2. Biofilm Response at Low Temperature**

As no significant changes occur between 10°C and 4°C with respect to the efficiency, the biofilm, biomass and microbiome responses of the nitrifying biofilm were assessed at 10°C, 4°C, 2°C and 1°C. As such, the changes in these responses are assessed above the kinetic threshold temperature at 10°C and 4°C; between the temperature range at which the kinetic threshold temperature was delineated at 4°C and 2°C; and below the kinetic threshold temperature at 2°C and 1°C. The biofilm response was characterized as the biofilm mass, the biofilm thickness and the biofilm densities, a function of the biofilm mass and thickness (Figure 4.3). Differences in nitrifying biofilm mass at 10°C, 4°C, 2°C and 1°C were not statistically relevant with initial biofilm masses at 10°C of  $32.05 \pm 2.06$  mg. The biofilm thickness measurements indicate an increase in thickness with decreasing temperatures. At the initial stage of 10°C, the nitrifying biofilm was measured at  $229.92 \pm 6.10$  μm and increased to  $308.50 \pm 8.56$  μm at 4°C, which represents an increase of 25.47% in biofilm thickness. At 2°C, the biofilm thickness increased by

6.54%, with a measured thickness of  $330.12 \pm 13.62 \mu\text{m}$ , followed by another increase of 9.60% at  $1^\circ\text{C}$  with a measured thickness of  $365.19 \pm 9.60 \mu\text{m}$ . Increases in biofilm thickness with decreasing temperatures were also observed in nitrifying biofilms operating at low temperatures with increases of 54% in biofilm thickness between  $20^\circ\text{C}$  and  $1^\circ\text{C}$  (Hoang et al., 2014a; Young et al., 2017a). Previous studies have stated that the overall lower metabolic activity of the biofilm allows for an increased substrate availability at lower biofilms depths, and thus biofilm growth, especially of filamentous bacteria and carbohydrate producers associated with increases in biofilm thickness (Bjornberg et al., 2009; Hoang et al., 2014a; Young et al., 2017a).

The nitrifying biofilm density is shown to decrease with exposure time at  $1^\circ\text{C}$  as a result of increasing biofilm thickness with no statistically significant change in biofilm mass being measured. The lower biofilm densities with increased biofilm thicknesses are typical of filamentous and porous biofilm morphologies and a higher polysaccharides content within the biofilm as previously described in low temperature nitrifying MBBR systems (Jang et al., 2003; Karizmeh et al., 2014; Young et al., 2016). As mentioned above, the biofilm responses in terms of density and decreasing temperatures support the biofilm response observed in low temperature nitrifying MBBR systems (Lapidou and Rittmann, 2004; Ahimou et al., 2007; Hoang et al., 2014a; Young et al., 2017a).

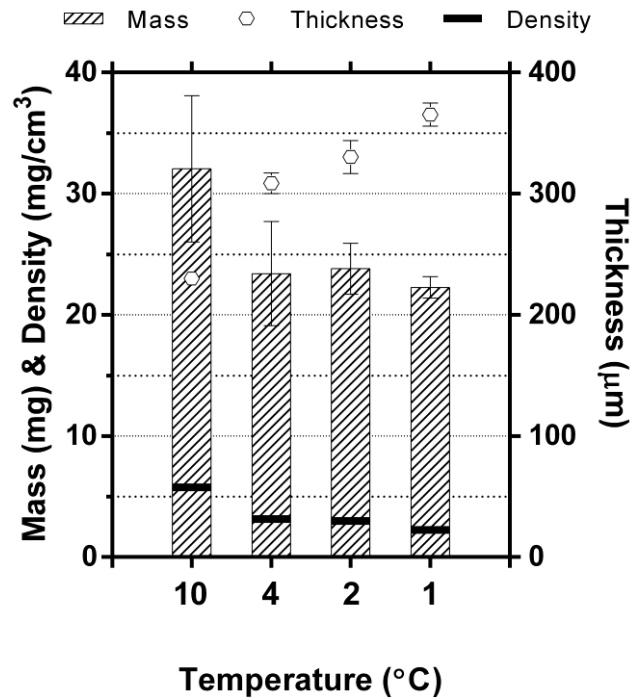


Figure 4.3. Nitrifying biofilm mass, density, and thickness across temperature.

#### 4.5.3. Biomass Cell Viability at Low Temperature

The biomass viability as viable cells percentage of the total cells was assessed again at the temperatures of 10°C, 4°C, 2°C and 1°C (Figure 4.4). The biomass viability measured at  $61.66 \pm 5.79\%$  at 10°C increased to  $85.67 \pm 4.96\%$  at 4°C, thus signifying an increase of 24.01% in viable cells above the kinetic threshold temperature ( $>4^\circ\text{C}$ ). Below 4°C, lesser and non-statistically significant increases in viable cells are recorded with the percentages of viable cells of  $90.13 \pm 4.47\%$  and  $94.01 \pm 1.40\%$  at 2°C and 1°C, respectively. The increase in biomass viability in the nitrifying biofilm at low temperatures correlate to the reported biomass response to low temperatures in nitrifying MBBR systems (Hoang et al., 2014a; Young et al., 2017a). An increase in cell viability at lower temperatures was previously attributed to an increase in substrate availability throughout the biofilm, especially at lower levels of the biofilm, as metabolic activities and thus the rate of substrate consumption of embedded cells decreases. Moreover, the decrease

in biofilm density also allows for an increase in substrate diffusion and an associated increase in substrate availability (Hoang et al., 2014a; Martín-Pascual et al., 2015; Young et al., 2017a).

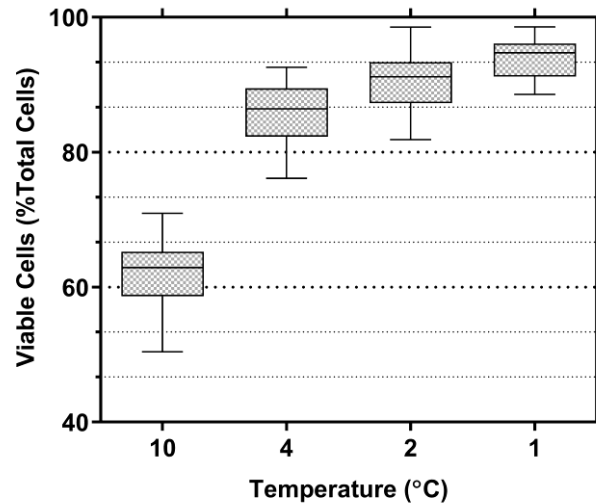


Figure 4.4. Nitrifying biofilm percent of viable cells at 10°C, 4°C, 2°C and 1°C.

#### 4.5.4. Microbiome Response at Low Temperature

The microbiome response was assessed the same as the biofilm and biomass at the temperatures of 10°C, 4°C, 2°C and 1°C. The total number of estimated species, measured as the  $\alpha$ -diversity, at the initial stage of 10°C was  $2532 \pm 78.61$  with no statistically relevant changes recorded in the number of estimated species at 4°C, 2°C at 1°C (Figure 4.5a). In particular, no loss of diversity due to low temperatures occurred to justify the decline in nitrification efficiency between 4°C and 2°C. Interestingly, the  $\beta$ -diversity and principal coordinate analysis (PCoA) analysis demonstrate shifts in microbial communities with decreasing temperatures in the nitrifying biofilms (Figure 4.5b). PCoA results indicate greater community shifts at temperatures occurring above the kinetic threshold temperature ( $> 4^\circ\text{C}$ ), while lesser microbial shifts occurred between 4°C and 2°C and below the kinetic threshold temperature between 2°C and 1°C. As such,

these results suggest that the bulk of the nitrifying microbiome adaptations to low temperature is occurring above the kinetic threshold temperature, correlating with the observed increases in biofilm thickness and biomass viability in the nitrifying biofilm above 4°C.

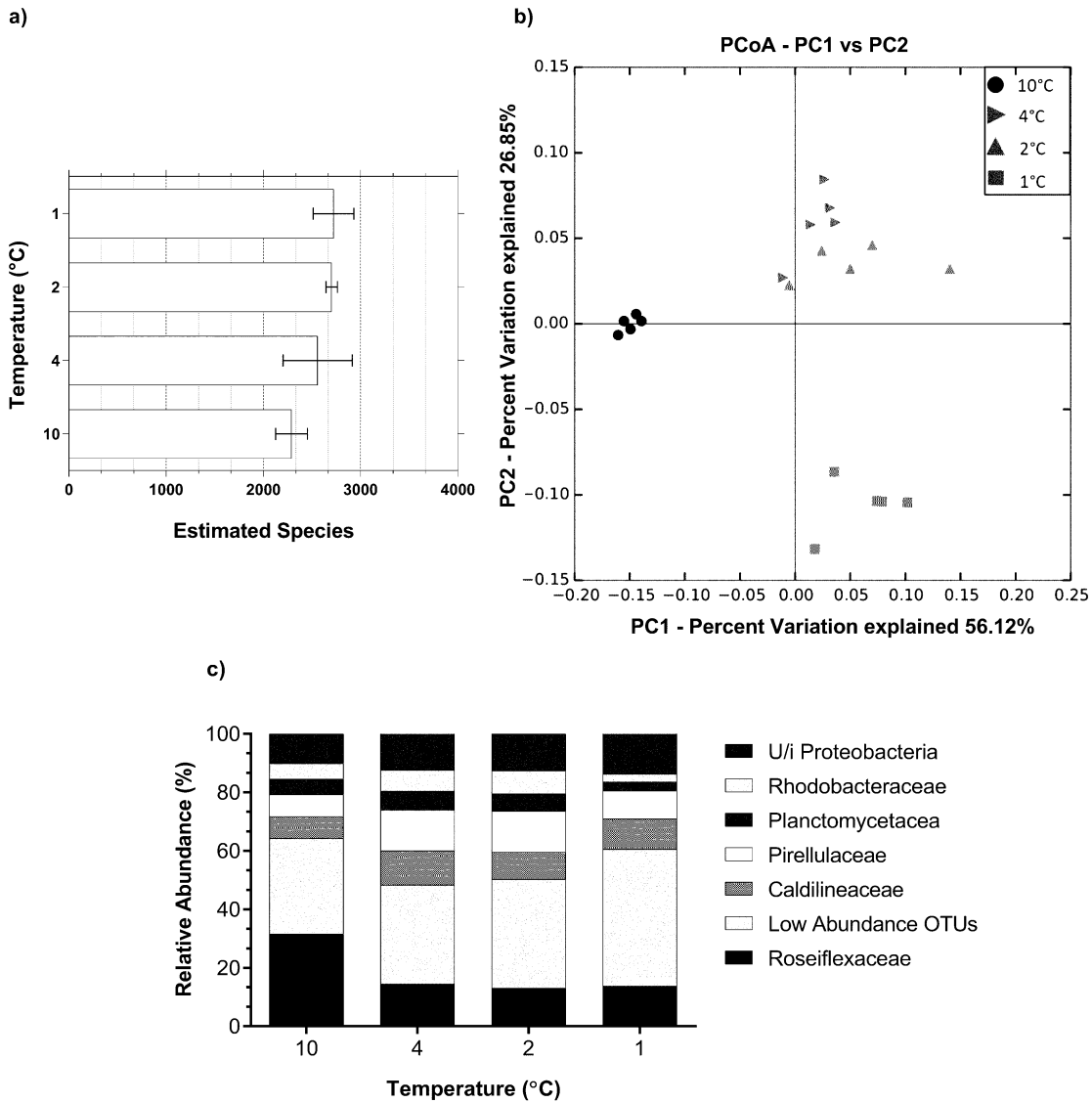


Figure 4.5. Nitrifying microbiome assessments across temperatures, a)  $\alpha$ -diversity, b)  $\beta$ -diversity, and c) family-level taxonomy.

Taxonomy assessment of the nitrifying biofilms indicates that the embedded bacteria of the nitrifying biofilms were predominantly mesophiles, suggesting that little to no bacterial growth occurred between the low temperatures of 10°C and 1°C. At the phylum level, *Actinobacteria*, *Proteobacteria*, *Planctomycetes*, *Acidobacteria* and *Chloroflexi* represented the major phylum groups at 10°C, 4°C, 2°C and 1°C. With respect to statistically relevant trends with decreasing temperatures, the bulk of the microbiome shifts occur again above the kinetic threshold temperature, or between 10°C and 4°C, at which *Chloroflexi*'s relative abundance decreased while an increase of *Actinobacteria* and *Proteobacteria* was recorded. At the family level, *Roseiflexaceae*, *Caldilineaceae*, *Pirellulaceae*, *Rhodobacteraceae*, *Planctomycetaceae*, and an unidentified *Proteobacteria* were identified as the major families while the minor families (<5%) were grouped under low abundance OTUs group (Figure 4.5c). As such, the 16s sequencing results demonstrate that the dominant bacteria identified in the nitrifying biofilm between 10°C and 1°C are involved in the metabolism of polysaccharides and, more importantly of amino acids, crucial for the maintenance of the biofilm's structure and extracellular polymeric substances (EPS) production. Thus, indicating the capacity of the phylogenetically diverse nitrifying biofilm to mitigate the adverse effects of environmental stressors such as low temperatures on the biofilm integrity (Ahimou et al., 2007; Nielsen et al., 2009; Whitman et al., 2015; Ren et al., 2016; Miao et al., 2017; Yu et al., 2018; Park et al., 2020b). For the statistically relevant changes with decreasing temperature, the fraction of filamentous bacteria, such as *Roseiflexaceae*, decreased by 17.25% above the kinetic threshold temperature, with a percent abundance of  $31.72 \pm 8.55\%$  at 10°C and  $14.47 \pm 1.92\%$  at 4°C (Beer et al., 2002; Ward et al., 2018). Despite this initial significant decrease in the fraction of filamentous bacteria, *Roseiflexaceae* remains unchanged below 4°C down to 1°C

and remains one of the dominant bacteria in the biofilm, which correlates with the thicker and less dense nitrifying biofilm observed above.

The *Nitrosomonas* and *Nitrosovibrio* genera from the *Nitrosomonadaceae* family and the *Nitrospira* genus from the *Nitrospiraceae* family were the only AOB and NOB identified with relative abundances of  $3.43 \pm 0.51\%$  and  $0.94 \pm 0.44\%$  respectively at  $10^\circ\text{C}$  (Cho et al., 2014; Young et al., 2017a; He et al., 2018b; Zhang et al., 2018). Although performing the metabolic process of interest, AOB and NOB represent only a small fraction of the microbiome, often demonstrated to be less than 12% of the species identified in low temperature nitrifying biofilms (Hoang et al., 2014a; Young et al., 2017a). The DNA sequencing data indicates no statistically relevant changes in the relative abundance of AOB between  $10^\circ\text{C}$  and  $1^\circ\text{C}$ , while NOB's percent abundance decreased above the kinetic threshold ( $> 4^\circ\text{C}$ ) from  $0.94 \pm 0.44\%$  to  $0.19 \pm 0.10\%$  with no further statistically relevant changes below  $4^\circ\text{C}$ . Thus, no changes in AOB's percent abundance was recorded to justify the decline in nitrification efficiency between the kinetic threshold temperature. Moreover, taking into account the observed increases in biofilm thickness, increases in biomass viability, and the relative abundance of nitrifiers in the biofilm, the estimated AOB per carrier (AOB/carrier) increases by 30.2% above the kinetic threshold temperature between  $10^\circ\text{C}$  and  $4^\circ\text{C}$ , followed by a lesser increase ( $<7\%$ ) below the  $2^\circ\text{C}$  (Hoang et al., 2014a; Young et al., 2017a). As such, these results identify the increase in estimated AOB/carrier within the nitrifying MBBR systems as a factor for the significant nitrification rates attained at low temperatures such as  $1^\circ\text{C}$ . On the other hand, the NOB/carrier decreases by 45.3% above the kinetic threshold temperature, between  $10^\circ\text{C}$  and  $4^\circ\text{C}$ , then increase by 5.9% between  $4^\circ\text{C}$  and  $2^\circ\text{C}$ , and finally increased again by 7.1% between  $2^\circ\text{C}$  and  $1^\circ\text{C}$ . A decrease in NOB/carrier above the kinetic threshold temperature is associated with the 66% decrease of the *Nitrospiraceae* family's relative

abundance between 10°C and 4°C. In addition, the decrease in the estimated NOB/carrier above 4°C correlates with the slight increase in nitrite accumulation in the bioreactors within the same temperature range recorded more specifically at 6°C.

The PICRUSt metabolic pathway and genes function predictions of the biofilm with KEGG indicate an upregulation of crucial functions above the kinetic threshold temperature ( $> 4^{\circ}\text{C}$ ), as displayed in Figure 4.6. Metabolic pathways and functional genes potential linked to EPS production increased above 4°C. Such pathways are characterized by an increase in the relative abundance of predicted functional genes linked to biofilm formation such as *remA* associated with EPS synthesis and carbohydrate-binding along with several other metabolic pathways linked to carbohydrate and amino acids metabolisms (Winkelman et al., 2013). The predicted upregulation of *IDH* and *ccpA* genes are also notable and essential to biofilms. The *ccpA* gene encoding for the *catabolite control protein A* increases the gene expression of *isocitrate dehydrogenase*, a crucial enzyme for polysaccharides intercellular adhesion (PIA) and EPS formation pathways (Arciola et al., 2015; Park et al., 2020a). However, it is worth noting the limitations of metabolic pathway predictions as the suppression of the relative functional potential of the citric acid cycle (TCA) pathways linked to an upregulation of the *ccpA* gene was not observed above 4°C. Overall, the functional genes potential changes above 4°C associated with the biofilm formation correlates with the observed increase in biofilm thickness, and the large fraction of polysaccharides producing families such as the *Roseiflexaceae* identified in the taxonomy results of the biofilms.

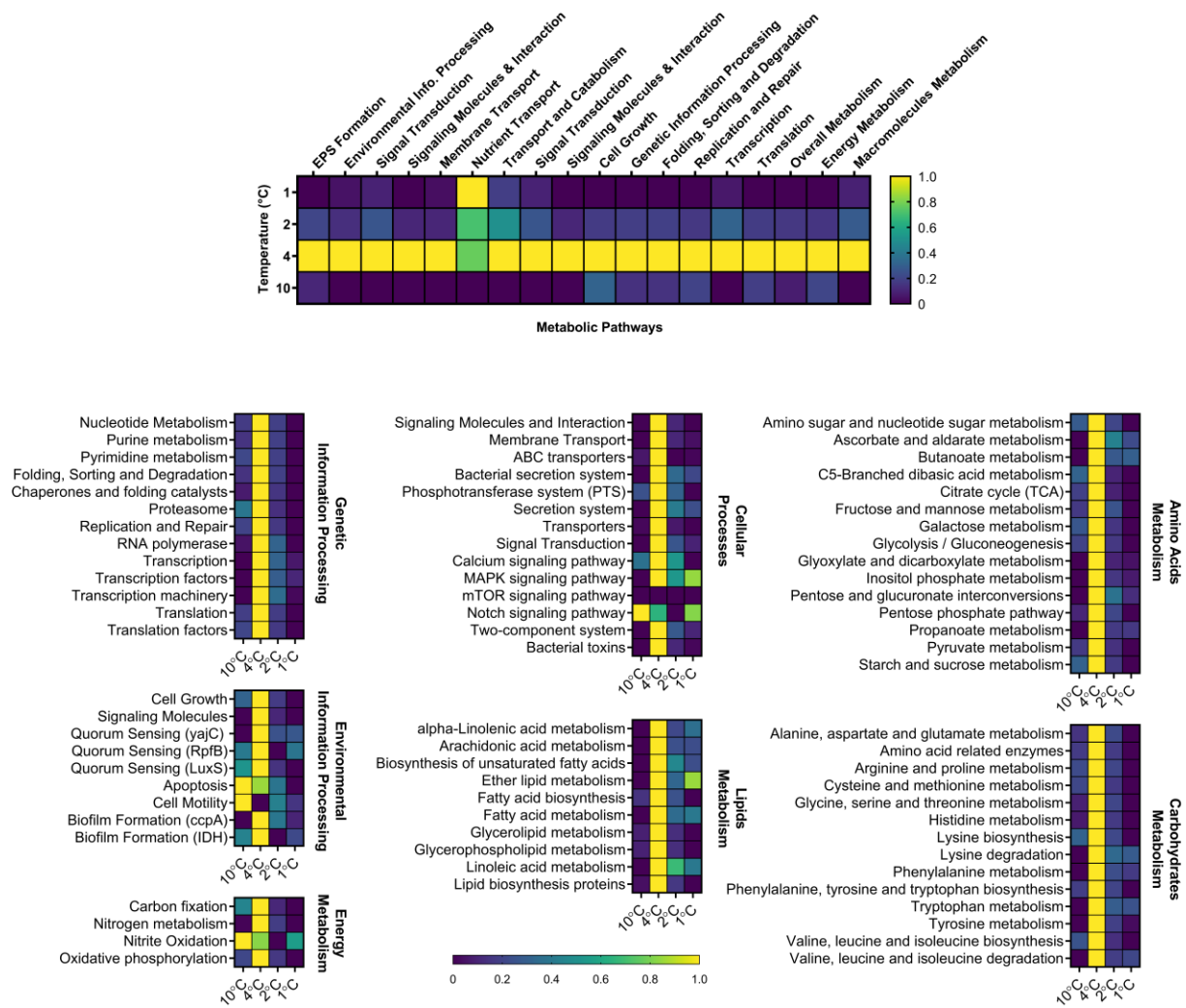


Figure 4.6. Predicted metabolic pathways and key metabolic processes in the nitrifying biofilm normalized across temperatures.

Cell communication pathways in the biofilm associated with the quorum sensing are up-regulated in the biofilm above the kinetic threshold temperature ( $>4^{\circ}\text{C}$ ) characterized by the upregulation of relative potentials of genes such *RpfB*, *LuxS* and *yajC* (Ham et al., 2019). These genes are integral parts of the biofilm responses and adaptation mechanisms to environmental stressors such as temperatures that correlate with the greater microbial shifts measured observed only above  $4^{\circ}\text{C}$  by  $\beta$ -diversity and PCoA analysis.

Energy metabolism within the biofilm also increases above 4°C, namely nitrogen metabolism pathways characterized by the two-step nitrification process, which correlates with the increase in estimated AOB/carriers within the reactors between 10°C and 4°C. The observed nitrite accumulation above the kinetic threshold temperature (at 6°C) is linked, according to PICRUSt analysis, to a down regulation of the *NorA* and *NorB* genes in the biofilm above 4°C. This decrease in the functional potential of these genes, encoding for the two subunits of the *nitrite oxidoreductase*, is the only pathway down regulated and associated with the nitrification reaction above 4°C (Spieck et al., 1998).

In addition, a similar pattern is observed for the carbon fixation potential within the biofilm, or the CO<sub>2</sub> utilization, mediated by autotrophs, such as the nitrifiers within the biofilm. Metabolic activities linked to genetic information processing demonstrate an upregulation above 4°C, correlating with the observed increase in cell viability between 10°C and 4°C within the biofilm. Also correlating with the increase in viable cells in the biofilm is the increase of mechanisms linked to biofilm growth mechanisms, stress response pathways protecting the DNA characterized by genes such as *dps* shielding the DNA from DNase activities, *btuE* and *trxA* mitigating oxidative stress (Almirón et al., 1992; Brenot et al., 2004). Other notable functional characteristics in the biofilm are the cell motility mechanisms characterized, for instance, by the *mTOR* pathways did not increase, suggesting that biofilm remains stable above 4°C; amino acids, lipids and carbohydrates biosynthesis linked to biofilm and EPS integrity (Flemming and Wingender, 2010; Zhu et al., 2015; Gu et al., 2018; Noureldein and Eid, 2018).

## 4.6. Conclusions

The biofilms, biomass and microbiome of attached growth systems were investigated between the temperatures of 10°C and 1°C in nitrifying MBBR systems fed with real wastewater

and operated at decreasing temperatures in a temperature-controlled chamber. The kinetic threshold temperature, characterized by a significant decline of 22.47% in attached growth nitrification efficiency, was delineated between the temperatures of 4°C and 2°C, above which the bulk of the biofilm, biomass, and microbiome acclimatization to low temperature occurred.

Taxonomy assessment indicated no statistically relevant changes in the relative abundance of AOB, mediating the rate-limiting step of the nitrification process between 10°C and 1°C. As such, the decline of 22.47% in nitrification efficiency between 4°C and 2°C is associated with a decrease in AOB's metabolic activities due to low temperatures. In contrast, NOB's relative abundance decreased above the kinetic threshold temperature ( $> 4^{\circ}\text{C}$ ), which correlates with a recorded increase in nitrite accumulation in the bioreactors at 6°C. The nitrifying biofilm increased in thickness and decreased in density above the kinetic threshold temperature ( $> 4^{\circ}\text{C}$ ), correlating with the dominant filamentous bacteria identified in the biofilm, and the predicted upregulation of metabolic pathways associated with biofilm and EPS formation. Similarly, the biofilm displayed a greater increase in biomass viability above 4°C. An increase in cell viability may be linked to an increase in substrate availability and diffusion throughout the biofilm linked to the lower cellular activity of the embedded bacteria and an increase in membrane rigidity at low temperatures. Moreover, in predicted metabolic pathways, apoptosis was down regulated while cell growth, transcription, and translation were up-regulated in the biofilm above 4°C. The  $\beta$ -diversity analysis indicates that the biofilm undergoes greater microbial shifts above 4°C as well, correlating with the predicted upregulation of key metabolic pathways such as those associated with quorum sensing. The estimate of nitrifiers/carrier, accounting for the increases in biofilm thickness and biomass viability, increases significantly above the kinetic threshold temperature ( $> 4^{\circ}\text{C}$ ), followed by lesser increases down to 1°C.

The sum of these observations indicates that the adaptation of nitrifying biofilms to low temperatures occurs above 4°C, preceding the low temperature-induced non-linear decline in nitrification efficiency. These responses to low temperatures in nitrifying MBBR systems, along with their capacity to retain slow growing bacteria inherent to biofilm technologies, allow these systems to maintain significant nitrification rates at temperatures as low as 1°C.

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## **Chapter 5. Microbial Response of Nitrifying Biofilms to Cold Shock**

### **5.1. Context:**

Chapter 5 is a version of the study submitted to the journal of Environmental Science: Water Research & Technology titled: *Microbial Response of Nitrifying Biofilms to Cold Shock* by W. Ahmed and R. Delatolla. This study includes an investigation of cold-shocked attached growth nitrification kinetics and an analysis of the effects of cold-shocks in nitrifying MBBR systems down to the microbiome level. This study delineates cold-shock responses down to 1°C with an assessment of the biofilm morphology (thickness, mass, and density), the biomass viability, and the embedded microbial communities. This study is the first and only study using modern molecular techniques to investigate the adverse effects of cold-shocks on nitrifying biofilms but also the first study assessing cold-shocked attached growth nitrification kinetics with extended exposures to 1°C.

### **5.2. Abstract**

The purpose of this study is to investigate the effects of cold-shocks on attached growth nitrification kinetics and the nitrifying biofilm between the temperatures of 10°C and 1°C. A comparison between two acclimatized MBBR reactor in-series system and a two cold-shocked reactors in-series resulted in 17.28% lower removal rates for the cold shocked system. Greater increases in biofilm thickness and decreases in density were recorded with the 10°C to 1°C transition for the acclimatized biofilms followed by lesser increases in thickness and decreases in density with elapsed time at 1°C contrary to the cold-shocked biofilm displaying an opposite pattern. The fraction of viable cells in the cold-shocked biofilm was shown to remain unchanged following the cold-shock and with exposure time at 1°C, contrary to the acclimatized biofilm that demonstrates an increase in viability of embedded cells at 1°C.  $\beta$ -diversity analysis results

indicated a greater community shift occurred in the acclimatized biofilm during the acclimatization as temperatures decreased from 10°C to 1°C, while the cold-shocked system underwent a significant microbiome shift following the shock event; during the operation at 1°C.

### **5.3. Introduction**

The critical nutrient levels recorded in surface waters in North America and worldwide are mainly due to the anthropogenic release from point source polluters such as water resources recovery facilities (WRRF)(Murdoch et al., 2000; Driscoll et al., 2003; Preston et al., 2011; Cao, 2015). The environmental and economic impacts of eutrophication and toxicity effects caused by elevated ammonia concentrations discharged into receiving water bodies are well reported and have resulted in increasingly stringent effluent regulations in numerous countries (EEC, 1991; Canada Gazette, 2012; USEPA, 2016). To mitigate this issue, the biological oxidation of ammonia through nitrification remains one of the most common ammonia treatment solutions in municipal treatment facilities (Balakrishnan and Eckenfelder, 1969b; Zhou et al., 2016a; Weißbach et al., 2017). However, the temperature-sensitive process of nitrification becomes hindered at low temperatures in conventional suspended growth systems such as the lagoon facility in which nitrification remains unachievable at temperatures as low as 1°C (van Dyke et al., 2003; Ducey et al., 2010; Chen et al., 2018).

On the other hand, attached growth systems such as the moving bed biofilm reactors (MBBR) have recently been shown to achieve significant nitrification rates at temperatures as low as 1°C (Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Delatolla et al., 2017; Young et al., 2017a; Ahmed et al., 2019). MBBR systems benefit from the inherent advantages of biofilm technologies, particularly the resiliency of biofilms to environmental stressors such as temperature and their inherent ability to retain slow growing organisms such as the bacteria mediating the two-

step process of nitrification (Wang et al., 2005; Barwal and Chaudhary, 2014; Dezotti et al., 2018). As such, MBBR systems are an appropriate upgrade technology to passive and low to moderate flow lagoon systems due to their hydraulic compatibility and low operational intensity compared to other technologies (Ødegaard, 2006; Zhou et al., 2018).

The biofilms attached to nitrifying MBBR carriers are composed of phylogenetically diverse bacteria, including the autotrophs identified as ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) responsible for the two-step nitrification process (Kindaichi et al., 2004; Monteiro et al., 2014). Several nitrifiers (AOB and NOB) were identified in WRRF with *Nitrosomonas* and *Nitrospira* reported as the dominant AOB and NOB families (Almstrand et al., 2013; Monteiro et al., 2014; Leyva-Díaz et al., 2017; Zhang et al., 2018). Although performing the metabolic process of interest, nitrifiers represent only a small fraction, often demonstrated to be less than 12%, of the species identified in the biofilms of low temperature nitrifying MBBR systems (Hoang et al., 2014a; Young et al., 2017a).

Although the impact of low temperatures on the performance of nitrifying MBBR has been shown in several studies there remains limited research available on the impact of rapid temperature decreases, known as cold-shocks, on nitrification kinetics and the nitrifying biofilm (Shammas, 1986; Hem et al., 1994; Rusten et al., 1995; Salvetti et al., 2006; Delatolla et al., 2010). Sudden decreases in wastewater facility temperatures may occur during periods of snowmelt and extreme weather events and cold air outbreaks, with the frequency of these occurrences being predicted to increase in specific geographic locations in the coming decades (Kanno et al., 2016; Champagne et al., 2017; Smith and Sheridan, 2018).

Cold-shocks cellular level responses are not fully understood as well as the extensively researched heat shocks; however, research on cold-shocks is occurring that aims to clarify the

cellular cold-shock response mechanisms in biofilms (Beales, 2004; Eshwar et al., 2017; Caruso et al., 2018; Coorey et al., 2018) . Rapid decreases in temperature have shown adverse effects on essential cellular functions from impeding transcription and translation mechanisms to hindering substrate uptake due to increased membrane rigidity(Horn et al., 2007; Phadtare and Severinov, 2010; Barria et al., 2013) . With respect to cold-shock effects on wastewater nitrifiers, current literature indicates that these rapid decrease in temperature have a severe impact on both suspended and attached growth nitrifying system's performance. (Hwang and Oleszkiewicz, 2007; Delatolla et al., 2010; Chen et al., 2018). The application of biofilm systems for the treatment of wastewater at 1°C is timely with new applications of nitrifying MBBR systems for passive treatment facility upgrades, the lowest temperature investigated in a cold-shock study using post-carbon treatment nitrifying MBBR to date has been at 4°C (Delatolla et al., 2010). However, studies have demonstrated that temperatures in lagoon facilities attain temperatures as low as 1°C for an extended period of time during the cold season (Almomani et al., 2014; Young et al., 2016; Young et al., 2017b, 2017a).

As such, no studies exist with respect to biofilm responses to rapid decreases of temperatures down to 1°C, indicating a crucial need for research not only on the impact of cold-shocks on attached growth nitrification kinetics but also the biofilm response to cold-shocks down to 1°C. For this aim, the purpose of this study is to investigate the effects of cold-shock to 1°C on nitrifying biofilm. Explicitly, the goals are to determine the effects of cold-shock to 1°C on the subsequent extended operation of nitrifying MBBR biofilms at 1°C and to determine the effects of a cold-shock to 1°C on the nitrifying biofilm thickness and density, cell viability of the embedded biomass as well as the response of the microbiome.

#### **5.4. Materials and Methods**

### 5.4.1. Experimental Setup, Start-Up and Operation

The experimental design consists of four laboratory scale MBBR systems separated into two treatment trains. Each treatment train consisting of two-stage MBBR reactors recommended for low temperature nitrifying MBBR systems operating at temperatures as low as 1°C (Ahmed et al., 2019). The first treatment train (AR1-AR2) is operated such that the two reactors in-series are acclimatized to 1°C and the second treatment train, again consisting of two reactors in-series (SR1-SR2), is cold-shocked from 10°C to 1°C (Figure 5.1). The reactors were placed in a temperature-controlled chamber to provide precise temperature control, and once the two treatment trains (four reactors) reached 1°C, they were operated for 52 days at 1°C. The reactors in each train are identical, with volumes of 2.2 L for AR1 and AR2 and 0.75 L for SR1 and SR2. Reactors with smaller volumes were selected for SR1 and SR2 due to the limited volume of collected municipal wastewater used for this study. AnoxKaldnes K5 seeded carriers (Lund, Sweden) with a diameter of 25 mm, a height of 4 mm and surface area to volume ratio of 800 m<sup>2</sup>/m<sup>3</sup> were used in all reactors at a fill percentage of 45%. The K5 carriers were harvested from a partially nitrifying integrated fixed-film activated sludge, secondary municipal wastewater treatment system located in Hawkesbury, Ontario, Canada. Air pumps and air diffusers were connected to the reactors to mix the media and supply dissolved oxygen (DO).

The acclimatized system, reactors AR1 and AR2, was slowly acclimatized down from 10°C to 1°C in decrements of 2°C (Hwang and Oleszkiewicz, 2007). This decrease in temperature was performed such that sufficient time was provided at each temperature of 10°C, 8°C, 4°C, 6°C, 2°C and 1°C for the reactors to reach steady-state kinetics prior to decreasing the temperature. Steady state was defined in this study as removal rate fluctuations within ± 10%. The reactors of

the cold-shocked system, SR1 and SR2, underwent a cold-shock, where the temperature was changing instantaneously from 10°C to 1°C without any gradual acclimatization period.

Both systems were fed with real wastewater collected from a partially aerated continuous flow multipond lagoon facility with a hydraulic retention time of 20.3 days. The wastewater fed to the MBBR systems were characterized by a total ammonia nitrogen ( $\text{NH}_4^+/\text{NH}_3\text{-N}$ ) concentration of  $39.97 \pm 0.36$  mg TAN/L; nitrate concentration of  $3.22 \pm 0.03$  mg  $\text{NO}_3^-$ -N/L; nitrite concentration of  $0.08 \pm 0.02$  mg  $\text{NO}_2^-$ -N/L; soluble chemical oxygen demand concentration of  $88.75 \pm 1.09$  mg sCOD/L; alkalinity of  $303.41 \pm 2.94$  mg  $\text{CaCO}_3$ /L, total suspended solids concentration of  $7.09 \pm 0.62$  mg TSS/L, and volatile suspended solids concentration of  $5.87 \pm 0.61$  mg VSS/L.

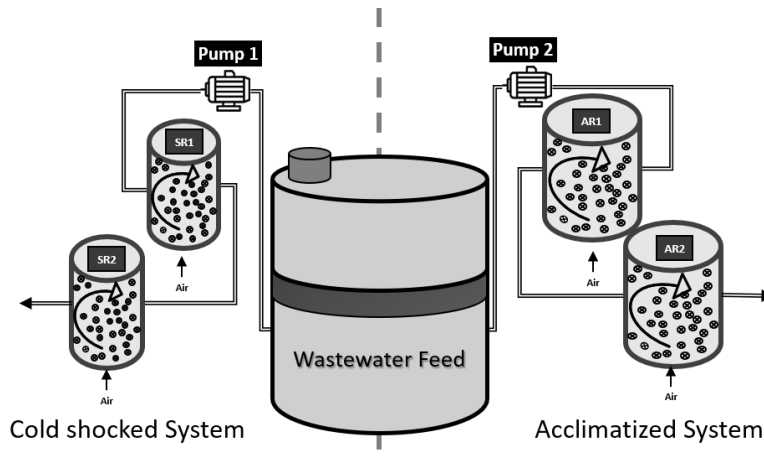


Figure 5.1 Experimental setup of the acclimatized system (AR1-AR2) and the cold-shocked system (SR1-SR2).

#### 5.4.2. Wastewater Constituent Analysis

The following standard methods were used to measure water constituents: 4500  $\text{NH}_3$ , 4500  $\text{NO}_2^-$ , 4500  $\text{NO}_3^-$ , 2540D-TSS (TSS dried at 103°C-105°C) and 2540E-VSS (fixed and volatile suspended solids ignited at 550°C) (APHA, 2005; Tian and Delatolla, 2019). sCOD and  $\text{CaCO}_3$  concentrations were measured using HACH methods 8000 and 10239 with a DR 5000

Spectrophotometer (HACH, CO, USA). Dissolved oxygen, pH and temperatures were measured using DO and pH probes (VWR, ON, Canada) and a temperature probe (Lascar Electronics, PA, USA). Ammonia and DO mass transfer limitations were determined using the Equation 5.1 where  $S_{ba}$  is the DO concentration in the bulk liquid,  $S_{bd}$  is the ammonia concentration in the bulk liquid,  $D_{wa}$  is the diffusivity constant of oxygen in water,  $D_{wd}$  is the diffusivity constant of ammonia in water,  $V_a$  is the molar stoichiometric reaction coefficient of oxygen,  $V_d$  is the molar stoichiometric reaction coefficient of ammonia,  $MW_a$  is the molecular weight of oxygen, and  $MW_d$  is the molecular weight of ammonia (WEF, 2010; Young et al., 2016).

$$S_{ba} < \frac{D_{wd} \cdot V_a \cdot MW_a}{D_{wa} \cdot V_d \cdot MW_d} \cdot S_{bd} \quad 5.1$$

### 5.4.3. Biofilm Morphology

The images used to assess the biofilm thickness were acquired by variable pressure scanning electron microscopy (VPSEM) with a Vega II-XMU SEM (Tescan USA Inc., PA, US). Twenty VPSEM images were acquired from triplicate carriers (Delatolla et al., 2009) with 100 measurements taken per image using NI Vision Assistant Machine Vision's Advanced Edge Detection tool (National Instruments, LabView 14, TX, US). Biofilm mass was determined using a modified Delatolla et al. (2009) method on triplicate carriers. Harvested carriers were dried at 105°C overnight then cooled down in a desiccator for 20 min before measuring the initial weight of each carrier and the attached biofilm. Next, the carriers were thoroughly abraded with a brush to remove the biofilm; the clean carriers were then dried at 105°C overnight and cooled down in a desiccator for 20 min before measuring their weight. Biofilm mass was calculated by subtracting the initial weight of the carrier with the attached biofilm by the weight of the clean carrier.

### 5.4.4. Cell Viability

The images used to assess the biofilm thickness were acquired by variable pressure scanning electron microscopy (VPSEM) with a Vega II-XMU SEM (Tescan USA Inc., PA, US). Twenty VPSEM images were acquired from triplicate carriers as per the Delatolla et al. (2009b) method with 100 measurements taken per image using NI Vision Assistant Machine Vision's Advanced Edge Detection tool (National Instruments, LabView 14, TX, US). Biofilm mass was determined using a modified Delatolla et al. (2009b) method on triplicate carriers. Harvested carriers were dried at 105°C overnight then cooled down in a desiccator for 20 min before measuring the initial weight of each carrier and the attached biofilm. Next, the carriers were thoroughly abraded with a brush to remove the biofilm; the clean carriers were then dried at 105°C overnight and cooled down in a desiccator for 20 min before measuring their weight. Biofilm mass was calculated by subtracting the initial weight of the carrier with the attached biofilm by the weight of the clean carrier. The density was calculated using Equation 5.2, where SA represents the specific surface area of Anoxkaldnes K5 carriers (m<sup>2</sup>).

$$\text{Biofilm Density (mg/cm}^3\text{)} = \frac{\text{Biofilm Mass}}{\text{Biofilm Thickness} \times \text{SA}} \quad 5.2$$

#### 5.4.5. Microbiome Analysis

The microbiome was analyzed following the Young et al. (2017a) method. For each sample, five replicate carriers were collected, and the DNA extracted using a FastDNA Spin Kit (MP Biomedicals, CA, US). The extracted DNA was amplified, targeting the 16s rRNA gene with a two-step polymerase chain reaction (PCR) using a Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc, MA, US). The first PCR amplifying the V6 hyper-variable region and adding a 4–6 nucleotides barcodes as well as the Illumina sequencing adapters at the 3' ends of the amplicons. The second PCR amplifying the product of the first PCR and adding Illumina

flow cell adapters at the 3' ends. A 2% agarose gel was used to inspect the amplicons before purifying them with a Montage PCR95 cleanup kit (EMD Millipore, Millipore Sigma, MA, US). The amplicons were quantified using a Quant-iT dsDNA HS Assay Kit (Life Technologies, ON, Canada) then a pooled sample containing 50 ng of DNA from each sample was sent for processing with a HiSeq2500 at the Center for Applied Genomics (ON, Canada). Sequenced reads assembly and quality filtering were performed using Fast length Adjustment of Short reads (FLASH) software with a minimum quality score of 20 over 90% of the sequences (Magoč and Salzberg, 2011). Using Novobarcode, the reads were demultiplexed and trimmed of their barcodes before analysis (Goecks et al., 2010).

Operational taxonomical units (OTU) clustering at 97% sequence similarity was performed with the Quantitative Insights into Microbial Ecology (QIIME version.1.9)(Caporaso et al., 2010b). The Taxonomy was assigned by closed reference using the UCLUST algorithm and Greengenes 13.5 as a reference database before performing  $\alpha$ -diversity analysis using the Chao1 index and  $\beta$ -diversity analyses (Kuczynski et al., 2012). The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed based on the 16S rRNA sequencing data (Langille et al., 2013). The gene counts for each predicted pathways were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa and Goto, 2000; Kanehisa et al., 2019).

#### **5.4.6. Statistical Analysis**

The student t-test was used to validate significant statistical differences for the constituent analysis result, removal rates, removal efficiencies with a *p*-value less than 0.05 indicating significance. Statistical significance was determined using a one-way ANOVA for the biofilm thickness, biofilm mass and cell viability with a *p*-value less than 0.05 indicating significance.

Kruskal Wallis sum-ranked was used to determine statistical significance for relative microbial abundance for the  $\alpha$ -diversity and ANOSIM for  $\beta$ -diversity's significance with a p-value less than 0.05 indicating significance. Error bars in figures signify 95% confidence intervals.

## 5.5. Results and Discussion

### 5.5.1. Cold Shock Attached Growth Nitrification Kinetics

To delineate the effects of cold-shock on attached growth nitrification kinetics, the removal rates of the two-stage acclimatized MBBR system (AR1-AR2) and the two-stage cold-shocked MBBR system, SR1-SR2, were compared at 1°C at a constant loading rate (Figure 5.2). The results indicate that the cold-shocked system's removal rates were on average 17.28% lower than the rates of the acclimatized system. An average removal rate of  $61.59 \pm 4.56 \text{ gN/m}^3 \cdot \text{d}$  was recorded for SR1-SR2 while AR1-AR2 has attained an average rate of  $75.84 \pm 1.85 \text{ gN/m}^3 \cdot \text{d}$ . In addition, the highest removal rate attained by the cold-shocked system was  $78.79 \text{ gN/m}^3 \cdot \text{d}$  compared to  $103.36 \text{ gN/m}^3 \cdot \text{d}$  for the acclimatized system; while the lowest rates recorded were  $29.65 \text{ gN/m}^3 \cdot \text{d}$  and  $64.05 \text{ gN/m}^3 \cdot \text{d}$  for the cold-shocked system and acclimatized system respectively. Furthermore, the acclimatized system's performance was more consistent, demonstrating less change and less variance throughout the operation at 1°C across 52 days. With respect to the removal efficiencies of the acclimatized system, AR1-AR2 reached higher efficiencies with an average of  $85.62 \pm 1.89\%$ , while the cold-shocked system, SR1-SR2, only attained an average efficiency of  $65.85 \pm 4.17\%$ . At 1°C, average sCOD removal efficiency of 28% and 13% were measured for the acclimatized and cold-shocked systems respectively, with effluents of  $63.5 \pm 1.72 \text{ mg/L}$  and  $77 \pm 2.68 \text{ mg/L}$ .

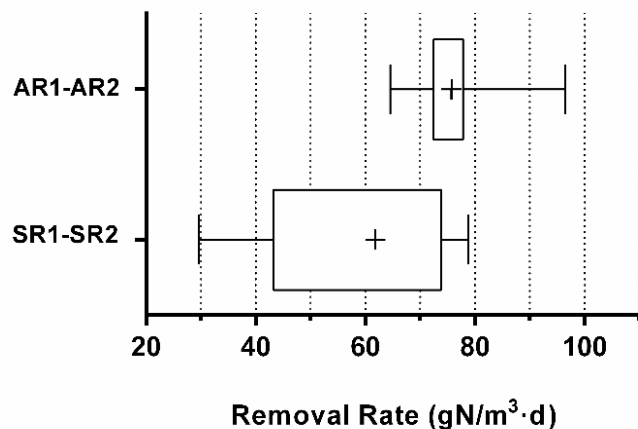


Figure 5.2. Removal rates for the acclimatized system (AR1-AR2) and the cold-shocked system (SR1-SR2) across a period of 52 days at 1°C; (+) average removal rate.

Figure 5.3 displays the average loading and removal rates for each reactor of both the acclimatized and cold-shocked MBBR systems. With loading rates of 181.98 g N/m<sup>3</sup>·d, the first reactors of both systems, AR1 and SR1, attained differing removal rates at 1°C. AR1 achieved an average removal rate of 98.44 ± 4.68 g N/m<sup>3</sup>·d while SR1 displayed an average removal rate 28% lower at 68.96 ± 9.19 g N/m<sup>3</sup>·d. For the second reactors of each system, average removal rates of 49.41 ± 1.34 g N/m<sup>3</sup>·d and 54.22 ± 9.04 g N/m<sup>3</sup>·d was recorded for AR2 and SR2 respectively. However, it is worth noting that the attained removal rates of AR2 and SR2 cannot be directly compared due to the difference in loading rates as the average loading rate of SR2 was 18% higher than AR2, with a rate of 93.53 ± 9.35 g N/m<sup>3</sup>·d compared to 73.87 g N/m<sup>3</sup>·d for AR1 benefiting from the enhanced performance of AR1. For the individual reactors within the two systems, efficiencies of 56.06 ± 1.87% and 40.38 ± 4.88% were recorded for AR1 and SR1 respectively. As for the second reactors of both systems, the efficiencies were 69.69 ± 2.14% for AR2 and 59.40 ± 9.47% for SR2. The removal efficiency comparison between the first reactor and the second reactor in each system shows that the second MBBR reaches higher efficiencies whether the system was acclimatized or cold-shocked down to 1°C. Explicitly, AR2 is 20.08% more efficient

than AR1, and SR2 is 30.92% more efficient than SR1. These second reactors, AR2 and SR2, are benefiting from lower loading rates compared to the first reactors, hence, resulting in ammonia mass transfer rate limited operational conditions as opposed to DO mass transfer rate-limited conditions that occurs at elevated loading rate.

The results hence indicate that the adverse effects of a cold-shock down to 1°C on the nitrification kinetics depends on the loading rate and whether the reactor is operating under ammonia or oxygen rate-limiting conditions. More precisely, cold shocks have a lesser impact on ammonia limited attached growth nitrification than on oxygen limited attached growth nitrification. All in all, the results indicate the cold-shock treatment has an adverse impact on attached growth nitrification kinetics and thus reactor performance. The kinetics of the cold-shocked reactor never recovered to reach the rates recorded for the acclimatized reactor suggesting a late onset of the cold-shock response or the inability of the cells in the cold-shocked MBBR system to alleviate the effects of the cold-shock down to 1°C. These observations correlate with previous findings on cold-shock treatments in both suspended and attached growth systems in which gradual acclimatization to low temperatures has been demonstrated to result in higher kinetics than a rapid decrease in temperature (Hwang and Oleszkiewicz, 2007; Delatolla et al., 2010; Chen et al., 2018; Ahmed et al., 2019).

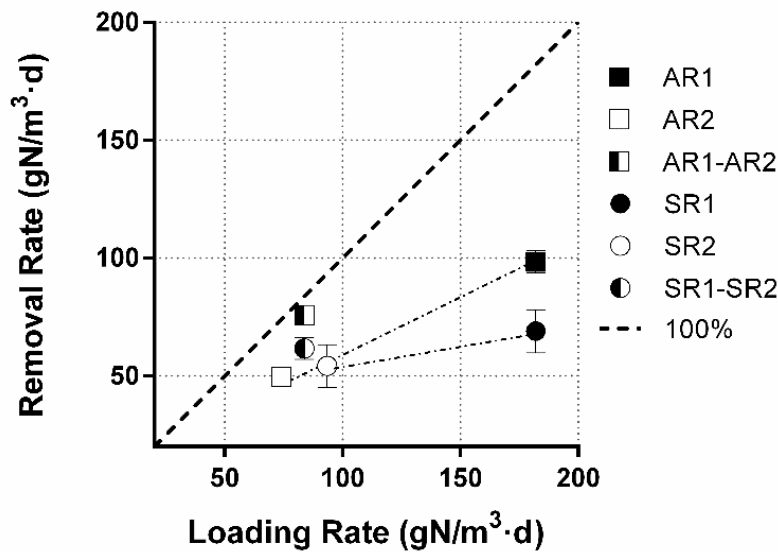


Figure 5.3. Average and 95% confidence intervals of removal rates for AR1-AR2 and SR1-SR2 systems and the individual reactors AR1, AR2, SR1 and SR2 at 52 days of operation at 1°C plotted across loading rates.

### 5.5.2. Cold Shock Biofilm Response

The nitrifying biofilms of the first reactors of the acclimatized and cold-shocked systems, AR1 and SR1, were compared due to their identical loading rates to isolate the biofilm responses for the transition down to 1°C from 10°C and the elapsed time at 1°C between Day 2 and Day 50 at 1°C (Figure 5.4). The initial biofilm thickness of AR1 ( $229.93 \pm 6.41 \mu\text{m}$ ) and SR1 ( $271.22 \pm 6.1 \mu\text{m}$ ) were shown to be slightly different likely due to the natural variance of biofilm thicknesses in different reactors; wherein this study the research focused on the change in thickness induced by temperature change in the systems. A statistically significant increase was observed for both the acclimatized and cold-shocked biofilm when comparing biofilm thickness at 10°C operation after two days of operation at 1°C. The biofilm thickness in AR1 significantly increased by 30.35% ( $p < 0.0001$ ) during the slow acclimatization down to 1°C from 10°C, followed by a lesser measured increase of 9.6% with extended elapsed time at 1°C (from the second day of operation to the fiftieth day of operation at 1°C). The cold-shocked biofilm of SR1 exhibits an increase in

thickness being observed following the sharp transition from 10°C down to 1°C, followed by a subsequent significant increase in thickness with exposure time at 1°C. However, in the cold-shocked biofilm, the greater increase in thickness occurs within the exposures time at 1°C, with the biofilm thickness increasing by 22.31% between the second day of operation and fiftieth day of operation at 1°C. As such, the acclimatized biofilm demonstrated a significant biofilm thickness increase during the transition from 10°C to 1°C, typical of the observations reported in low temperature nitrifying biofilms. The overall lower metabolic activity of the embedded nitrifying cells at low temperatures decreases substrate scarcity at biofilm depths, which likely, in turn, leads to biofilm growth and the biofilm thickness being increased (Bjornberg et al., 2009; Hoang et al., 2014a; Young et al., 2016). The biofilm responses with respect to the thickness for the second reactors, AR2 and SR2, followed a similar pattern to AR1 and SR1 (Supplemental Figure.5.7a).

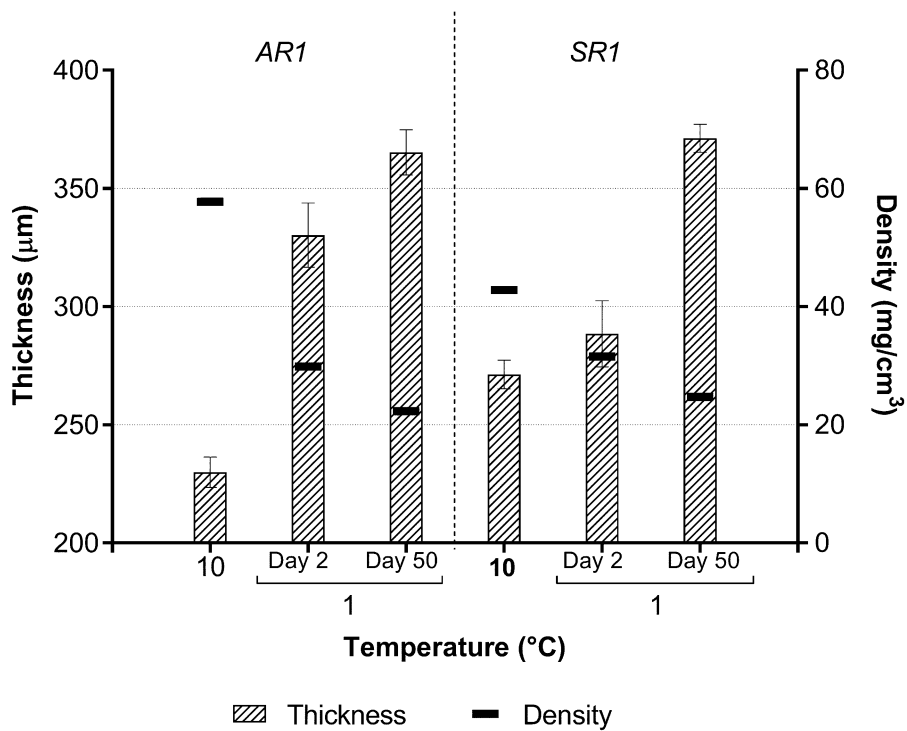
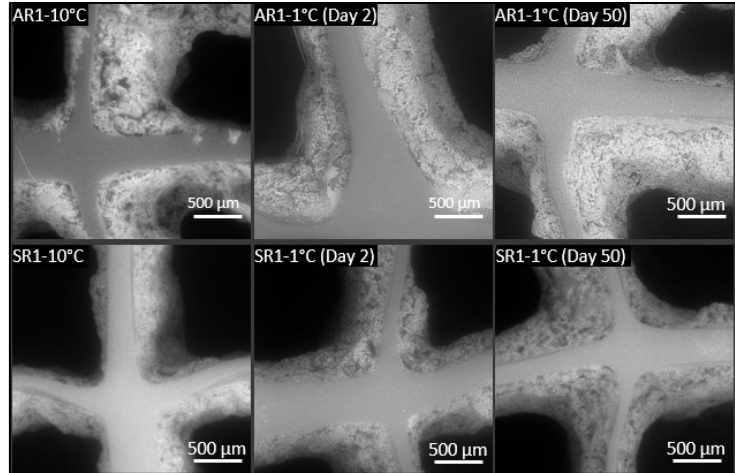


Figure 5.4. Biofilm VPSEM images at  $\times 60$  magnifications, biofilm thickness, and density at  $10^{\circ}\text{C}$ ,  $1^{\circ}\text{C}$  on day 2 and  $1^{\circ}\text{C}$  on day 50.

The biofilm density in both AR1 and SR1 decreased with exposure time at  $1^{\circ}\text{C}$ . The decrease in density results from the increasing biofilm thickness at  $1^{\circ}\text{C}$  with no statistically significant changes in biofilm mass for both the acclimatized and cold-shocked biofilms. During the slow acclimatization down to  $1^{\circ}\text{C}$ , biofilm density in AR1 decreased by 48.27% followed by a second decrease of 15.48% between the 2<sup>nd</sup> and 50<sup>th</sup> day at  $1^{\circ}\text{C}$ . For SR1, the biofilm density

decreased by 26.43% following the cold-shock then by 21.53% between the 2<sup>nd</sup> and 50<sup>th</sup> day at 1°C. Lower densities with increased biofilm thicknesses may be due to the filamentous biofilm morphology observed at 1°C (Karizmeh et al., 2014; Young et al., 2016). Previous research also indicates that a higher polysaccharides content is measured in thicker biofilms, including 1°C nitrifying biofilms, which display more porosity and thus lower densities (Jang et al., 2003; Young et al., 2016). Previous studies also demonstrate that bacterial cold-shock response triggers a series of metabolic pathways initiated in response to rapid downshifts in temperatures, such as cold shock biofilm dispersal (Bester et al., 2013; Toyofuku et al., 2016; Díaz-Salazar et al., 2017; Lin Chua et al., 2017; Wang et al., 2017). However, despite the cold-shock down to 1°C the biofilm in SR1 remained within the typical normal biofilm density suggesting the stability of nitrifying MBBR systems to cold-shock events (Laspidou and Rittmann, 2004; Ahimou et al., 2007; Young et al., 2016).

### **5.5.3. Cold Shock Biomass Response**

The biomass viability, as the percentage of viable cells in the biofilm, was assessed for 10°C to 1°C transition and the elapsed time at 1°C between 2<sup>nd</sup> day and 50<sup>th</sup> day at 1°C (Figure 5.5). The results indicate that the acclimatized and cold-shocked biofilms significantly differ in biomass viability after two days of operation at 1°C. Further, the cell viability of the embedded biomass differed again between the acclimatized and cold-shocked reactors after 50 days of operation at 1°C. It should be noted that no statistical difference in cell viability of the embedded biomass was observed between the acclimatized and cold-shocked reactor at steady state 10°C operation. A significant increase of 31.05% ( $p < 0.0001$ ) in viable cells is recorded for AR1 on the 2<sup>nd</sup> day at 1°C and no statistical difference in cell viability on the 50<sup>th</sup> day compared to the 2<sup>nd</sup> day of operation at 1°C. On the other hand, no statistically relevant change in the percentage of viable

cells is observed for the cold-shocked biofilm, whether it is after the transition to 1°C or with elapsed time at 1°C. The increase in viable cells in the acclimatized biofilm is supported by previous studies on 1°C nitrifying MBBR systems (Hoang et al., 2014a; Young et al., 2017a). The low metabolic activity of the embedded biomass in the biofilm at low temperatures of 1°C may increase substrate availability that likely promotes the viability of numerous cells with low metabolic activities. In other words, an increase in cell viability at lower temperatures was previously attributed to an increase in substrate availability throughout the biofilm, especially at higher depths of the biofilm, as metabolic activities, and thus the rate of substrate consumption of embedded cells decreases. In addition, the decrease in biofilm density recorded above, allowing the increase in substrate diffusion coupled with the decrease in substrate uptake of the embedded bacteria due to membrane rigidity at lower temperatures, both contribute to the increased substrate availability.

In the cold-shocked biofilm, the previously described negative impacts of rapid decreases in temperature, the general impairment of metabolic activities may justify the lack of an increase in cell viability with exposure time at 1°C. The cold-shock response mechanism in phylogenetically diverse cells can also trigger an increase in viability with the assistance of molecular chaperones such as the trigger factor protein, or in general, mitigates the cold-shock adverse effects and cells regain their metabolic activity (Kandror and Goldberg, 1997; Phadtare, 2004; Phadtare and Severinov, 2010). Despite being operated for an extended period after the cold-shock treatment down to 1°C, no increase in cell viability was observed. These observations for AR1 and SR1 were like those made for the second reactors, AR2 and SR2 (Supplemental Figure.5.7b)

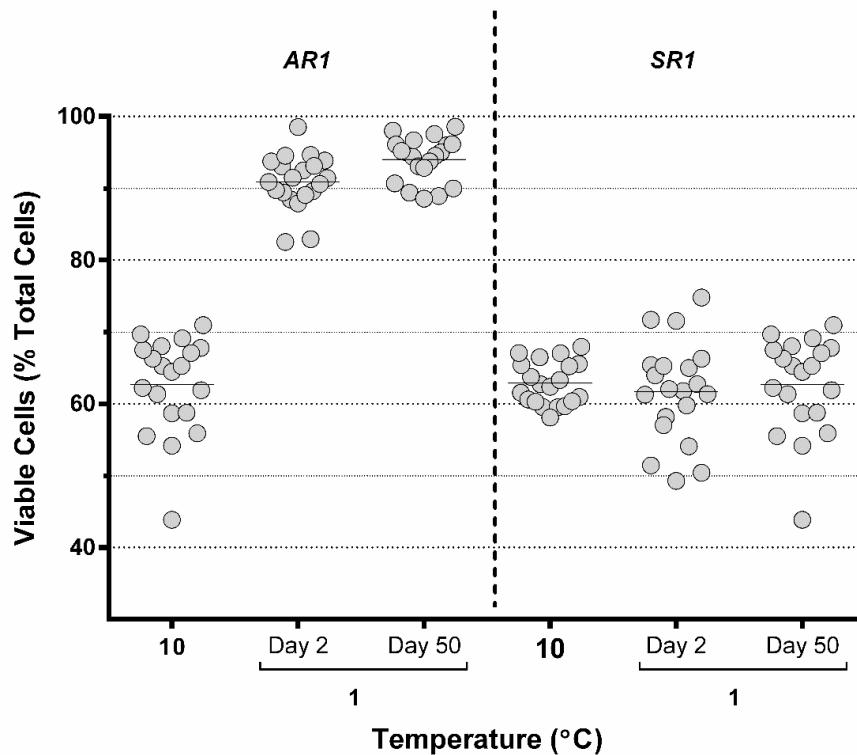
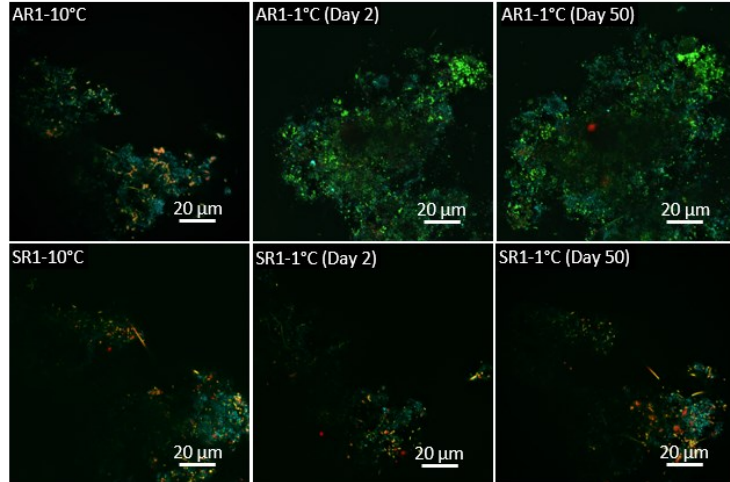


Figure 5.5. CLSM images at  $\times 630$  magnification and cell viability data for AR1 and SR1 at 10°C, 1°C on day 2 and 1°C on day 50.

#### 5.5.4. Cold Shock Microbiome Response

The microbiome response was analyzed using next generation DNA sequencing. High-throughput Illumina sequencing of the biomass embedded in the biofilm of the AR1 and SR1 carriers generated an average read of  $713656 \pm 232893$ . An overall analysis of the estimated

species indicated no significant change in the total number of estimated species between 10°C and 1°C and elapsed time at 1°C between the second and fiftieth days for both AR1 and SR1 (). The acclimatized biofilm at 10°C demonstrated that a total of 41 distinct bacterial phyla were detected with *Firmicutes* ( $18.87 \pm 0.02\%$ ), *Proteobacteria* ( $18.49 \pm 0.04\%$ ), *Chloroflexi* ( $13.83 \pm 0.07\%$ ), *Chlorobi* ( $13.61 \pm 0.02\%$ ) and representing the major sequenced phyla in the biofilm. No significant change occurred in the total number of phyla sequenced in the biofilm remained statistically similar throughout the operation at 1°C. For the cold-shocked biofilm, a total of 35 phyla were detected at the initial stage of 10°C, which remained unchanged with the exposure time at 1°C. Overall the major phylum listed above for AR1 was observed in the cold-shocked biofilm as well, which are common to conventional suspended growth and attached growth systems (Cho et al., 2014; Young et al., 2017a; He et al., 2018a; Zhang et al., 2018).

The ammonia oxidizing bacteria family *Nitrosomonadaceae* is the only AOB family, commonly observed in WRRF, identified in both AR1 and SR1 with relative abundances in the biofilms of  $3.13 \pm 0.01\%$  and  $4.10 \pm 0.001$  at 10°C for AR1 and SR1 respectively. No statistically significant change in the relative abundance of *Nitrosomonadaceae* is observed following two days of operation at 1°C and with the extended operation on the 50<sup>th</sup> day for both AR1 and SR1. Nitrite oxidizing bacteria were also detected in the AR1 and SR1 from the *Nitrospiraceae* and *Bradyrhizobiaceae* families, which include the *Nitrospira* and *Nitrobacter* genera, respectively. Within the acclimatized biofilm, NOB accounted for  $0.62 \pm 0.001\%$  of the communities in the biofilm at 10°C. This relative abundance increased to  $5.13 \pm 0.01\%$  during the acclimatization down to 1°C with no statistically relevant changes observed with the elapsed time at 1°C. An increase in NOB relative abundance during the acclimatization from 10°C to 1°C is likely due to the reported higher growth rates of these bacteria at low temperatures compared to AOB (Helling,

1998; Bollon et al., 2016). The NOB relative abundances did not follow the same trend in the cold-shocked reactor as the fractions of NOB in the biofilm remained unchanged following the cold-shock treatment or with elapsed time to 1°C. A difference between 10°C and 1°C in NOB's relative abundances for the acclimatized and cold-shocked biofilms is associated with the lack of acclimatization steps for the cold-shocked reactor.

The  $\beta$ -diversity using ANISOM metrics, or multiple-site dissimilarity, based on phylogeny within the biofilms of AR1 and SR1 following the transition down to 1°C and with the exposure time at 1°C was also investigated (Figure 5.6). Principle coordinate analysis (PCoA) was conducted to identify the microbiome shifts occurring within the biofilm and the extent of these shifts. For the acclimatized biofilm, the results indicate that a greater microbiome shift occurred during the 10°C to 1°C transition. PCoA analysis at 1°C was also assessed to identify which of the two reactors exhibit community shifts, or adaptation, with the elapsed time at 1°C (Figure 5.6b). As operation at 1°C progresses, SR1 exhibit greater changes within its biofilm compared to AR1 as greater Unifrac distances are recorded for cold-shocked biofilm compared to the acclimatized biofilm. The results suggest that the lesser shifts in the microbiome of AR1 compared to SR1 during the exposure at 1°C may indicate the adaptation of the biofilm to low temperatures occurred at temperatures above 1°C.

Assessing essential metabolic pathways within the biofilms through PICRUSt indicates that the bulk of the acclimatized biofilm responses occurs during the 10°C to 1°C compared to the cold-shocked biofilm (Fig. 5.6c). The upregulation of functional genes associated with biofilm and EPS formation pathways such as *yfiQ* and *RemA* occurred with the 10°C to 1°C transition, thus correlating with the measured increases in biofilm thickness (Park et al., 2020a). Moreover, the potential of macromolecules synthesis pathways such as carbohydrates and proteins, which are

crucial for the biofilm structure, increased between 10°C and 1°C (Winkelman et al., 2013; Arciola et al., 2015). Cell membrane functions including nutrient uptake, signal transductions, and secretion systems essential for the quorum sensing, also increased during this acclimatization phase. The increase of genetic information processing pathways, including DNA repair and replication, is noted during the 10°C to 1°C transition alongside a down regulation of cell death pathways potential characterized by the *mazEF*, *pezAT* and *recA* functional genes (Khoo et al., 2007; Peeters and Jonge, 2018). These metabolic pathways predictions as temperatures decreased from 10°C to 1°C correlate with the recorded increases in cell viability in the acclimatized biofilm. The cold-shocked biofilm displayed a different profile as most of the metabolic pathways increased in potential with elapsed time at 1°C instead of the 10°C to 1°C transition.

Predictions of metabolic pathways have their limitations exemplified by the upregulation of nitrogen metabolism pathways, including ammonia and nitrite oxidation, although removal rates did not improve with elapsed time at 1°C in the cold-shocked reactor. Considering the rapid decrease in temperature between 10°C and 1°C, the cold-shocked biofilm, the similarity in predicted metabolic pathways profiles at 10°C and the 2<sup>nd</sup> day at 1°C is to be expected. However, this similarity highlights the differences with respect to the adverse effects of low temperatures and cold shocks on nitrifying biofilms as not only the nitrification kinetics are drastically different between 10°C and 1°C, but they are also significantly lower than AR1 measured rates. Moreover, between the acclimatized and cold-shocked biofilms, the differences in predicted metabolic potentials with elapsed time at 1°C indicate the existence of a biofilm adaptation to low temperatures occurring between 10°C and 1°C. A crucial adaptation to low temperatures exemplified by the acclimatized reactors and their performance compared to the cold-shocked reactors at the low temperature of 1°C.

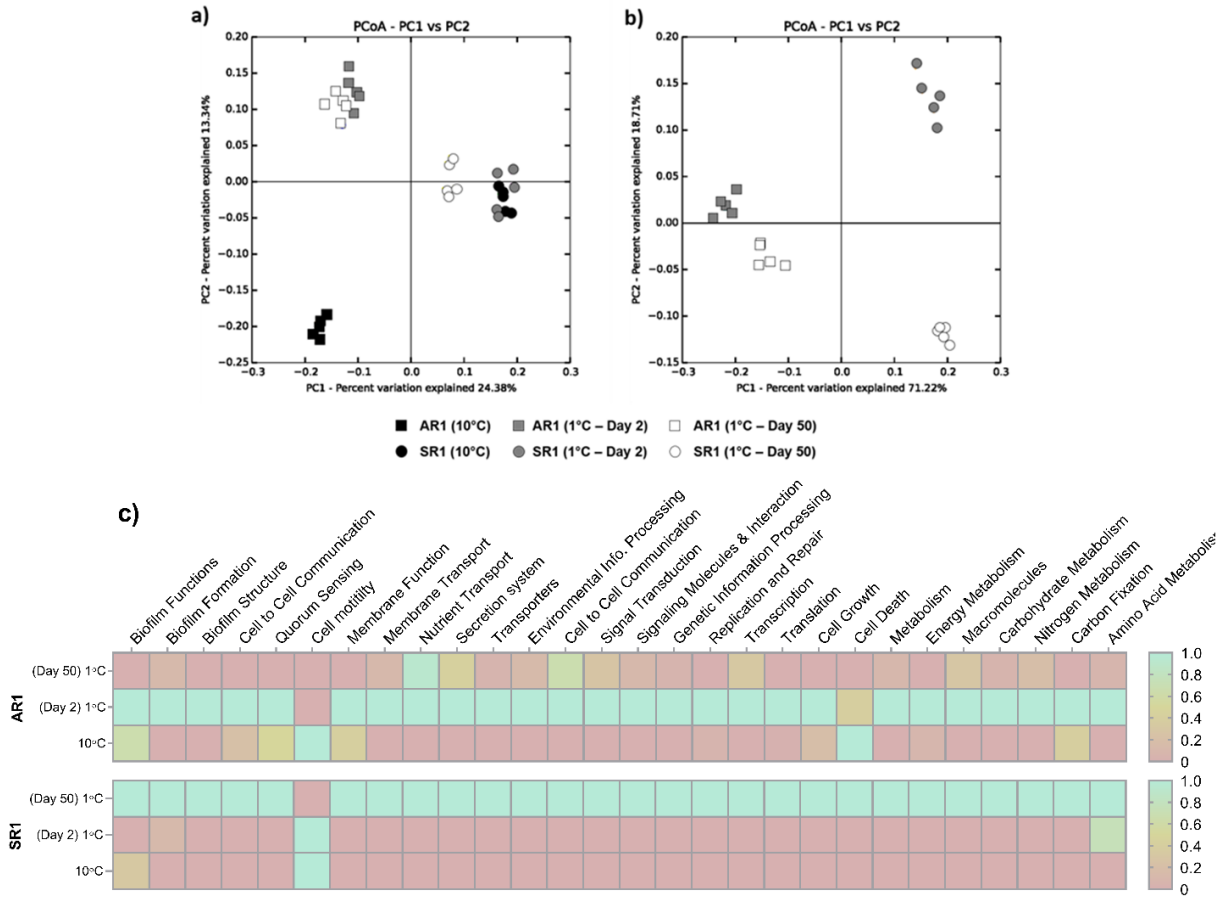


Figure 5.6.  $\beta$ -diversity analysis for AR1 and SR1, a) 10°C, 1°C on day 2 and 1°C on day 50, b) 1°C on day 2 and 1°C on day 50 only, c) PICRUSt metabolic pathway predictions at 10°C, 1°C on day 2 and 1°C on day 50

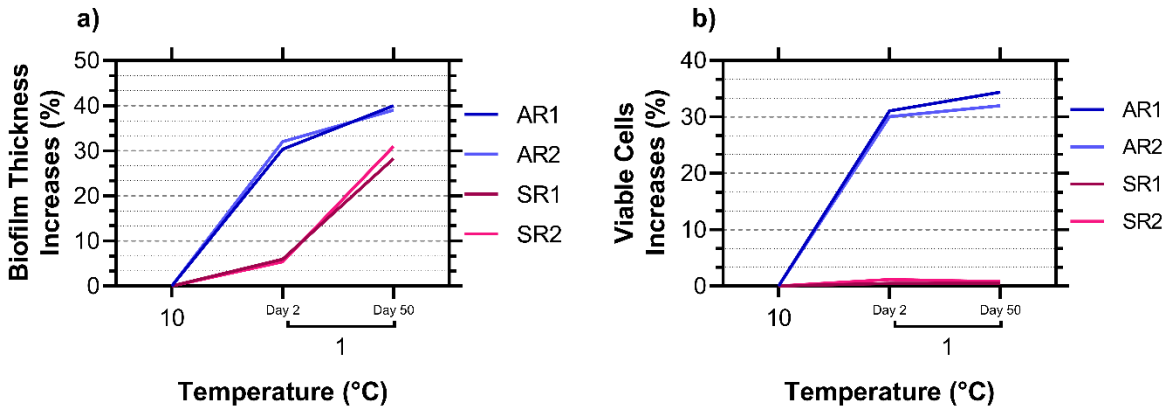
## 5.6. Conclusions

The effect of cold-shocks, or rapid decreases in temperatures, on attached growth systems was investigated using acclimatized and cold-shocked MBBR systems. The cold-shocked MBBR systems in-series, SR1-SR2, displayed, on average 17.28% lower TAN removal rates compared to the acclimatized system, AR1-AR2. Moreover, the first reactors of both acclimatized and cold-shocked systems, AR1 and SR1, with similar loading rates attained different 1°C removal efficiencies with AR1, attaining 28% higher efficiencies than SR1. In addition, SR1 never attained rates similar to the rates recorded in AR1 at 1°C following the cold-shock, despite an extended

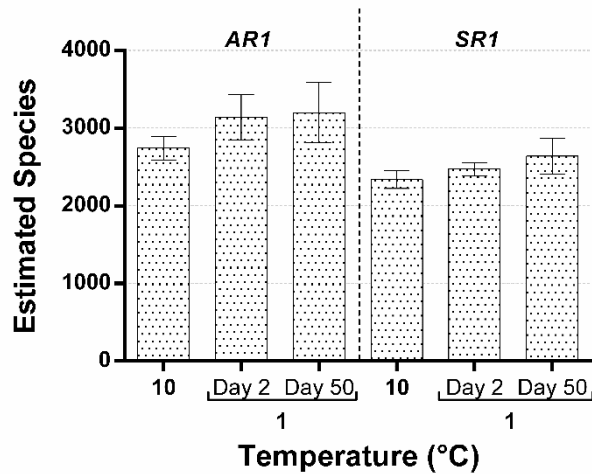
operation. The biofilms in both AR1 and SR1 increased in thickness while their densities decreased between 10°C and 1°C, as reported in previous low temperature nitrifying MBBR systems. Assessment of biomass viability indicated a difference between the acclimatized and cold-shocked reactors. The biomass viability increased by 31.05% in AR1 during the transition from 10°C to 1°C, contrary to SR1, which displayed no statistically significant change in cell viability from the initial stage of 10°C. The  $\alpha$ -diversity results indicated no statistically relevant changes for the transition from 10°C to 1°C and with elapsed time at 1°C with respect to the estimated number of species in both the biofilms of AR1 and SR1. The  $\beta$ -diversity analysis demonstrated that contrary to SR1, greater community shifts occurred in the biofilm of AR1 during its acclimatization down to 1°C from 10°C, followed by lesser community shifts with elapsed time at 1°C. In contrast, the cold-shocked biofilm of SR1 displayed greater microbiome shifts with elapsed time at 1°C. This dissimilarity correlates with the predicted metabolic pathways potentials in both biofilms. The bulk of metabolic pathways upregulations occurs between 10°C and 1°C for the acclimatized biofilm while it occurs with elapsed time at 1°C in the cold-shocked biofilm.

The overall analysis of the results of this study isolates and explains the response of nitrifying biofilms to cold-shock treatment. A rapid decrease in temperature from 10°C to 1°C was characterized by lower nitrification kinetics and differences in biomass and microbiome responses compared to a parallel temperature acclimatized system. Despite the observed changes in performance along with changes in biomass characteristics and the microbial communities, the cold-shocked system exemplified an absence of cold-shock response mechanisms. Although the cold-shocked reactor demonstrated a lower performance compared to the acclimatized system, the cold-shocked system showed an ability to maintain a viable nitrifying community and a significant rate of nitrification.

## 5.7. Supplemental Materials



Supplemental Figure.5.7. Biofilm and biomass responses for the AR1-AR2 and SR1-SR2 a) biofilm thickness increases, and b) viable cells increases.



Supplemental Figure 5.8.  $\alpha$ -diversity analysis for AR1 and SR1 at 10°C and on Day 2 and 50 at 1°C.

## 5.8. References

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# **Chapter 6. Review of Biofilm Technologies as Upgrade Systems to Low Temperature Passive Treatment Systems**

## **6.1. Context**

Chapter 6 is a version of the manuscript in preparation for submission to the journal of Biotechnology titled: *Review of Biofilm Technologies as Upgrade Systems to Low Temperature Passive Treatment Systems* by W. Ahmed and R. Delatolla. This publication summarizes currently available nitrifying biofilm technologies as add-on solutions to passive treatment systems to attain nitrification in low temperatures. In addition, this study synthesizes current knowledge with respect to low temperature nitrifying MBBR systems for their optimal design and operations as upgrade post-carbon removal units.

## **6.2. Abstract**

Increasingly stringent effluent regulations around the globe aim to mitigate the severe adverse effects of nutrient loads on surface waters such as effluent ammonia discharged by wastewater resources recovery facilities (WRRF). The biological mediated process of nitrification remains the most common ammonia treatment method in WRRF. However, this temperature-sensitive process becomes impeded at low temperatures (<15°C) especially in conventional suspended growth systems, with more severe impact observed in the widespread passive treatment systems. While impeded by low temperatures as well, attached growth systems have been shown to be affected to a lesser extent. From the historic century-old trickling filters (TF) to the more modern moving bed biofilm reactors (MBBR), biofilm technologies have been proven capable of attaining significant nitrification rates at lower temperatures than suspended growth systems. The inherent advantages of the attached growth systems allow them to mitigate the adverse effects of environmental stressors such as the temperatures. In fact, at the low temperature of 1°C at which

nitrification ceases in the commonly used lagoon facilities, the nitrifying MBBR technology, can maintain rates between 96 gN/m<sup>3</sup>d and 168 gN/m<sup>3</sup>d as demonstrated in several studies. Moreover, in addition to the innate advantages shared amongst biofilm technologies, the MBBR systems with its small footprints and more importantly its low operational intensity compared to other biofilm technologies presents itself as a suitable upgrade solution to lagoon facilities for year-long nitrification at low temperatures.

### **6.3. Introduction**

The elevated ammonia levels measured in surface water in North America and around the world are due to the anthropogenic release of ammonia from various point source polluters, with water resources recovery facilities (WRRF) being identified as major contributors (Murdoch et al., 2000; Driscoll et al., 2003; Preston et al., 2011). The environmental and economic impacts of water toxicity and eutrophication caused by ammonia discharged into receiving water bodies are resulting in increasingly stringent effluent regulations in most regions of the world (EEC, 1991; Canada Gazette, 2012; USEPA, 2016). To meet these effluent regulations the process of nitrification, the biological oxidation of ammonia to nitrite and subsequently to nitrate, remains one of the most economical treatment solutions in wastewater treatment (Balakrishnan and Eckenfelder, 1969; Zhou et al., 2016). However, as a temperature-sensitive process, nitrification in WRRF becomes hindered at low temperatures leading to untreated ammonia loads discharges in receiving water bodies (van Dyke et al., 2003; Ducey et al., 2010; Chen et al., 2018). Nitrification in lagoon facilities which represent over 50% of Canadian Treatment Facilities, as passive treatment systems are even affected to a greater extent by low temperatures due to long hydraulic retention times (HRT) and severe heat loss which can lead to extended periods of ice covering (Houweling et al., 2007). Moreover, several studies have demonstrated that the growth

rate of nitrifying organisms responsible for the oxidation of ammonia decreases at lower temperature to eventually cease below 4°C, and as such the crucial factor to attain nitrification at low temperatures is contingent on the capacity to retain these organisms in bioreactors in the form of biofilms (Buswell et al., 1954; Painter and Loveless, 1983; Rittman and Snoeyink, 1984; McCartney and Oleszkiewicz, 1990).

The purpose of this review is to synthesize the current available attached growth systems available for the treatment of ammonia in WRRF that operate seasonally or year-round at low temperatures. More specifically this review focuses on conventional and modern biofilm technologies reported to have reached significant nitrification rates at temperatures lower than 15°C. For this aim, the performance and the operational parameters of low temperature nitrification are synthesized for nitrifying biological contactors (RBC), trickling filters (TF), biological aerated filters (BAF), and moving bed biofilm reactors (MBBR); as well as less commonly investigated technologies such as the submerged attached growth reactors (SAGR) or emerging technologies such as the biofilm airlift suspension reactors (BASR).

#### **6.4. Low Temperature Nitrifying Biofilm Technologies Studies below 15°C**

Passive treatment accounted for over half of WRRF in operation since the 1960s, their low operational intensity and land availability to build them made lagoon systems a suitable option for wastewater treatment needs in rural areas (Heinke et al. 1991). However, the hindered nitrification process in suspended growth systems such as lagoons during cold seasons and ever stringent effluent regulations demonstrates the need for suitable upgrade solution to attain ammonia treatment targets. Nitrifying attached growth system demonstrated, on the other hand, the capacity to maintain nitrification at low temperatures defined as temperatures below 15°C in this review (Andreadakis et al., 1993; Rusten et al., 1995; Choi et al., 2008; Matthews et al., 2009; Delatolla

et al., 2010; Kishimoto et al., 2014; Ahmed et al., 2019). It is well established throughout the studies on biofilm technologies, that these bioreactors offer numerous advantages over conventional suspended growth systems which include amongst other their higher treatment efficiencies, low operation costs, small footprints, and foremost for some of them their low operational intensity crucial in the search of lagoon upgrade systems (Rittman 1992; USEPA 1993; Ødegaard, 1999; WEF 2011). Moreover, biofilm technologies have been reported capable of maintaining nitrification as low as 1°C, which is the case of pilot scale nitrifying MBBR systems with volumetric loading rates (VLR) of  $\sim 609.48 \text{ gN/m}^3\text{d}$  or surface area loading rates (SALR) of  $1.25 \pm 0.01 \text{ gN/m}^2\text{d}$ , attained significant volumetric removal rates (VRR) of  $167.24 \text{ gN/m}^3\text{d}$  or surface area removal rates (SARR) of  $0.35 \pm 0.05 \text{ gN/m}^2\text{d}$  (Young et al., 2016; Young et al., 2017) The sum of these advantages indicating that these systems may represent potential upgrade solutions to the common lagoon facility for low temperature ammonia treatment needs as reported in several studies conducted below 15°C (Table 6.1).

Table 6.1. Overview of studies on nitrifying biofilm technologies performed below 15°C

Biofilm Technology and Study Scale	Process Parameters	Temperatures (°C)	Key Findings		References
			Kinetics	Arrhenius correction coefficients ( $\theta$ )	
RBC Pilot scale	Polystyrene foam discs: total surface area of 6.5 m <sup>2</sup> HRT: 5 - 10 hrs pH: 6.7 -7.1	12 - 24		$E_{12^{\circ}\text{C}}^b = 20\%$ (two-stage RBC systems)  $E_{12^{\circ}\text{C}} = 15\%$ (single-stage RBC systems)	(Tawfik et al., 2006)
RBC Full and Pilot scale	Single-stage tertiary RBC $SALR_{10^{\circ}\text{C}}$ $= 4.0 - 5.0 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: 0.31 and 1.37 hrs	~10	$SARR_{10^{\circ}\text{C}}$ $= 1.6 - 2.2 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Boller et al., 1990)
RBC Pilot scale	Discs; total surface area of 3.6 m <sup>2</sup> HRT: 0.02–0.06 hrs pH: 6–9	8 - 21	$E_{10^{\circ}\text{C}} = 68.1\%$	$\theta_{\geq 18^{\circ}\text{C}} = 1.053$	(Andreadakis et al., 1993)
RBC Full scale	HDPE discs: total surface area of 23500 m <sup>2</sup> HRT: <5 hrs pH: 6.9	5 - 15	$SARR_{5^{\circ}\text{C}} = 0.235 \frac{\text{gN}}{\text{m}^2\text{d}}$	$\theta_{15^{\circ}\text{C} \leftrightarrow \leq 7^{\circ}\text{C}} = 1.02$	(Andreottola et al., 2000)
RBC Full scale	Polystyrene discs media; Bionet & Biospiral Polystyrene packages: SA of 260 m <sup>2</sup> /m <sup>3</sup>	4 - 19	$SARR_{13^{\circ}\text{C}} = 1.8 \frac{\text{gN}}{\text{m}^2\text{d}}$  $SARR_{8^{\circ}\text{C}} = 1.5 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Nowak, 2000)

RBC Pilot scale	pH: 6.2 – 7 Polyethylene discs: total surface area of 7.2 m <sup>2</sup> $SALR = 1.8 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: 1.7 and 3.2 hrs pH: 7.5 - 8	4.5 - 24	$E_{8^\circ\text{C}} = 95\%$ $SARR_{8^\circ\text{C}} = 1.71 \frac{\text{gN}}{\text{m}^2\text{d}}$ $SARR_{4.5^\circ\text{C}} = 0.898 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Kapoor et al., 2003)
TF Pilot scale	Vertical flow plastic media FilterPack: SA of 200 m <sup>2</sup> /m <sup>3</sup> pH: 7	15 - 25	$SARR_{15^\circ\text{C}} = 0.25 \frac{\text{gN}}{\text{m}^2\text{d}}$ (Oxygen Limiting condition) $SARR_{<15^\circ\text{C}} = 0.14 - 0.25 \frac{\text{gN}}{\text{m}^2\text{d}}$ (Ammonia Limiting condition)	$\Theta_{25^\circ\text{C} \leftrightarrow 15^\circ\text{C}} = 1.08$	(Eding et al., 2006)
TF Pilot scale	Vertical flow plastic media: SA of 140 m <sup>2</sup> /m <sup>3</sup> $SALR_{13.4^\circ\text{C}} = 1.9 \frac{\text{gN}}{\text{m}^2\text{d}}$	13.4 - 17.6	$SARR_{13.4^\circ\text{C}} = 1.8 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Anderson et al., 1994)
TF Pilot scale	Cross flow Polyurethane media; SA of 230 m <sup>2</sup> /m <sup>3</sup> $SALR_{11^\circ\text{C}} = 3.2 \frac{\text{gN}}{\text{m}^2\text{d}}$	11 - 21.5	$SARR_{11^\circ\text{C}} = 2.6 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Parker et al., 1989)
TF Pilot scale	Vertical flow Polyethylene LT-15 media: SA of 200 m <sup>2</sup> /m <sup>3</sup> pH: 6.7 ± 0.5	11 - 25	$E_{15^\circ\text{C}} = 61.5\%$	$\Theta_{25^\circ\text{C} \leftrightarrow 11^\circ\text{C}} = 1.1$	(Kanda et al., 2016)

TF Lab scale	Vertical flow rough and smooth media: SA of 500 m <sup>2</sup> /m <sup>3</sup> Cross flow Polyurethane media: SA of 223 m <sup>2</sup> /m <sup>3</sup> $VLR_{11^{\circ}\text{C}} = 0.32 \frac{\text{gN}}{\text{m}^3\text{d}}$ HRT: 0.38 - 0.86 hrs pH: 7-8	10 - 17	$E_{10^{\circ}\text{C}} = 0.27 * E_{17^{\circ}\text{C}}$	$\Theta_{17^{\circ}\text{C} \leftrightarrow \leq 10^{\circ}\text{C}} = 1.096$	(Kishimoto et al., 2014)
TF Full scale	Cross flow media	10	$SARR_{10^{\circ}\text{C}} = 1.20 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Gullicks and Cleasby, 1986, 1990)
TF Full scale	Cross flow Polyurethane media: SA of 138 m <sup>2</sup> /m <sup>3</sup>	~ 7	$SARR_{7^{\circ}\text{C}} = 1.2 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Parker et al., 1990)
TF Pilot scale	Vertical flow coarse and fine plastic media: total surface area of 3.68 m <sup>2</sup> HRT: 0.08 hrs pH: 6.0 ± 0.2	7 - 19	$SARR_{7^{\circ}\text{C} \leftrightarrow 11^{\circ}\text{C}} = 0.47 \frac{\text{gN}}{\text{m}^2\text{d}}$ $E_{15^{\circ}\text{C}} = 61.5\%$		(Stone et al., 1975)
TF Pilot scale	Vertical flow polypropylene MT Cascade Filterpak media: SA of 220 m <sup>2</sup> /m <sup>3</sup> pH: 7.73 - 8.24	3 - 30	$SARR_{3^{\circ}\text{C}} = 0.029 \frac{\text{gN}}{\text{m}^2\text{d}}$ $SARR_{3^{\circ}\text{C}} = 0.09 * SARR_{30^{\circ}\text{C}}$		(Matthews et al., 2009)
BAF Pilot scale	Downflow BAF media: total surface area of 28 m <sup>2</sup>	12 - 19.9	$VRR_{12^{\circ}\text{C}-13^{\circ}\text{C}} = 500 \frac{\text{gN}}{\text{m}^3\text{d}}$	$\Theta_{20^{\circ}\text{C} \leftrightarrow 12^{\circ}\text{C}} = 1.05$	(Paffoni et al., 1990)

	$VLR^g$ $= 670 - 1000 \frac{\text{gN}}{\text{m}^3\text{d}}$		$E_{12^\circ\text{C}-13^\circ\text{C}} = 74.62\%$	
BAF Lab scale	Downflow BAF Gravel media: SA of $0.93 \times 10^4$ $\text{m}^2/\text{m}^3$ ; Lava rock media: SA of $1.81 \times 10^4$ $\text{m}^2/\text{m}^3$ ; and plastic ring media: SA of $3.67 \times 10^4$ $\text{m}^2/\text{m}^3$  $VLR_{\sim 13^\circ\text{C}} = 600 \frac{\text{gN}}{\text{m}^3\text{d}}$ HRT: 0.5, 1, and 2 hrs pH: $8.0 \pm 0.1$	$\sim 13^\circ\text{C}$	$E_{13^\circ\text{C}} = 97.8 - 98.5\%$  $VRR_{\sim 13^\circ\text{C}} = \sim 589 \frac{\text{gN}}{\text{m}^3\text{d}}$	(Ha et al., 2010a)
BAF Pilot scale	Upflow BAF Vitrified Clay media $VLR$ $= 270 - 569 \frac{\text{gN}}{\text{m}^3\text{d}}$ HRT: 0.43 - 0.73 hrs	10 - 19.2	$VRR_{15.3^\circ\text{C}} = 350 \frac{\text{gN}}{\text{m}^3\text{d}}$  $VRR_{13.2^\circ\text{C}} = 380 \frac{\text{gN}}{\text{m}^3\text{d}}$  $VRR_{10.9^\circ\text{C}} = 270 \frac{\text{gN}}{\text{m}^3\text{d}}$  $E_{10.9^\circ\text{C}} = 88\%$	(Stensel and Brenner, 1988)
BAF Pilot Scale	Upflow BAF Zeolite beads media $VRR_{7^\circ\text{C}} = \sim 692 \frac{\text{gN}}{\text{m}^3\text{d}}$ HRT: 1, 2 and 4 hrs	7 - 19	$VRR_{7^\circ\text{C}} = 450 \frac{\text{gN}}{\text{m}^3\text{d}}$ (C/N ratio: 15:1)  $VRR_{7^\circ\text{C}} = 200 \frac{\text{gN}}{\text{m}^3\text{d}}$	(Jiang et al., 2009)

(C/N ratio: 18:1)

$$VRR_{7^{\circ}\text{C}} = 160 \frac{\text{gN}}{\text{m}^3\text{d}}$$

(C/N ratio: 20:1)

$$E_{7^{\circ}\text{C}} = 80\%$$

(HRT: 4 hrs)

$$E_{7^{\circ}\text{C}} = 38\%$$

(HRT: 1 hr)

BAF Pilot scale	Upflow Biostyr BAF polystyrene beads media: $\text{Ø} = 3.8 \pm 1.2$ media	7 - 10	$VRR_{7^{\circ}\text{C}} = \geq 799 \frac{\text{gN}}{\text{m}^3\text{d}}$	(Payraudeau et al., 2001)
BAF Pilot scale	Upflow BAF Expanded clay media: SA of 4.57 $\text{m}^2/\text{g}$ and Zeolite media: SA of 6.84 $\text{m}^2/\text{g}$ HRT: 0.95-1.43 hrs pH: 6.5 - 7.9	7 - 10	$VRR_{7^{\circ}\text{C}} = 0.1 *$ $VRR_{20^{\circ}\text{C}}$ (Expanded clay media)  $VRR_{7^{\circ}\text{C}} = 0.26 *$ $VRR_{20^{\circ}\text{C}}$ (Zeolite media)	(He et al., 2007)
BAF Lab scale	Downflow BAF plastic media: SA of 9300 $\text{m}^2/\text{m}^3$ VLR $= 500 - 750 \frac{\text{gN}}{\text{m}^3\text{d}}$ HRT: 0.5, 1, and 2 hrs pH: $7.0 \pm 0.2 - 8.1 \pm 0.2$	6.5 - 13	$VRR_{13^{\circ}\text{C}} = 600 \frac{\text{gN}}{\text{m}^3\text{d}}$  $E_{6.5^{\circ}\text{C}} = 95\%$ (HRT = 2 hrs)  $E_{6.5^{\circ}\text{C}} = 92\%$ (HRT = 1 hr and 200% effluent recirculation)	(Ha et al., 2010b)

BAF Pilot scale	Upflow Biostyr BAF polystyrene beads media: $\emptyset = 3.8 \pm 1.2$ mm HRT: 1 - 8 hrs pH: 7.2 - 7.8	4 - 20	$VRR_{14^{\circ}\text{C}}$ $= 800 - 600 \frac{\text{gN}}{\text{m}^3\text{d}}$ $VRR_{\sim 4^{\circ}\text{C}}$ $= 160 - 200 \frac{\text{gN}}{\text{m}^3\text{d}}$ $VRR_{4^{\circ}\text{C}}$ $= 0.16 * VRR_{20^{\circ}\text{C}}$	$\theta = 391 \times 10^2 \cdot \ln(t)$ $+ 9.83 \times 10^{-1}$ $\Theta_{20^{\circ}\text{C} \leftrightarrow 4^{\circ}\text{C} (\text{Day } 1)}$ $= 1.002$ $- 1.018$ $\Theta_{20^{\circ}\text{C} \leftrightarrow 4^{\circ}\text{C} (\text{Day } 115)}$ $= 1.123$ $- 1.048$ $\Theta_{20^{\circ}\text{C} \leftrightarrow 4^{\circ}\text{C} (\text{Day } 115)}$ $= 1.123$ $- 1.048$ $\Theta_{4^{\circ}\text{C}} = 391 \times 10^2 \cdot \ln(t)$ $+ 9.83 \times 10^{-1}$	(Delatolla et al., 2009)
MBBR Pilot and Lab-scale	Polyethylene carriers: SA of $300 \text{ m}^2/\text{m}^3$ pH: 7 - 7.5	15	$SARR_{15^{\circ}\text{C}}$ $= 0.1 - 1.3 \frac{\text{gN}}{\text{m}^2\text{d}}$ $\left( DO^i = \frac{6 \text{ gO}_2}{\text{m}^3} \right)$ $SARR_{15^{\circ}\text{C}}$ $= 0.1 - 1.7 \frac{\text{gN}}{\text{m}^2\text{d}}$ $\left( DO = \frac{8 \text{ gO}_2}{\text{m}^3} \right)$		(Hem et al., 1994)
MBBR Full scale	Hydroxyl-Pac: SA of $568 \text{ m}^2/\text{m}^3$	8 - 21	$E_{15^{\circ}\text{C}-12^{\circ}\text{C}} = 92.4\%$		(Bjornberg et al., 2009)

	$SALR_{<15^{\circ}\text{C}}$ $= 0.059 - 0.078 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: ~3.14 hrs		$E_{<12^{\circ}\text{C}} = 91.4\%$		
MBBR Pilot scale	Plastic Carriers: SA of $310 \text{ m}^2/\text{m}^3$ $SALR_{18^{\circ}\text{C}-7^{\circ}\text{C}} =$ $\geq 1 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: <3 - 6 hrs	7 - 18.3	$SARR_{10^{\circ}\text{C}} = 1.01 \frac{\text{gN}}{\text{m}^2\text{d}}$ <i>(Pre-denitrification)</i>  $SARR_{10^{\circ}\text{C}} = 1.24 \frac{\text{gN}}{\text{m}^2\text{d}}$ <i>(Post-nitrification)</i>	$\Theta_{12.4^{\circ}\text{C} \leftrightarrow 7.8^{\circ}\text{C} (\text{Day } 1)} = 1.09$	(Rusten et al., 1995)
MBBR Pilot scale	Plastic carriers: SA of $350 \text{ m}^2/\text{m}^3$ $SALR_{10^{\circ}\text{C}} = \sim 1.17 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: <1 hrs	3 - 14	$SARR_{10^{\circ}\text{C}} = 1.17 \frac{\text{gN}}{\text{m}^2\text{d}}$		(Ødegaard, 2006)
MBBR Pilot scale	Kaldness media: $\text{Ø} = 10$ mm and height of 7 mm HRT: 1.8 - 2 hrs pH: 7 - 7.5	3 - 15	$VRR_{8^{\circ}\text{C}} = 210 \frac{\text{gN}}{\text{m}^3\text{d}}$  $VRR_{4^{\circ}\text{C}} = 140 \frac{\text{gN}}{\text{m}^3\text{d}}$		(Delatolla et al., 2010)
MBBR Full scale	Anoxkadnes K1 carriers: SA of $350$ $\text{m}^2/\text{m}^3$ $SALR_{4.5^{\circ}\text{C}} = 0.15 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: 10.32 hrs pH: 7 - 9	2 - 8	$SARR_{4.5^{\circ}\text{C}} = 0.06 \frac{\text{gN}}{\text{m}^2\text{d}}$  $E_{4.2^{\circ}\text{C}} = 40\%$		(Wessman and Johnson, 2006)

MBBR Lab scale	AnoxKaldnes K3 carriers; SA of 500 m <sup>2</sup> /m <sup>3</sup> HRT: 2.1 - 6 hrs pH: 7.2 -7.8	1 -20	$VRR_{1^{\circ}C}$ $= 110 - 120 \frac{gN}{m^3d}$	(Almomani et al., 2014)
	$VLR_{1^{\circ}C}$ $= 324.48$			
	$\pm 34.56 \frac{gN}{m^3d}$			
MBBR Lab scale	AnoxKaldnes K3 carriers; SA of 500 m <sup>2</sup> /m <sup>3</sup> HRT: 4 - 6 hrs pH: 7 - 8	1 - 20	$VRR_{1^{\circ}C} = 108 \frac{gN}{m^3d}$	(Hoang et al., 2014a)
	$VLR_{1^{\circ}C}$ $= 275.34 \pm 10 \frac{gN}{m^3d}$			
MBBR Pilot scale	AnoxKaldnes K5 carriers; SA of 800 m <sup>2</sup> /m <sup>3</sup> HRT: 1.5 – 2 hrs pH: 6.98 - 7.2	1 - 20	$VRR_{1^{\circ}C}$ $= 96.37 - 167.34 \frac{gN}{m^3d}$	(Young et al., 2017)
	$VLR_{1^{\circ}C}$ $= 275 - 678 \frac{gN}{m^3d}$			

MBBR Lab scale	AnoxKaldnes K5 carriers; SA of 800 $\text{m}^2/\text{m}^3$ $VLR_{1^\circ\text{C}}$ $= 275.34 \pm 10 \frac{\text{gN}}{\text{m}^3\text{d}}$	1 - 10	$VRR_{1^\circ\text{C}}$ $= 98.44 \pm 4.69 \frac{\text{gN}}{\text{m}^3\text{d}}$	(Ahmed et al., 2019)
BASR Lab scale	Polyethylene media: SA of 1150 - 1475 $\text{m}^2/\text{m}^3$ pH: 7.0 - 8.6	13 - 30	$\tau_{13^\circ\text{C}} = \frac{40.73}{d}$ $VTR_{13^\circ\text{C}}$ $= 40.73x \frac{\text{gN}}{\text{m}^3\text{d}}$	(Saidu et al., 2018)
SAGR Pilot scale	Sand media: SA of 820 $\text{m}^2/\text{m}^3$ $VLR_{15^\circ\text{C}}$ $= 500 - 1800 \frac{\text{gN}}{\text{m}^3\text{d}}$ pH: 8.14 - 8.47	15 - 20	$VRR_{15^\circ\text{C}}$ $= 420 - 1450 \frac{\text{gN}}{\text{m}^3\text{d}}$ $E_{15^\circ\text{C}} = 91\%$	(Onnis-Hayden et al., 2007)
SAGR Full Scale	Submerged media: total surface area of 56 000 $\text{m}^2$ $SALR_{\sim 10^\circ\text{C}}$ $= 0.025 \frac{\text{gN}}{\text{m}^2\text{d}}$ HRT: 13.5 hrs pH: 6.5 - 9	~10	$SARR_{\sim 10^\circ\text{C}}$ $= 0.024 \frac{\text{gN}}{\text{m}^2\text{d}}$	(Mattson et al., 2018)
SAGR Full scale	Mino-silicate mineral zeolite media pH: 8.55 $\pm$ 0.07	4 - 12	$E_{4^\circ\text{C}} \leq 38\% * E_{12^\circ\text{C}}$ (without zeolite media)	(Miazga-Rodriguez et al., 2012)

$$E_{4^{\circ}\text{C}} = 42 - 133\% * E_{12^{\circ}\text{C}}$$

(with zeolite media)

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## 6.5. Nitrifying Rotating Biological Contactors

RBC systems are one of the oldest biofilm technology still employed for biological treatment of wastewater in WRRF, these systems are in continuous operation in most parts of Europe since 1958 (Hittlebaugh and Miller, 1981). Originally, RBC systems were used for cBOD removal, but the use of the process was later expanded to include the treatment of TAN and total nitrogen removal. Conventionally, these bioreactors consist of rotating discs made from various materials mounted on a rotating horizontal shaft partially submerged in wastewater; typically, submergences of ~40% (Dutta et al., 2007; Ravi et al., 2013). Nitrifying RBC parameters such as media types, rotation speed, staging configuration, organic loads, recycling regime, hydraulic retention times and media submergence are all factors of TAN removal efficiencies in this type of attached growth system (Ishiguro, 1983; Peters and Wu, 1984; Patwardhan, 2003; Bagchi et al., 2012; Hassard et al., 2015; Pal et al., 2016; Feng et al., 2017; Torretta et al., 2017). Compared to the TF systems, this biofilm technology requires 1/10th of the TF's footprint and require lower operating costs than conventional activated sludge systems (Allen, 1929; Hassard et al., 2015). However, inconsistent performances due to several factors such as excessive biomass growth, growth of biofilm predators, and frequent breakage of the rotating mechanisms amongst other issues have led to very few installations of these older systems in the last decades (Spuhier, 2012; USEPA, 1993).

Although the research on low temperature optimization of nitrifying RBC is limited compared to other biofilm technologies; the proper design and operation of these systems can improve low temperature nitrification efficiencies as demonstrated by several studies with 4.5°C as the lowest temperatures investigated in the current literature (Kapoor et al., 2003). Nitrifying RBC employed in municipal treatment plants, at an average temperature of 13°C, have been

reported capable of maintaining significant nitrification (Miller et al., 1981). Nowak (2000) reported a rate of 1.8 gN/m<sup>2</sup>d at full scale nitrifying RBC systems at 13°C. At temperatures below 13°C, a significant decline in nitrification rates are observed in nitrifying RBC systems as approximately 2.5 times more surface area will be required in order to maintain the same removal rates recorded at 13°C (Rodgers and Zhan, 2003).

It is estimated that a performance loss of 4.5%/°C occurs in nitrifying RBC as temperatures decline furthermore (Nowak, 2000). At 10°C and elevated TAN loading rates, removal rates as elevated as 3 gN/m<sup>2</sup>d can be attained in tertiary nitrifying RBC systems (Boller et al., 1990; Gujer and Boller, 1990). Moreover, Boller et al. (1990) reported that at such temperatures pre-filtration of influents can drastically improve the TAN removal rates at low temperatures. Nowak (2000) reported a rate of 0.8 gN/m<sup>2</sup>d at 8°C with this biofilm technology proven capable of maintaining this same rate down ~4°C according to Kapoor et al. (2003). In their investigation between 4.5°C and 24°C, Kapoor et al. (2003) demonstrate that with proper design and monitoring of nitrifying RBC systems a significant nitrification rates and efficiencies can be attained at temperatures below 5°C.

These bioreactors can be operated as mixed biomass systems for the combined treatment of cBOD and TAN or as single biomass bioreactors used as tertiary treatment solutions for TAN removal only (Gujer and Boller, 1990; Tawfik et al., 2006). It is well understood that elevated organic loads can affect nitrification rates; for this reason, RBC staging strategies are a crucial parameter for nitrifying RBC destined for low temperature operation as demonstrated by Tawfik et al. (2006) at 12°C as 5% greater efficiencies were obtained in two RBC bioreactors in-series as opposed to a single-stage RBC system. For this reason, it is recommended to utilize more than a single RBC to attain nitrification at the later stages with less heterotrophic growth and considering

the optimal C:N ratio of below 1.5 (Lehman, 1983). Moreover, RBC staging also mitigates the reduced nitrification kinetics at low temperatures as the overall loading rates decrease with the number of RBC systems in series. Alternatively, single-stage RBC systems, effluent recycling strategies can be implemented to dilute the organic and TAN loads while the use of tank covers can prevent excessive heat loss during periods of low temperature operations (Cortez et al., 2008; USEPA, 1993).

The oxygen transfer rates (ORT) in the biofilm are regulated by the rotation speed of the support media and the media submergence, two linked parameters to consider at low temperatures specifically in oxygen limited conditions (Andreadakis et al., 1993). The partially exposed RBC media represents a challenge as the biofilm is exposed to the harsh cold air temperatures while rotating out of the wastewater with each revolution of the shaft. Increasing the rotation speed to reduce the biofilm exposure time to cold air and increase ORT for oxygen limited conditions (Cortez et al., 2008). Studies indicate that for nitrifying RBC that optimal peripheral velocities for the support media are between 0.15 m/s to 0.34 m/s beyond which the adhesion of the biofilm to the support media may be hindered (Lehman, 1983; Nowak, 2000). Alternatively, increasing the submergence of the media to reduce the biofilm exposure to the elements may also be beneficial; however, can reportedly reduce the transfer rate of oxygen and DO concentrations in the bulk liquid.

## **6.6. Nitrifying Trickling Filters**

TF systems were introduced more than a century ago in the 1890s are still in operation to this date in WRRF with the initial system employed for cBOD removal in wastewater (Bitton, 2005). TF systems are categorized as non-submerged attached growth bioreactors composed of a non-submerged fixed media, a wastewater distribution system to deliver the influent on the media

and a drainage system to collect the effluent as main components (Wik, 2003; Vianna et al., 2012; Ali et al., 2017). These bioreactors were eventually optimized to perform nitrification with efficiencies depending on several factors namely temperature, DO, pH, depth of media and type, TAN loading rates, and organic loads (Balakrishnan and Eckenfelder, 1969). Nitrifying TF technology is favored over conventional suspended growth systems such as the activated sludge systems due to their lower operational cost and greater nitrification efficiencies at low temperatures (Stone et al., 1975; Parker et al., 1990). As demonstrated by several studies performed at temperatures below 15°C, significant nitrification rates can be attained by nitrifying TF systems with the lowest investigated temperature with this type of technology being 3°C (Matthews et al., 2009).

Gullicks and Cleasby (1986) reported rates of 0.5 -1 gN/m<sup>2</sup>d at temperatures of 14°C and above. Anderson et al. (1994) in an investigation of tertiary vertical flow pilot nitrifying TF systems have reported stable removal rates of 1.8 gN/m<sup>2</sup>d at 13.4°C highlighting the importance of biofilm predator control as a factor for maintaining stable nitrification efficiencies. The same authors obtained higher efficiencies (87% - 94.7%) between the temperatures of 15°C and 13.4°C with alternating two-stage TF bioreactors in-series benefiting from overall lower loading rates compared to single-stage nitrifying TF bioreactors. Below 13°C, Parker et al. (1990) have demonstrated that crossflow media are capable of withstanding elevated TAN loadings and attaining higher efficiencies especially in oxygen-limited conditions at which rates of 2.6 gN/m<sup>2</sup>d are attainable at 11°C due to the increased ORT over vertical flow media. Kanda et al. (2016) proposed correction coefficients of 1.1 to model removal rates at temperatures from 25°C down to 11°C. Between 11°C and 10°C, with reduced nitrification kinetics a 100% recirculation of nitrifying TF systems' effluents improves TAN removal efficiencies as long as organic loads

remain below 0.20 kgCOD/m<sup>3</sup>d (Kishimoto et al., 2014). Gullicks and Cleasby (1990) in their pilot scale investigation of nitrifying TF systems recorded rates of 0.75 gN/m<sup>2</sup>d at 10°C under oxygen limiting conditions; moreover, in such conditions, it is reported that bulk liquid DO saturations below 60 - 65% or concentrations below 5.5-7.5 mgO<sub>2</sub>/L, drastically reduces nitrifying TF systems' efficiencies. The same authors, in an assessment of conventional nitrifying TF design curves obtained rates of 0.95 gN/m<sup>2</sup>d and 0.88 gN/m<sup>2</sup>d at 7°C under ammonia and oxygen-limiting conditions with the later lower rate attributed to low hydraulic loading rate as well as inadequate wetting of the media. Nonetheless, increasing ORT in the biofilm at the same temperature of 7°C can result in 30% higher removal rates with rates of 1.2 gN/m<sup>2</sup>d in oxygen-limiting conditions. As temperatures decrease down to 7°C, it is estimated that 37% more surface area is required for the oxidation of 1 lb of TAN compared to attached growth nitrification above 11°C (Stone et al., 1975). Efficiencies as high as 95% are attainable in single nitrifying systems at loading rates of 0.45 gN/m<sup>2</sup>d and temperatures as low as 4°C as demonstrated in a pilot study; however, organic loading rates were relatively low in this study for heterotrophs to outcompete the nitrifiers on the media and thus hinder the nitrification process (Duddles et al., 1974).

## **6.7. Nitrifying Biological Aerated Filters**

Nitrifying BAF systems are described as completely submerged fixed-film bioreactors employed for the process of nitrification for several decades in WRRF (Payraudeau et al., 2001; Chang et al., 2009). This type of reactor can be defined by the direction of the flow; as Downflow or Upflow BAF systems, and further characterized by their operational parameters such as their fluid velocities; the types of media and their backwashing regimes (Farabegoli et al., 2009; Feng et al., 2010). Nitrifying BAF performance in low temperature settings has been proven in several studies below 15°C with 4°C as the lowest temperature investigated to date (Delatolla et al., 2009).

In low temperature settings, nitrifying BAF systems have displayed better efficiencies than the conventional activated sludge systems (Payraudeau et al., 2001). Moreover, this biofilm technology is more efficient than other attached growth systems such as the nitrifying TF and RBC technologies reviewed above as reported by Hansen et al. (2007) in their long-term study. Ha et al. (2010b) have investigated nitrifying BAF systems as upgrade units to lagoon facilities to reduce TAN effluent below 15°C and reported at 13°C efficiencies of 98.5% at loading rates of 600 gN/m<sup>3</sup>d. Investigations on polishing nitrifying BAF systems between 12°C and 13°C, have identified this technology as efficient add-ons as tertiary treatment solutions with rates of 500 gN/m<sup>3</sup>d (Paffoni et al., 1990). Nitrification efficiency at 10°C is reported to reach 88% at approximately loading rates of 508 gN/m<sup>3</sup>d in mixed biomass BAF bioreactors treating BOD and TAN with backwashing frequency of 1 cycle per day identified as key design factor in improving nitrification rates (Stensel and Brenner, 1988). A finding confirmed in several studies including Han et al. (2010) confirming that backwashing cycles are crucial in maintaining significant nitrification rates at temperatures as low as 9°C. Below this temperature, BAF systems can still maintain significant nitrification rates with rates of 260 kgN/m<sup>3</sup>d at 8°C (Delatolla et al. 2010).

As heterotroph growth remains a concern for the TAN treatment efficiencies same as any other biofilm technology, Jiang et al. (2009) observed in mixed biomass BAF systems a decrease in removal rates with increasing C:N ratios; with obtained rates of 450, 200 and 160 gN/m<sup>3</sup>d at C:N ratios of <15:1, 18:1 and 20:1 at 7°C. At this temperature, removal rates range between 10 % to 26% of the rate attainable at more optimal temperatures for nitrification such as 20°C, according to He et al. (2007). Jiang et al. (2009) also highlight the importance of optimizing recycling strategies to improve low temperature nitrification efficiencies as supported by Ha et al. (2010a) in their investigation down to 6.5°C.

Delatolla et al. (2009) demonstrated that these bioreactors are capable of maintaining nitrification despite long exposures to low temperatures down to 4°C with removal rates approximately 16% of the rates recorded at 20°C and ranging between 160 gN/m<sup>3</sup>d and 200 gN/m<sup>3</sup>d. These same authors identified the elapsed time at low temperatures as an additional crucial factor of nitrification and proposed a Theta model accounting for exposure time at 4°C. As reported in suspended growth systems, rapid decreases in temperatures or cold shocks, have severe lasting effects on nitrification kinetics on attached growth systems as well as demonstrated in nitrifying BAF systems cold shocked down from 20°C to 4°C which displayed a significant decrease in efficiency of 56% (Delatolla et al. 2009).

## **6.8. Nitrifying Moving Bed Biofilm Reactors**

The MBBR systems are recent attached growth systems developed four decades ago with free-floating carriers, serving as support media for the biofilm; mixed with aeration also providing DO requirements in those bioreactors for processes such as nitrification (Oleszkiewicz and Barnard, 2006). The search for a compact and economical treatment solution has led to the design of MBBR systems which, in addition to all the benefits inherent to biofilm technologies, possess their own advantages such as their small footprint and more importantly their simplistic operation (Ødegaard et al., 1994; Ødegaard, 1999; Ødegaard, 2006). This low operational intensity of MBBR systems presents this biofilm technology as good candidates for the upgrade of lagoon facilities for year-long nitrification including cold seasons (Ødegaard, 2006; Houweling et al., 2007; Delatolla et al., 2010). The capability of MBBR systems to perform nitrification at low temperatures has been demonstrated in numerous studies below 15°C down to temperatures as low as 1°C (Almomani et al., 2014; Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017; Ahmed et al., 2019).

Below 15°C, nitrifying MBBR systems have been proven more efficient than other biofilm technologies such as the RBC technology as reported by Andreotolla et al. (2000) in their study, between 15°C and 5°C. While measuring the performance of the bioreactors, the authors determined that their pilot scale MBBR systems maintained stable performances below 8°C with an average removal rate of 0.9 gN/m<sup>2</sup>d as opposed to a less efficient RBC system. Assessments of nitrifiers' growth below their optimal growth rate temperatures in MBBR reactors have determined that at 6°C, AOB and NOB require 7.11 and 3.56 times longer to grow respectively compared to their growth rates at 26°C (Bian et al., 2017). Below this temperature, nitrifying MBBR systems can reach significant removal rates of 140 gN/m<sup>3</sup>d at 4°C, rates notably comparable to the more operationally intensive nitrifying BAF reactor assessed in the same study (Delatolla et al., 2010). The same authors have demonstrated that MBBR reactors' efficiencies can withstand extended exposures to low temperatures as BAF reactors; moreover, the Delatolla et al. (2009) Theta model accounting for the elapsed time at 4°C in BAF reactors also correctly predicted the nitrifying MBBR reactor's kinetics as well. This finding thus suggesting that low temperatures do not have a greater impact on nitrifying MBBR reactors compared to nitrifying BAF systems.

Studies on 1°C nitrifying MBBR systems, have been published in recent years. This type of biofilm technology has been shown capable of attaining nitrification rates at 1°C between 98.44 gN/m<sup>3</sup>d and 167.24 gN/m<sup>3</sup>d or 1°C efficiencies 20% of those attainable at 20°C at both the pilot and laboratory scales (Hoang et al., 2014a; Hoang et al., 2014b; Young et al., 2016; Young et al., 2017). In a 1°C pilot study, Young et al. (2017) have investigated four loading rates; 59.13 gN/m<sup>3</sup>d, 245.86 gN/m<sup>3</sup>d, 609.48 gN/m<sup>3</sup>d and 678.27 gN/m<sup>3</sup>d and have proposed 167.24 gN/m<sup>3</sup>d as the 1°C removal rate for nitrifying MBBR reactors; a rate obtained a VLR of 609.48 gN/m<sup>3</sup>d with nitrification kinetics decreasing above that loading rates in their bioreactors (SALR = 678.

gN/m<sup>3</sup>d). Although the bulk liquid-free ammonia concentrations in the MBBR systems of this study were below the inhibition concentrations of 4 mgN/L – 20 mgN/L reported at 20°C, an explanation of such decrease in kinetics at high loading rates at 1°C has yet to be determined (Anthonisen et al., 1976; Van Hulle et al., 2010). In the laboratory setting, Hoang et al. (2014a) reported, using synthetic wastewater, rates approximately 35% to 38% lower than the removal rate in the above-mentioned pilot study. Similarly, Ahmed et al. (2019) also reported lower 1°C rates of 98.44 gN/m<sup>3</sup>d in their laboratory-scale temperature-controlled study. A discrepancy attainable rate at 1°C likely due to the limited temperature control available in pilot studies and thus nitrifying MBBR systems benefiting from slight increases in temperature contrary to laboratory-scale MBBR reactors strictly operating at 1°C. Nonetheless, 1°C design curve for nitrifying MBBR systems is obtained from these different studies at 1°C as displayed in Figure 6.1 which sets the obtainable removal rate at 144.5 gN/m<sup>3</sup>d under zero order condition at 1°C or the first order rate at 72.75 gN/m<sup>3</sup>d.

Temperature dependent rate modeling of MBBR reactors between the temperatures of 17°C and 1°C, using the conventional Arrhenius correction coefficients of  $\theta = 1.072$  and  $1.09$  resulted in modeled rates 30% higher than the measured rates; however,  $\theta = 1.086$  for the temperatures between 5°C and 1°C resulted in a higher correlation between modeled and measured rates down to 1°C (Young et al., 2017). A discrepancy due to the existence of a kinetic threshold temperature delineated between 6°C and 2°C in several studies (Hoang et al., 2014a; Young et al., 2017; Delatolla et al., 2010; Ahmed et al., 2019). More recently, Ahmed et al. (2019) delineated this kinetic threshold temperature between 4°C and 2°C in their temperature-controlled study and thus reported more accurate correction coefficients of  $\theta = 1.049$  above this threshold temperature (between 10°C and 4°C) and  $\theta = 1.149$  below this threshold temperature and at 1°C.

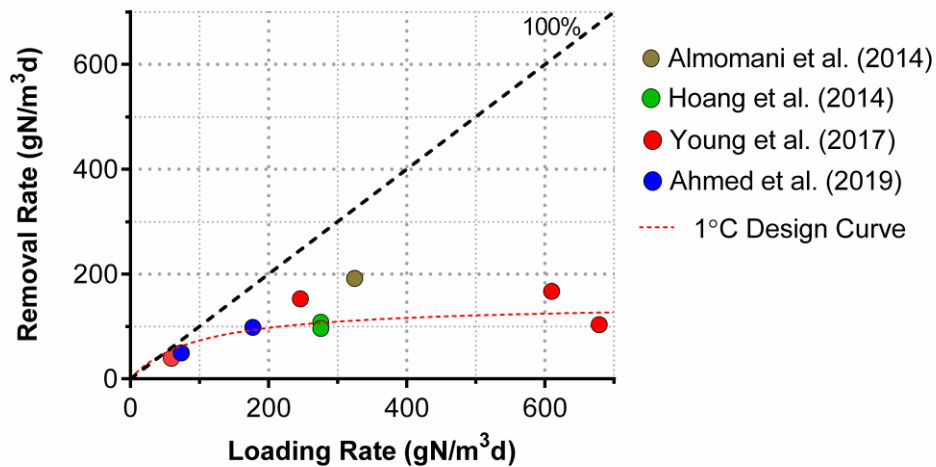


Figure 6.1. Removal, loading rates and design curves of 1°C nitrifying MBBR systems (modified from Almomani et al., 2014; Hoang et al., 2014a; Young et al., 2017; Ahmed et al., 2019).

With respect to the biofilm, biomass and microbiome in nitrifying MBBR systems, most studies reported similar responses to the low temperature of 1°C (Table 6.2). Starting with the biofilm mass, no significant increases are observed with decreasing temperatures between 20°C and 1°C (Almomani et al., 2014; Hoang et al., 2014a;); although, increases in biofilm mass were reported in a pilot study investigating the same temperature range (Young et al., 2016). Between 20°C and 1°C, the biofilm thickness decreases as the biofilm thickness increases with no statistically significant changes in biofilm thickness correlating with increases in loading rates at 1°C (Hoang et al., 2014b; Young et al., 2017). Regarding the microbiome of 1°C nitrifying MBBR reactors, the main ammonia-oxidizing bacteria identified at both 20°C and 1°C were from Nitrosomonadacea family (which includes the Nitrosomonas, Nitrospira and Nitrosovibrio genera) with no statistically significant changes in relative abundance decreasing temperatures or with loading rates at 1°C (Hoang et al., 2014b; Young et al., 2017). In these same studies, the main NOB identified in the nitrifying biofilms were from the *Nitrospiraceae* family (which includes the *Nitrospira* genus) at both 20°C and 1°C although there is a discrepancy between the above-

mentioned laboratory scale and pilot scale studies on whether the relative abundance increases or decreases down to 1°C.

Table 6.2. Low carbon nitrifying MBBR systems' biofilm, biomass, microbiome responses at 20°C and 1°C.

Biofilm and Microbiome Characteristics	Hoang et al. (2014b)		Young et al. (2017)	
	20°C	1°C	20°C	1°C
Biofilm Mass (mg)	23.0 ± 3	19.0 ± 0.9	9.2 ± 0.9	30.2 ± 1.8
Biofilm thickness (µm)	216 ± 19.51	267 ± 27.01	91	297 ± 110
Biomass Viability (%)	3.06 ± 0.11*	3.51 ± 0.08*	71**	82.9 ± 2.7**
Dominant AOB (%)	Nitrosomonadacea (-)	Nitrosomonadacea (-)	Nitrosomonadacea (7.1 ± 5.0)	Nitrosomonadacea (7.0 ± 1.8)
Dominant NOB (%)	Nitrospiraceae (3.8)	Nitrospiraceae (7.9)	Nitrospiraceae (8)	Nitrospiraceae (4)

\*Biomass viability as ratio of dead and live cells in the biofilm.

\*\* Biomass viability as live cells coverage in the biofilm.

## 6.9. Alternative Nitrifying Biofilm Technologies

In addition to the attached growth systems reviewed above, other less widespread biofilm technologies are available to attain nitrification; these attached growth systems include emerging technologies but also biotechnologies already in operation in WRRF in limited numbers. However, there is currently limited published studies on these attached growth systems and their operation in cold temperatures settings. Two examples of such biotechnologies are the biofilm airlift suspension reactors (BASR) and submerged attached growth reactors (SAGR) systems. The BASR systems were designed to withstand high loading regimes compared to the attached growth systems reviewed above (Heijnen et al., 1993). In typical BASR design, air is supplied at the bottom of the bioreactor providing mixing, carrier lift and DO for nitrification to occur, and the washout of small

particulates. This emerging technology offer advantaged over the common types of bioreactors reviewed above; especially its efficiency under oxygen limiting conditions which is typically the case of nitrification at lower temperatures. However, they are operationally intensive compared to biofilm technologies reviewed so far (Van Benthum et al., 1996; Van Benthum et al., 1997). Nonetheless, Saidu et al. (2018) in their investigation between 13°C and 30°C have reported the recirculation regimes as a crucial factor to improve the nitrification process with nitrification efficiencies improvements of 13% and 27% at 100% and 200% recirculation of the effluent. At lower temperatures, BASR can reach nitrification rates of 0.3 gN/m<sup>2</sup>d at 8°C with nitrification becoming oxygen limited at TAN concentration above 10 mg/L (Choi et al., 2008). The SAGR systems, despite limited available research, have been employed as suitable upgrade solutions for lagoon facilities to attain nitrification during cold seasons with rates of 0.024 gN/m<sup>2</sup>d at temperatures below 10°C (Mattson et al., 2018). These SAGR upgrade units consist of aerated cells filled with a bed of gravel as the support media for the biofilm to grow on. Miazga-Rodriguez et al. (2012) have investigated nitrifying SAGR systems down to 4°C and concluded that attainable nitrification rates are 10 times greater with the addition of zeolite media in lagoons; In addition, the authors have highlighted the importance of allowing the growth of nitrifiers at temperatures above 4°C, or more precisely at approximately 12°C before the onset of the cold season as they recorded no improvement in nitrification with the addition of support media once the temperatures declined to 4°C.

## **6.10. Conclusions**

The biologically mediated process of ammonia removal from wastewater remains an economical and viable solution to mitigate the adverse effects of ammonia discharged into receiving waters by WRRF. As in suspended growth nitrification, the attached growth nitrification

process is adversely affected by low temperatures; however, it is well accepted that biofilm technologies remain hindered to a lesser extent and capable of maintaining significant nitrification rates at lower temperatures than conventional suspended growth systems (Gullicks and Cleasby, 1986; Parker et al., 1989; Kapoor et al., 2003; He et al., 2007; Delatolla et al., 2010; Young et al., 2017).

Studies on low temperature nitrifying biofilm technologies indicate that the crucial factor in attaining nitrification at low temperatures includes not only taking into account fundamental factors affecting the nitrification process (organic loads, pH, etc.) but also considering the design and operational parameters inherent to each type of attached growth system such as the media type, HRT, recycling rates and backwashing frequencies, etc. Research on the optimization of established and historic attached growth systems, such as the century-old TF systems reported capable of maintaining nitrification rates at temperatures as low as 3°C or the RBC system performing nitrification down 4.5°C. Whereas continued research on nitrifying biofilm has led to the development of newer biofilm technologies, as in the case of BAF systems stemming from continued research and innovations on trickling filters, which has demonstrated to maintain significant rates during extended operation at 4°C.

Investigations conducted on more recent biotechnologies such as the MBBR technology have demonstrated the ability of these systems in maintaining significant nitrification rates despite extended exposures to low temperatures without any notable declines in efficiency over time. Moreover, assessments of the biofilm, biomass and microbiome responses down to 1°C in nitrifying MBBR systems indicate that the capability of these bioreactors to retain viable embedded cells including the nitrifying bacteria responsible for the nitrification process. As most of the biofilm technologies reviewed above have been evaluated as add-on solutions to lagoon

facilities; the MBBR technology's low operational intensity in addition to the inherent advantages of biofilm technologies may represent the ideal upgrade solution to successfully retrofit existing passive treatment systems for year-long ammonia treatment objectives in northern countries and colder climates.

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## **Chapter 7. Discussion, Conclusion, and Future Direction**

This research was performed to answer critical research questions on the design and operation of post-carbon, low temperature nitrifying MBBR systems. The timeliness of this research is important as the first system has been built in Canada and is scheduled to begin operation in the fall of 2020. Low temperature attached growth nitrification kinetics, as well as cold shocked attached growth nitrification kinetics in MBBR systems, were investigated down to 1°C. In addition, the effects of low temperatures and cold shocks on the nitrifying MBBR system's biofilms and their embedded cells were investigated to delineate the biofilm, biomass, and microbiome responses down to 1°C.

### **7.1. Low Temperature Attached Growth Nitrification**

To study the attached growth nitrification kinetics down to 1°C, an investigation was conducted in an enhanced temperature-controlled chamber to isolate the effects of low temperatures nitrifying MBBR systems (Chapter 3). This study is the only temperature-controlled investigation of low temperature nitrifying MBBR systems using real wastewater and elevated total ammonia nitrogen (TAN) concentrations typical of sanitary sewer systems to be performed in a precise temperature-controlled environment. The loading rate for the nitrifying MBBR systems at 1°C was set to 186.89 gN/m<sup>3</sup>d; above the previously reported attainable removal rate of 167.24 gN/m<sup>3</sup>d for nitrifying MBBR systems at 1°C reported in pilot studies. Under the temperature-controlled setting at 1°C of this research, the average removal rate attained was 40% lower than the above-mentioned rate with an average removal rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d. The lower observed rate, relative to pilot studies, is likely due to the precision and stability of the temperature in the temperature-controlled chamber of this research; hence, eliminating slight temperature fluctuations observed in pilot systems that enhance the kinetics of ammonia removal.

During on-site operation, systems may benefit from slight increases in temperatures above 1°C during winter operation compared to the MBBR systems strictly operating at 1°C in this research. As such, the enhanced temperature control rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d quantified in this research is reported as the 1°C intrinsic removal rate of nitrifying MBBR systems compared to the previously 1°C removal rates. According to the 1°C design curves in Figure 6.1 of Chapter 6, this 1°C intrinsic removal rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d may also represent the conservative rate to consider in the design of nitrifying MBBR systems destined for low temperature operation.

The intrinsic 1°C removal rate of  $98.44 \pm 4.69$  gN/m<sup>3</sup>d has been shown to produce effluent concentrations below the common effluent regulation value of 10 mgTAN/L. Two nitrifying MBBR systems in-series has been shown to increase the overall removal efficiency of the MBBR upgrade system. As such, this research identifies this configuration of two MBBR in-series for the treatment of TAN concentrations in non-combined sewer lagoon effluent at temperatures that reach 1°C.

The kinetics of nitrifying MBBR systems were investigated at 10°C, 8°C, 6°C, 4°C, 2°C and 1°C to study the effects of low temperatures with incremental decreases in temperatures. The key findings, in Chapters 3 and 4, indicate a non-linear relationship between decreasing temperatures and declining nitrification kinetics with the existence of a temperature threshold between 4°C and 2°C. Removal efficiencies remained above 90% between 10°C and 4°C, with a first significant decrease in efficiency of 19.55% recorded below 4°C. This finding delineates the temperature range around which to expect a significant decline in nitrifying MBBR performance during the onset of winter operation.

Whereas low temperature attached growth nitrification kinetics were investigated in Chapter 3, the biofilm, biomass and microbiome responses to low temperature were assessed in

Chapter 4 to identify the cellular level changes occurring in nitrifying MBBR during operation at temperatures as low as 1°C, and specifically above and below the kinetic threshold temperature delineated between 4°C and 2°C. Assessment of the biofilm responses to low temperatures in Chapter 4 indicates a greater increase in biofilm thickness and greater decreases in biofilm density above the kinetic threshold temperature (above 4°C) compared to changes occurring below 2°C. Similarly, the biomass cell viability demonstrated greater increases in the percentage of viable cells above 4°C as opposed to the lesser decreases observed below 2°C. At the microbiome level, beta diversity analysis shows greater microbial shifts are occurring again above the kinetic threshold temperature or above 4°C compared to the lesser microbial shifts below 2°C. Therefore, the biofilm, biomass and microbiome responses seem to indicate that in acclimatized systems that experience a steady decline in operational temperature, the bulk of nitrifying biofilm adaptation to low temperatures may occur above 4°C, before the first significant decrease kinetics due to the low temperatures is observed.

With the finding of a non-linear decline in low temperature kinetics between 10°C and 1°C, the temperature dependent rate modeling with Arrhenius correction coefficients was investigated with temperature-controlled experimentation. Above the kinetics' threshold temperature, between 10°C and 4°C, the Arrhenius correction coefficient was determined to be 1.049 while a second coefficient of 1.149 is proposed below kinetics' threshold temperature to model the rates at 1°C. Taking into account the exposure time to low temperature, which is another critical factor of nitrification kinetics have yielded the Equation 3.3, an adjusted Delatolla et al. (2009) Theta model which produces modeled rates correlating with the measured rates between 4°C and 2°C.

## **7.2. Cold Shocked Attached Growth Nitrification**

As reported for both nitrifying attached growth and suspended growth systems, the cold shock investigations in Chapters 3 and 5 have determined that cold shocked nitrifying MBBR systems at 1°C underperformed compared to the slowly acclimatized systems with a difference of 21% in average TAN removal rate. Analysis of solid production in both cold-shocked and acclimatized systems indicates that the lower cold shocked kinetics are likely not due to commonly referenced cold shocked triggered events in the nitrifying biofilms, such as the cellular response to cold shocks triggering a shift from the sessile state to the planktonic state in embedded cells, which eventually leads to biofilm dispersal. It is hence likely that the adaptation mechanisms of embedded nitrifying communities are incapable of mitigating rapid decreases in temperatures of 10°C down to 1°C.

Adding to the findings in Chapter 4, Chapter 5 presents the findings with respect to the nitrifying biofilm, biomass, and microbiome responses to cold shocks down to 1°C. The effect of cold shock treatments on nitrifying MBBR down to the molecular level was investigated using slowly acclimatized MBBR systems to low temperatures and cold shocked MBBR systems. Assessment of the biofilm response down to 1°C with respect to biofilm thickness and density due to low temperatures and cold shocks demonstrated similar increases in biofilm thickness and decreases in biofilm density resulting from increases in thickness while no statistically relevant changes in biofilm mass in both systems were measured. However, the nitrifying biomass response in terms of cell viability indicates that cold shocked biomass does not exhibit the same increases in viable cells as observed in slowly acclimatized biomass to low temperatures down to 1°C. A discrepancy between the cold shocked and slowly acclimatized nitrifying microbiome was also observed. Whereas statistically relevant changes were recorded in the total number of estimated species in both acclimatized and cold shocked biofilms between 10°C and 1°C, and beta diversity

analysis indicates that nitrifying biofilms undergo microbial shifts due to low temperature above the temperature of 1°C. The largest community shifts were observed during the acclimatization down to 1°C from 10°C with a lesser shift during the operation at 1°C in nitrifying biofilms slowly acclimatized down 1°C. This finding confirms the observations of Chapter 4 that the adaptation of nitrifying biofilms to low temperatures occur above 1°C during the transition from warmer temperature to these low temperatures. As the extended operation at 1°C of this investigation did not result in the kinetic recovery of the cold shocked MBBR system to match the acclimatized systems, the sum of these findings on the lasting adverse effects of cold shocks indicates the importance of incorporating design to minimize cold shocks to nitrifying MBBR systems during periods of snowmelt and cold air outbreaks.

### **7.3. Future Directions**

The capacity of MBBR systems to maintain nitrification at low temperatures down to 1°C was confirmed in this research. This research was conducted to address the remaining and crucial research questions on nitrifying MBBR systems for their optimal design and operations as upgrade solutions to lagoon facilities at a time when low temperature nitrifying MBBR technologies are entering the marketplace. Nonetheless, the findings of this research have raised questions to be answered in future research.

First, an assessment of nitrifying microbiome beyond the scope of DNA sequencing and relative abundance results with molecular techniques such as droplet digital PCR to determine any changes in the number of nitrifiers down to 1°C in biofilms acclimatized to low temperatures as well as cold shocked biofilms. Secondly, proteomics investigations will be necessary to readily determine shifts in metabolic activities occurring in nitrifying biofilms' embedded cells in response to low temperatures and cold shocks. For instance, in the case of low temperatures, the

assessment of key proteins' levels such as the ammonia monooxygenase (amoA) and hydroxylamine oxidoreductase (HAO) to assess whether the lower nitrification kinetic rates are due to a down-regulation at the transcription or translation level; inhibition of their activity; or, their degradation. Furthermore, targeting additional proteins in this assessment should provide the crucial missing knowledge on nitrifying biofilms molecular responses to low temperatures, notably investigating common protein function regulators should delineate cellular adaptations to low temperatures; membrane proteins to identify shifts in membrane rigidity, nutrient uptake and cell to cell communications; biofilm formation and dispersal regulators to identify the main enzymes involved in these mechanisms and their pathways in nitrifying biofilms. Explicitly in relations to the findings presented in this dissertation, a study of the proteome will define the shifts in metabolic activities occurring with decreases in temperatures and associated decreases in nitrification kinetics; especially the molecular shifts at nitrification kinetics' threshold temperature between 4°C and 2°C; the differences in metabolic activities between cold shocked and slowly acclimatized biofilms to low temperatures; the cold shock response mechanisms in nitrifying biofilms; and, to determine whether the lack of cold shock response mechanisms observed in this research is due to a late onset of these mechanisms in nitrifying biofilms or their inability to mitigate the severe adverse effects of rapid decreases in temperatures.

Finally, further investigations are recommended on an add-on nitrifying and denitrifying MBBR system in-series to passive treatment systems for complete nitrogen removal down to 1°C. Future kinetic and molecular studies will provide needed knowledge on biofilm mediated total nitrogen (TN) removal down to 1°C. These studies will provide a fundamental understanding for the design of upgrade MBBR systems to passive treatment systems for TN removal, including an

assessment of denitrifying biofilms, biomass, and microbiome responses to temperatures as low as 1°C.