UNIVERSITY OF OTTAWA

DEPARTMENT OF CIVIL ENGINEERING

UNDERSTANDING EXTRACELLULAR POLYMERIC SUBSTANCES IN NITRIFYING MOVING BED BIOFILM REACTOR

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

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Abstract

Water and wastewater treatment solutions incorporating biofilm systems are becoming increasingly popular due to more stringent regulations pertaining to drinking water and wastewater effluent discharge in Canada and in other parts of the world. As a major component of biofilm, extracellular polymeric substances (EPS) have been considered as an important factor affecting the physical and chemical properties of biofilm. Further, the selected method of EPS extraction and the methods of detecting the composition of the EPS have shown to affect the results of EPS measurements.

In this research, protocols for EPS extraction and EPS composition analysis were investigated and optimized for nitrifying moving bed biofilm reactor (MBBR) biofilm. In addition, the confocal Raman microscopy (CRM) spectra of EPS in nitrifying MBBR biofilm and the protein, polysaccharide and extracellular DNA (eDNA) percent concentrations of the EPS were investigated at various operating temperatures. Further, the CRM spectra and the protein, polysaccharide and eDNA percent concentration of EPS in nitrifying MBBR biofilm along with the biofilm morphology and thickness and the viability of the embedded cells were investigated at various hydraulic retention times (HRTs). The EPS was characterized at various temperatures and HRTs in order to investigate potential correlation between the EPS components of the nitrifying biofilm and the ammonia removal kinetics. The biofilm morphology and thickness along with the bacterial viability of the biofilm were also investigated at various HRTs. Biofilm morphology images and thickness measurements were acquired using a variable pressure scanning electron microscope (VPSEM). The percentages of viable embedded cells in the biofilm were quantified using live/dead staining in combination with confocal laser microscopy (CLSM) imaging.

The research demonstrates that an increase in protein content and subsequently a decrease in polysaccharides and eDNA contents in the

EPS of nitrifying MBBR biofilm were observed at the lowest operational HRT and the highest temperature in this work. In particular, the EPS protein to polysaccharide (PN/PS) ratio of nitrifying MBBR systems was shown to significantly decrease below a value of 3 when the system was underloaded (observed at the highest operational temperature in this study) or hydraulically overloaded (observed at the lowest HRT in this study). As such, data obtained at lower operational temperatures, with the system no longer underloaded, and at longer HRTs, with the system no longer hydraulically overloaded, all demonstrate an EPS PN/PS ratio of approximately 3. Correlations were observed between the chemically measured EPS PN/PS ratios and the measured Raman spectra intensity ratios; supporting the concept of higher PN/PS ratios of EPS in more optimal nitrifying MBBR operations. Further, the ammonia removal kinetics and EPS response at HRT values of 0.75 and 1.0 h indicate that nitrifying MBBR systems may be optimized to operate at HRTs as low as 0.75 to 1.0 hour as opposed to conventional HRTs of 2.0 to 6.0 h.

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List of Abbreviations

asym	asymetric
AOB	Ammonia Oxidizing Bacteria
BAF	Biological Aerated Filter
BCA	Bicinchoninic Acid
BOD	Biochemical Oxygen Demand
BOD_7	7-day Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
breath	breathing
CAS	Conventional Activated Sludge
CBBG	Coomassie Brilliant Blue G-250
CEPA	Canadian Environmental Protection Agency
CER	Cation Exchange Resins
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
CRM	Confocal Raman Microscope
def	deformation
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
eDNA	Extracellular DNA
EDTA	Ethylene Diamine Tetraacetic Acid
EPS	Extracellular Polymeric Substances
ESEM	Environmental Scanning Electron Microscopy

FCP	Folin Ciocalteau Phenol
F/M ratio	Food to Microorganism Ratio
G	Guanine
HRT	Hydraulic Retention Time
LB-EPS	Loosely Bound EPS
LR	Loading Rate
MBBR	Moving Bed Biofilm Reactor
NOB	Nitrite-oxidizing Bacteria
Phe	Phenylalanine
PN	Protein
PS	Polysaccharide
PVC	Polyvinyl Chloride
RBC	Rotating Biological Contactor
RM	Raman Microscope
RNA	Ribonucleic Acid
RR	Removal Rate
scis	scissoring
SEM	Scanning Electron Microscopy
SRT	Solids Retention Time
str	stretching
sym	symmetric
T	Thymine
TB-EPS	Tightly Bound EPS

TEM	Transmission Electron Microscopy
TF	Trickling Filter
TS	Total Solids
TSS	Total Suspended Solids
Tyr	Tyrosine
VPSEM	Variable Pressure Scanning Electron Microscope
VS	Volatile Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant
XPS	X-ray Photoelectron Spectroscopy

List of Parameters

A_{Blind}	absorbance of protein sample at 570 μm without $CuSO_4$ added (a.u)
A_{Humic}	calculated absorbance of humic substances (a.u)
$A_{Protein}$	calculated absorbance of protein (a.u)
A_{Total}	absorbance of protein sample at 570 μm with $CuSO_4$ added (a.u)
b	endogenous decay coefficient (g cells/g cells d)
C_{in}	influent ammonia concentration (mg/m^3)
$fill\%$	carriers fill percentage
K	saturation coefficient (g/m^3)
k	maximum specific substrate utilization rate (g substrate/g cells d)
k_T	bacterial growth rate (g new cells/g cells d)
k_{TR}	bacterial growth rate at reference temperature (g new cells/g cells d)
Q	wastewater influent flow rate (m^3/d)
pKa	the acid dissociation constant (=9.5)
S	substrate concentration (g/m^3)
SA_{bs}	bulk specific surface area of carrier (m^2 of biofilm/m^3 of carrier)
T	temperature (°C)
T_R	reference temperature (typically 20°C)
θ	temperature coefficient (1.02 to 0.6)
μ_m	maximum specific growth rate (g new cells/g cells d)
V	reactor volume (m^3)
V_m	carriers volume (m^3)
X	biomass concentration (g/m^3)

1 Introduction

1.1 Background

Biofilm technology has been widely adopted as a means of biological wastewater treatment over the past decades. Biofilm treatment systems are often efficient and economical, with lower sludge production compared to suspended growth systems. In particular, biofilm treatment systems are the most economical means of performing nitrification (Metcalf and Eddy, 2003). In order to improve global water supply and solve operational problems associated with wastewater treatment plants (WWTPs), it is important to further improve our understanding of the development, structure and dynamics of biofilms (Flemming et al., 2000). Recently, particular attention has been given to the extracellular polymeric substances (EPS) component of biofilms, with EPS being the largest component of many biofilms and hence the component that significantly effects the fundamental properties of biofilms, including the kinetically controlling mass transfer dynamics, surface characteristics and adsorption abilities (Sheng et al., 2010). Therefore, enhancing the research of EPS and developing a deeper understanding of EPS constituents, their functional performance and the relation between the composition of EPS and the performance of biofilm technologies are critical for the design of the next generation of biofilm technologies.

In Canada, ammonia has been identified and included on the Canadian Environmental Protection Agency (CEPA) list of toxic substances since 1999 due to its toxic effects to aquatic ecosystems. Ammonia releases from WWTPs has been identified as a significant source of ammonia discharge into natural water and hence a major contributor to aquatic ecosystem toxicity (Env. Canada, 2003). The inclusion of ammonia on the toxic substance list in Canada has recently resulted in more stringent federal regulations pertaining to the discharge of ammonia from Canadian WWTPs (Canada Gazette, 2012). With the publication of the federal regulations, the Canadian

Council of Ministers of the Environment (CCME) has identified over 800 WWTPs across Canada that potentially may not be able to meet the federal regulations (Canada Gazette, 2012).

The moving bed biofilm reactor (MBBR) system is a robust biofilm wastewater treatment technology with a strong adaptability to shock and influent loading, along with a capability to achieve high removal efficiencies of nitrogenous and carbonaceous pollutants (WEF, 2011). The MBBR technology has been proven for secondary treatment of municipal wastewaters. MBBR systems require simple maintenance, low operational intensity and a lower operating cost than other biofilm systems. The MBBR technology operates with the help of a mechanical mixer or an aerator to keep biocarriers in motion as a moving bed in a basin. The biocarriers housed in MBBR systems provide a high surface attachment area as well as preservation capabilities for the attached bacterial growth (WEF, 2011). Recently, the MBBR technology has been demonstrated as an upgrade unit capable of achieving nitrification at very cold temperatures for prolonged periods of cold temperature exposure (Delatolla et al., 2011; Hoang, et al., 2013). Although this finding positions the MBBR technology as a cost-feasible upgrade solution to non-nitrifying systems that operate in cold climate countries such as Canada, the current understanding of nitrifying MBBR biofilms and in particular the EPS component of the biofilms is limited.

EPS research has led to the development of numerous extraction techniques to isolate EPS from biofilm and new analytical techniques to measure the chemical composition of the EPS (Flemming et al., 2000; Sheng et al., 2010). EPS extraction is the focus of many of these studies and has been characterized as the most important component in the study of EPS characteristics of microbial aggregates and biofilms; however, as of today there exists are no standard methods to extract EPS efficiently while completely avoiding cell lysis (Frølund et al., 1996). Due to the shortcomings of

current EPS analytical methods, it is crucial to optimize current EPS extraction methods for specific biofilms, continue to develop new analytical techniques for EPS composition and continue to explore *in-situ* analytical techniques for EPS characterization (Sheng et al., 2010).

1.2 Aim of Study

The overall purpose of this work is to characterize the EPS of nitrifying MBBR biofilm at various operational conditions. The effects of temperature and HRT on the EPS components and cellular activity in nitrifying MBBR biofilms are investigated using both chemical and *in-situ* analyses. Specifically, a confocal Raman microscope (CRM) is applied as a new *in-situ* method to identify the EPS components of nitrifying biofilm and is used as a complemental analytical method to the modified traditional chemical analytical methods of EPS characterization. The specific objectives for this research are as follows:

- Summarize and optimize existing chemical methods for nitrifying MBBR biofilm EPS extraction and the EPS protein, polysaccharide and eDNA determination;
- Extract EPS from nitrifying MBBR biofilm and characterize the protein, polysaccharide and eDNA percent concentration of extracted EPS at various temperatures and HRTs;
- Investigate the Raman spectra of EPS in nitrifying MBBR biofilms at various temperatures and HRTs and identify the dominant Raman bands and chemical assignment of these bands;
- Characterize the effects of temperature in MBBR wastewater treatment systems on the EPS components, the protein to polysaccharide (PN/PS) ratio in EPS and the nitrifying kinetics;

- Characterize the effects of HRT in MBBR wastewater treatment systems on the EPS components, the PN/PS ratio in EPS, the nitrifying kinetics, the biofilm thickness and morphology along with the embedded cells viability.

1.3 Thesis Organization

The following thesis is comprised of five chapters. Chapter 1 presents the background information on the significance of this research and the objectives of this study. Chapter 2 presents a literature review on the fundamentals of biological wastewater treatment, nitrification microbiology, nitrifying MBBRs, biofilms and an overview of EPS in biofilms. Chapter 3 describes the experimental design of the work and the optimized method of EPS extraction as well as the protein, polysaccharide and eDNA quantification methods. In Chapter 4, the effects of temperature and HRT on the EPS of nitrifying MBBR biofilms are investigated. Particular attention is given to the correlation between the nitrifying kinetics and the PN/PS ratios in EPS of nitrifying MBBR biofilms at varying temperatures and HRTs. Finally, Chapter 5 offers the conclusion of this study and suggestions for future related work.

1.4 References

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2 Literature Review

Chapter 2 is a review about the background and fundamental knowledge related to this project. It mainly consists of 6 sections including the introductions of biological wastewater treatment, biological treatment systems, nitrification, biofilm and EPS.

2.1 Biological Wastewater Treatment

Wastewater treatment processes are designed to reduce the contaminant concentrations in wastewaters and reduce the risk of deleterious constituents in natural waters. As an integral part in wastewater treatment plants (WWTPs), biological treatment is applied as secondary or tertiary treatment after heavy solids are allowed to settle by physical screening in preliminary treatment and sedimentation in primary treatment. The main objective of biological treatment is to utilize microbial metabolic functioning to transform biodegradable contaminants into less deleterious end products.

2.1.1 Bacterial Metabolism

Metabolism is the total sum of the chemical processes for bacteria growth with the requirement of specific substrate, adequate pH, temperature and dissolved oxygen (DO) concentrations. Figure 2.1 shows a schematic of bacterial metabolism (Rittmann and McCarty 2001).

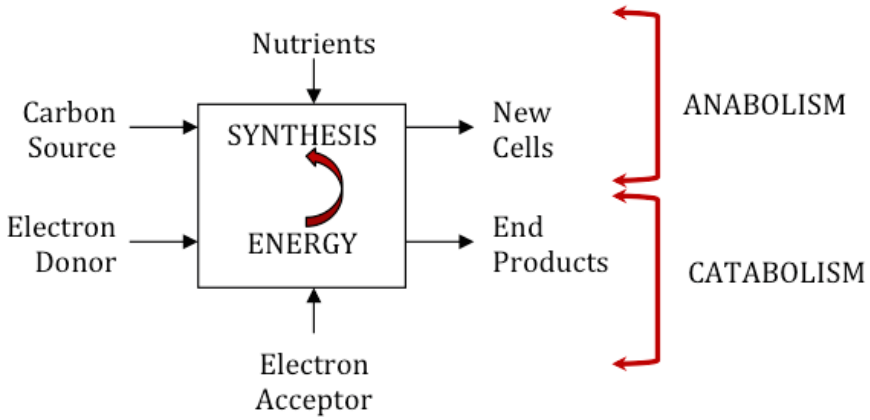


Figure 2.1 A schematic of bacterial metabolism (interpreted from Rittmann and McCarty 2001)

Bacteria metabolism involves the energy coupling of catabolism and anabolism (Rittmann and McCarty, 2001). The carbon source, the nutrients, the energy source and the electron acceptor are the four essential elements for an integrated metabolic process, in which a series of redox reactions regulate energy required for cell synthesis, maintenance and endogenous decay. Generally, anabolism results in the synthesis of new cells and supports existing cells. It includes the uptake of a carbon source and nutrients with the supply of energy produced by catabolism. Meanwhile, catabolism is a process of reaction happens between the electron donor and electron acceptor. During the metabolic process, bacterial enzymes act as catalysts during the metabolic processes (Grady et al., 1999).

Microorganism classification based on metabolic mechanisms is presented in Figure 2.1 (Rittmann and McCarty, 2001). Energy and carbon source as well as electron donor and accepter could be various for different microorganism. In conventional municipal biological wastewater treatment systems, aerobic heterotrophic bacteria oxidize organic carbon, of which the concentrations are measured as the biochemical oxygen demand (BOD) or the chemical oxygen demand (COD). While inorganic substances, such as ammonia (NH_4^+) and nitrite (NO_2^-), are oxidized by aerobic

autotrophic bacteria. Biomass generation is traditionally measured as volatile suspended solids (VSS) in suspended growth treatment systems or as volatile solids (VS) in attached growth biological treatment systems.

Table 2.1 Classification of microorganism (Rittmann and McCarty, 2001)

Type	Energy source	Carbon source	Electron donor	Electron acceptor
Phototrophs	Light	-	-	-
Chemotrophs	Chemical compounds	-	-	-
Heterotrophs	-	Organic C	-	-
Autotrophs	-	Inorganic C	-	-
Organotrophs	-	-	Organic compound	-
Lithotrophs	-	-	Inorganic compound	-
Aerobic	-	-	-	O_2
Anoxic	-	-	-	NO_2^- or NO_3^-
Anaerobic	-	-	-	Not O_2, NO_2^- or NO_3^-
Facultative	-	-	-	O_2, NO_2^- or NO_3^-

2.1.2 Bacterial Growth

2.1.2.1 Bacterial Growth Curve

In pure cultures, the pattern of bacteria growth in a batch culture with the initial condition of unlimited nutrients can be divided into six phases (Figure 2.2) (WEF, 2011): lag, increasing growth, logarithmic growth, declining growth, stationary and death.

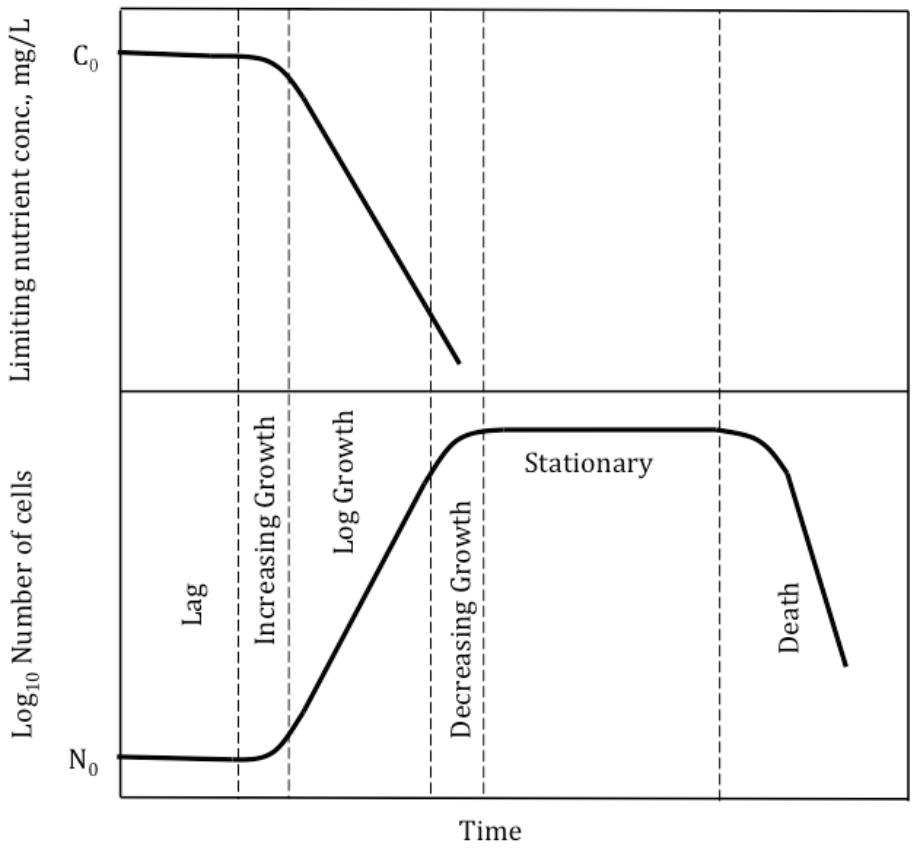


Figure 2.2 Bacterial growth curve (WEF, 2011)

Lag is an acclimatization stage where cells adapt to the new environment. During the following increasing growth stage, the bacteria growth rate changes from zero to a maximum value in a short period of time, where the bacteria population shifts from a non-growth population to a growth phase one. Exponential growth is observed in the log growth phase until the nutrient concentration starts decreasing. As the nutrient concentration approaches zero, the growth rate slows down and cell division approaches a complete stop; this is called the decreasing growth phase. The stationary stage is characterized by zero growth while the cells functions are maintained by catabolism. Finally, the cells start to die when the surviving cells' only nutrient sources are the components released via cell lysis of other cells, which have died in the death phase (WEF, 2011).

2.1.2.2 Bacterial Growth Kinetics

Bacterial growth is the results of a large number of extracellular and intracellular reactions. The bacterial growth and nutrient removal rates are traditionally measured by lumped and surrogate parameters in the field of wastewater treatment. Such parameters include total suspended solids (TSS), total solids (TS), VSS, VS, BOD and COD. The specific growth rate, μ (g new cells/g cells d), growth rate, μ_g (g/m^{3}d), specific substrate utilization rate, q (g substrate/g cells d), and substrate utilization rate, r_s (g substrate/m^{3}d), can be described by using the Monod equations below (Metcalf and Eddy, 2003):

$$\mu = \frac{\mu_m S}{K + S} - b \qquad \textbf{Eq. 2.1}$$

$$\mu_g = \frac{\mu_m S}{K + S} X - bX \qquad \textbf{Eq. 2.2}$$

$$q = -\frac{kS}{K + S} \qquad \textbf{Eq. 2.3}$$

$$r_s = -\frac{kS}{K + S} X \qquad \textbf{Eq. 2.4}$$

where μ_m = maximum specific growth rate (g new cells/g cells d);

K = saturation coefficient (g substrate/m^3);

S = substrate concentration (g substrate/m^3);

b = endogenous decay coefficient (g cells/g cells d);

X = biomass concentration (g cells/m^3);

k = maximum specific substrate utilization rate (g substrate/g cells d).

In biological WWTPs, biomass is produced as compounds are consumed, and the cell yield Y is described as the relative biomass produced to substrate consumed (Equation 2.5).

$$Y = \frac{g\ cells\ produced}{g\ substrate\ consumed} \qquad \textbf{Eq. 2.5}$$

In addition, the bacterial growth rate and substrate consumption rate can be strongly affected by temperature. The rate-temperature relationship can be indicated by the equation below:

$$k_T = k_{T_R}\theta^{T_R - T} \qquad \textbf{Eq. 2.6}$$

where T = temperature (°C);

k_T = bacterial growth rate (g new cells/g cells d);

k_{TR} = bacterial growth rate at the reference temperature (g new cells/g cells d);

T_R = reference temperature (typically 20°C);

θ = temperature coefficient (1.02 to 0.6).

2.2 Biological Treatment Systems

Biological wastewater treatment systems are classified as suspended growth and attached growth systems.

2.2.1 Suspended Growth Systems

Aerated lagoon and activated sludge treatment systems are the conventional rural and urban treatment systems in Canada and both are suspended growth systems. They are the most common methods of secondary wastewater treatment in Canada.

2.2.1.1 Lagoon Treatment Systems

Lagoons are one of the oldest forms of wastewater treatment in the world. They are in-ground earthen basins that are used for wastewater treatment. Lagoon treatment systems rely on natural processes that often include both algae and bacteria

pathways of constituent utilization. Figure 2.3 is a schematic diagram of an aerated lagoon treatment system.

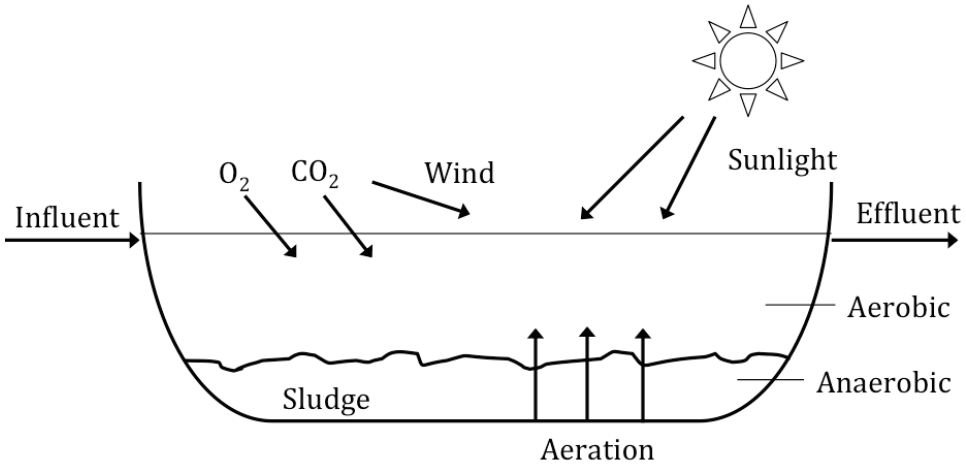


Figure 2.3 A schematic of an aerated lagoon treatment system

Lagoons are mechanically simple and inexpensive to operate and maintain. Until 2009, 67% of the total wastewater treatment plants in Canada were lagoon treatment systems; they are generally used to serve smaller communities in most provinces and territories. Compared to conventional mechanical treatment systems, such as activated sludge treatment, lagoons only represent a small portion of the total wastewater volume treated per day in Canada. Poor ammonia removal has been demonstrated in lagoons, especially in cold Canadian winter conditions, which can disadvantageously affect the aquatic ecosystem due to the direct release of untreated ammonia (FCMNRC, 2004). In addition, the lagoon systems are not as efficient in removing various contaminants compared to other modern treatment facilities and they require significantly larger areas of land.

2.2.1.2 Conventional Activated Sludge System

The activated sludge system is also a conventional wastewater treatment approach, mainly applied in large population centers of Canada. The typical activated

sludge (CAS) system is an aerobic suspended growth system consisting of an aerated tank, a clarifier and a sludge recycle, in which microorganisms are grown for the purpose of organic matter (BOD/COD) biodegradation with or without ammonia oxidation (Figure 2.4). It is a highly controllable process in which a high degree of BOD/COD removal is achieved with nitrification being achieved by increasing the solid retention time (SRT).

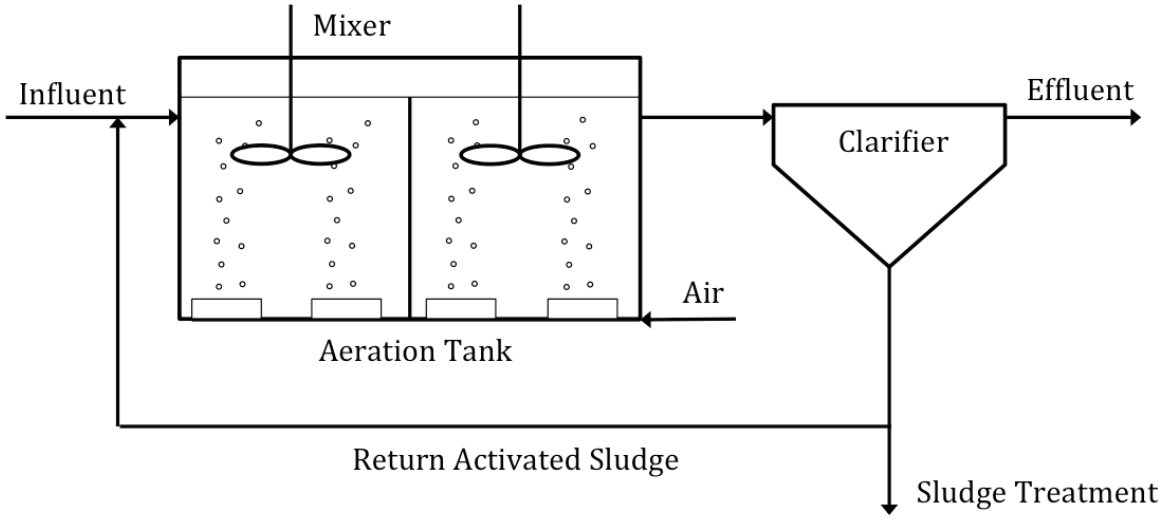


Figure 2.4 A schematic of a CAS process

However, nitrification requires a significantly higher SRT. The minimum SRT for ammonia removal is from 3 to 18 h, which is much longer then the SRT for BOD removal, which is around 1 to 2 h. Additionally, in CAS systems the bioflocs are suspended freely in the bulk phase; air or DO is supplied to promote aerobic respiration and keep the flocs in suspension. Clarification units separate the solids (comprised largely of microbial biomass) from the treated liquid (Grady et al., 1999), thus the equipment for sludge disposal is always needed, which causes a wastage of actived sludge.

2.2.2 Attached Growth System

Another class of biological treatment processes are the attached growth or fixed growth wastewater treatment systems, which have been used extensively in environmental engineering practice. The key distinguishing characteristic of these systems is that the microorganisms exist in a sessile form as biofilms attached to an engineered carrier. The substrate and nutrients must therefore be transported to the biofilm and they must diffuse through within the biofilm by mass transfer process (Grady et al., 1999). In particular, in attached growth systems, the bacteria attach to fixed or movable carriers and detached biofilm fragments exit the system with the effluent stream. Due to the lack of suspended biomass in the effluent (no mixed liquor), attached growth systems do not require as extensive sludge disposal and hence reduce energy and operational costs. In addition, attached growth systems also are less susceptible to losing specific bacterial species due to washout events (WEF, 2011; Grady et al., 1999).

Numerous designs of attached growth systems exist with common types being trickling filters (TFs), rotating biological contactors (RBCs), biological aerated filters (BAFs) and moving bed biofilm reactors (MBBRs). Similar to the CAS, the BAF and MBBR systems can be used for BOD removal as well as a combination of BOD removal and nitrification; or they can be used solely for tertiary nitrification (WEF, 2009).

2.2.2.1 Trickling Filter

TFs have been used extensively in wastewater treatment for over 80 years. They are comprised of a packed tower which contains a bed of rocks, gravel or polyvinyl chloride (PVC) with wastewater flowing downwards over the biofilm surface and an upward flow of air diffusing through the liquid and into the biofilm (WEF, 2011). Figure 2.5 (WEF, 2009) shows a schematic of a TF system. Acting as a single filter, the top portion of the filter often contains thicker biofilms with mainly heterotrophic bacteria

and the bottom portion often contains thinner biofilms of primarily autotrophic bacteria.

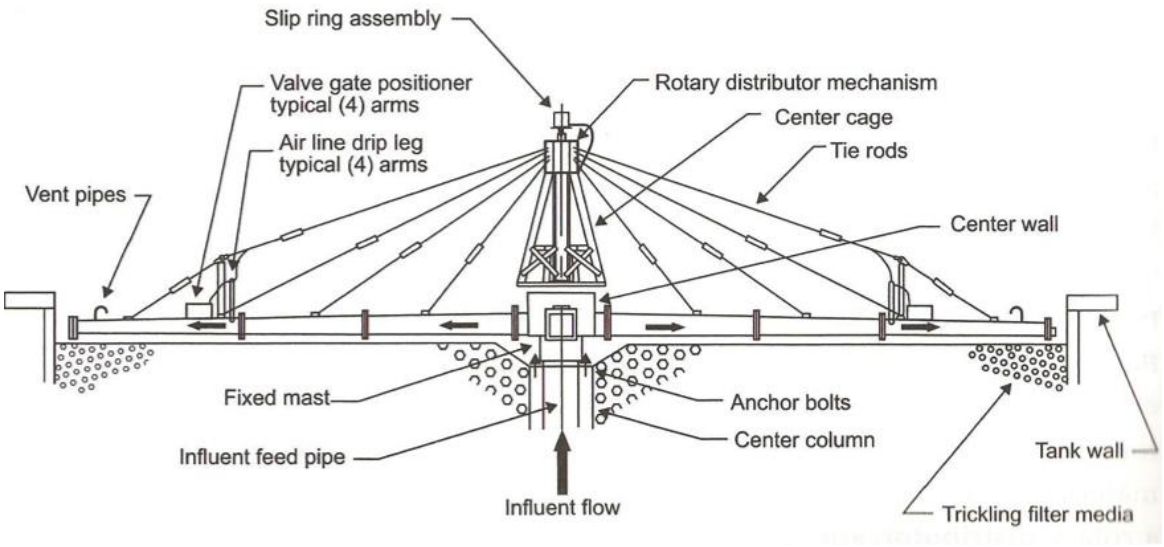


Figure 2.5 A schematic of the TF system (WEF, 2009)

TFs are easy to operate and require less energy than CAS, however, some disadvantages exist. Due to the uneven distribution of biofilm, clogging can occur in the pores of the media and can affect the hydraulic retention time (HRT) of the system, resulting in a high value of TSS in the effluent, thus the biomass removal process is needed. In addition, TFs require relatively low loadings and they potentially have vector and odor problems (Metcalf and Eddy, 2003). In Canada, it is impossible to operate TFs in freezing weather because of the exposure of facilities and media to cold temperature.

2.2.2.2 Rotating Biological Contactors

The earliest RBC system was installed in Germany in the 1960s. A RBC consists of a circular disk of polystyrene or polyvinyl chloride as packing media mounted on a horizontal shaft, which is partially submerged (typically 40%) in a tank of wastewater undergoing treatment. The biofilm grown on the media is exposed to the nutrient and

oxygen by rotating the media slowly with a speed of 1 to 1.6 rpm on the direction of influent flow (WEF, 2011). Figure 2.6 (WEF, 2009) shows a schematic of RBC system.

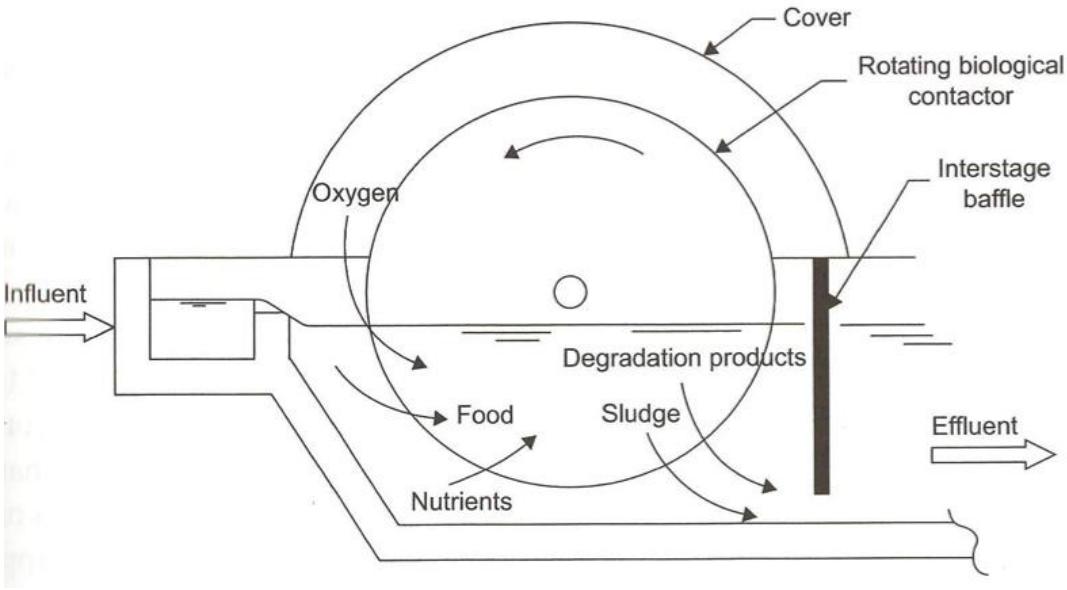


Figure 2.6 A schematic of the RBC system (WEF, 2009)

Similar to the TF system, excess biomass should be removed from wastewater via clarification, and a low BOD loading is required to initiate and perform nitrification (Env. Canada, 2003). The RBC process has a low operational intensity and is energy efficient. Numerous documented RBC system failures have however occurred, usually resulting from inappropriate shaft design. Nonetheless, these failures have caused RBC system implementations to be restricted and prevented by some regulatory agencies (WEF, 2011).

2.2.2.3 Biological Aerated Filters

BAFs were initially used for wastewater nitrate removal under anoxic conditions in the late 1970s (WEF, 2011). With a long time development, BAFs can be operated under both aerobic and anoxic conditions for BOD removal, nitrification and denitrification as a full secondary or tertiary treatment process. In a BAF reactor,

packing material of sand, gravel, activated carbon or polystyrene beads is submerged in a vertical cell, and the media provides a high surface area for biofilm growth (Env. Canada, 2003).

The BAF reactors can be characterized into the downflow BAF with media heavier than water, the upflow BAF with media heavier than water, the BAF with floating media, the continuous backwashing filters and the nonbackwashing submerged filters (WEF, 2009). Figure 2.7 gives an example of an up flow BAF System. Due to the fast clogging in the media in BAFs, backwashing is necessary to guarantee a proper effluent, while a higher backwashing frequency is required for the downflow BAFs than the upflow BAFs.

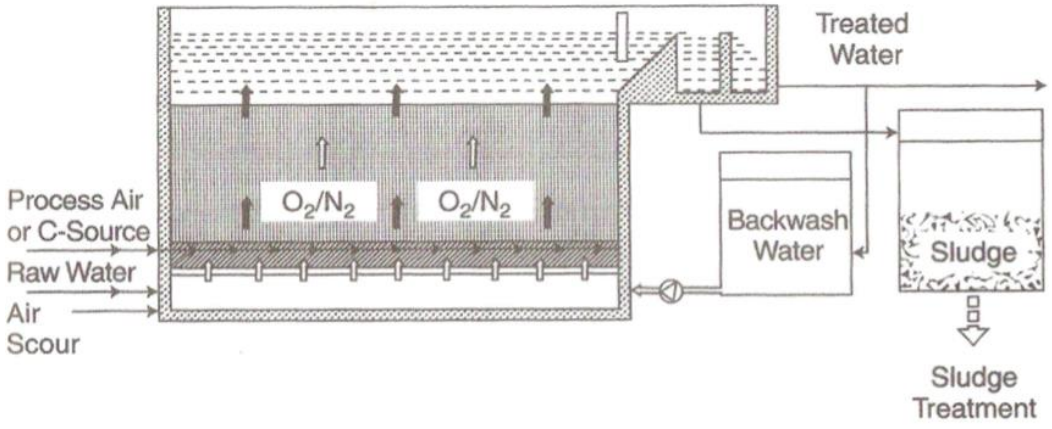
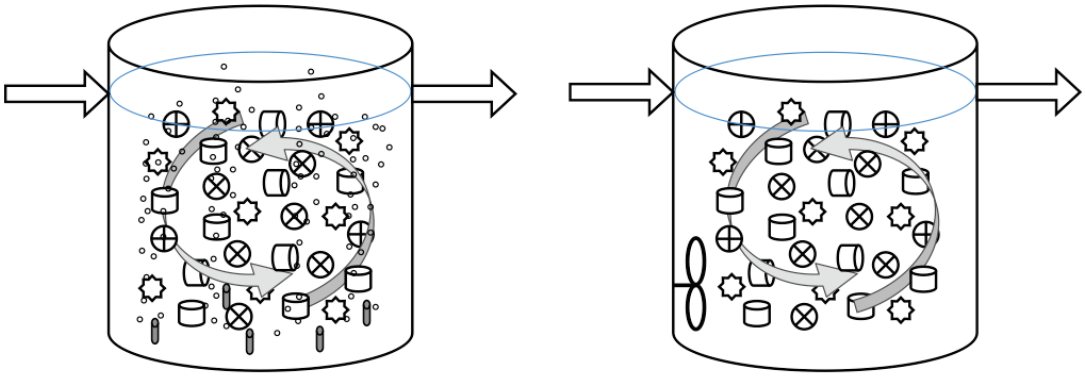


Figure 2.7 A schematic of an upflow BAF (WEF, 2009)

2.2.2.4 Moving Bed Biofilm Reactors

MBBRs are a type of biofilm reactor where biofilm grows attached to carriers that are continuously moving in the MBBR basin. The MBBR technology was firstly developed in Norway in the late 1980s to reduce the nitrogen discharge to the North Sea and has been established worldwide in the past decades (Ødegaard, 1999; WEF, 2011). In Canada, the MBBR technology has been proved to be a well-suited upgrade for municipal aerated lagoons (Hoang et al., 2013).

The unique design of using suspended carriers in motion in MBBRs allows the activated biofilms to grow upon the inner surfaces of the carriers, protecting the biofilms from physical abrasion, while being subjected to bulk liquid shearing forces that stabilize the thickness of the biofilm. The reactors are generally filled with carriers to between one to two thirds of the reactor volume. Aerators or mechanical mixers are used to circulate the carriers in the tank (Figure 2.8).



a. Aerobic Reactor b. Anoxic Reactor

Figure 2.8 Schematic of the MBBR technology

The MBBR process has many advantages. The biofilm grows on the inner side of the carriers, which protects biofilm from external forces, while the continuous movement in the reactor eliminates the need for backwashing or cleaning of overgrowth from the carriers. The treatment capacity of operating MBBR systems can be increased by simply increasing the fill fraction of carriers in the reactor. Therefore, compared to other biological treatment systems, MBBRs are relatively simple to operate with low operational intensity and low maintenance, which make them have lower operating costs then other technologies (Metcalf and Eddy, 2003; WEF, 2011).

Generally, MBBR reactors are filled to between 25 to 67% of their volume with carriers. Conventional HRT values are greater than 0.5 h for carbon removal systems

and greater than 1 h for nitrifying and denitrifying systems; with excessively high HRTs (larger than 6 h) being shown to lead to biofilm detachment (WEF, 2009).

2.3 Nitrification

Ammonia released in wastewater effluent can be harmful to the aquatic ecosystem of the receiving waters due to the ammonia's oxygen demand, propensity for eutrophication initiation and toxicity (Ward et al., 2011). The acute toxicity of ammonia can directly kill 50% of rainbow trout over 96 h period of exposure while chronic toxicity of ammonia may not only reduce the reproductive capacity of fish but also cause fish growth retardation. As such, the concentration of unionized ammonia released by wastewater treatment plants has been limited to 1.25 mg-N/L at 15°C in effluent wastewaters across Canada (Canada Gazette, 2012). Due to this regulation, ammonia removal achieved by biological nitrification is becoming increasing popular throughout Canada.

2.3.1 Nitrification Processes

Nitrification is a two-step biological oxidation process which first coverts ammonia (NH_3/NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-) under aerobic conditions. This process can be described using the following stoichiometric equations:

$$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \textbf{Eq. 2.7}$$

$$NO_2^- + 1.5\,O_2 \rightarrow NO_3^- \qquad \textbf{Eq. 2.8}$$

The biomass generated during nitrification is associated with the growth of the autotrophs; including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (both referred to as nitrifiers). The nitrifiers oxidize ammonia or nitrite to obtain energy for cell synthesis and oxidize inorganic substances as a carbon source (WEF, 1998). These nitrifiers are known to be very sensitive to various environmental factors

(including temperature, pH, DO, COD and alkalinity) and have much lower growth yields than heterotroph species responsible for BOD/COD removal in wastewater treatment systems (Ward et al., 2011).

2.3.2 Nitrification Operation in Wastewater

2.3.2.1 Ammonia Concentration and pH/Alkalinity

Ammonia existing in wastewater can be free/unionized ammonia (NH_3) or ionized ammonium (NH_4^+). The relationship between pH and the speciation of ammonia is illustrated by the following Henderson-Hasslebalch equation (Pertrucci et al., 2007):

$$pH = pKa + log\frac{[NH_3]}{[NH_4^+]} \qquad \textbf{Eq. 2.9}$$

where *pKa* is the acid dissociation constant equals to 9.5.

In biological nitrifying processes, the pH value is a key parameter governing speciation of both NH_3 to NH_4^+ and NO_2^- to HNO_2 (Ward et al., 2011); where both NH_3 and HNO_2 are toxic to nitrifiers. According to the nitrification stoichiometric equations (Eqs. 2.7 and 2.8), two equivalents of H^+ are produced when one mole of ammonia is oxidized. Hence, 7.14 g of alkalinity (as $CaCO_3$) are needed for per gram of oxidized NH_4^+-N in order to maintain the pH wastewater treatment systems. The relationship is shown in the following equation (Eq. 2.10) (Metcalf and Eddy, 2003):

$$NH_4^+ + 2O_2 + 2HCO_3^- \rightarrow NO_3^- + 2H_2O + 2CO_2 \qquad \textbf{Eq. 2.10}$$

2.3.2.2 Dissolved Oxygen

As nitrification is the aerobic oxidation of nitrogen compounds, DO concentration is a key parameter for maintaining stable nitrification. It can be observed from the nitrification stoichiometric equations (Eqs. 2.7 and 2.8) that 3.43 g O_2/g NH_4^+-N and

1.14 g O_2/g NO_2^--N are required for ammonia and nitrite oxidation respectively; which amounts to 4.57 g O_2/g NH_4^+-N for complete nitrification to NO_3^-.

Since nitrifiers as well as heterotrophs and solids constitute biological flocs in wastewater treatment systems, the floc density and size/depth ratio are the main factors affecting DO mass transfer. In attached growth, biofilm. In treatment systems the mass transfer of DO to the nitrifying biomass is dependent upon the thickness, density, morphology and structure of the biofilm. Due to a low DO inhibition effects being more prevalent for NOB as compared to AOB, partial nitrification (the oxidation of ammonia to nitrite) has been observed at low DO concentration operational conditions (Metcalf and Eddy, 2003).

2.3.2.3 Temperature

As mentioned in the last section, the growth rate of nitrifiers and the ammonia removal rate in wastewater can be strongly affected by temperature according to the equation 2.6. The optimum temperature for nitrification is between 30°C to 36°C (WEF, 2010). As nitrifiers are very sensitive to lower temperature conditions, a significant decrease in nitrification rate has been observed when the temperature drop to below 6 to 8°C (Choi et al., 1998; Head and Oleszkiewicz, 2004). Therefore, maintaining a stable nitrification process at low temperature conditions in cold climate regions has been a major issue in northern countries, such as in Canada, and has limited the regulation of ammonia discharge into Canadian natural waters.

2.3.3 Nitrifying MBBR

The MBBR technology was developed at a time when nitrogen removal was a critical need for certain treatment facilities, and as such the odds of approval for a new nitrifying possess were better, especially when coupled with either chemical coagulation or biological carbonaceous removal (Ødegaard, 1999). Generally, the MBBR

nitrification rate is influenced by the organic loading, DO concentration, ammonia concentration, temperature and pH/alkanility (WEF, 2011).

Figure 2.9 (Hem et al., 1994; Ødegaard, 1999) shows the influence of 7-day biological oxygen demand (BOD_7), DO and ammonia concentration on nitrification rate of MBBR systems. In order to achieve nitrification, higher oxygen concentrations are required at higher organic loadings (Figure 2.9 a). Above a critical supply of organic matter, the nitrifiers' growth could become inhibited by the growth of the heterotrophs (BOD/COD oxidizers); therefore organic loading should be below 5 g $BOD/m^2 \cdot d$, the influent soluble BOD should lower than 12 mg/L and the TKN: BOD ratio of the influent wastewater should be less than 1 (WEF, 2011). In addition for nitrification to occur readily in a wastewater treatment basin, the DO concentration should be above 2 to 3 mg O_2/L. Figure 2.9 b) shows that the ammonia concentration is the mass transfer limiting factor for nitrification below around 0.7, 1.7 and 2.7 g NH_4^+-N/L for DO concentrations of 9, 6 and 3 mg/L; with DO being the mass transfer rate limiting factor above the listed ammonia concentrations. Although poor nitrification has been observed at low temperatures below 6 to 8°C in conventional suspended growth system, researches have showed that the MBBR systems have the ability to maintain significant nitrification rates at lower temperatures (Houweling et al. 2007, Delatolla et al., 2011 and Hoang et al., 2013).

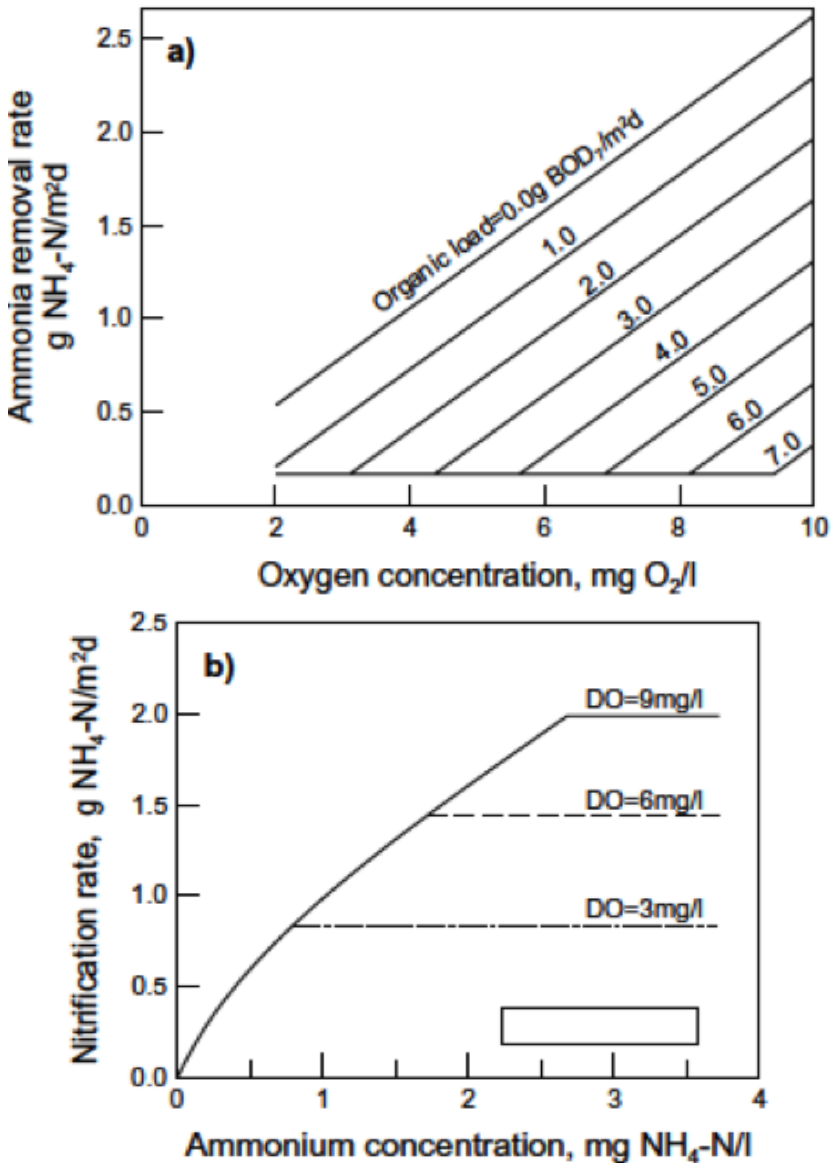


Figure 2.9 BOD_7, DO and ammonia concentration effects on MBBR nitrification kinetics (Hem et al., 1994; Ødegaard, 1999)

2.4 Biofilm

In the early twentieth century, biofilms were first observed to have the tendency of allowing bacteria to adhere in natural aquatic ecosystems (ZoBell et al., 1943). It has been found since that biofilms exist universally anywhere in the word, such as in soil,

on plants and animals, within artificial constructions and within human bodies, as long as conditions are proper for proliferation. Biofilms are now recognized as a critical element to water quality and a key component of biological reactions in wastewater treatment systems. In particular, in the last decades, the understanding and management of biofilm development, structure and dynamics have become an important concept in improving water and wastewater treatment and supply (Flemming et al., 2000).

Among the numerous studies on the subject, one of the best definitions used for describing biofilm is as follows: "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" (Costerton et al, 1999). This definition clearly indicates that the three essential components of biofilms are microbes (community of cells), polymeric substances and suitable surfaces. The inclination of bacterial adhesion to surfaces suggests a strong survival instinct and provides an advantage to adhered bacteria over suspended bacteria (Dunne, 2002). As the main component, microbes aggregate and remain attached through the excretion of polymeric substances. Properly conditioned surfaces provide a specific location for growth as well as a protection, keeping cells away from predation and harmful substances in the bulk liquid. In biofilms, the excreted polymeric substances surrounding the cells account for far more biofilm mass than the mass of the microorganisms themselves (Flemming and Wingender, 2010). The polymeric matrix consists of many complex extracellular polymeric substances (EPS) that keep cells together in a three-dimensional structure. Organisms often produce most of the EPS themselves, and the EPS have been found crucially affect the physic-chemical properties of microbial aggregates (Sheng et al., 2010).

The process of biofilm formation is generally characterized into three distinct phases (Figure 2.10): initial attachment events, the growth and maturation of complex biofilms and detachment events (Dunne, 2002).

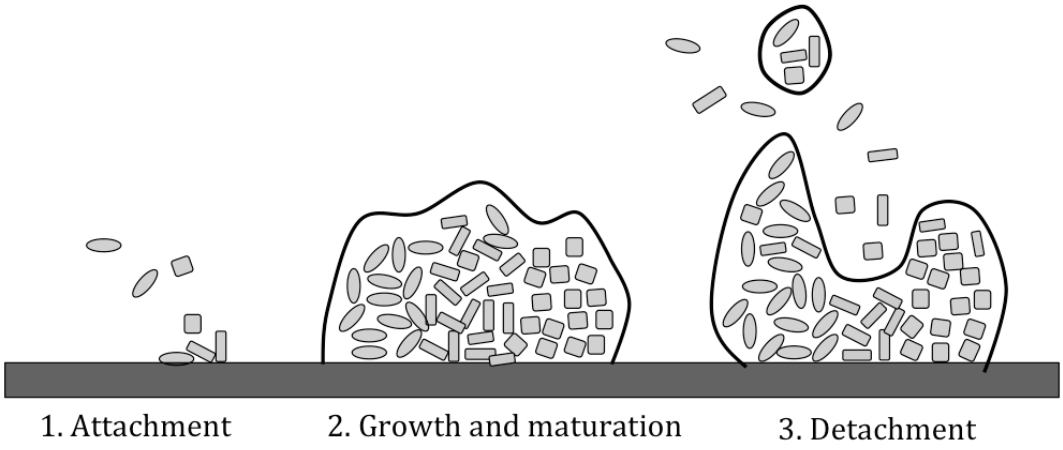


Figure 2.10 Biofilm formation processes

2.4.1 Attachment

Bacterial initial attachment constitutes the contact and immobilization that occurs between planktonic microorganisms and a conditioned surface. The attachment process consists of a two-step sequence involving reversible and irreversible attachment processes. In the first stage – the primary or docking stage: microorganisms have to be in close proximity to the surface to promote attractive forces or repulsive forces, such as electrostatic and hydrophobic interactions, steric hindrances and van der Waals forces, which are the final driving forces of adhesion (Dunne, 2002). If the conditions were not favorable for microbial adhesion, the attachment would be classified as reversible as the cells would eventually detach from the surface (Ghannoum and O'Toole, 2004). A conditioned surface is preferably rough and hydrophobic (Palmer and White et al., 1997), and will become coated by conditioning film in most waters (Schwartz et al., 1998). In WWTPs, the solid-liquid interface

provides nearly an ideal environment for microorganism attachment (Flemming, 1998).

During the second stage, also called the locking stage, bacterial adhesion becomes irreversible due to the loosely bound organisms consolidating the adhesion process by producing EPS. The metabolic byproducts of heterogeneous species can promote adhesion of the other microorganisms on the surface, thus planktons and different species can adhere to each other, producing multiple adhesions and forming microbial aggregates on the substratum (Dunne, 2002).

2.4.2 Growth and Maturation

The biofilm maturation process starts when the surface-bound organisms begin to actively replicate. During this time, the EPS surrounding cells and excreted EPS starts to interact with organic and inorganic substances in the immediate environment to create the glycocalyx (the coumponds of proteins and polysaccharides). During this stage, the overall density and complexity of biofilm increases. The growth potential is limited by the availability of nutrients, liquid dynamics and the bulk phase conditions such as internal pH, oxygen, carbon source, temperature and electrolyte concentration. When the biofilm gets to a thickness of 10 to 25 um, conditions near its base may begin to become anaerobic and biofilms with a high species diversity and stability will generally reach a critical mass and a dynamic equilibrium. Therefore, the outermost layers of growth begin to generate planktonic organisms and cells nearest the surface become inactive or die due to limitation of growth conditions (Hamilton 1987; Dunne, 2002). As biofilms become fully matured, altered patterns of bacterial growth, physiological cooperation and metabolic efficiency occur within the EPS matrix, which enables a form of functional communal coordination within the biofilm that mimics primitive eukaryotic issue (Costerton et al., 1995).

2.4.3 Detachment

Cell detachment refers to an interfacial transfer process involving the transfer of cells and other components of the biofilm to the bulk liquid phase (Characklis and Marshal, 1990). The process can be divided into three distinctive categories: abrasion, erosion and sloughing. Abrasion and erosion are similar in that they are both a loss of small groups of cells and biofilm components. However, what distinguishes these processes from one another is that abrasion is initiated by particle collision while erosion occurs due to the hydrodynamic shearing of the biofilm surface. Sloughing, on the other hand, is the loss of large group of cells and components due to sudden changes in hydrodynamic stress, nutrient limitation or other factors (WEF, 2011). Biofilms with a large fraction of active bacteria have been found to have a greater detachment rate. Moderated detachment processes are generally regarded as a positive event as it not only promotes genetic diversity, but also aids the trapped microorganisms near the biofilm/liquid interface, removing them from a potentially unfavorable habitat and allowing them to restart the biofilm growth process at a new location (Prakash et al., 2003; Percival et al., 2011).

2.4.4 Mass Transfer in Biofilms

In suspended growth systems, dissolved constituents are available to the suspended microorganisms at an approximate uniform concentration, whereas in biofilms, the availability of nutrients and substrate to the attached bacteria is controlled by mass transport mechanisms through the biofilm matrix (Grady et al., 1999). Mass transfer is thus the mechanistic driving force of substrate and nutrients from the bulk liquid phase to the biofilms and to the embedded cells. As schematic of the transformation process is shown in the Figure 2.11 (WEF, 2011).

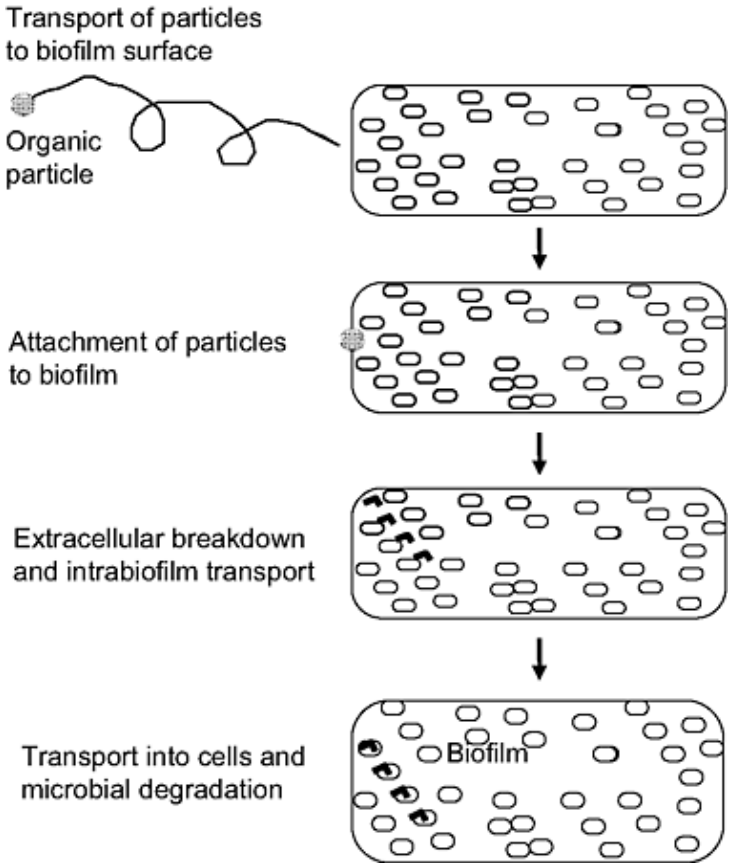


Figure 2.11 Mass transfer process in biofilms (WEF, 2011)

The substrate particles come into contact with the surface of the biofilm by turbulent transport and can become attached to the biofilm surface. Smaller molecules can directly enter the biofilm structure by coming not contacting with bound enzymes or cell surfaces at the biofilm surface, while larger molecules require hydrolysis by extracellular enzymes until they can be transported through the biofilm and absorbed by cells. Finally, microbial degradation by the cells embedded in the biofilm completes the biotransformation process of biofilms (WEF, 2011). Due to the heavy reliance on mass transfer mechanisms, low temperature effects are believed to be limited in nitrifying biofilms (Wijffels et al., 1991).

2.4.5 Nitrifying MBBR Biofilms

In order to better understand and optimize the nitrification performance in MBBR processes, studies in the nitrifying MBBR biofilms are becoming more important. With a serve of different-degrade-leveled substances, different fractions of heterotrophs in detached biofilms were observed (Ferrai et al., 2010). A correlation was found between the nitrification rate and the attached biomass as well as the fractions of different biofilm components (Zhang et al., 2013). By estimating biofilm morphology, a significant increase of biofilm thickness was observed for biofilm subjected to 1 °C for long periods of time, compared to long exposures to 20 °C (Hoang et al., 2014). Obviously, the nitrifying MBBR biofilm development and the components in biomass need to be roundly clarified in future work. A study of EPS of MBBR biofilms could be an entry point as the EPS are the major part of biofilms.

2.5 EPS

A broad definition of EPS is that it is a general and comprehensive term used to describe the polymeric components within microbial aggregates. EPS are a complex mixture of constituents such as proteins, polysaccharides, lipids and nucleic acids (Wingender et al., 1999). EPS are characterized as having a high-molecular-weight (Mw ＞10,000 g/mol) (Sheng et al., 2010). EPS are mostly produced by organisms themselves, while complex organic matter derived from the bulk liquid may also be absorbed to the biofilm and become a component of the EPS matrix (Nielson and Jahn, 1999; Liu and Fang, 2002). Microorganism accounts for less than 10% of the total dry mass, with up to 90% of biofilms being comprised of EPS (Flemming and Wingender, 2010). However, in the field of biological treatment, far more research focused on the cells in biological flocs from activited sludge rather than biofilms and in particular on the EPS component of the biofilm.

Generally, EPS can be classified as bound EPS and soluble EPS (Nielson and Jahn, 1999; Laspidou and Rittmann, 2002). The bound EPS are mainly comprised of organic materials in close proximity to the embedded cells, while soluble EPS, or so called "slime", are weakly bound to cells or are dissolved in solution. Based on their proximity to cells, bound EPS can be also classified as either tightly bound EPS (TB-EPS), located in the inner layers of the biofilm near the cells, and loosely bound EPS (LB-EPS), located in the outer layers of the biofilm (Figure 2.12).

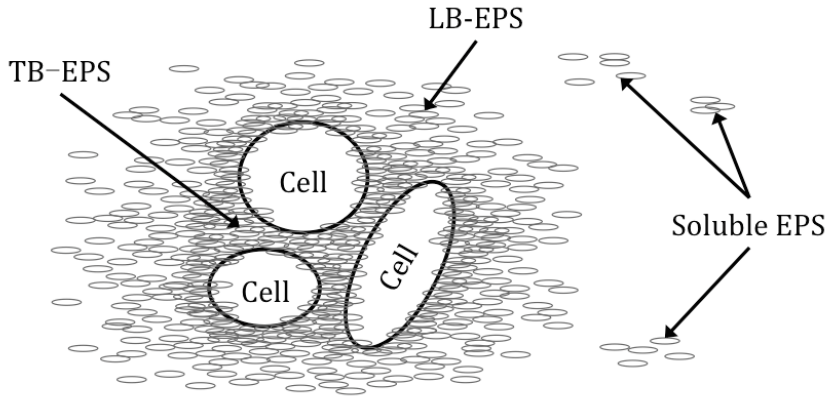


Figure 2.12 EPS classification bases on the structure of EPS

2.5.1 Functions of EPS in Biofilms

In the early stages of EPS research in the field of environmental engineering, most of the studies on the functioning of EPS were focused on flocs that occur in activated sludge wastewater treatment systems. The findings of EPS research on activated sludge flocs have led to an improved understanding of the physico-chemical properties of EPS in activated sludge flocs and subsequently important advancements with respect to the sorption processes, settling, purification, bioflocculation and dewatering processes for activated sludge treatment systems (Brown and Lester, 1982; Eriksson and Hairdin, 1984; Horan and Eccles, 1986; Ryssov, 1986).

It is believed that the EPS are the single largest contributor to biofilm maintenance and the mode of life. The functions and processes occurring within EPS have been found to be very similar to those in activated sludge flocs. Table 2.2 (Flemming and Wingender, 2010) explains the various functions of EPS. Generally, the EPS of biofilms enable the biofilm to operate normally, have a proper structure, good nutrient availability, and they provide protection and communication pathways (Karatan and Watnik, 2009). Due to such comprehensive functions, the EPS have been considered to have a significant influence on the physical and chemical properties of microbial aggregates. Therefore the in-depth study of EPS in wastewater biofilms will help improve our understanding of biological wastewater treatment processes.

Table 2.2 Functions of EPS in bacterial biofilms (Flemming and Wingender, 2010)

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins

Table 2.2 Functions of EPS in bacterial biofilms (continue) (Flemming and Wingender, 2010)

Function	Relevance for biofilms	EPS components involved
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Faciliates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

2.5.2 Composition of EPS

The composition of EPS can be varied based on the microbial species present, the physiological state of the bacteria, and the environment or operating conditions of the system. Further, the EPS extraction method is also known to affect the measured composition of EPS (Urbain et al., 1993; Liu and Fang, 2002). The major constituents of EPS are carbohydrates and proteins, with minor constituents being nucleic acids, uronic acids and humic substances (Wingender et al., 1999).

2.5.3.1 Extracellular Proteins

Proteins comprise a major fraction of the components of EPS. A portion of the extracellular proteins are enzymes, which have been shown to be responsible for a significant amount of the enzymatic activity of activated sludge flocs (Frølund et al., 1995). As mentioned in section *2.4.4 Mass Transfer in Biofilms*, extracellular enzymes play a very important role in promoting substrate absorption by cells. These extracellular enzymes primarily accelerate the conversion of substrate particles in wastewater into low-mass-molecular products, which can then be up taken into the cells for further breakdown by intracellular enzymes. Extracellular enzymes can also degrade EPS components such as polysaccharides during periods of starvation (Wingender et al., 2001; Zhang and Bishop, 2003). This degradation ability has caused biofilms to be widely applied in biological wastewater and drinking water treatment processes (Grady et al., 1999; Flemming and Wingender, 2010). In addition, when environmental condition changes, the extracellular enzymes help break down structural EPS to promote bacterial detachment (Sauer et al., 2004; Gjermansen et al., 2005), which allows for new biofilms to be formed.

Non-enzymatic proteins of EPS serve as structural components of the EPS and are hence called structural proteins (Flemming and Wingender, 2010). For example,

the glucan-binding and lectin-like proteins have been shown to format and stabilize the extracellular matrix of biofilms and establish connections between the cell surfaces and EPS (Lynch et al., 2007; Higgins and Novak, 1997). A group of proteins called "biofilm-associate surface proteins" are high molecular mass proteins, which may potentially contribute to the formation of various bacterial biofilms (Lasa and Penadés, 2006). In addition to these two categories of proteins, some proteinaceous components and proteinaceous appendages in the EPS have also been shown to act as structural elements (Flemming and Wingender, 2010).

2.5.3.2 Extracellular Polysaccharides

Polysaccharides comprise a major fractional component of EPS. Most of the polysaccharides are heteropolysaccharides (long chains of single sugar units bound together) while a small portion are homopolysaccharides (single type of sugar) (Flemming and Wingender, 2010). Polysaccharides are mostly involved in the biofilm development and architecture. For example alginate, an extensively studied heteropolysaccharide in biofilm, helps establish microcolonies at the beginning of biofilm formation as well as keep mechanical stability mature biofilms; other heteropolysaccarides such as *Pel* and *Psl* are related to biofilm attachment or promoting interaction between cells (Ryder et al., 2007; Byrd et al., 2009; Ma et al., 2009). In addition, those non-polysaccharide-producing species are only able to form mix-species biofilms with the help of exopolysaccharides (Watnik and Kolter, 1999; Danese et al., 2000; Sutherland, 2001; Ma et al., 2009).

2.5.3.3 Extracellular DNA

Extracellular DNA (eDNA) have been observed as a significant component in numerous wastewater biofilm samples (Frølund et al., 1996). eDNA were initially regarded as a residual substance resulting from cell lysis and more recently are

being regarded as an integral part of the EPS matrix of biofilms (Wingender et al., 1999) that may be actively excreted from live cells (Flemming and Wingender, 2010). In different species based biofilms, the quantity and role of eDNA varies significantly. The nucleic acids of *Rhodovulum* have been shown to promote the flocculation of cells (Watanabe et al., 1998); eDNA produced by *P. aeruginosa* biofilms is very big in amount and contribute to the functions of intercellular connection (Yang et al., 2007); *Bacillus cereus* eDNA acts as an adhesion for various external constituents to the biofilm (Vilain et al., 2009). In addition, active excretion could be another source of eDNA besides of lysed cells (Flemming and Wingender, 2010).

2.5.3.4 Other Components in EPS

Additional components such as humic substances, lipids, uronic acids have also been observed in EPS from various matrixes (Frølund et al., 1996; Wingender et al., 2010). Humic substances in the EPS of activated sludge flocs were shown to be involved in esterase activity, and their mass fraction was measured to be approximately 10 to 15% (which was similar to the measured mass fraction of polysaccharides in the sample) (Frølund et al., 1995; Conrad et al., 2003). Lipids are believed to play an important role in the hydrophobic properties of activated sludge EPS, however, the fraction of lipids (1.8%) is often significantly lower than the protein fraction (40%) or polysaccharide fraction (9.9%) (Conrad et al., 2003).

2.5.4 Analysis Approach of EPS

To date, numerous EPS analytical methods have been developed to quantify or qualify of the composition of the exopolymeric substances. Conventional chemical spectrophotometric methods are commonly applied as the final step to analyze the composition of the EPS. Conventional methods include the Lowry and Bradford

methods for protein determination along with the phenol-sulfuric acid and anthrone methods for polysaccharide determination (Raunkjaer et al., 1994). As the EPS of biofilms are complex mixtures of numerous components, interference of these components with conventional analytical methods is common. Therefore optimization/modification of classical methods and development of new methods are often required for the analysis of EPS extracted from a new set of biofilm samples (Sheng et al., 2010).

The EPS extraction approach has also been shown to have a direct effect on the subsequent analysis of the EPS components. An important first step in optimizing the analysis of EPS is to maximize the extraction efficiency of the EPS from the biofilm while minimizing cell lysis. Thus selection and optimization of an optimal extraction method is critical to being able to accurately measure the components of EPS for a study (Flemming et al., 2000).

Aside from chemical analyses, numerous *in-situ* methods of EPS analyses have been investigated and applied with the aim of characterizing the EPS structure and the distribution of complex components of the EPS. For example, microbial aggregates with the EPS were generally observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Li and Ganczarczyk, 1990; Bura et al., 1998). The spatial distribution of EPS and total EPS content can be obtained using confocal laser scanning microscopy (CLSM) in combination with various fluorescence (Zhang and Fang, 2001; Kawaguchi and Decho, 2002; Staudt et al., 2004). X-ray photoelectron spectroscopy (XPS) has been used to study the interaction between EPS and metals as well as the role of EPS for microbial adhesion process (Dufrene and Rouxhet, 1996; Omoike and Chorover, 2004; Ortega-Morales et al., 2007). Raman microscopy (RM), another relatively novel technique to the field of EPS analysis, can be used to investigate the chemical composition of the EPS, and

it is promising to establish a component identification process for EPS and biofilm by using RM along or in combination with other microscopic methods (Ivleva et al., 2009; Wagner et al., 2009; Kniggendorf and Meinhardt-Wollweber, 2011; Virdis et al., 2012; Chen et al., 2013; Zhang et al., 2014). Overall, in order to gain a comprehensive understanding of EPS, it is common to use a combination of analytical methods and to employ new approaches with combine conventional methods.

2.5.5 Factors Affecting EPS in Wastewater Treatment

Several characteristics of wastewater along with operational parameters of wastewater treatment plants have been found to have substantial effects on EPS production of wastewater flocs. Activated sludge microorganism fed with glucose demonstrated a higher quantity of EPS production than microorganisms fed with acetate (Li and Yang, 2007). Increases in food to microorganism (F/M) ratios or reducing the supply of phosphor as in the feed have been shown to enhance the production of EPS by cells (Jang et al., 2007; Liu et al., 2006). Further, in activated sludge systems, the solid retention time (SRT) has shown to effect EPS production. For example, when SRT increased from 4 to 16 days, the total quantity of EPS increased (Sesay et al., 2006), and Li and Yang (2007) found that the LB-EPS production decreased at longer SRT. Other external conditions, such as metal concentrations, the presence of toxic substances, the shear ratio of the reactors and the presence of aerobic/anaerobic conditions, were also shown to influence EPS production (Sheng et al., 2010).

Apart from the EPS production, the composition of the EPS and in particular the protein and polysaccharide contents, along with the protein to polysaccharide (PN/PS) ratio, has been also reported to change with different wastewater treatment operational conditions. The amount of polysaccharides has been shown to

increase as the phosphorus supply is reduced in activated sludge systems (Bura et al., 1998). The quantity of protein has been shown to increase as the carbon to nitrogen ratio of influent changed from 5 to 40 (Durmaz and Sanin, 2001). Sesay et al. (2006) also reported that the PN/PS ratio increased as SRT increased. Liang et al. (2010) found higher PN/PS ratio in biofilms than in activated sludge, and that appears to be the reason why the bacteria surface is more hydrophobic and adhesive in biofilms.

Bassin et al. (2012) showed that polysaccharide and protein concentrations proved to be good indicators of biomass development and detachment in nitrifying MBBR system. However, little work has been done on operational effects of EPS production in nitrifying biofilm systems and in particular little work has been done on temperature and HRT effects on EPS production of nitrifying biofilm systems. Therefore, in this study EPS in nitrifying MBBR biofilms were characterized under different operational conditions. Temperature and HRT are the two most important parameters expected to have a significant effect on EPS compositions. It is believed that the major effects would be associated with the variation of the EPS compositions and the PN/PS ratio, or may also affect the biofilm morphology and cell viability in biofilms.

2.6 References

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3 Methodology

This is a chapter about the systematic methodology of the project, including the design of the whole experiment process, the summarization and comparison of existing methods, as well as the description of each method procedure. It mainly consists of 5 sections: experimental design, constituent measurements, EPS chemical analyses, EPS *in-situ* analyses and statistical analysis.

3.1 Experimental Design

Currently over 1000 wastewater treatment lagoons are in operation in Canada and designed to reduce the impact of the biological oxygen demand (BOD) and total suspended solids (TSS) of discharged wastewater to the receiving waters. In order to investigate tertiary ammonia removal, a pilot-scale nitrifying moving-bed bioreactor (MBBR) plant was constructed in a joint effort between the City of Gatineau, the University of Ottawa and Veolia Water Technologies Canada at the Masson-Angers aerated lagoon treatment plant, approximately 37 km from the City of Ottawa, ON, Canada. A schematic of the pilot plant portion that was used for this research is shown in Figure 3.1.

Wastewater effluent of the Masson-Angers aerated lagoon enters the housing structure of the pilot plant and passes through bars and screens to remove large objects. The wastewater is then sent to an influent wastewater tank, which enables control of the influent flow rate to the MBBR reactors. Wastewater is successively sent to the two MBBR treatment trains. The two cylindrical MBBR reactors are identical, with a volume of 223 L each. The reactors housed AnoxKaldnes™ K5 carriers. These carriers have a bulk specific surface area of 800 m^2/m^3, a diameter of 25 mm and a height of 7 mm.

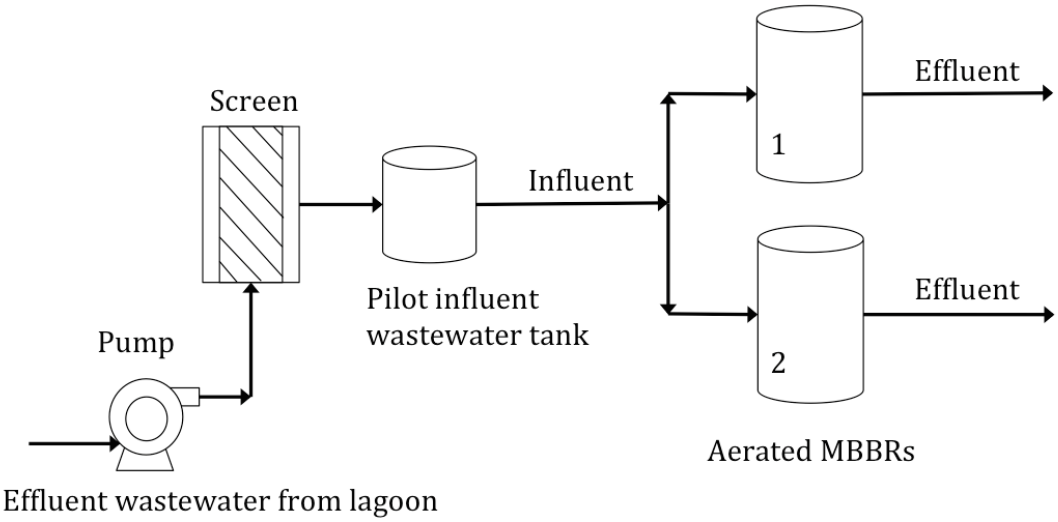

Figure 3.1 Schematic of the nitrifying MBBR pilot plant used for this research

This study uses chemical and *in-situ* analyses to characterize the effects of temperature and HRT on EPS composition, biofilm characteristics and cellular activity. Figure 3.2 illustrates the processing of the samples in this study. The biofilm carriers were subject to chemical analyses that consist of EPS extraction and protein, polysaccharide and extracellular DNA (eDNA) determination, while the *in-situ* investigation includes confocal Raman microscopy (CRM), confocal laser scanning microscopy (CLSM) and variable pressure scanning electron microscopy (VPSEM).

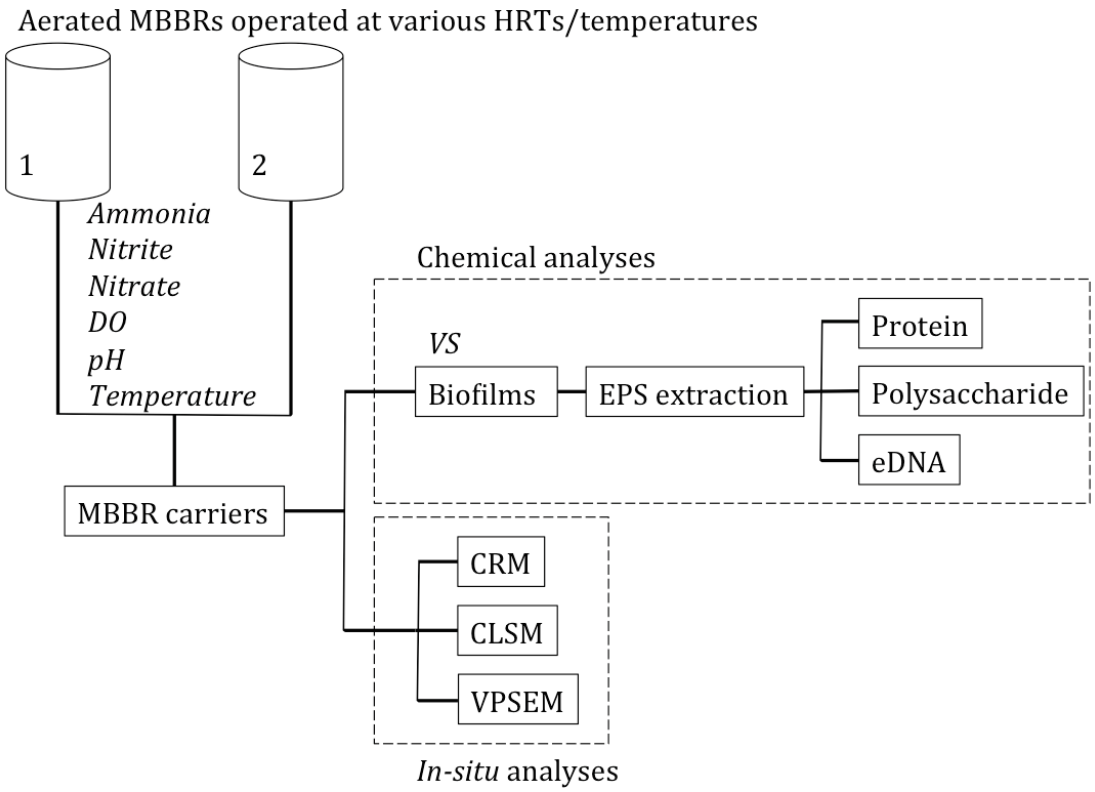

Figure 3.2 Experimental processing of samples

Reactors 1 and 2 were operated at various temperatures and HRTs while all other operating conditions such as loading rates and aeration rates were kept constant. The influent COD concentration and ammonia concentration of the reactors were 20 ± 2.0 mg/L and 19.7 ± 0.2 mg N/L respectively. Samples from reactor 1 were harvested and analyzed as the reactor was operated at a loading rate of 442 ± 24 gN/m^3d (m^3 in the unit refers to the volume of total carriers), a DO concentration of 4.5 ± 0.3 mg/L, a pH of 7 ± 0.1, a constant HRT of 2.2 h and at temperatures of 22, 15, 10 and 5°C. Meanwhile, samples from reactor 2 were harvested and analyzed as the reactor was operated at a loading rate of 864 ± 14 gN/m^3d, a DO concentration of 5.5 ± 0.3 mg/L, a pH of 7 ± 0.1, a constant temperature of 20°C and at HRTs of 0.5, 0.75, 1.0 and 3.0 h.

HRT (h) and loading rate (gN/m^{3}d) are calculated as the equations below (Eqs. 3.1 and 3.2):

$$HRT = \frac{V}{Q} \quad \textbf{Eq. 3.1}$$

$$Loading\ rate\ = \frac{Q \times C_{in}}{SA_{bs} \times V \times fill\%} \quad \textbf{Eq. 3.2}$$

where Q = wastewater influent flow rate (m^3/d);

V = reactor volume (m^3);

C_{in} = influent ammonia concentration (mg/m^3);

SA_{bs} = bulk specific surface area of carrier (m^2 of biofilm/m^3 of carrier);

fill% = carriers fill percentage.

Therefore, the HRT can be adjusted by changing the influent flow rate. However, the loading rate was kept constantly, thus the HRT in reactor 2 was adjusted by changing both the flow rate and the carriers fill percentage of the reactor. In addition, the reactors were operated for a minimum of two weeks after steady state operation was established in order to ensure stabilization of the biofilm before sampling. Steady state operation was defined by a variance in effluent ammonia and nitrate concentrations of less than ± 10%.

3.2 Constituent Measurements

The influent and effluent ammonia, nitrite and nitrate concentrations were measured in accordance with Standard Methods 4500C-NH_3, 4500B-NO_2^- and 4500B-NO_3^- (APHA, 1995). The DO concentration and temperature within the reactors were measured using an HACH LDO® probe (HACH, ON, Canada), and the pH was measured using a CORNING® Pinnacle 530 pH meter (Corning MA, US). The measurements were all carried out in triplicate.

3.3 EPS Chemical Analyses

The biofilm was physically removed from the MBBR carriers and subsequently the harvested biofilm was subjected to chemical analyses (Figure 3.2). The EPS was then extracted from the harvested biofilm followed by the quantification of proteins, polysaccharides and eDNA. The volatile solids were also quantified as the components of the EPS were determined as g/g VS. The sample collection, extraction and testing methods are described below.

3.3.1 Biofilm Harvesting

Biofilm developed on MBBR carriers grow attached to the interior surfaces of the carriers; whereas the biofilm attached to the exterior surfaces of the carriers are subject to abrasion due to carrier collision during the MBBR treatment process and hence the exterior attached biofilm is often very thin with minimal mass compared to biofilm attached to the interior surfaces of the carriers. Hence, to remove all biofilm from the inner section of the carriers is a difficult task (Bassin et al., 2012).

Preliminary work demonstrated that the nitrifying biofilm attached to MBBR carriers is very difficult to extract from the carriers by centrifugation, and that sonication may induce cell lysis (Liu and Fang, 2002); therefore a different approach was utilized in this research to obtain workable quantities of extracted biofilm. Biofilm was harvested from the carriers by manually, gently scraping off the biofilm from the carriers (although this is labor intensive it proved to be the best method of acquiring biofilm without compromising the integrity of the embedded cells in the biofilm). Prior to removing the biofilm from the interior surfaces of the carriers, MBBR carriers were placed on an absorbent surface (such as Kimwipes™) for 30 seconds in order to remove excess water on the biofilm surface. The carriers were then cut to access the biofilm growing on the interior surfaces of the carriers. The biofilm extracted from the carriers was collected in a 1.50 ml micro centrifuge tube

and the mass of the biofilm was immediately tested followed by the extraction of the EPS to preserve the integrity of the sample.

3.3.2 Volatile Solids

The volatile solids (VS) concentration (g/g biofilm) was measured according to Standard Method 2540E for VS measurements (APHA, 1995).

3.3.3 EPS Extraction

3.3.3.1 Review of Conventional EPS Extraction Methods

EPS extraction has been applied extensively in previous research for biopolymer detection and quantification in activated sludge (Flemming et al., 2000). The use of different EPS extraction methods has been found to change the structure and functional groups in EPS as well as vary the EPS composition (Wingender et al., 1999; Eriksson and Alm, 1991; Urbain et al., 1993; and Frølund et al., 1994). As such, no EPS extraction method has currently been established as the standard across the discipline of environmental engineering.

Three important factors should be considered when selecting a proper EPS extraction method: the amount of the harvested EPS that is required for subsequent analyses, the EPS yield (mg EPS/mg VS) and the quantity of cell lysis. Extracted EPS, typically based on conventional methods, account for approximately 15% of the measured dry mass of the sample (Urbain et al., 1993; Frølund et al., 1994). Additionally, current extraction methods are not capable of producing consistent EPS extraction quantities (Brown and Lester, 1980). Cell lysis, which is the destruction of cells, has been shown to cause contamination of the extracellular material by releasing intracellular polymers and producing false positive EPS protein measurements. As such, cell lysis has been shown to severely affect subsequent EPS analyses (Gehr and Henry, 1983; Frølund et al., 1995). All methods

of EPS extraction have reported various degrees of cell lysis (Flemming et al., 2000). Since maximizing the EPS yield generally results in increased the degree of cell lysis, extraction methods for specific samples are often selected and modified to produce a reasonably effective EPS yield while limiting cell lysis and exopolymer disruption (Frølund et al., 1996).

The EPS are bound to the substratum and cell surfaces through numerous binding patterns in the matrix, including weaker forces such as van der Waals forces, electrostatic forces between individual components and bridging between polymeric chains provided by divalent cations such as magnesium (Mg^{2+}) and calcium (Ca^{2+}). Additionally, one must consider the strength of interactions between hydrophobic substances of extracellular biopolymers and cell surface. All the extraction methods have been developed on the basis of destruction of these binding forces (Flemming et al., 2000). The extraction methods are either classified as physical or chemical. A variety of methods used today are based on either one of these principles or a combination of both (Tapia et al., 2009). The physical methods include ultrasound, centrifugation and heating. These methods disrupt the EPS structure through mechanical vibrations or physical manipulation, whereas the chemical methods aim at destabilizing the EPS matrix by sequestering or modifying the molecules connecting the different components in EPS; as such, the chemical methods involve the addition of chemicals (Sheng et al., 2010). Tapia et al. (2009) identifies the following five common extraction methods: EDTA extraction, NaOH extraction, the use of cation exchange resins (CER), heating (thermal treatment) and centrifugation. Additional methods that have also been recently used include ultra-sonication and formaldehyde.

As mentioned in Chapter 2, EPS are categorized into TB-EPS, LB-EPS and soluble EPS. Previous work that focused on differentiating the two fractions of

bound EPS have extracted LB-EPS first by using relatively gentle physical methods (such as centrifugation and heating) following by TB-EPS extraction by using chemical methods (Li and Yang, 2007; Liang et al., 2010). However, once the EPS were extracted by the relative methods, the three categories of EPS were shown to not be distinguishable from one another (Flemming et al., 2000).

3.3.3.2 Review of CER Exchange Method

The CER exchange method was chosen as the EPS extraction method in this study as this method has been frequently used in recent studies and proven to be efficient to this end (More et al., 2014; Zhang et al., 2014; Wang et al., 2014). The CER exchange method did not produce EPS yields as high as the formaldehyde-NaOH and the EDTA methods, however it is not the least efficient approach either (Liu and Fang, 2002). Unlike the sonication method, the formaldehyde/formamide-NaOH method and the formaldehyde-sonication method, little to no cell lysis was reported from the CER exchange method (Frølund et al, 1996; Liu and Fang, 2002). As this study was not limited by the quantity of EPS that can be harvested from samples, the CER method was deemed a conservative and adequate method for this work. The CER exchange method is hence is expected to provide more accurate results in the quantification of EPS composition by avoiding interference with intracellular substances while yielding a significant amount of EPS.

The principle of CER exchange relies on the removal of cations from the sludge/biofilm matrix, leading to the break-up of the flocs, and subsequent release of EPS (Frølund et al., 1996). In EPS, multivalent cations such as Ca^{2+} and Mg^{2+} have been observed to play an important role in cross-linking the charged polymers (Sobeck and Higgins, 2002); therefore EPS can be extracted through the exchange of multivalent cations with monovalent cations such as Na^{+} (Wingender et al., 1999). Furthermore, due to the enriched sodium carbonate and hydroxide used in

conventional protein analytical methods such as the Lowry method, divalent cations of Ca^{2+} and Mg^{2+} are prone to form precipitates, which induce a decline in absorbance during protein determination (Shen et al., 2013). Hence, the CER exchange process does not only enable EPS to be isolated from biofilms, but it also replaces divalent ions that negatively affect subsequent analyses.

In addition, the resin contact times of the CER exchange method can be modified to induce higher EPS yields or less cell lysis; with resin contact times of 12 hours (effective method) showing higher effective EPS yields and contact times of 1 hour (mild method) inducing less cell lysis (Frølund et al., 1996). In this study, both of the resin contact times were tested. However, the 'effective method' did not show a distinct advantage in the yielded EPS amount, with the "mild method" demonstrating similar quantities of proteins and polysaccharides in the tested nitrifying MBBR biofilm EPS samples (Table 3.1). The "mild method" simply required a larger quantity of extracted biofilm mass to be harvested, which was readily available in this study. In this case, to reduce potential cell lysis, the mild method, with a 1 h resin contact time, was applied as a means of EPS extractions to all samples in this research.

Table 3.1 Results of the CER exchange method using various resin contact times

Mass of biofilm (g)	**Effective method (12 h)**		**Mild method (1 h)**	
	Protein (mg/g VS)	Polysaccharides con. (mg/g VS)	Protein (mg/g VS)	Polysaccharides con. (mg/g VS)
0.10	87.24 ± 2.8	41.51 ± 1.2	-	-
0.17	-	-	87.71 ± 5.8	42.08 ± 1.3

3.3.3.3 CER Exchange Method Procedure

Figure 3.3 illustrates the steps of the CER exchange procedure used in this research. Prior to use, the CER (Dowex Marathon C, 20-50 nm mesh, sodium form, Fluka 91973) was washed for one hour with an extraction buffer (2 mM Na_3PO_4, 4 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl at pH 7) at a ratio of 35 g CER/150 ml buffer. The biofilm was scraped off fresh MBBR carriers and the VS of the extracted biofilm was measured. The number of carriers used to acquire the biofilm sample depended on the mass of biofilm attached to each carrier, with an average of 0.2 g of harvested biofilm (obtained from approximately 3 to 6 carriers) being used for EPS extraction. The extracted biofilm was subsequently suspended in a beaker with approximately 40 ml of extraction buffer. The washed CER was then added to the suspended harvested biofilm at a dose of 75 ± 15 g CER/g VS, depending on the quantity of harvested biofilm. The suspension was stirred with a magnet bar for 1 h at a stirring intensity of 600 rpm and a temperature of 4°C. After ten minutes of settling, with the CER particles fully settled at the bottom of the beaker, the supernatant was pipetted into eighteen 1.50 ml centrifuge tubes. The supernatant was centrifuged for 0.5 h at 12,000 g at a temperature of 4°C, with a floc component of the supernatant being removed from solution. The supernatant of this final centrifuge step constitutes the extracted EPS dissolved in solution, termed the EPS solution.

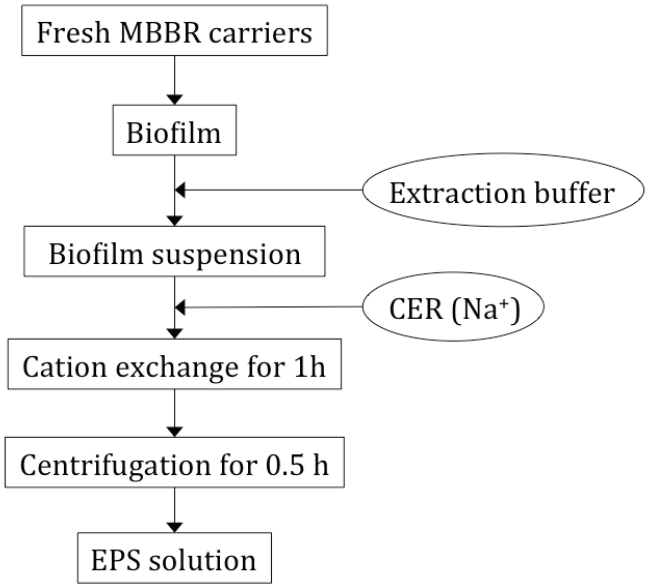

Figure 3.3 Schemetic of CER exchange method procedure used for EPS extraction

3.3.4 Protein Determination

3.3.4.1 Review of Conventional Protein Measurement Methods

Proteins are considered a main component of exopolymers and can be measured by several methods. The Kjeldahl-N method involves measuring the released ammonia from acid digestion of organic solids, with the protein content being calculated based on the assumption that proteins contain 16.5% (w/w) of nitrogen. However, organic solids include components other than proteins that contain nitrogen (such as humic substances and urea) and the nitrogen quantity can vary in different proteins (Raunkjear et al., 1994). Thus, the Kjeldahl-N method was deemed not appropriate for this research. Other methods to analyze proteins mainly rely on colorimetric techniques, such as the biuret method, the Lowry method (Lowry et al., 1951), the Bradford method (Bradford, 1976) and the bicinchoninic acid (BCA) method (Smith et al., 1985). For the biuret method, the peptide bonds in proteins give off a purple color after treatment with copper sulfate in an alkaline

solution. However, this method is only precise at high protein quantities (1-20 mg) (Ramanathan et al., 1968) rather than low quantities that are measured in extracted EPS solution. The Lowry method is more sensitive and is based on the reaction of peptides with copper sulfate and a Folin Ciocalteau phenol (FCP) reagent. A disadvantage of this method is the interference caused by lipids, fatty acids and humic substances (Lowry et al, 1951; Peterson, 1979; Box, 1983; Davis, 1988). The BCA method is similar to the Lowry method. The BCA reagent used in this method has been shown to be more stable for specific proteins and more tolerant to interfering substances as compared to the FCP reagent used in the Lowry method. However, the BCA method is sensitive to interferences from sugars (Smith et al., 1985), which eliminates its utility for biofilm EPS analysis as biofilm are inevitably composed of polysaccharides. The Bradford method uses Coomassie Brilliant Blue G-250 (CBBG) as a dye to analyze protein spectrophotometrically. However, only the macromolecular protein structures of biofilms bind to the CBBG, which makes this method impropriate for biofilm analysis (Raunkjear et al., 1994). Amongst all these methods, the Lowry method is the most commonly applied to wastewater samples due to the requirement of sensitivity and specificity for protein, and it is stable to measure different proteins.

In 1995, Frølund et al. developed a modified Lowry method (referred herein as the modified Lowry method) by discovering that both proteins and humic substances demonstrate absorbance through a reaction with $CuSO_4$ In the classic Lowry method, $CuSO_4$ is added as a portion of the reagent, whereas the modified Lowry method enables protein quantities to be calculated from light absorbance of samples with and without $CuSO_4$ addition. The modified Lowry method therefore effectively reduces the overestimation of protein content by the classic Lowry

method due to the effects of humic substance and it is the most appropriate for protein determination in this study.

3.3.4.2 Modified Lowry Method Procedure

As mentioned above, the method chosen for protein determination of EPS in this study is the modified Lowry method. This method requires six reagents: reagent A (2% Na_2CO_3 in 0.10 N NaOH), reagent B (1% NaK Tartrate in H_2O) and reagent C (0.5% $CuSO_4$ H_2O in H_2O), along with the other reagents are reagent D_1 (9.6 ml of reagent A mixed with 0.2 ml of reagent B and 0.2 ml of reagent C), reagent D_2 (9.6 ml of reagent A mixed with 0.2 ml of reagent B and 0.2 ml of distilled water) and reagent E (1 ml of Folin-Phenol mixed with 1 ml of distilled water). Reagents A, B and C were stored at 4°C for no longer than 3 months prior to use, while reagents D_1, D_2 and E were prepared fresh just prior to analysis. For each sample, two disposable test tubes were set up, followed by the addition of 1 ml of EPS solution into each tube. 2 ml of reagent D_1 and reagent D_2 were added into these two test tubes respectively. After incubating at room temperature for 10 minutes, 0.2 ml of reagent E was added, and the tubes were vortexed immediately. The solutions were allowed to stand for 30 minutes at room temperature and were then measured using a Hach DR5000 spectrophotometer, at a wavelength of 570 nm. The true absorbance of protein was then calculated according to the equations 3.1, 3.2 and 3.3 (Frølund et al., 1995). The protein concentrations were determined with a standard curve prepared using the BSA (Bovine Serum Albumin) (Sigma, Canada) reagent at concentrations of 10 to 100 mg/L. Distilled water was used as a blank. All the samples were tested in triplicate (samples were put in 3 tubes of and each tube was measured for 3 times) and the standard curve was made every three and half months. Figure 3.4 shows a sample of the protein standard curve.

$$A_{Total} = A_{Protein} + A_{Humic} \quad \textbf{Eq. 3.3}$$

$$A_{Blind} = 0.2A_{Protein} + A_{Humic} \quad \textbf{Eq. 3.4}$$

$$A_{Protein} = 1.25(A_{Total} - A_{Blind}) \quad \textbf{Eq. 3.5}$$

where A_{Total} = absorbance of sample at 570 μm with reagent D_1 added (a.u);

A_{Blind} = absorbance of sample at 570 μm with reagent D_2 added (a.u);

$A_{Protein}$ = calculated absorbance of protein (a.u);

A_{Humic} = calculated absorbance of humic substances (a.u).

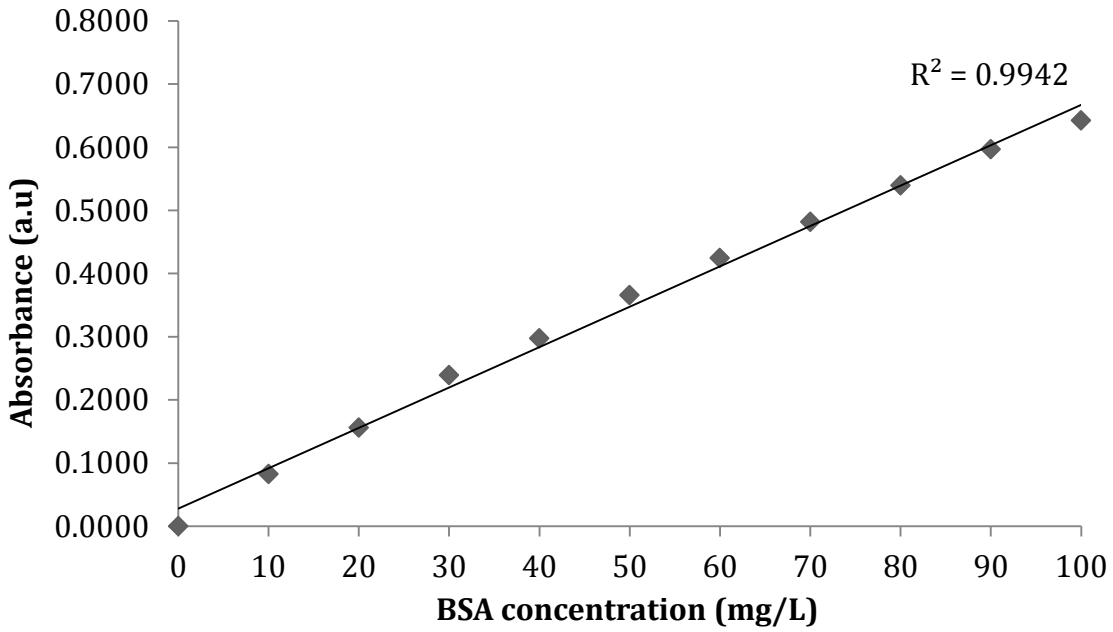

Figure 3.4 A sample of the protein standard curve

3.3.5 Polysaccharide Determination

3.3.5.1 Review of Conventional Polysaccharide Measurement Methods

Another major component of EPS are polysaccharides. Total polysaccharides are traditionally measured using either the anthrone method (Dreywood, 1946) or

the phenol-sulfuric acid method (DuBois et al., 1956). These two traditional methods are based on the same spectrophotometric principles and both methods include the step of hydrolyzing the samples polysaccharides to monosaccharides using strong acids. Both methods have demonstrated a high specificity for polysaccharides with an overall limited interference by other substances (Raunkjear et al., 1994). The anthrone method, however, has demonstrated a higher level of sensitivity towards specific carbohydrates; where the color intensity would be higher for hexoses and lower for pentoses and heptoses (Koehler, 1952). In order to avoid the use of the corrosive and poisonous phenol reagent and improve the accuracy of current methods, Albalasmeh et al. (2013) developed the UV-sulfuric acid method. The principle of this method is based on the establishment of a direct correlation between UV light (315 nm) absorbance and total polysaccharide carbon concentration as compared to as compared to visible light (490 nm) and polysaccharide concentration. The UV-sulfuric acid method is safer due to the lack of phenol usage and faster due to a shorter incubation time. However, the interferences on the absorbance of polysaccharides from other UV absorbable substances (such as proteins) have not been established (Albalasmeh et al. 2013).

Overall, both the phenol-sulfuric acid method and the UV-sulfuric acid method were used in this research to measure polysaccharides in EPS. The linear regression coefficient (R-squared) of the UV-sulfuric acid standard curve (0.99937) is similar to the linear regression coefficient (R-squared) for the phenol-sulfuric acid standard curve (0.99457) (Figure 3.5), with glucose used as a standard. Also both curves demonstrated similar ranges of linearity. However, the results of EPS samples analyzed using both methods (Figure 3.6) demonstrate that the value of polysaccharide concentrations measured using the UV-sulfuric acid method was consistently higher than the phenol-sulfuric acid method. This increase in

polysaccharide quantity measured using the UV-sulfuric acid is likely caused by absorbance interferences (Albalasmeh et al. 2013). Hence for this research, the phenol-sulfuric acid method was chosen as the most appropriate polysaccharide determination method.

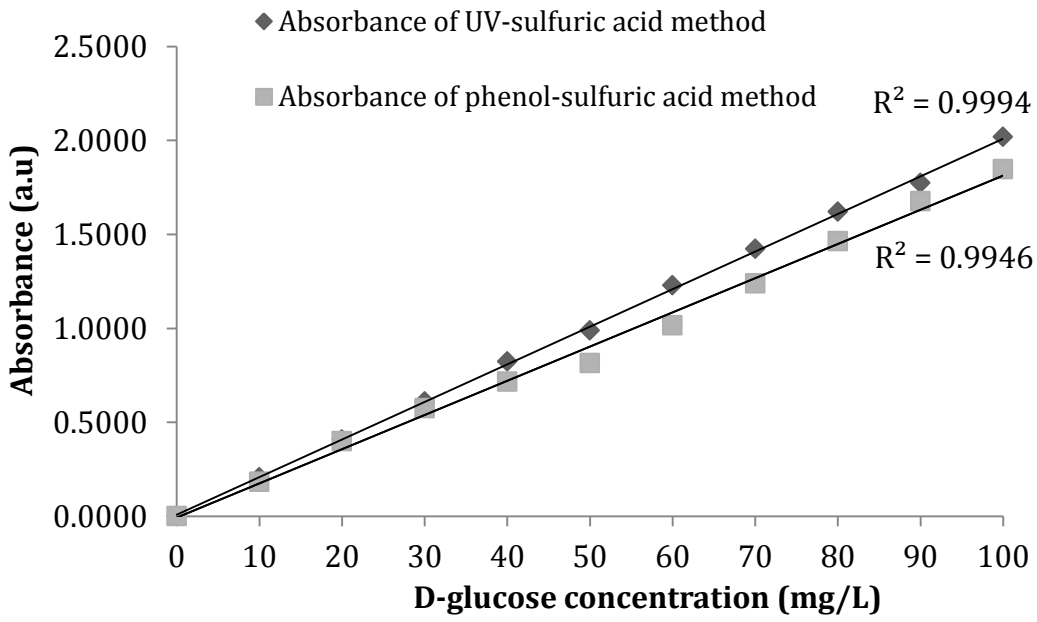

Figure 3.5 Standard curves of UV-sulfuric acid method and phenol-sulfuric acid method

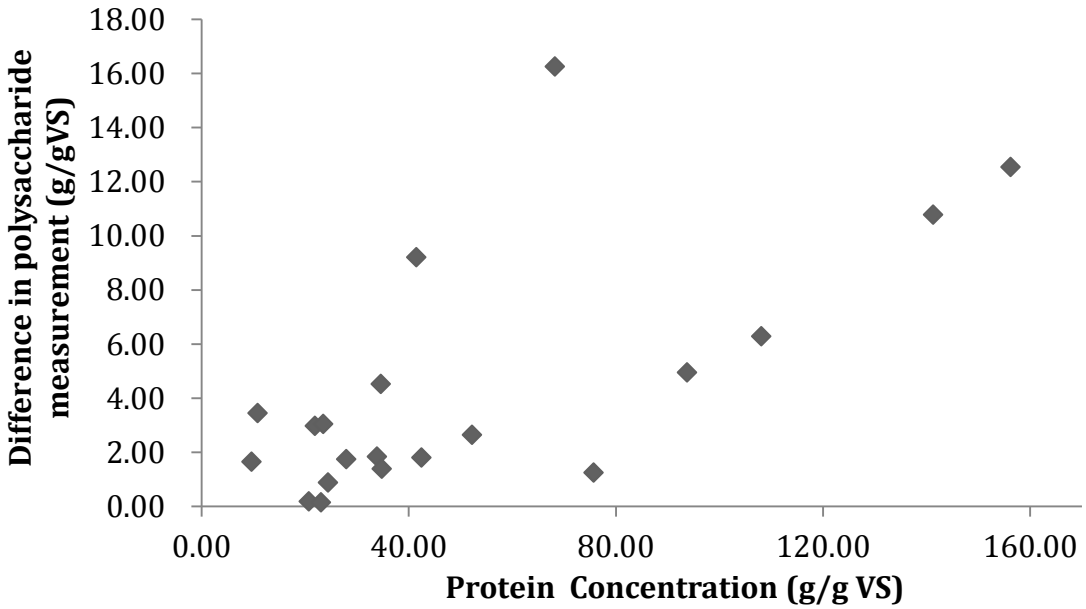

Figure 3.6 The difference in polysaccharide concentrations measured using UV-sulfuric acid method and phenol-sulfuric acid method (g/g VS) at various protein concentrations

3.3.5.2 Phenol-Sulfuric Acid Method Procedure

The phenol-sulfuric acid method was performed as follows. First, 2 ml of EPS supernatant was pipetted to a disposable test tube. 0.05 ml of 80% phenol solution was added to the tube followed by the rapid addition of 5 ml of 95.5% concentrated sulfuric acid. The tube was allowed to rest for 10 minutes and was then transferred to a 30°C water bath for 15 minute. The absorbance of the solution was then read using a Hach DR5000 spectrophotometer, at a wavelength of 490 nm. The polysaccharide concentrations were determined by making reference to a standard curve prepared by dissolving a D-glucose stock solution (10 g/L) to the concentrations of 10, 20, etc. to 100 mg/L; distilled water was used as the blank and in dilutions. All the samples were prepared and tested in triplicate (samples were

put in 3 tubes of and each tube was measured for 3 times) and the standard curve was made each three and half months.

3.3.6 eDNA Determination

The diphenylamine colorimetric method is the most popular method for determining eDNA concentration in solution (Liu and Fang, 2002; Adav and Lee, 2008; Wang et al., 2014). This method was modified by capitalizing on the Burton procedure established in 1956. The main difference between the conventional and the modified Burton methods is that the modified Burton method has a shorter incubating time for the samples (Burton, 1956; Gendimenico et al., 1988). In this study the modified diphenylamine colorimetric method was used to be efficient to reduce the time intensity of the EPS measurements.

The procedure of the diphenylamine colorimetric test was performed according to the following steps as discussed by Sun et al. (1999). First, the diphenylamine reagent is prepared using 1.5 g of diphenylamine (Sigma-Aldrich, Canada), 100 ml of glacial acetic acid, 1.5 ml of concentrated sulfuric acid and 0.5 ml of 1.6% acetaldehyde (Fluka, Canada) (the 1.6% acetaldehyde solution was prepared weekly and stored in an amber glass bottle, at 4°C). 1 ml of EPS solution being tested was then pipetted into a test tube, followed by the addition of 2 ml of the diphenylamine reagent. The tube was then incubated in a 50°C water bath for 3.0 h. The absorbance was measured using a Hach DR5000 spectrophotometer at a wavelength of 570 nm. The DNA concentration was calculated using a previously constructed standard curve. All the samples were prepared and tested in triplicate (samples were put in 3 tubes of and each tube was measured for 3 times). Calf thymus DNA (Sigma, Canada) was used as the standard DNA solution. Standards were prepared at concentrations of 10 to 100 mg/L, in 10 mg/L increments; distilled

water was used as a blank. The standard curve was prepared each three and half months. A sample of the standard curve is shown in Figure 3.7.

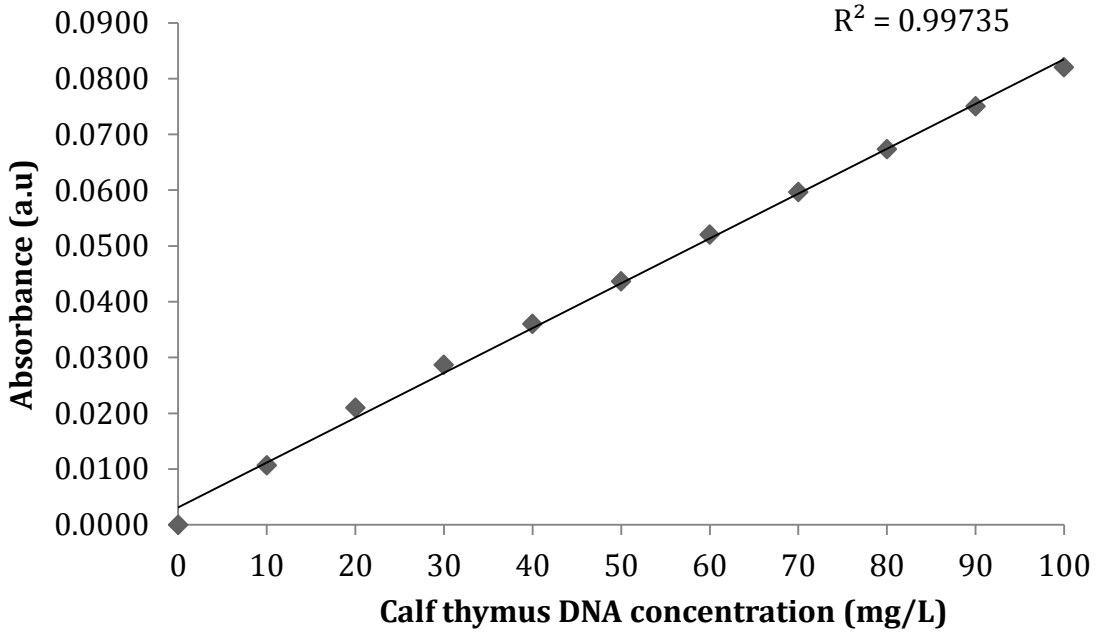

Figure 3.7 A sample of the DNA standard curve

3.4 EPS *In-situ* Analyses

CRM was used for *in-situ* EPS characterization of all the samples in this study. The other microscopic methods of CLSM and VPSEM were only applied for the HRT experiments as these methods were added to the experimental protocol during the later stages of the research.

3.4.1 EPS Raman Spectra Acquisition

Confocal Raman microscopy (CRM) is a non-destructive analytical technique, which provides high-resolution imaging of the chemical composition of the analyzed sample. It is widely used for the chemical characterization of materials and specific chemical species, without labeling or other sample preparations. In this technique, the laser emitted by the CRM is scattered un-elastically by the molecules of the

sample resulting in a Raman spectrum, which provides information regarding the chemical composition of the sample, chemical bonds existing within the sample and the relative distribution of the bonds. The emitted Raman spectra can be used to approximate relative concentrations of the chemical compounds of the sample. A CRM analysis was used to investigate all MBBR biofilm samples in this study as a complementary method to the chemical analyses of the EPS and for *in-situ* chemical characterization of EPS components, in an attempt to gather detailed information about the EPS of nitrifying MBBR biofilms.

MBBR carriers with attached biofilm were carefully sliced and fixed on a petri dish, and then submerged in distilled water. Raman spectra were acquired using a CRM (CRM200, WITec, GmbH, Ulm, Germany) equipped with a 63× water immersion objective (Carl Zeiss Canada). A He-Ne green laser (532 nm) was used for excitation. Raman signals were detected using a P-500 piezo-scanner (Physik Instrumente, Karlsruhe, Germany) and a high sensitive CCD camera (Princeton Instruments Inc., Trenton, NJ). Three Raman spectra were acquired for each sample at random locations on the sample surface with an integration time of 10 s and 10 accumulations. This operation was repeated three times, totaling 9 spectra for each sample set acquired at specific MBBR operational conditions investigated in this study. Raman spectra of new plastic carriers (without attached biofilm) were collected to characterize the background signal of the plastic carriers; with this background signal being normalized prior to EPS spectra acquisition.

The Raman spectra were analyzed using the WITec Project (v.2.10, WITec GmbH, Ulm, Germany) and OriginPro software (OriginLab, US). Raman peaks were fitted with Gaussian curves after linear baseline subtraction. Spectra were normalized in regard to the intensity of the polysaccharide peak at 362 cm^{-1} in order to allow peak intensity ratios of specific bands to be compared throughout the study.

3.4.2 Biomass Viability

Five random MBBR carriers were harvested from reactor 2 at the various HRT operational conditions of this study. The carriers were cut along their diameter, and then fixed on a petri dish to expose the interior section of the carrier for staining and subsequent microscopy. A Zeiss LSM 510 (Carl Zeiss Canada) confocal microscope was used with the film Tracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies, Ontario, Canada) to analyze cell viability in biofilms on the MBBR carriers. SYTO 9 and propidium iodide are the fluorescent stains used to fluoresce live cells green and dead cells red upon microscopy excitation.

The stained samples were protected from light exposure for 40 minutes prior to being rinsed with distilled water; to remove the excess stain before microscopy. CLSM uses pinpoint illumination to eliminate unfocused background details and obtain the spatial distribution of the sample by capturing stacked images at various depths in the biofilm without compromising the physical integrity of the biofilm. A 63× water immersion lens was used for image viewing with the samples immersed in distilled water. The equipped Argon laser (488 mm; 514 mm) and He-Ne laser (543 mm) were used for florescence excitation. Five stacked images with an area of 214 × 143 μm and a depth interval of approximately 5 to 6 μm were captured at four depths per sample slice; thus 20 images were taken in total for each set of carriers. NI Vision Assistant 7.1 (LabView 8.0 – National Instruments Canada) was used to identify and count the live and dead cells per acquired CLSM image. The biofilm area (cell coverage) on images was measured with the pore space and unfocused areas excluded. Based on verifying the range of a real cell size, the live and dead cell were identified and their numbers per biofilm area were obtained by measuring the green and red fluorescence, respectively.

3.4.3 Biofilm Thickness and Morphology

VPSEM is a modification to traditional scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM) used to visualize biofilm EPS morphology and thickness. The VPSEM uses a variable pressure chamber instead of the high-pressure chamber used for traditional SEM and hence eliminates the need for destructive preparation associated with the SEM (Flemming et al., 2000).

Four fresh carriers were harvested from reactor 2 during each HRT phase. Approximately five images with three biofilm thickness measurements per image were taken at random points using a Tescan Vega II-XMU VPSEM (Tescan USA Inc., USA, Pennsylvania) to quantify the biofilm thickness and observe the biofilm morphology. Little preparation work is required for VPSEM imaging; samples were analyzed at a pressure of 40 Pa with a magnification of 60×.

3.5 Statistical Analysis

The chemical and microscopic data measured from the same reactor at various temperatures and HRTs were tested for statistical differences using the student t-test with a *p*-value of 0.05 signifying statistical significance. Combined error calculations were used for errors that include more than one variable (**Table 3.2**). Correlations between various data sets were tested for statistical significance using Pearson's correlation with *p*-value of 0.1 indicating a significant correlated relationship.

Table 3.2 Rules for combined errors calculation

Relation between Z, A and B	Combined error calculation for Z
$Z = A + B;\ Z = A - B$	$(\Delta Z)^2 = (\Delta A)^2 + (\Delta B)^2$
$Z = A \times B;\ Z = A/B$	$(\frac{\Delta Z}{Z})^2 = (\frac{\Delta A}{A})^2 + (\frac{\Delta B}{B})^2$
$Z = A^n$	$\frac{\Delta Z}{Z} = n(\frac{\Delta A}{A})$

(Z = the variable depends on one or more variables A or/and B; ΔA and ΔB = the errors of A and B; ΔZ = the combined error.)

3.6 References

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4 Temperature and Hydraulic Retention Time Effects on Nitrifying MBBR Biofilm

4.1 Setting the Context

Chapter 4 of this thesis is presented in the format of a manuscript that has been prepared for submission to the journal of *Chemosphere*. The article presented in Chapter 4 is entitled *Temperature and HRT Effects on Nitrifying MBBR Extracellular Polymeric Substances*; and is authored by Baisha Ren, Bradley Young, Fabio Variola and Robert Delatolla. This article focuses on investigating the changes in EPS composition and PN/PS ratio of EPS in tertiary nitrifying MBBR biofilms affected by temperature and HRT. The manuscript also relates cell viability and biofilm thickness to biofilm changes at various HRTs.

The roles of authors are herein defined for the research performed in this chapter. Baisha Ren performed all EPS chemical analyses along with the CRM and VPSEM investigation of the biofilm samples. She also optimized the chemical and microscopic protocols herein used for EPS characterization. Further, Ms. Ren performed the analyses of all the data presented in this chapter and wrote the chapter. Bradley Young is a PhD graduate student under the supervision of Dr. Robert Delatolla. Mr. Young was partially responsible for maintaining the operation of the MBBR pilot plant, he sampled the carriers from the pilot and he performed the ammonia and nitrate analyses of the reactors along with the CLSM investigation of the carriers as well as processing the CLSM images for analyses. Drs. Fabio Variola and Robert Delatolla co-supervised the research of Ms. Ren and the writing of this manuscript.

4.2 Introduction

Wastewater treatment solutions incorporating biofilm systems are becoming increasingly popular due to more stringent regulations pertaining to wastewater

effluent discharge (Ward et al., 2011; Gardner et al., 2012). Biofilm treatment systems are often relatively efficient and economical, with lower sludge yield production compared to suspended growth systems; with biofilm treatment systems being the most economical means of performing wastewater nitrification (Chen et al., 2012; Hoang et al., 2014). As a major component of biofilms, extracellular polymeric substances (EPS) are considered an important factor affecting the physical and chemical properties of biofilm and the performance of the treatment technology. In particular, EPS has been shown to kinetically control substrate mass transfer dynamics, biofilm surface characteristics and the adsorption ability of biofilms (Sheng et al., 2010; Flemming and Wingender, 2010). Therefore, enhancing the research of EPS and developing a deeper understanding of EPS constituents, their functional performance and the relation between the composition of EPS and the performance of biofilm technologies is critical for the design of the next generation of biofilm technologies.

The moving bed biofilm reactor (MBBR) system is a robust biofilm wastewater treatment technology, with a strong adaptability to shock and influent loading, along with a capability to achieve high removal efficiencies of nitrogenous and carbonaceous pollutants (Ødegaard, 2006). This technology is proven for secondary treatment of municipal wastewaters (Rusten et al., 1998), and it requires lower maintenance, operational intensity and operating cost as compare to other biofilm systems (WEF, 2011). The MBBRs operate with the help of mechanical mixer or aerator to keep biocarriers in motion as a moving bed in basin. The biocarriers employed housed by MBBR systems provide a higher working surface attachment area as well as certain preservation capabilities for the attached bacterial growth (WEF, 2011).

The overall purpose of this work is to investigate the effects of temperature and HRT on the EPS composition and kinetics of nitrifying MBBR biofilms. Also the work investigates the effects of HRT on biofilm morphology and thickness along with embedded cells viability. In particular, the study uses both chemical analyses and confocal Raman microscope (CRM) as an *in-situ* method to characterize the EPS of the nitrifying biofilm.

4.3 Methods

4.3.1 Nitrifying MBBR Pilot Plant Configuration and Operation

Wastewater effluent of the Masson-Angers aerated lagoon treatment plant was diverted into a tertiary nitrifying MBBR pilot plant that was installed as an upgrade nitrifying treatment unit. The pilot plant is comprised of two 223 L cylindrical aerated reactors, which were filled with the AnoxKaldnes™ K5 carriers (Figure 3.1). The carriers have a bulk specific surface area of 800 m^2/m^3, a diameter of 10 mm and a depth of 7 mm. The pilot reactors were in operation for approximately a year prior to the experimental work of this research; hence the harvested carriers are assumed to contain mature biofilms. The two pilot reactors, which were harvested for the biofilm samples investigated in this study, were operated at the conditions outlined in Table 4.1. In particular, reactor 1 was operated at a constant HRT of 2.2 h, a constant ammonia loading rate of 442 ± 24 gN/m^3d and at four different temperatures, namely 22°C, 15°C, 10°C and 5°C (between the months of August and December). Conversely, reactor 2 was operated at a constant temperature of 20°C, a constant ammonia loading rate of 864 ± 14 gN/m^3d and at various HRTs of 0.5 h, 0.75 h, 1.0 h, and 3.0 h (between the months of June and July). The dissolved oxygen (DO) concentration in the MBBR reactors were adjusted to maintain a stable DO concentration between 4 to 6 mg/L at all operational conditions of the study. In order to allow the biofilm to acclimatize to all operational conditions, the two

reactors were operated for at least two weeks at each set of conditions before kinetics, biofilm EPS, biofilm thickness and embedded cell viability were tested.

Table 4.1 The operational conditions of MBBR pilot reactors 1 and 2

Conditions	Reactor 1	Reactor 2
COD loading rate (g/m^3d)	494 ± 45	965 ± 25
Ammonia loading rate ($g\ N/m^3d$)	442 ± 24	864 ± 14
Influent COD concentration (mg/L)	22 ± 2.0	22 ± 2.0
Influent ammonia concentration (mg N/L)	19.7 ± 0.2	19.7 ± 0.2
DO (mg/L)	4.5 ± 0.3	5.5 ± 0.3
pH	7 ± 0.1	7 ± 0.1
Temperature (°C)	22, 15, 10, 5	20 ± 2
HRT (h)	2.2	0.5, 0.75, 1.0, 3.0

4.3.2 Constituent Measurements

The influent and effluent ammonia (NH_4^+/NH_3-N) and nitrate (NO_3^-) concentrations within the reactors were measured using standard methods 4500C-NH_3 and 4500B-NO_3^- (APHA, 1995). DO concentration and temperature within the reactors were measured by using a HACH LDO® probe (HACH, ON, Canada), and the pH was measured using a CORNING® Pinnacle 530 pH meter (Corning, MA, US).

4.3.3 EPS Extraction and Chemical Analysis

Biofilms were abraded from harvested MBBR carriers and weighed, and the volatile solids (VS) concentration (g VS/g biofilm) was quantified by standard method 2540E (APHA, 1995).

The EPS extraction procedure was carried out according to the CER exchange method (Frølund et al., 1996). Biofilms were first suspended in 40 ml of extraction buffer and then CER (Dowex Marathon C, 20-50 nm mesh, sodium form, Fluka 91973), which was previously prewashed for 1.0 h, was added at a dose of 75 ± 15 g g CER/g VS. Cation exchange was performed at 4°C with the magnetic stirrer speed of 600 rmp and resin contact time of 1.0 h. The extracted EPS were harvested by centrifugation with a rotational speed of 12000 g for 0.5 h at 4°C.

The EPS components were quantified in terms of protein, polysaccharide and eDNA. All the tests were performed in triplicate to quantify statistical significance. The modified Lowry method (Frølund et al., 1995) was applied for protein determination and BSA (Bovine Serum Albumin) (Sigma, Canada) was used as the standard. The polysaccharide content was measured by the phenol-sulfuric acid method (DuBois et al., 1956) using D-glucose as the standard. The eDNA content was measured using the diphenylamine colorimetric method (Sun et al., 1999) and used Calf thymus DNA (Sigma, Canada) as the standard.

4.3.4 Raman Spectra and Analysis

Raman spectra were acquired on samples from both pilot reactors during the aforementioned operating conditions. All the measurements were performed at room temperature, using a WITec CRM (WITec, GmbH, Ulm, Germany) equipped with a 63× water immersion objective (Carl Zeiss Canada) and a He-Ne laser (532 nm) was used for excitation. MBBR carriers were harvested from the reactors, then

sliced longitudinally and fixed on a petri dish (47 mm, Fisher, Canada). Three locations along the interior surface of the carriers were randomly selected on each sample for Raman spectra; with three Raman spectra being collected at random points at each field of view using an integration time of 10 s and 10 accumulations (total of 9 spectra per sample). Spectral analysis was carried out using OriginPro software (OriginLab, US).

4.3.5 CLSM Image Acquisition and Analysis

Samples were taken from reactor 2 when it was operated at different HRTs: 0.5 h, 0.75 h, 1.0 h, and 3.0 h. A Zeiss LSM 510/Axio imager M.1 confocal microscope (Carl Zeiss Canada) was used for CLSM imaging at various wavelengths (488, 514 and 543 nm). For each measurement, 5 sample slices were fixed on a petri dish and stained using live and dead cells stains (SYTO 9 and propidium iodide). A 63× water immersion objective was used for image viewing. Five stacked images with an area of 214×143 μm and a depth interval of around 5 to 6 μm were captured, totaling 25 images per sample. Live and dead cells numbers on the images were counted by using NI Vision Assistant 7.1 (LabView 8.0-National Instruments Canada). The biofilm area (cell coverage) on images was measured with the pore space and unfocused areas excluded. Based on verifying the range of a real cell size, the live and dead cell were identified and their numbers per biofilm area were obtained by measuring the green and red fluorescence respectively (Figure 4.7). Thus the percentage of live cells and the live cell numbers per carrier were calculated using Equation 4.1 and 4.2.

$$Percent\ live\ cells = \frac{live\ cell\ count}{total\ cell\ count} \qquad \textbf{Eq. 4.1}$$

$$Live\ cells\ per\ carrier = Live\ cells\ per\ biofilm\ area \times SA_{bs} \times V_m \times Biofilm\ thickness \qquad \textbf{Eq. 4.2}$$

where SA_{bs} = bulk specific surface area of carrier (m^2 of biofilm/m^3 of carrier).

V_m = carrier volume (m^3).

4.3.6 VPSEM Image Acquisition and Analysis

Samples were taken from reactor 2 when it was operated at different HRTs: 0.5, 0.75, 1.0 and 3.0 h. A Tescan Vega II-XMU variable pressure scanning electron microscopy (VPSEM) (Tescan USA Inc., USA, Pennsylvania) was used to obtain the biofilm morphology and thickness. Four sample carriers were analyzed per time, at a pressure of 40 Pa with magnifications of 60×. Images were taken at five randomly selected points on each sample carrier. The biofilm thickness was measured on the acquired images by using the software Axio Vision LE (Carl Zeiss Canada).

4.3.7 Statistical Analysis

The statistical significance of changes in constituent concentrations, content of EPS, protein/polysaccharide (PN/PS) ratio, biofilm thickness and cell viability percent were tested using the t-test with a *p*-value lower than 0.05 indicating statistical relevance. Correlation was tested using Pearson's correlation with *p*-value of 0.1 indicating a significant correlated relationship.

4.4 Results and Discussion

4.4.1 Effect of Temperature on Nitrifying MBBR Biofilm

4.4.1.1 Raman spectra of EPS and EPS percent composition

Figure 4.1 illustrates the Raman spectra of nitrifying MBBR biofilm EPS obtained at varying temperatures. All spectra were normalized based on the 362 cm^{-1} carbohydrate peak, which was present in all samples and hence used for normalization. Table 4.2 shows the Raman band assignment summarized from previous research (Maquelin et al., 2002; Ivleva et al., 2009; Wagner et al., 2009).

Raman bands in the 350 - 500 cm^{-1} region were attributed to carbohydrates. The peak at 862 cm^{-1} was assigned to C-C stretching and C-O-C glycosidic link of carbohydrates. The S-S bond of proteins was detected at 505 cm^{-1}. The bands at 1635 and 1278 cm^{-1} were assigned to Amide I and Amide III, and used to identify the presence of proteins. These peaks could also be indicative of the asymmetric stretching of the COO^- group of carbohydrates and/or the CH_2 twist and =C-H bonds of lipids. The prominent C-H deformation band located at approximately 1455 cm^{-1} mainly originates from the amino acid side chains of proteins and carbohydrates as well as the $-CH_3$, $-CH_2$ and C-H functional groups in lipids. In the 1000-1200 cm^{-1} region, the band at 1030 cm^{-1} was assigned to the C-C and C-O vibrations of lipids or the C-H bond of proteins; the band at approximately 1116 cm^{-1} was assigned to the C-C and C-N stretching vibration of proteins or lipids or the C-C and C-O-C glycosidic link of carbohydrates; the band at approximately 1161 cm^{-1} originated from C-C and C-N stretching vibration of proteins or C-C and C-O ring of carbohydrates. The band at 1752 cm^{-1} may originate from the C=O stretching of carbohydrates or the C=O ester of lipids. In addition, several bands were assigned to the amino acid bands of phenylalanine (Phe) and tyrosine (Tyr) and the RNA/DNA nucleotide base-ring bands of guanine (G) and thymine (T) were also observed in the Raman spectra. No qualitative differences (the differences of Raman band positions) were found among the Raman bands identified in the Raman spectra of the nitrifying MBBR biofilm EPS at different operating temperatures (Figure 4.1).

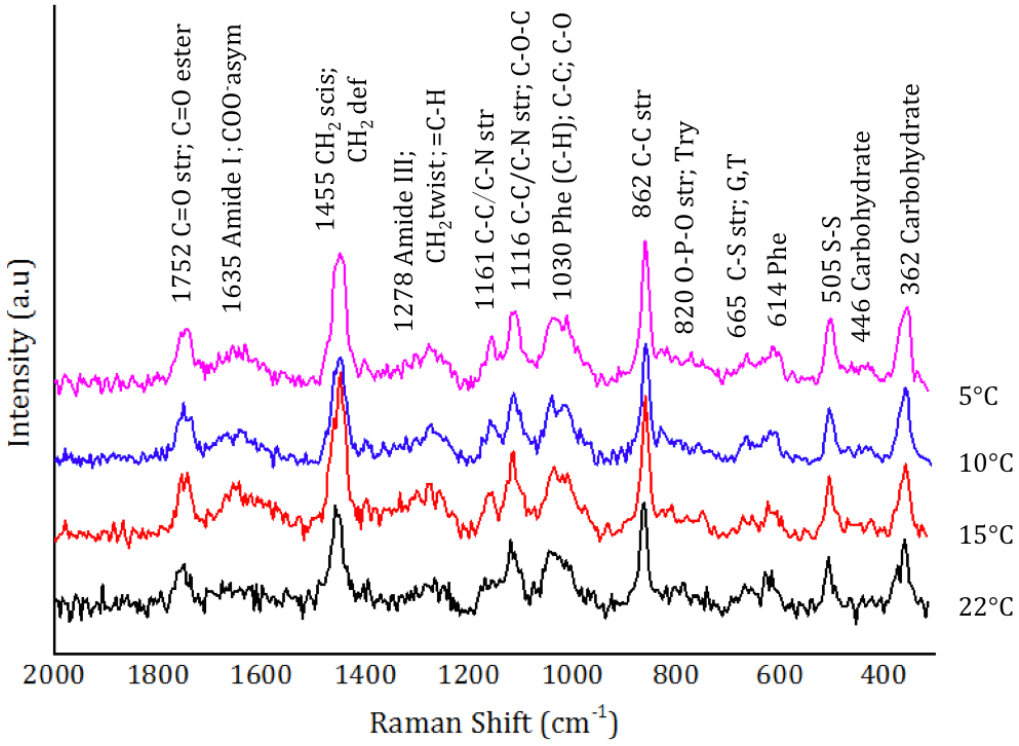

Figure 4.1 Stacked Raman spectra of EPS at various temperatures

Table 4.2 Assignment of Raman bands for EPS of nitrifying MBBR biofilms

Peak positions	**Assignment**			
(cm^{-1})	**Carbohydrate**	**Protein**	**DNA/RNA**	**Lipids**
362	Carbohydrate			
446	Carbohydrate			
505		S-S		
614		Phe; C-C twisting		
665		C-S str	G, T	
820		Try	O-P-O str	
862	C-C str, C-O-C 1,4-glycosidic link			
1030	Carbohydrates C-C; C-O	C-H in plane phe		C-C; C-O
1116	C-C str, C-O-C 1,4-glycosidic link; ring breath, sym	C-N, C-C str		C-C str
1161	C-C, C-O ring breath, asym	C-C/C-N str		
1278		Amide III		CH_2 twist; =C-H
1455	$\delta(CH_2)$ scis; CH_2 def	$\delta(CH_2)$ scis; CH_2 def		$\delta(CH_2)$ scis; CH_2 def
1635	COO^- asym	Amind I		
1752	C=O str			C=O ester

The protein, polysaccharide and eDNA content of the nitrifying MBBR biofilm EPS at varying temperatures were measured using modified chemical analytical methods (Figure 4.2). The protein percentage of the nitrifying MBBR EPS was shown to significantly increase from 39.3 ± 3.2% at a temperature of 22°C to a 58.3 ± 1.7% at a temperature of 15°C. As expected, as the protein percentage increased the polysaccharide and eDNA percentage exhibited the opposite trend. The content of polysaccharide significantly decreased from 33.63 ± 3.4% at 22°C to 19.04 ± 0.8% at 15°C and the content of eDNA significantly decreased from 27.09 ± 0.2% at 22°C to 22.67 ± 1.0% at 15°C. As the temperature of the operating nitrifying MBBR system continually decreased from 15°C to 5°C, no measured statistical change were observed in the protein and polysaccharide percentages, however, the eDNA percentage showed a significant decrease from 22.67 ± 0.2% to 17.58 ± 0.9%.

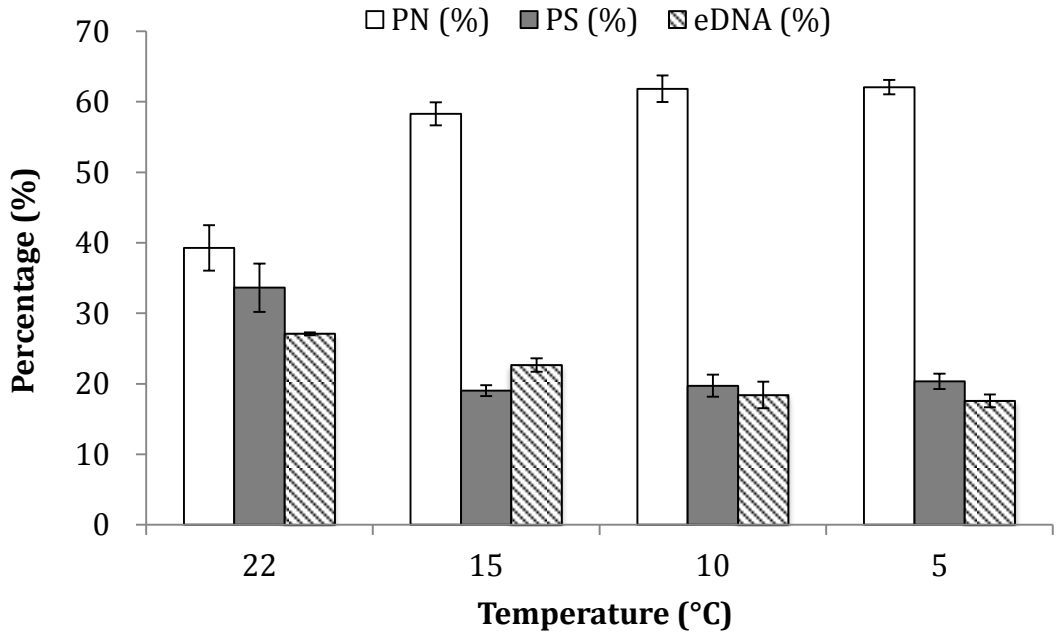

Figure 4.2 Percentage of PN, PS and eDNA in EPS of nitrifying MBBR biofilm at various temperatures

4.4.1.2 PN/PS Ratio and Ammonia Removal Efficiency

Figure 4.3 compares the PN/PS ratio of nitrifying MBBR biofilm EPS to the corresponding ammonia removal efficiency at various operational temperatures. A constant loading rate (442 ± 24 gN/m^3d) and HRT (2.2 h) were set in reactor 1 for operation at both warm and cold temperatures (with cold temperatures expectantly showing a significant decrease in ammonia removal kinetics). The loading rate at 22°C is considered low for an MBBR nitrifying system operating at very warm temperatures; which is supported by the low effluent ammonia concentration at this temperature (Table 4.3). As such, at 22°C the intrinsic ammonia removal kinetics of the cells are the highest relative to the other temperatures studied in this research, which potentially results in a strong competition among microorganisms embedded in the biofilm matrix and subsequently the cells approaching a state of famine. Hence, the underloaded conditions at 22°C results in a lower availability of substrate that may be causing a lower rate of extracellular enzymatic proteins production, an enhanced production of polysaccharides due to low substrate availability (Ahimou et al., 2007) and eDNA excretion due to cell lysis; which ultimately results in the observed low EPS PN/PS ratio of 1.21 ± 0.20. The low PN/PS ratio of 1.21 ± 0.20 appears to indicative of a nitrifying biofilm that is underloaded.

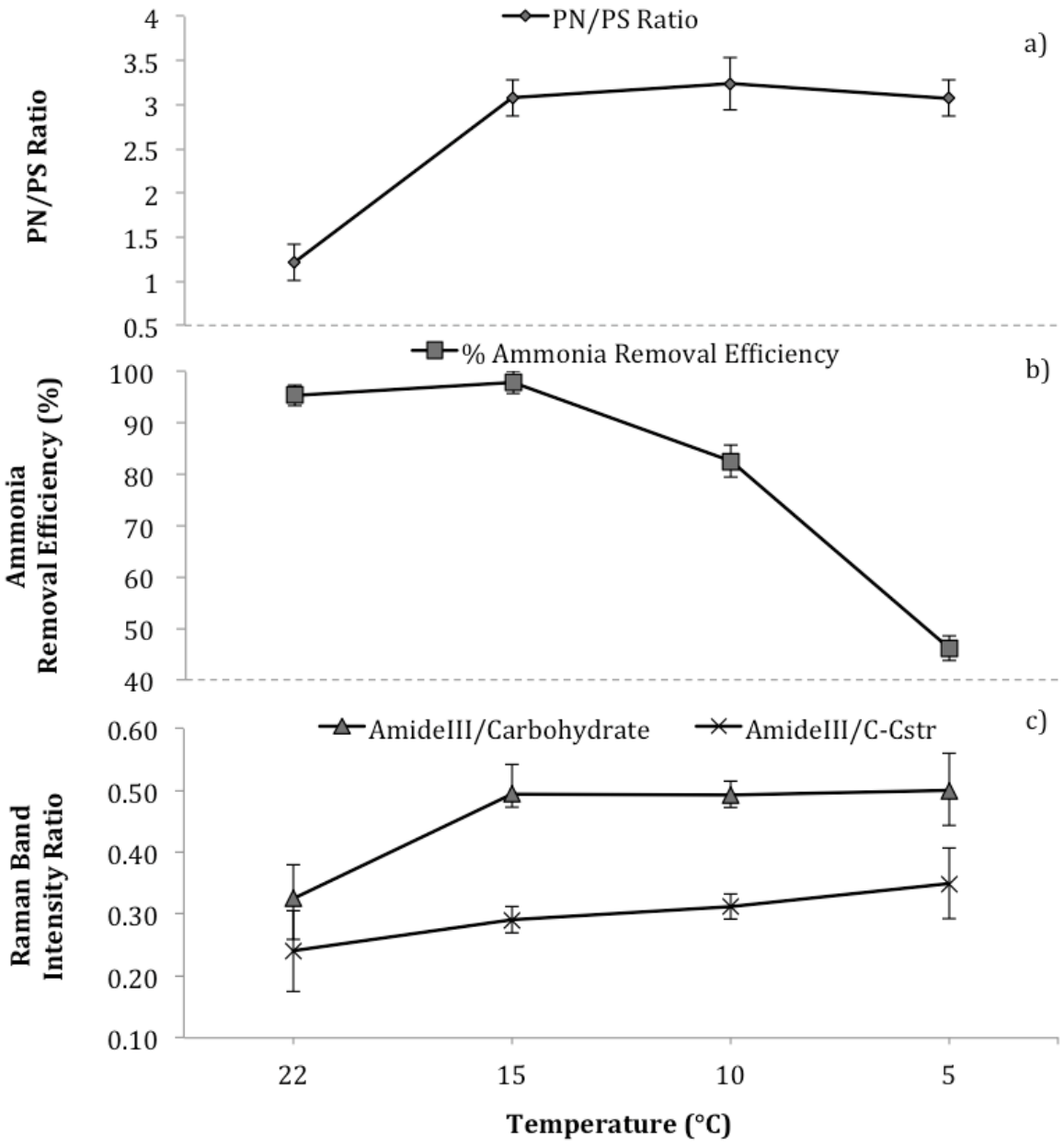

Figure 4.3 PN/PS ratios in EPS, ammonia removal efficiency and Raman band intensity ratios at various temperatures

Table 4.3 Effluent ammonia concentration at various temperatures

Temperature (°C)	Effluent ammonia concentration (mg N/L)
22	0.9 ± 0.2
15	0.5 ± 0.1
10	3.57 ± 0.0
5	10.9 ± 0.1

A decrease in temperature from 22 to 15°C does not demonstrate a significant change in the ammonia removal efficiency of the system, hence supporting the notion that the system was underloaded at 22°C. The transition from an underloaded condition at 22°C to a more optimized operation of the MBBR system at 15°C is marked by a higher EPS PN/PS ratio of 3.08 ± 0.21 (Figure 4.3 b) and a low effluent ammonia concentration at this temperature (Table 4.3). The measured PN/PS ratio of the nitrifying MBBR EPS remains stable at a value of approximately 3 at temperatures lower than 15°C (Figure 4.3 a). At lower temperatures the cell kinetics decrease, resulting in significantly higher effluent ammonia concentrations at 10°C and 5°C (3.6 ± 0.0 mg N/L and 10.9 ± 0.1 mg N/L) as compared to 15°C (0.5 ± 0.1 mg N/L) and in turn significantly lower ammonia removal efficiencies at 10°C and 5°C (82.6 ± 3.2% and 46.2 ± 2.4%) as compared to 15°C (97.9 ± 2.0%). Hence at temperatures of 15°C and lower the MBBR system appears to be adequately loaded; which corresponds to an EPS PN/PS ratio of approximately 3.

Protein-to-polysaccharide spectra band ratios were also quantified in this study from selected Raman bands and used to compare the trend of Raman intensity ratios to the chemically measured PN/PS ratios at varying temperatures and HRTs. Although quantitative analysis of the Raman spectra bands poses various challenges, the Raman band intensity can be considered proportional to the concentration of

associated assigned scattering species (Dieing et al., 2011). As such, the intensity ratio of the Amide III band (1278 cm^{-1}) to the carbohydrate bands at 362 and 862 cm^{-1} were calculated and are compared to the chemically measured PN/PS ratios (Figure 4.3 c). Although the Amide III band may be associated with protein and lipid content, it is assumed that the Amide III band (1260 cm^{-1}) is mostly attributed to EPS proteins in biofilm based on the relatively low lipid to protein content that is characteristic of biofilm EPS (Conrad et al., 2013; More et al., 2014). As the Raman spectra were normalized based on a representative band peak, the magnitudes of the intensity ratios are not similar to the magnitudes of the chemically measured PN/PS ratios. A Pearson's correlation shows that the EPS PN/PS ratio shows a significant relationship to the intensity ratio of Amide III to carbohydrate at 362 cm^{-1}, with a correlation coefficient of R = 0.98 and p = 0.02. However, the EPS PN/PS ratio to the intensity ratio of Amide III to carbohydrate at 862 cm^{-1} dose not show a significant correlation, R = 0.886 and p =0.11 (Figure 4.3 c).

4.4.2 Effect of HRT on Nitrifying MBBR Biofilm

4.4.2.1 Raman spectra of EPS and EPS percent composition

The Raman spectra of nitrifying MBBR biofilm EPS obtained at varying HRTs are shown in Figure 4.4. The Raman bands assignment of these spectra are summarized in Table 4.2. The spectra were normalized based on the 362 cm^{-1} carbohydrate peak and no qualitative differences were found. The Raman bands assignment of these spectra can be also summarized as Table 4.2.

The EPS percent composition determined by chemical analyses demonstrates that as the HRT value increases from 0.5 to 0.75 h, the protein percentage of EPS shows a significant increase from 33.1 ± 4.5% to 60.0 ± 3.4%, while the eDNA percentage decreases significantly from 35.9 ± 2.2% to 13.4 ± 1.3% (Figure 4.5). No

discernable change was observed in percent polysaccharide composition between 0.5 and 0.75 h, however a statistical change in percent polysaccharide was observed between HRTs of 0.5 and 3.0 h; 31.05 ± 2.3% and 20.69 ± 0.2% respectively. The percentages of proteins, polysaccharides and eDNA at HRTs of 1.0 and 3.0 h were shown to remain stable.

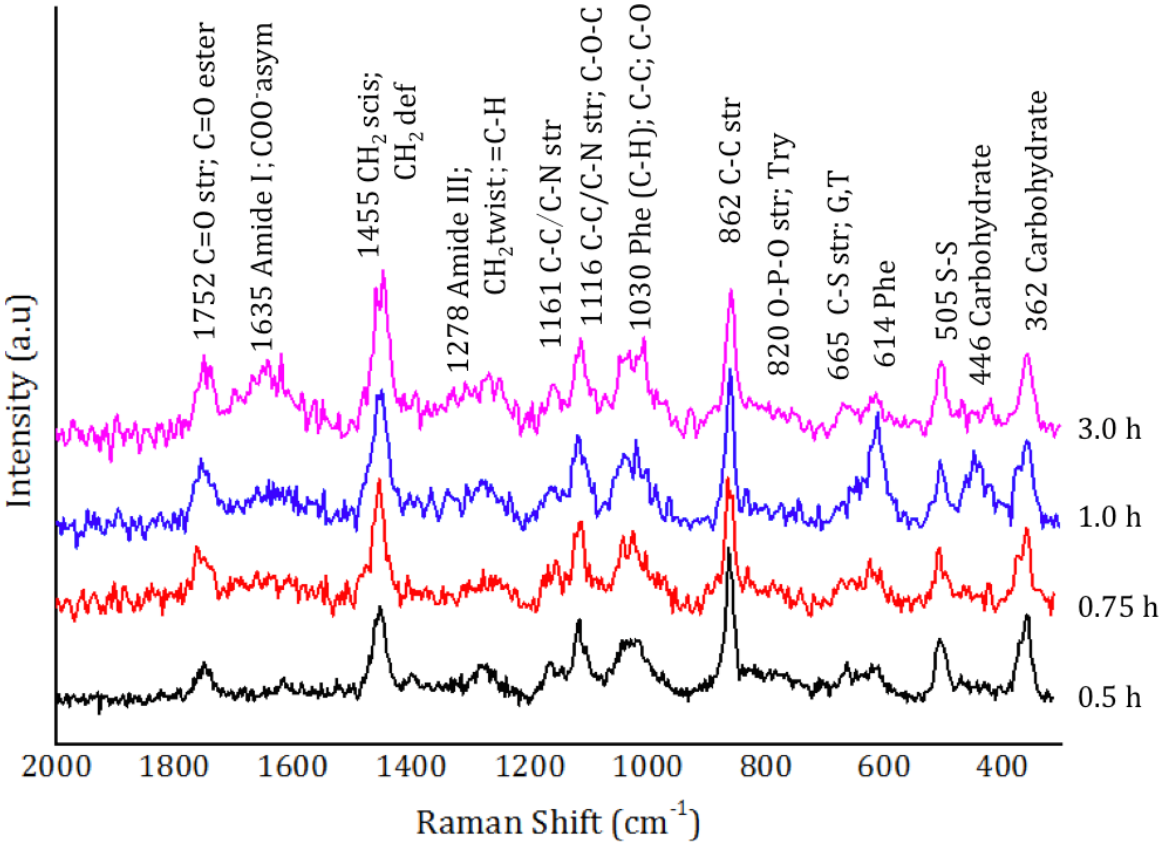

Figure 4.4 Stacked Raman spectra of EPS at various HRTs

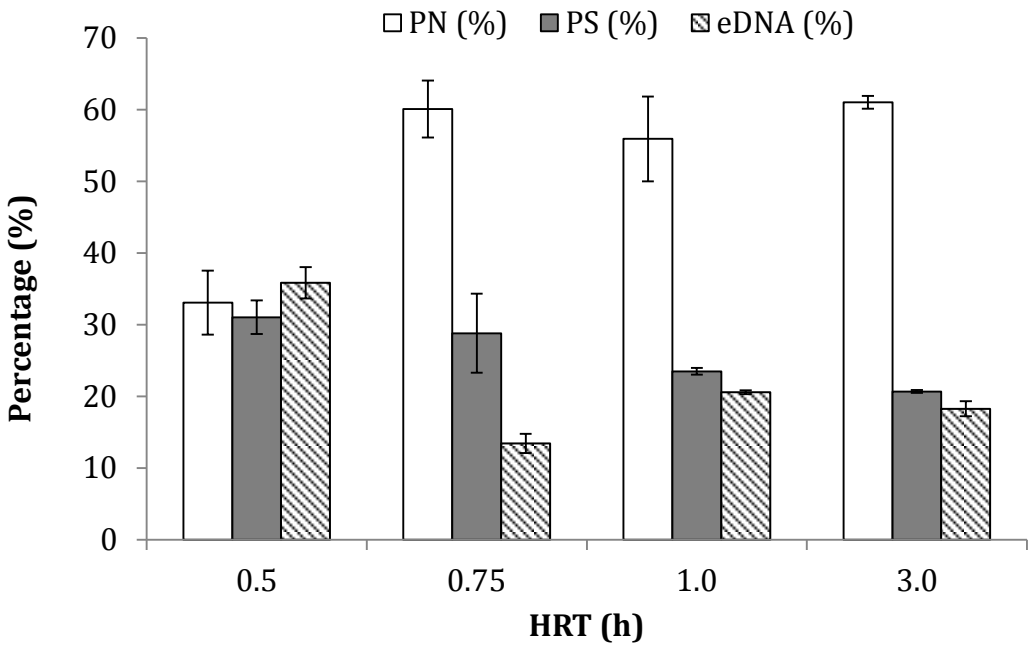

Figure 4.5 Percentage of PN, PS and eDNA in EPS of nitrifying MBBR biofilm at various HRTs

4.4.2.2 PN/PS Ratio and Ammonia Removal Efficiency

Figure 4.6 shows the effects of HRT with constant hydraulic loading rate of 864 ± 14 gN/m^3d and temperature of 20 ± 2°C. At an HRT of 0.5 h, the dissolved ammonia residence time in the system appears to be too short to allow for complete enzymatic reaction of the ammonia and metabolic uptake of this substrate by the nitrifying bacteria embedded in the biofilm. As such, operation at an HRT of 0.5 h produces an effluent ammonia concentration that is significantly larger than the effluent at HRTs of 0.75, 1.0 and 3.0 h (Table 4.4). Hence, an HRT of 0.5 h is shown to hydraulically overload the nitrifying MBBR system and restrict the availability of substrate to the nitrifying bacteria. At these conditions the response of the EPS PN/PS ratio is similar to the results observed during the 22°C temperature experiments; where the system was underloaded and the EPS composition may be

due to a lack of extracellular enzymes an enhanced production of polysaccharides due to a restricted substrate availability (Ahimou et al., 2007) and possibly eDNA excretion due to cell lysis. As conventional HRT values for ammonia removal MBBR systems are between 2 to 6 h, it is expected that as the HRT increased to 3.0 h the ammonia removal efficiency increased. The kinetic and EPS response at HRT values of 0.75 and 1.0 h hour indicate that nitrifying MBBR systems may be optimized to operate at HRTs as low as 0.75 to 1.0 h. Subsequently, these results show that once again as the nitrifying MBBR system approaches optimal operation, the EPS PN/PS ratio approaches a value of approximately 3 (Figure 4.6 a). Hence, the EPS PN/PS ratio of nitrifying MBBR systems has shown to significantly decrease below a threshold value of 3 when the systems are substrate underloaded or hydraulically overloaded. Using Pearson's correlation, the EPS PN/PS ratio to the ammonia removal efficiency shows a significant correlation (R = 0.935 and p = 0.06), while the EPS PN/PS ratio to the effluent ammonia concentration shows a significant negative correlation (R = -0.935 and p = 0.07). As was demonstrated at various temperatures, the Raman band intensity ratio of the Amide III band to the carbohydrate bands were calculated, and the values to the chemically measured EPS PN/PS ratios shows significant correlations with the coefficients values of R = 0.922 and p = 0.08 for carbohydrate at 362 cm^{-1} and R = 0.932 and p = 0.07 for carbohydrate at 862 cm^{-1} (Figure 4.6 c).

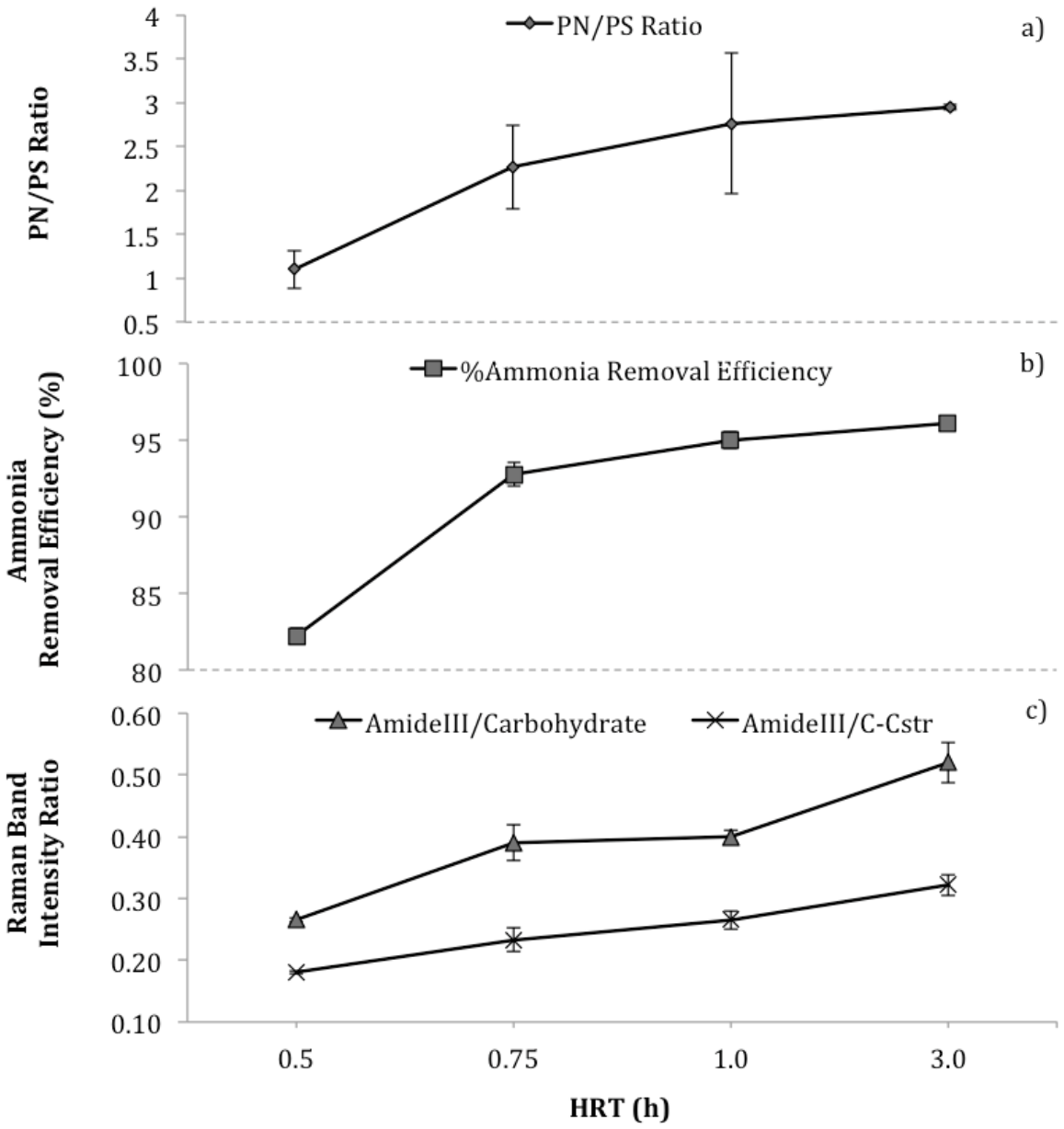

Figure 4.6 PN/PS ratios in EPS, ammonia removal efficiency and Raman band intensity ratios at various HRTs

Table 4.4 Effluent ammonia concentration at various HRTs

HRT (h)	Effluent ammonia concentration (mg N/L)
0.5	3.8 ± 0.1
0.75	1.1 ± 0.0
1.0	0.9 ± 0.1
3.0	1.0 ± 0.0

4.4.2.3 Cell Viability and Biofilm Thickness

The second phase of the study moved beyond chemical and CRM analysis of the EPS response to varying HRTs and incorporated an analysis of the embedded cell viability and biofilm morphology and thickness. In the CLSM images shown in Figure 4.7, the dead cells fluoresce red (propidium iodide stained) and the live cells fluoresce green (SYTO 9 stained). The percentages of live cells per total cells were calculated (Figure 4.8). The results demonstrate that as the system's HRT increases the percentage of bacterial community that is viable also increases; with statistically greater percentage of live cell values being measured at 1.0 and 3.0 h (76.0 ± 3.7% and 89.2 ± 2.8%) as compared to the percentage of live cells measured at an HRT of 0.5 h (54.5 ± 2.2%). Hence, the observed percentage of live cells supports the findings of statistically greater ammonia removal rates and PN/PS ratios being observed at HRTs of 1.0 and 3.0 h (2.76 ± 0.80 and 2.95 ± 0.03) as compared to 0.5 h (1.10 ± 0.22). Further, the highest live cell percentage was observed at the highest kinetics and as the PN/PS ratio approached the threshold value of 3.

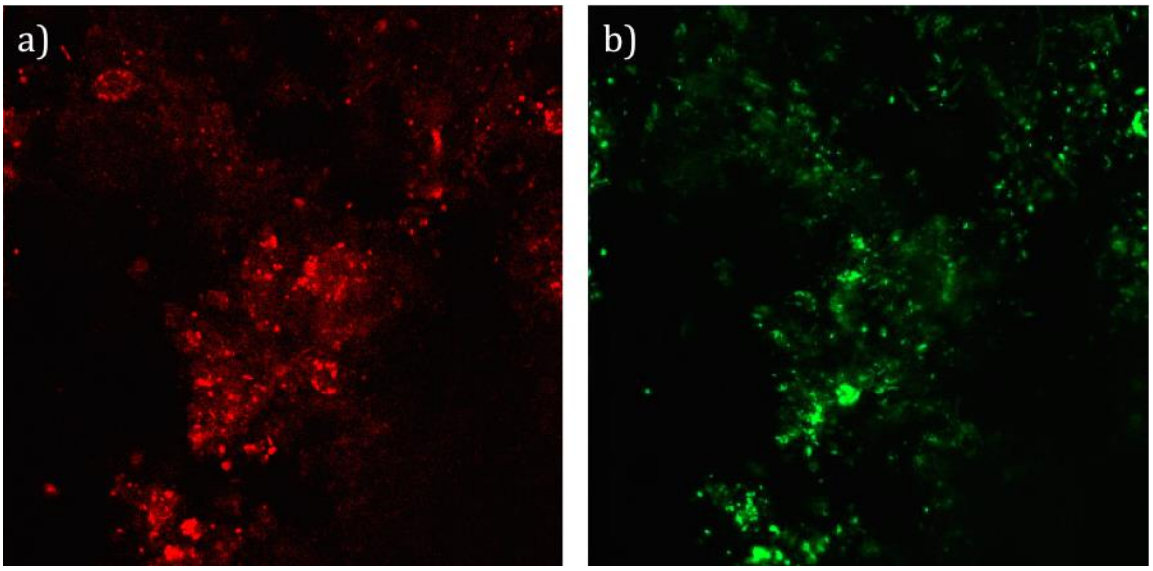

Figure 4.7 CLSM images of nitrifying MBBR biofilm, a) dead cells stained with propium iodide (red); b) live cells stained with SYTO 9 (green)

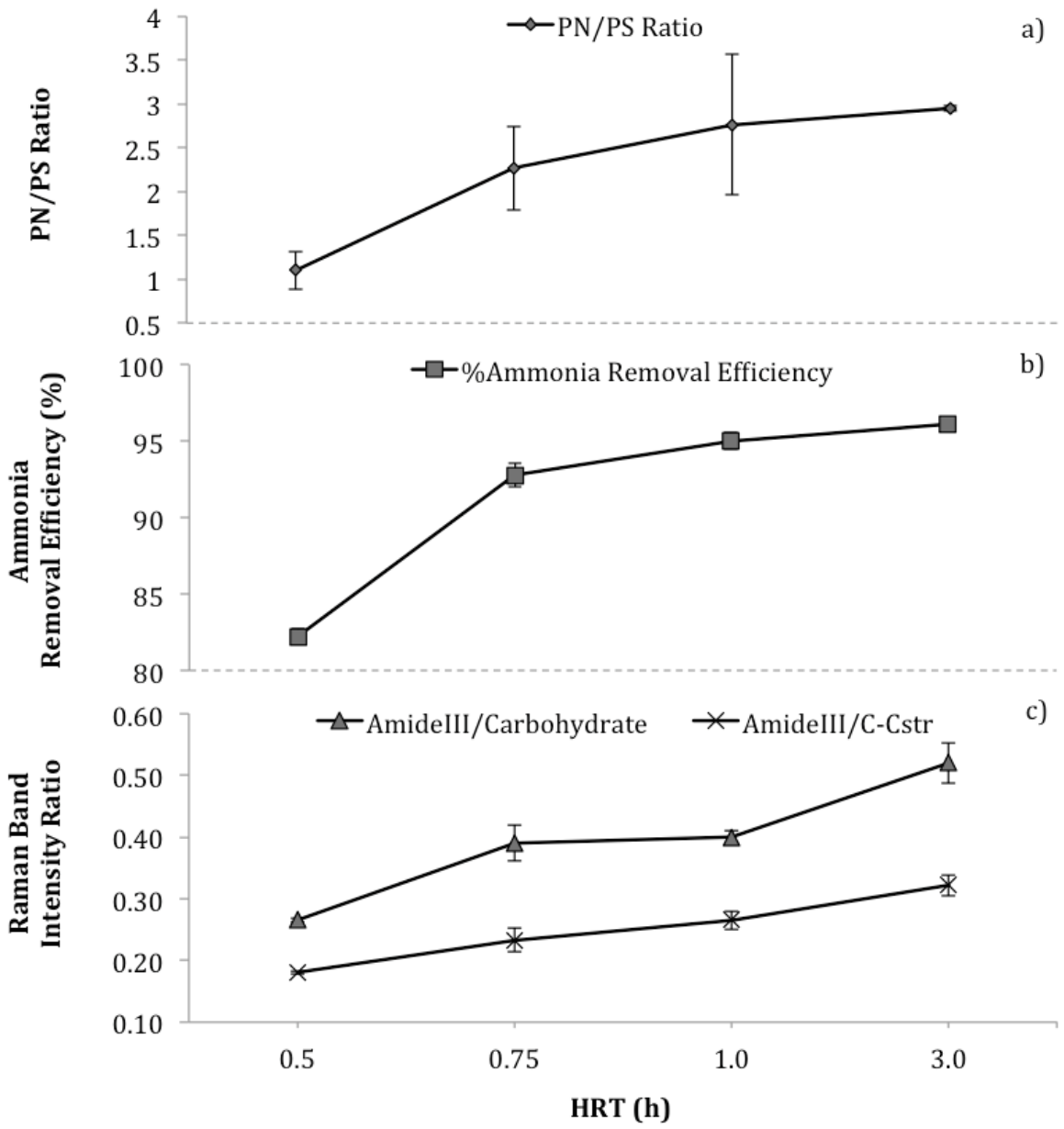

Figure 4.8 Percent live cells, live cells number per carrier and biofilm thickness at various HRTs

VPSEM images were acquired to investigate changes in biofilm morphology at various HRTs while the system was operated at a constant ammonia loading rate

and temperature (Figure 4.9). The morphology of the MBBR nitrifying biofilm did not appear to significantly differ at the various HRTs. The biofilm remained porous and with limited filamentous structure across the HRTs investigated in this study. VPSEM images were also acquired to investigate changes in biofilm thickness at different hydraulic loading rates (Figure 4.9). The biofilm thicknesses at 0.5, 0.75, 1.0 and 3.0 h were very stable and were measured as 102 ± 14 μm, 104 ± 6 μm, 104 ± 8 μm and 88 ± 7 μm, respectively. These values of approximately 100 μm are similar to thickness values observed in past research (Hoang et al., 2014; Almomani et al., 2014).

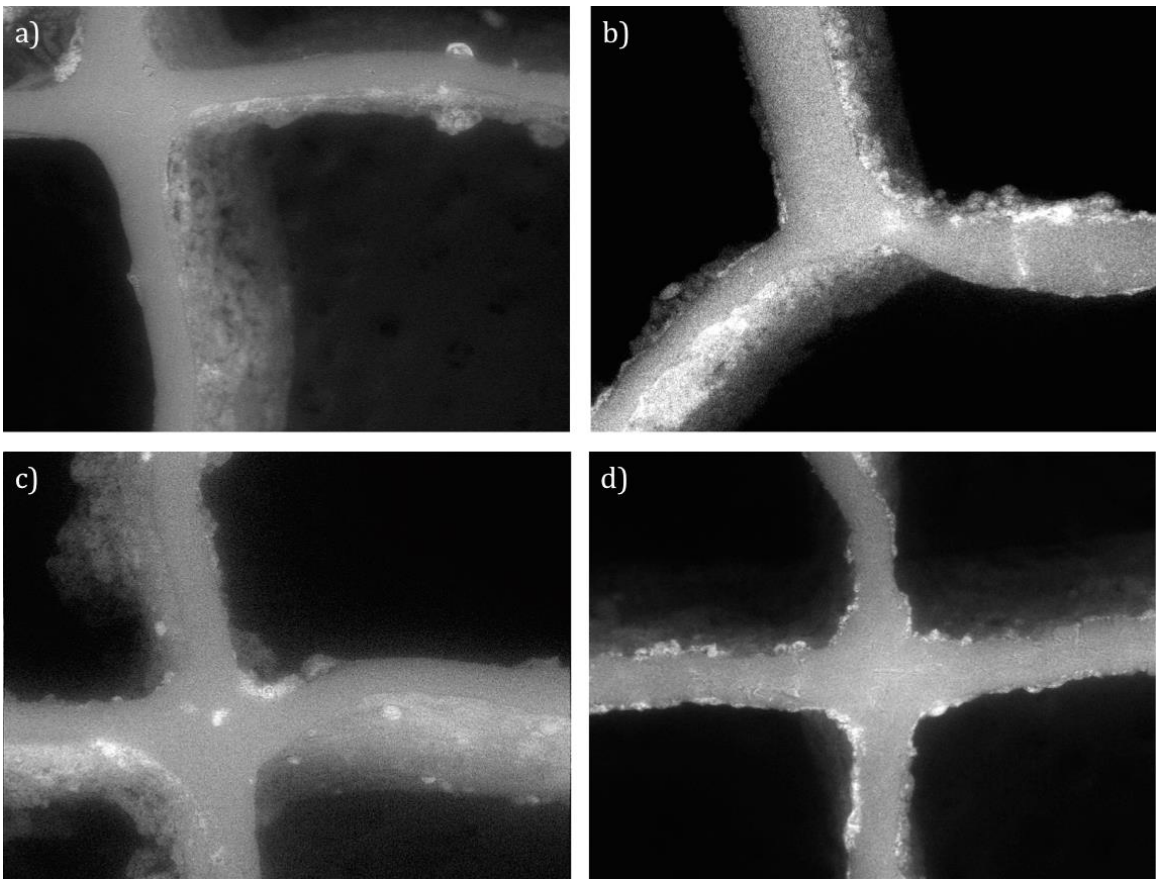

Figure 4.9 VPSEM Images (60× magnification) of nitrifying biofilm attached to MBBR carriers, a) HRT=0.5 h; b) HRT=0.75 h; c) HRT=1.0 h; d) HRT=3.0 h

The total live cell numbers per carrier were calculated based on the counted live cells per biofilm area in the acquired CLSM images and the biofilm thicknesses measurements on the VPSEM images. The live cell number per carrier shows no significant difference in the quantity of live cells per carrier across various HRTs. Hence, although the percentage of live cells in the bacterial communities increased with increasing HRT, the total viable cell counts showed no statistical change at these various HRT; indicating that the size of the viable bacterial community remained stable.

4.5 Conclusion

In this study, a better understanding on nitrifying MBBR biofilms was investigated by identifying temperature and HRT effects on biofilm's characteristics and performance via traditional chemical tests and *in-situ* analyses. At a constant HRT, constant loading and varying temperature, it was found that the percent protein in the biofilms increased when temperature was reduced from 22°C (39.3 ± 3.2%) to 15°C (58.3 ± 1.7%). While percent polysaccharides decreased from 33.6 ± 3.4% to 19.0 ± 0.8% and percent eDNA decreased from 27.1 ± 0.2% to 22.7 ± 1.0%. As temperatures continued to decrease (from 15°C down to 5°C), no significant change in EPS composition was observed; with the results indicating that the biofilm of more optimally loaded nitrifying MBBR systems demonstrate EPS PN/PS ratios of approximately 3.

At a constant temperature, constant loading and varying HRT, it was found that the percent protein in the biofilms increased significantly from 33.1 ± 4.5% at 0.5 h to 60.1 ± 4.0% at 0.75 h. At the same time, eDNA percentage decreased from 35.9 ± 2.2% to 13.4 ± 1.3%, with no observable trend in percent polysaccharides. As HRT values increased from 0.5 to 1.0 h, the percent polysaccharides showed a slight, statistically significant decrease from 31.1 ± 2.3% to 23.5 ± 0.5%. However, as HRTs of changed from 1.0 to 3.0 h, the percentage of all three components remained stable, with the EPS PN/PS ratios approaching a value of 3. Hence, the EPS composition of nitrifying MBBR biofilm at various HRTs demonstrate that more optimally hydraulically loaded nitrifying MBBR systems demonstrate EPS PN/PS ratios of approximately 3; which is similar to the findings observed in this study at more optimally loaded operation. Hence, this study demonstrates that an EPS PN/PS ratio of approximately 3 for nitrifying MBBR biofilms is indicative of well-operated systems.

In addition, as Raman spectra were acquired and analyzed for the EPS of the nitrifying MBBR biofilm at all operating temperatures and HRTs, the results also show that a correlation exists between the trend of the *in-situ* Raman spectra intensity ratios and the chemically measured PN/PS ratios. Further, based on the kinetics and EPS response results, the study demonstrates that tertiary nitrifying MBBR systems may be optimized by operating at HRTs as low as 0.75 to 1.0 h as opposed to conventional HRTs of 2.0 to 6.0 h.

4.6 References

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5 Conclusions and Recommendations

5.1 Conclusions

Nitrifying MBBR biofilm EPS were characterized in this study via traditional chemical analysis and an *in-situ* confocal Raman microscope (CRM) as a complementary method. A variable pressure scanning electron microscope (VPSEM) and a confocal laser scanning microscope (CLSM) in combination with viability staining were applied to investigate the biofilm morphology and thickness as well as the viability of cells embedded in the nitrifying MBBR biofilm. The main conclusions drawn from this work are the following:

- percent protein, polysaccharide and eDNA content of the EPS demonstrated a significant change from a temperature of 22°C to a temperature of 15°C, with the operation at 22°C being likely underloaded;
- as temperature continually decreased from 15°C to 5°C, no measured statistical change in EPS component percentage was observed may because of enough substrate supplement;
- at and below the temperature of 15°C, the system was adequately loaded, which could be the reason of the PN/PS ratio in EPS being stable at approximately 3;
- protein percentage showed significant change as the HRT increased from 0.5 to 0.75 h, while no discernable change was observed in polysaccharide content and the percent eDNA decreased, with the operation at an HRT of 0.5 h being likely hydraulically overloaded;
- percent protein, polysaccharide and eDNA at HRT of 1.0 and 3.0 h were shown to remain stable maybe caused by more optimal hydraulically loading;

- the result of chemical analyses of EPS in nitrifying MBBR biofilms showed a correlation with the result of CRM, which proved that CRM is a promising approach for EPS analysis;
- the highest live cell percentage was observed at the highest HRTs (1.0 and 3.0 h) and as the PN/PS ration approached the threshold value of 3, which indicate the system was optimized as HRT increased;
- the kinetics and EPS response at HRT values of 0.75 and 1.0 h indicate that nitrifying MBBR systems may be optimized by operating at HRTs as low as 0.75 to 1.0 h;
- potential magnitude of PN/PS ratio in EPS lower than 3 is indicative of under optimal operation of the tertiary MBBR biofilm.

5.2 Future Recommendations

The recommendations for future work on the EPS in nitrifying MBBR biofilms are as follows:

- wastewater in Canada is typically exposed to very low temperature in the winter, the EPS analyses for the nitrifying MBBR biofilms could be carried out at the temperature even lower than 5°C; perhaps as low as 0.1 to 1°C;
- the same analyses can be done at longer HRTs (>3.0 h) to observe how the EPS and cellular activity change when biofilm detachment is initiated;
- additional operational conditions such as the loading rate could be studied as another contributing factor of EPS composition in nitrifying MBBR biofilms;

- the concentrations of the EPS components can be obtained from the Raman spectra by using calculations based on an intensity-concentration calibration curve.