Tertiary Nitrifying Moving Bed-Biofilm Reactor: A Study of Carrier and Loading Effects on Nitrifying Kinetics, Biologically Produced Solids and Microbial Community

By

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Abstract

There is an increasing need for tertiary level wastewater treatment in Canada, driven in some cases by both provincial and federal regulation (Canada Gazette, 2012). Tertiary nitrification is the biologically mediated oxidation of nitrogen in the form of ammonia to nitrate following secondary treatment of carbonaceous material (Barnes & Bliss, 1983). The application of tertiary nitrification can prove challenging in the Canadian climate because of the temperature sensitive nature of nitrifiers (Hwang & Oleszkiewicz, 2007). Hence the greater than 1000 lagoon treatment plants currently in operation throughout country are susceptible to the full onslaught of weather effects and as such their nitrification processes become non-existent during the winter months (Delatolla et al., 2011,Hoang et al., 2014).

The moving bed biofilm reactor (MBBR) system has been studied and shows promise for continuous nitrification with prolonged exposure to cold temperatures (Hoang et al., 2014). They are marketed as cost effective and low operation intensive upgrade options for existing treatment plants as well as effective stand-alone systems and are currently in operation in many countries worldwide (WEF, 2011).

Despite the MBBRs initial development as a nitrification technology, recent research has been focused on COD removal systems. Studies showing that MBBR performance is directly related to surface area loading rates (SALRs) and not carrier type or shape have been performed exclusively on COD removal systems. The influence of MBBR carrier type on system solids production has also been solely studied for COD removal and the principles learnt have been transferred to tertiary nitrification systems without confirmation that they hold true. There is an

absence of research on tertiary nitrifying kinetics; the effect of loading and carrier type, the nature of the solids produced and the carrier biofilm characteristics.

This study investigated three MBBR carrier types, the K3, M and P Anoxkaldnes carriers in an effort to quantify the effects of carrier type on nitrifying kinetics, biologically – produced solids and the bacterial community at normal and high loading conditions. Four tertiary nitrifying laboratory scale MBBRs were fed with synthetic wastewater and operated at a high loading condition (HLC) with a SALR of 1.89 ± 0.10 g-N/m^2·d and a normal loading condition (NLC) with SALR of 0.91 ± 0.1 g-N/m^2·d. At both HLC and NLC, results show no difference in the ammonia removal rates obtained by the different carrier types. It was however noticed that stressed operational conditions developed for the P and M carrier at the HLC due to the clogging of carrier pore spaces with biofilm and subsequent reductions in removal efficiency were observed. Despite the fact that larger surface area to volume carriers (such as the M and P) may lead to MBBR designs with smaller footprints and lower operational cost, the study revealed their greater propensity to become clogged under high loading conditions than the smaller surface area carriers (such as the K3). In addition the larger surface area carriers demonstrated longer transitional periods from high loading conditions to lower loading conditions.

A reduction in effluent total suspended solids (TSS) concentrations and improved solids settleability was observed with the shift from HLC to NLC. These results suggest the avoidance of high loading conditions in tertiary nitrifying MBBR operation. If low loading rates are not achievable then system design may have to consider the incorporation of coagulant use or an advanced solids separation technique to meet effluent solids regulation.

Variable pressure scanning electron microscope (VPSEM) images at HLC showed the presence of water mites on the K3 carrier and nematodes and ciliates on the M and P carriers. While NLC images do not show these organisms. VPSEM also measured thicker biofilms during the HLC than the NLC for all carriers. The results demonstrate a difference in the meso-environments and suggest a difference in the micro-environments of the biofilm attached to each carrier.

Microbial analysis showed no shifts in the dominant nitrifying species between the loading conditions, as well as no differences in the percent live /dead cell coverage. *Nitrosomonas* and *Nitrospira* were identified as the dominant AOB and NOB genera respectively at both the HLC and the NLC. Clear shifts in the microbial populations were observed for specific bacteria; with filamentous bacteria being observed at greater relative abundance at HLC than HLC. The increased relative abundance of filamentous organisms are also associated with the significantly poorer effluent settling characteristics observed at HLC.

Acknowledgement

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Finally I dedicate this thesis to my dearest friend Pvt. Yannick Gregory.

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List of Abbreviations

AOB - Ammonia Oxidizing Bacteria

ASP - Activated Sludge Process

BOD - Biochemical Oxygen Demand

BVRR - Biofilm Volume Removal Rate

C - Carbon

CAS - Conventional Activated Sludge

cBOD - Carbonaceous Biochemical Oxygen Demand

CEPA - Canadian Environmental Protection Agency

COD - Chemical Oxygen Demand

CLSM - Confocal Laser Scanning Microscope

DO - Dissolved Oxygen

DNA - Deoxyribonucleic Acid

EPS - Extracellular Polymeric Substances

HRT - Hydraulic Retention Time

HLC - High Loading Condition

IFAS - Integrated Fixed-Film Activated Sludge

LDA – Linear Discriminate Analysis

nBOD - Nitrogenous Biochemical Oxygen Demand

NLC - Normal Loading Condition

MBBR - Moving Bed Biofilm Reactor

N - Nitrogen

NOB - Nitrite Oxidizing Bacteria

OTE - Oxygen Transfer Efficiency

OUT - Operational Taxonomic Unit

R1 - Reactor 1

R2 - Reactor 2

R3 - Reactor 3

R4 - Reactor 4

RBC - Rotating Biological Contactor

SALR - Surface Area Loading Rate

SARR - Surface Area Removal Rate

SEM - Scanning Electron Microscope

TF - Trickling Filter

TSS - Total Suspended Solids

VCRR - Viable Cell Removal Rate

VPSEM - Variable Pressure Scanning Electron Microscope

VSS - Volatile Suspended Solids

WWTPs - Wastewater Treatment Plants

Chapter 1- Introduction

1.1. Background

The major quantifiable source of ammonia released to aquatic environments in Canada is municipal wastewater treatment plants (Environment Canada, 2001). These releases tend not to pose a problem as it relates to eutrophication with phosphorus being the more active contributor in fresh water systems, however they are harmful due to their toxic effects on fresh water organisms (Environment Canada, 2001). Ammonia was added to the Canadian Environmental Protection Agency (CEPA) list of toxic substances in 1999 (Environment Canada, 2001) and presently its concentration in wastewater treatment plant discharge is controlled by federal regulation. The Federal wastewater effluent regulations came into effect in 2012 and it affects all treatment plants with a daily discharge of greater than 100 m^3. The regulations limit un-ionized ammonia to 1.25 mg/l expressed as nitrogen at 15˚C at intrinsic pH (Canada Gazette, 2012). Unionized ammonia concentrations are understood to be function of temperature and pH small changes in either may result in significant changes in the speciation of the ammonia.

In response to this regulation many provinces are now putting into law equivalent or stricter effluent regulations, thereby qualifying for federal equivalency with respect to wastewater effluent regulations. For example, Quebec in response has written new provincial regulations which now make it illegal to release an acutely lethal wastewater effluent (Environment Canada, 2014). Acute lethality refers to a quality assurance test for wastewater toxicity; the death of 50% or more rainbow trout after 96 hours of exposure to an undiluted effluent is characterised as acutely lethal (Environment Canada, 2013). Ammonia is toxic to all vertebrates causing cell death in the nervous system, in freshwater species of algae, trout and salmon the effects of un-

ionized ammonia ranged from reduced development to death. Furthermore Regina, Saskatchewan is aiming to remove nitrogen from their wastewater effluents by 2016 (AECOM & City of Regina, 2014). The need to reduce nitrogen in Saskatchewan is because the soil has high phosphorus content, this combined with nitrogen releases from wastewater treatment plants results in eutrophication problems in their streams and rivers.

The new federal and provincial regulations to restrict ammonia discharge in Canada demonstrate a pressing need for tertiary treatment which may prove challenging to the over 1000 lagoon systems spread throughout the country. The moving bed biofilm reactor (MBBR) is thought to be the response to this need. It was developed in Norway in the 1980's from a need to reduce their nitrogen discharges to the North Sea by 50 % (Rusten et al., 1994). The potential of the MBBR system as a tertiary nitrifying upgrade unit to lagoon treatment systems in Canada has been demonstrated by Hoang et al.,(2014).

Research on COD removal MBBR systems has based system removal efficiency on the surface area for biofilm attachment and not the type of carrier used (Ødegaard et al., 2000). COD studies have also concluded that higher loading conditions result in higher total suspended solids (TSS) and reduced setlleability of the reactor effluent (Ivanovic et al., 2006). These findings have not been confirmed as applicable to tertiary nitrifying MBBR units. There is also a gap in the microbial research, there are no studies investigating the effect of carrier type and loading changes on the morphology and thickness of nitrifying MBBR biofilm as well as the dynamics of its microbial population.

1.2. Aim of Study

The overall goal of this work is to determine the effects of three specific MBBR carrier types on the performance of tertiary nitrifying MBBR under normal and high loading conditions. This work investigates the effects of carriers on nitrifying kinetics, the biologically – produced solids, the biofilm attached to the carriers and the cells embedded in the biofilm with the specific objectives as follows:

- quantify and compare nitrifying kinetics and solids production rates for each carrier,
- characterise the size distribution of particles produced by each carrier before and after settling,
- qualify observable changes in biofilm morphology and quantify biofilm thickness for each carrier,
- quantify the viability of the bacterial cells and population shifts of bacterial communities.

1.3. Thesis Organization

This thesis is comprised of 6 chapters: Chapter 1 is introductory material and includes an abstract, background information and the aims of the research. Chapter 2 is a literature review of the fundamentals of wastewater treatment, the moving bed biofilm process and tertiary nitrification. Chapter 3 outlines the research methods, reactor design and testing procedures. Chapter 4 examines the effect of MBBR carrier type on tertiary nitrifying MBBR systems under high and normal loading conditions. In particular quantifying and comparing nitrifying kinetics and solids production rates for each carrier type as well as characterisation of the size distribution of particles produced by each carrier before and after settling. Chapter 5 investigates

the meso and micro-scale effects of carrier type and loading. In particular it aims to qualify observable changes in microbial communities and quantify the viability of the bacterial cells present within the biofilm of each carrier. Chapters 4 and 5 have been prepared for submission as journal articles. Finally chapter 6 is a presentation of the overall research findings and study conclusions.

1.4. Contribution of Authors

1.4.1 Article 1

Forrest, D., Delatolla R., Kennedy, K. *Carrier Effects on Tertiary Nitrifying MBBRs- An examination of Performance, Biofilm and Biologically Produced Particles*.

Forrest, D. – Reviewed literature, contributed to the experimental design, developed and conducted the testing procedure, analysed results and prepared manuscript.

Delatolla, R. – Supervised development of the testing procedure, finalized and approved the experimental design and analysis of results, reviewed manuscript.

Kennedy, K. - Supervised analysis of results and reviewed manuscript.

1.4.2 Article 2

Forrest, D., Young, B., Delatolla, R., Kennedy K. *Meso and Micro-scale response of tertiary nitrifying MBBR biofilm to variations in loading and carrier type*.

Forrest, D. – Reviewed literature, contributed to the experimental design, developed and conducted the testing procedure, analysed results and prepared manuscript.

Young, B. – Carried out DNA testing on biofilm samples, prepared DNA procedure and result graphs for manuscript and contributed to DNA result interpretation.

Delatolla, R. – Supervised development of the testing procedure, finalized and approved the experimental design and analysis of results, reviewed manuscript.

Kennedy, K. – Supervised analysis of results and reviewed manuscript.

Stintzi, A – Supervised development of DNA testing procedure and microbial data analysis.

1.5 References

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Chapter 2- Literature Review

2.1 Biological Wastewater Treatment

Wastewater treatment aims at the reduction of contaminant concentrations to protect public health and the environment in such a manner that is commensurate with political and economic concerns (Metcalf & Eddy, 2002). The extent of treatment required is often dependent on the intended re-use or the sensitivity of the release environment. In general wastewater treatment plants (WWTPs) in North America are concerned with the following 2 major effluent components: total suspended solids (TSS), and carbonaceous biochemical oxygen demand (cBOD). In Ontario the average effluent TSS and cBOD standard for secondary municipal WWTPs are both 25 mg/L. Canadian wastewater treatment systems discharging 100 m^3 or more effluent daily are affected by federal legislation that requires secondary level of treatment. In particular federal legislation regulates allowable ammonia effluent concentration (NH_4^+-N) to 1.25 mg/l expressed as nitrogen at 15 ± 1˚C at intrinsic pH (Canada Gazette, 2012).

Biological treatment as opposed to physical and chemical treatment has established itself as the standard for secondary treatment because it is a cost effective and environmentally friendly nature (Mulkerrins et al., 2004). In addition to its efficient removal of organics (secondary treatment), biological treatment is also currently promoted as a favourable option for tertiary treatment such as nitrification, denitrification and phosphorus removal.

Essentially biological wastewater treatment employs ubiquitous micro-organisms to transform contaminants into acceptable end-products through aerobic or anaerobic respiration. Successful operation requires the presence and maintenance of microorganisms in the treatment system

through the continued provision of favourable microbial environmental conditions. Biological wastewater treatment processes may utilise either attached or suspended microbial growth systems, where suspended growth processes are currently the convention in both urban and rural areas of North America.

2.2 Suspended Growth Systems

2.2.1 Conventional Activated Sludge Systems

The AS system was developed in England at the turn of the 20th century in response to water pollution problems resulting from its dense populations and industrial growth, the first full scale installation was built in 1926 (University of Duisburg-Essen, 2014).The conventional activated sludge process (CAS) as shown in figure 2.1 is now the most widely used biological process for the treatment of municipal and industrial carbonaceous waste (Rittmann & McCarty, 2001; Guibaud, et al., 2003).

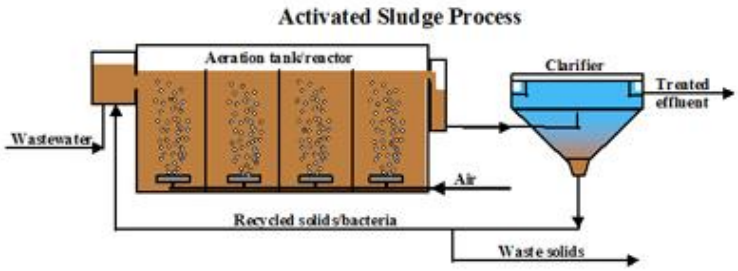

Figure 2.1 Schematic of the CAS process (Scottish Water, 2008)

It is a treatment system with operational cBOD removal efficiency dependent on the growth and retention of an obligate and facultative aerobic micro-organism suspension (Raoet al., 2012). These micro-organisms when provided with favourable environmental conditions (as it relates to

pH and DO) will remove the biodegradable cBOD present through their microbial metabolic processes as well as a portion of the particulate organic and inorganic substances via sorption. Provided sufficient SRT and aeration CAS treatment can also achieve biological nitrification (ammonia oxidation to nitrate). Many configurations and flow pattern variations of CAS treatment exist including an anaerobic suspended growth process train for denitrification (WEF, 2011).

A better understanding of the operational principles of the CAS process has brought about better C and N removal performance but some problems still remain (Rittmann & McCarty, 2001). Most major among them is sludge bulking. Sludge bulking is the inability of microbial flocs to settle or compact well during secondary clarification, resulting in the loss of system biomass and resultant decrease in process efficiency (Rittmann & McCarty, 2001).

2.2.2 Lagoons

Lagoons have been built since the 1950's using existing lakes or through excavation work. They are another suspended growth system and may be aerobic, facultative or anaerobic. When correctly designed, maintained and operated lagoons are able to provide satisfactorily effluent quality with respect to BOD and TSS. In Canada during the 1960s lagoons accounted for almost half of all wastewater treatment facilities (Heinke et al., 1988), however with increased urbanization they now account for about 25% of wastewater treated in Canada and are considered as conventional treatment for rural Canadian communities. Lagoons are generally used by small and medium size communities because their construction and operation is less expensive than CAS treatment plants and there is usually a surplus of inexpensive land available for their construction. Similar to the CAS system they operate through the maintenance of a

microbial community in ponds with long solids and hydraulic retention times to allow for the uptake of organics and in-organics through microbial metabolism. Figure 2.2 shows a lagoon schematic.

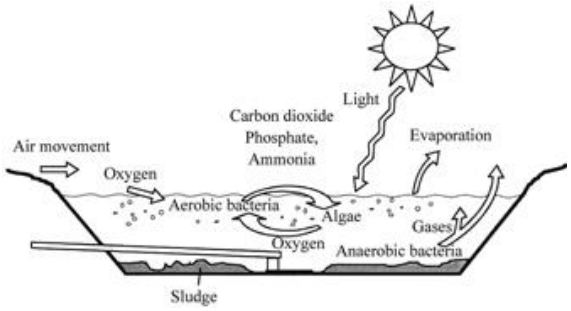

Figure 2.2 Lagoon schematic (Hygnstrom et al., 2010)

Though they offer a great option to rural communities the lack of process control, large exposed surface areas and slow flow leaves them vulnerable to the full onslaught of weather effects which tend to have a particularly negative impact on effluent quality during the winter months (Heinke et al., 1988). In general lagoons demonstrate low nitrification rates when exposed to cold temperatures and seasonal fluctuations in influent quality (Delatolla et al., 2011).

2.3Attached Growth Systems

2.3.1 Advances Associated with Attached Growth Systems

The application of attached growth systems originated in the early 1880's with the full scale operation of the trickling filter (TF) in Wales (Lazarova & Manem, 2000), the rotating biological contactor (RBC) is also one of the older technologies. As it relates to more recent development the moving bed biofilm reactor (MBBR) and the bio-cord system show great promise. They are discussed in this chapter.

There is a trend toward more advanced wastewater treatment driven in some cases by more stringent legislation, such as the new Canadian federal regulations and the resultant provincial responses. Attached growth systems are quite possibly the Canadian solution to upgrade and advanced treatment needs. These systems like suspended growth systems employ the use of micro-organisms to produce treated effluent. Whereas the activated sludge system uses free suspended organisms, the functional unit of attached growth systems is the biofilm structure; a thin layer accumulation of micro-organisms adhered to a sub-stratum (Rittmann & McCarty, 2001). The basic metabolic processes are the same but the biofilm structure lends many unique properties to these fixed film systems. Major among them is reduced energy and operational costs, process simplification due to the lack of mixed liquor inventories or sludge wasting and the smaller reactor volumes used (WEF, 2011).

In addition the complexity of the biofilm structure allows for better response to organic concentration, flow and temperature fluctuations or to a sudden toxin release to the wastewater stream, as such attached growth systems are offered protection and are able to survive harsh environments (Wang et al., 2005). As it relates to the Canadian legislation biofilm technologies promise to WWTP operators consistency in performance and the ability to satisfy the regulations even at the low temperatures experienced during the winter months.

Nitrification is the rate- limiting step in combined cBOD and nBOD biological wastewater treatment, it is also the most temperature-sensitive (Hwang & Oleszkiewicz, 2007; Bassin et al., 2012). Compared to average operational temperatures significant drops in nitrification rates are observed below 15˚C and 50 % reductions at 12˚C (Andersson et al., 2001). At one point efficient nitrification under long term exposure to low temperatures was not thought to be feasible, but recent research has shown that with attached growth systems consistent removals

were obtained even at temperatures of $5^{\circ}C$ and $3^{\circ}C$ (Hoang, 2014; Young, 2014; Rusten et al., 1994).

2.3.2 Trickling Filter

This fixed-film reactor has been in use for over 100 years treating municipal and industrial waste. Initially the reactors were packed with rocks but now virtually all are constructed with plastic packing media which is lighter and has allowed for the lengthening of the treatment column (Metcalf & Eddy, 2002). Wastewater is distributed at the top of the column and as it trickles downward organic material in the wastewater is adsorbed and used for metabolic processes by a community of micro-organisms including; fungi, algae, aerobic and anaerobic bacteria and protozoa which are attached to the packing media as a film or slime. They are advantageous in the simplicity, effectiveness and reliability of their biological processes and have been described as efficient nitrification units (EPA, 2000) a schematic is presented below in figure 2.3.

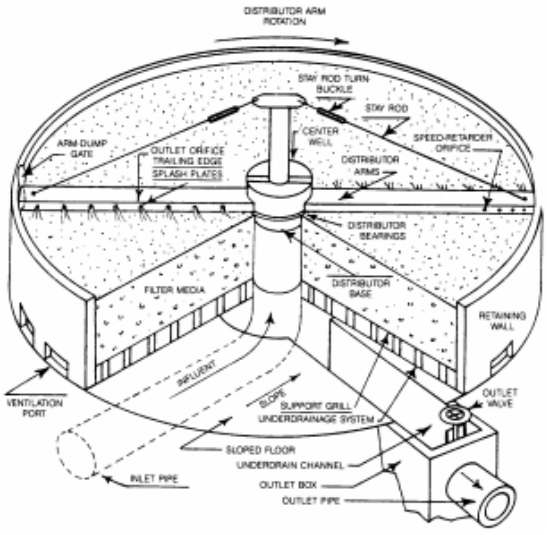

Figure 2.3 Trickling filter schematic (Metcalfe & Eddy, 1991)

The trickling filter (TF) has a major disadvantage in temperate regions where summer months bring large scale loss of reactor biomass due to insect and predator activity. In cold climates the TF must be placed indoors to maintain temperature for if kept outdoors freezing of the biofilm will occur. In both circumstances the efficiency of the treatment process is greatly reduced and the effluent often has higher BOD and TSS values than the influent (Metcalf & Eddy, 2002).

2.3.3 Rotating Biological Contactors (RBCs)

The first installation of RBCs was in west Germany in the 1960's ,because the system design and installation is not as simple as many of the other reactor types, and because there was a lack of understanding of the structure , mechanics and biology of their operation RBC systems developed a history of installation and operational problems (Metcalfe & Eddy, 2003). Despite this these systems have established themselves as the Canadian conventional attached growth system.

RBCs consist of circular disks positioned on a horizontal shaft and rotated through wastewater. Oxygen for the biological processes enters the system when the discs are exposed to the atmosphere. These circular discs have biofilm growth and as the water flows through the disc treatment occurs (Metcalfe & Eddy, 2003). An operation system is shown in figure 2.4.

Figure 2.4 Rotating biological contactor (Triveni Engineering and Indutries liminted, 2013)

2.3.4 Bio-Cord

Bio-cord happens to be one of the most recent biofilm technologies; its simplicity makes it extremely cost effective and versatile as an upgrade option to existing systems. It is a cord covered with threads of various densities which can give it an extremely high surface area that allows for the attachment of a heterogeneous microbial community (Fukui North America, 2004) an imge of two bio-cords is shown below in figure 2.5.

Figure 2.5 Bio-cord (Bishop Water Technologies, 2012)

Bio-cord has been marketed as an easy add-on system to lagoons, as well as a component of Integrated Fixed-film Activated Sludge (IFAS) systems to improve efficiency and even as stand-

alone systems (Bishop Water Technologies, 2012). To date there are no installations in North America.

2.3.5 MBBR

The MBBR was developed in the 1980's in Norway in response to a directive from the North Sea Protection Conference to reduce its nitrogen discharges to the North Sea (Rusten et al., 1994). The system has evolved to also include COD and nutrient removal and many carrier types have been developed. It has established itself as an effective upgrade or stand-alone system and is in operation in many countries worldwide (WEF, 2011) schematic of aerobic and anaerobic configurations is shown below in figure 2.6.

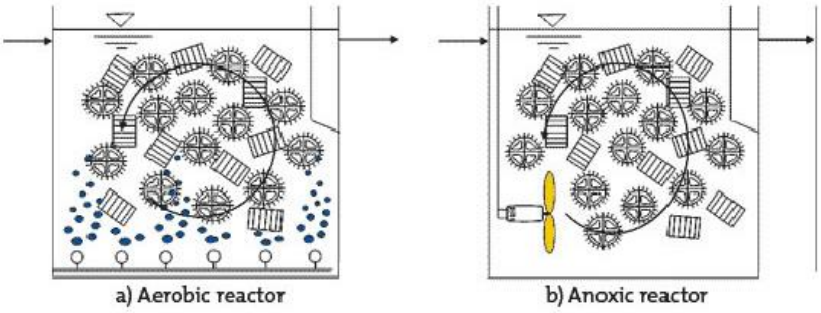

Figure 2.6 MBBR schematic for aerobic and anaerobic set-up (Veolia Water Solutions & Technologies, 2001)

MBBR carriers are made of plastic material (usually polyethylene) with density slightly less than water. The carriers provide a surface for biofilm attachment and protection from shear and abrasion forces. Aerators or mechanical mixers circulate the carriers throughout the wastewater tank and the attached biofilm removes the organics and inorganics through its metabolic

processes (WEF, 2011). The MBBR process is the focus of this research and is described further in section 2.6.

2.4 Biofilms

The biofilm structure is the functional unit behind attached growth systems, it is thought to be a response to nutrient constraints and unfavourable environmental conditions, where energy expenditure in nutrient gathering is minimised and maximum energy is channelled towards reproduction (WEF, 2011). Simpler reasons for this growth behaviour have also been expressed; nutrients in the aqueous environment tend to concentrate around solid surfaces (ZoBell & Allen, 1935).

Biofilm has been described as a functional consortium of micro-organisms organised within an extensive exo-polymer surface (Elder et al., 1995). In fact in most cases less than 10% of the biofilm is actually made up of micro-organisms, the remaining matrix consists of water, entrapped particles and most notably extra-polymeric substances EPS (Flemming & Wingender, 2010). The complete functionality of the EPS layer is still not fully understood, but scientists agree that it confers many benefits to the micro-organisms living within the biofilm though they are unsure whether it confers these advantages to all the micro-organisms especially those far from the film surface (Dunne, 2002). The EPS layer is thought to be largely responsible for the three dimensional structure of the biofilm , its ability to immobilize the cells allows for a closer clustering and more effective cell to cell communication resulting in a faster more uniform community response to changes in the environment (Flemming & Wingender, 2010). The matrix retains many of the enzymes released from the cells and so it acts as an external digestive system

making nutrients more readily available to the cells, it also protects the micro-organisms from desiccation and biocides (Dunne, 2002; Wang et al., 2005).

Researchers have linked biofilm structure and performance to reactor conditions; Hoang et al., (2014) measured increased biofilm thickness with reductions in temperature, postulating increased optimum biofilm thickness due to increased oxygen solubility (WEF, 2011). Wang et al., (2005) observed rough, fluffy biofilms at low carrier fill percentages and thinner more dense biofilms at high carrier fill due to increased carrier collision and resultant biofilm detachment. The study observed smaller oxygen limitations in the fluffy films and resultant higher microbial activity rates than that within the dense smooth films.

Researchers have also begun to realise that detachment is a very important factor that controls the biofilm structure and process efficiency hinges on the attainment and maintenance of an optimum film thickness (Rittman, 2007). Erosion is one of the mechanisms useful in this regard it is the detachment of small pieces of biofilm as a result of the shear forces associated with the bulk liquid and in some cases the aeration mechanisms. Other methods include abrasion due to carrier collisions within the reactor and moderate predator activity. All three allow for small continuous loss of biomass from the outer surface of the biofilm and are considered as norms in process operation (WEF, 2011). Sloughing however is the uncontrollable, undesirable detachment of biomass from the substratum interface. The treatment system experiences increased BOD and TSS outputs and reductions in substrate removal rates (WEF, 2011).

One of the most notable features of biofilms is the presence of substrate concentration gradients. As a result organisms at different layers within the film will have different substrate utilisation and growth rates. In some cases the different environmental conditions occurring throughout the

film will lead to the existence of varied microbial communities moving from aerobic to anoxic conditions (WEF, 2011).

Biofilm kinetics is dependent on many rates; substrate transfer rates through the bulk liquid to the liquid- biofilm interface, transfer rates through the biofilm to the cells, rate of substrate utilization by the cells (Melo, 2005). Often times the internal mass transfer rate (through the biofilm) is the slowest and is therefore the rate limiting process (WEF, 2011).

2.5 Nitrification

Nitrification is the process by which ammonia is converted to nitrites which are then further oxidised to nitrates. In many cases nitrification is all that is required as the aim is to prevent ammonia toxicity and oxygen depletion (Barnes & Bliss, 1983). The aim of treatment is to ensure that nitrogen released into waterways is in a desired form and concentration. Nitrogen in aquatic environments is of concern, because untreated release poses a risk not only to the environment but also to human health.

The oxidation of ammonia to nitrite is carried out by ammonia oxidising bacteria most commonly identified to be *Nitrobacter* while the conversion of nitrite to nitrate is the function of nitrite oxidising bacteria of which *Nitrospira* is recognised as most common. The overall reaction has low energy yields while the incorporation of carbon dioxide into cellular material requires large amounts, thus explaining the slow growth rate of nitrifiers (Gerardi, 2002).

2.6 MBBR

2.61. History and Principles of MBBR

Much of the initial work on the MBBR was carried out in Norway in the 1980's in an effort to significantly reduce nitrogen discharges to the North Sea (Rusten et al., 1994). Norwegian researchers realized that the most cost-effective upgrade option would be a compact biofilm process and as a result the plastic carrier moving bed bioreactor was developed. MBBR systems have established themselves as a simple, robust, effective technology capable of removing nitrogen and BOD. MBBRs have been found to be versatile and so can be considered for a variety of reactor geometries; as such they are well suited for use in existing facilities where treatment upgrades are required (WEF, 2011).

The principle behind MBBR operation is the growth of a biofilm upon the inner surfaces of plastic carriers, where it is protected from physical abrasion , carrier types and properties are shown in figure 2.7. These carriers are circulated through the wastewater tank by course bubble aerators in aerobic treatment and by mechanical mixers in anaerobic treatment. It is this constant motion that allows for optimum interaction between the biofilm, the bulk liquid dissolved oxygen and the substrate (the pollutant to be removed). Compared to other fixed film systems these reactors are reported to show no clogging and exhibit lower head loss while the reduced operation and sludge processing cost make it a favourable alternative to the activated sludge process (Andreottola et al., 2000).

Over the years many companies and designs have emerged and the produced carrier types have varied in material, shape and size. An established manufacturer is Anox Kaldness responsible for the production of the carriers shown:

Manufacturer	Name	Bulk Specific Surface Area[1]	Dimensions (Depth; Diameter)	Carrier Photograph
Veolia Inc.	AnoxKaldnes™ K1 or K1 Heavy	500 m^2/m^3	7 mm; 10 mm	
	AnoxKaldnes™ K3	500 m^2/m^3	12 mm; 25 mm	
	AnoxKaldnes™ Biofilm Chip (M)	1,200 m^2/m^3	2 mm; 48 mm	
	AnoxKaldnes™ Biofilm Chip (P)	900 m^2/m^3	3 mm; 45 mm	
	AnoxKaldnes™ Matrix™ Sol	800 m^2/m^3	4 mm; 25 mm	

Figure 2.7 Anoxkaldnes biofilm support media (WEF, 2011)

2.6.2 Nitrifying MBBR

The nitrifying MBBR was the first of the MBBR systems developed and has been studied exhaustively. Efficiency of nitrification in the moving bed biofilm reactor depends on the BOD loading, ammonium concentration and dissolved oxygen concentration (Slawomir et al., 2010).

Insufficient removal of organic matter before flow into nitrifying MBBR's will allow heterotrophic micro-organisms to outcompete nitrifiers for both space and oxygen. Research has shown that as the organic loading is decreased the rate of nitrification increases until dissolved oxygen becomes the rate-limiting substrate. Ammonia will only become the rate limiting

substrate when its concentrations are extremely low; below 2 mg/l. As long as the ratio of oxygen to ammonia is greater than 2, oxygen will remain as the rate limiting substrate (WEF, 2011).

It has previously been mentioned that nitrification rates are affected by temperature but this does necessarily result in a loss of efficiency in the treatment process. Research has shown that cold wastewater temperatures may sustain thicker biofilm layers and higher bulk dissolved oxygen (Hoang et al., 2014). The reduced temperatures result in increased oxygen solubility. The higher bulk DO concentrations allow for increased oxygen penetration depths thus supporting thicker biofilm. Therefore the reduced nitrification rates in cold weather may be offset and effective operation maintained without increased aeration.

2.6.3 Effect of Carrier Type on MBBR Performance

Few studies have been carried out investigating the effect of MBBR carrier type on system performance. One of the more significant studies was carried out by ØDeegaard et al., (2000) which studied the performance of two different Anoxkaldnes biofilm media K1 and K2 under COD removal conditions. This study defines the design technique used for MBBR processes today; Ødegaard reported that substrate removal rates are dependent on surface area loading not carrier type, size or reactor volume. However the study did find inexplicable differences in the settleability of the MBBR produced solids; with the K2 carrier showing better settleability than the K1carrier.

More recently Levstek & Plazi (2009) investigated two distinct MBBR carrier types under nitrifying conditions; Anoxkaldnes K1 and the polyvinyl alcohol (PVA) gel carrier. The PVA

gel has a network of minute pores about 20 microns in diameter tunneling throughout each bead, this porous microstructure allows microorganisms to penetrate and colonize the entire gel material and provides favourable conditions for the growth and retention of slow growing microbes (Rouse et al., 2005; Kuraray- Aqua Co., 2008).At the recommended fill percentages the removal rates of the two carrier types were very similar. They however noted the difference in required fill percentage with the K1 carrier recommended at 67% and the PVA gel at 15% reactor volume because of PVA's larger surface area (Levstek & Plazi, 2009). They found that under the manufacturer recommended operating conditions the measured nitrification rates and biofilm mass were unaffected by carrier type but found the PVA gel carrier to have the ability to grow more biomass per volumetric carrier filling than the K1 carrier. Their speculation is that higher fill percentages for the PVA gel could yield to higher nitrification rates compared to the K1 carrier (Levstek & Plazi, 2009).

Levstek and Plazi's finding is significant because researchers have also investigated carrier effects as it relates to carrier fill percentages and their effect on oxygen transfer efficiency (OTE). While research findings are contradictory, increased MBBR performance has also been postulated as a result of increased oxygen transfer efficiency due to increased carrier fill, thereby giving small surface area carriers an advantage over larger surface area to volume carriers. (Pham et al., 2008; Piculell et al., 2013).

Though studies have investigated carrier effects on COD removal, nitrification and even oxygen transfer efficiency, little work has been done tertiary nitrifying systems and the effect of carrier type on system performance.

2.6.4 Effect of Reactor Loading and Operation on Particle Production

Loading of the moving bed biofilm reactor is based on surface area and is calculated as follows:

$$SALR = \frac{(C_i \, x \, Q_i)}{V_i \, x \, A_{sc} \, x \, \alpha} \quad \textbf{2.1}$$

Where SALR (g-N/ m^2day) is surface area loading rate, Ci (g/m3) is the influent concentration , Q_i (m^3/day) is the influent flow rate, V_i (m^3) is reactor volume, A_{sc} (m^2/m^3) surface area to volume ratio and α (%) is the percent fill.

Researchers studying COD removal in MBBR systems have shown that increased surface area loading rates (SALR) result in increased biosolids production (Aygun , et al., 2008). This increase is accompanied by reductions in biosolids settleability which have been explained by microscopic observations showing inherently different floc structures at high and low loading rates (Ivanovic et al., 2006; Ivanovic & Leiknes, 2012).

Essentially increased MBBR loading rates stress the agglomeration and settlement processes and significantly increase the number of particles produced; specifically smaller particles. Due to the poor settleability of the reactor effluent under high loading conditions metal coagulant or cationic polymer may be necessary to meet effluent solids standards. (Ivanovic, Leiknes, & Ødegaard, 2006; Ivanovic & Leiknes, 2012). Similar studies have not been carried out for tertiary nitrifying MBBRs, therefore it is uncertain whether the principles hold true for these systems.

2.7 Microbiology

For optimization of wastewater treatment processes a full understanding of the microbial consortia involved in treatment is necessary as well as their response to changes in loading and reactor conditions (Boltz et al., 2011). This type of research is lacking for tertiary nitrifying MBBR systems.

In a biofilm reactor Volcke et al. (2008) noticed that the same NOB was present in two reactors with distinctly different loading patterns, however the NOB concentration was lower in the reactor with the longer HRT and lower SALR than in the reactor with the shorter HRT and higher loading rate. In addition the major AOB differed between the reactors, the long HRT - low loading condition was dominated by *Nitrosomonas Europaea* while *Nitrosomonas sp.* dominated the reactor with short HRT- high loading (Volcke et al., 2008). Researchers have also noticed greater concentrations of filamentous bacteria under high reactor loading, and it is thought that these organisms negatively affect settling (Ivanovic, et al., 2006). Furthermore the MBBR system has shown increased microbial diversity under higher loading conditions (Bernet et al., 2004; Calderon et al., 2012), diverse microbial populations are viewed as important for resilience of the treatment system (Almstrand et al., 2013).

Ammonia oxidisers within the *Nitrosomonas* genus show different responses to increases in substrate availability after starvation in trickling filters. *N. Europaea* quickly responds while *N. Oligotropha* takes longer periods before they start nitrifying (Almstrand et al., 2013).

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Chapter 3- Materials and Methods

3.1. Experimental Design

Four laboratory scale moving bed biofilm reactors (MBBRs), containing three different carrier types, were fed with the same influent synthetic wastewater and operated at identical surface area loading rates (SALRs), temperature, pH, dissolved oxygen (DO) and hydraulic retention time (HRT) of 3 hours during two experimental phases. High concentrations of DO; averaging 9.0 ± 0.9 mg/l were maintained in each reactor. Optimum microorganism performance is between pH values of 8 and 9 (Metcalf & Eddy, 2002); therefore sodium bicarbonate (286 mg/l) was used in the feed as a buffer to allow pH to be maintained at an average value of 8.0 ± 0.1 within the reactors. Bubble diffusers were installed in each reactor to provide oxygen for biological nitrification and mixing of the carriers.

Figure 3.1 highlights the 4 study reactors and the use of K3 carriers in reactors 1 and 2 (R1, R2), P carriers in reactor 3 (R3) and M carriers in reactor 4 (R4); all carriers are high density polyethene biofilm support media supplied by Anoxkaldnes.

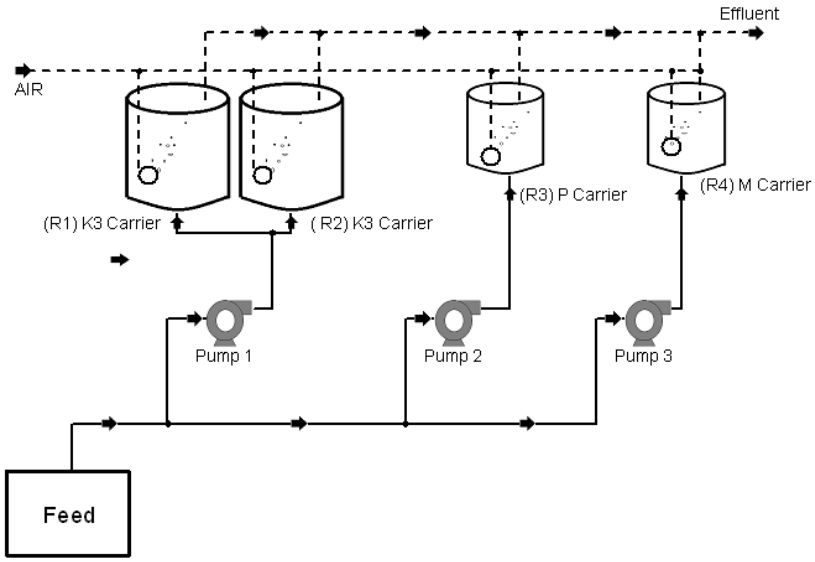

Figure 3.1 Experimental set-up

Phase one was a high loading condition (HLC) with SALR of 2 g-N/m^2·day while phase 2 was the normal loading condition (NLC) with SALR of 1 g-N/m^2·day. At HLC influent COD was 10 mg/l. Influent COD was reduced to 5 mg/l for NLC. In both phases no temperature control mechanism was used as the reactors were operated in a thermostat controlled building with experiment temperatures averaging 22˚C ± 1˚C.

Carrier biofilm in the reactors was inoculated with sludge from COD removal MBBR systems and was allowed to acclimatize at a SALR of 1.2 g-N/m^2·day with a HRT of 3 hours over a 17 day period. Following the achievement of steady state conditions in all reactors subsequent changes in loading were carried out gradually to prevent shock to the systems. Steady state was defined as variations in measured effluent nitrogen constituents being no greater than 10% over a one week period. Increases in loading necessary for the achievement of HLC were carried out by increasing the feed incrementally by 0.3g-N/m^2·d. Decreases from HLC to NLC were achieved by lowering the loading rate by 0.3g-N/m^2·d every week. For HLC and NLC, steady state

conditions were validated prior to data collection in total the systems were operated for over 9 months or 2160 HRTs.

3.2 Reactors

3.2.1 Reactors 1 and 2

R1 and R2 were identical 2 litre cylindrical Perspex reactors containing 50% by volume K3 Anoxkaldnes carriers. The internal diameter of the two reactors was 12 cm and the height was 17 cm resulting in a diameter to height ratio of 0.71. Aeration was provided by a single air-line attached to a diffuser that kept the carriers in rotation and completely mixed the reactor. The K3 carrier is 7 mm in depth with a diameter 10 mm and an available surface area of 500 m^2/m^3 (WEF, 2011). Influent was introduced at the base of the reactors and effluent overflowed through the liquid level control effluent line at the top.

3.2.2 Reactor 3

R3 was a 1.2 litre cylindrical reactor with 30% by volume fill of Anoxkaldnes P carriers. The reactor had an internal diameter of 10 cm and height of 15 cm resulting in a diameter to height ratio of 0.67. Aeration and mixing was achieved by the same diffuser configuration afore described for R1 and R2. Preliminary design work found improved P carrier rotation with more narrow reactors than that required for effective K3 movement. Therefore a smaller diameter to height ratio was used for R3. The P carrier is 2 mm in depth, 48 mm wide and has an available surface area of 900 m^2/m^3 (WEF, 2011). Influent was introduced at the base of the reactors and effluent overflowed through the liquid level control effluent line at the top of the reactor.

3.2.3 Reactor 4

R4 was a 1.2 litre cylindrical reactor with 20% fill of Anoxkaldnes M carriers. R3 had in internal diameter of 10 cm and height of 15 cm, resulting in a diameter to height ratio of 0.67. Aeration and mixing were achieved as described previously for R1, R2 and R3. Preliminary design work found better M carrier rotation with more narrow reactors than that required for effective K3 movement. Therefore a smaller diameter to height ratio was used for R4. The M carrier is 2 mm in depth, 48 mm in diameter and has a surface area for biofilm attachment of 1200 m^2/m^3 (WEF, 2011). Influent was introduced at the base of the reactors and effluent overflowed through the liquid level control effluent line at the top. Table 3.1 shown below is a summary of the reactor design.

Operating Condition	**Reactor 1**	**Reactor 2**	**Reactor 3**	**Reactor 4**
Carrier type	K3	K3	P	M
Carrier Surface Area (m^2/m^3)	500	500	900	1200
Reactor Volume (L)	2	2	1.2	1.2
Percent fill (%)	50	50	30	20

Table 3.1 Reactor set-up

3.2.4 Reactor Influent

Synthetic wastewater was used throughout the study period because it allowed for the maintenance of stable loading rates with limited variations and it prevented the introduction of suspended solids to the reactors thus enabling an effective investigation of the biologically-produced solids without influent solids interaction effects. The synthetic wastewater was based on a variety of sources (De Beer et al., 1993; Rusten et al., 1994; Gieseke et al., 2001; Lee et al., 2004 Rochex, et al., 2008; Karizmeh et al., 2014) and is as follows for NLC: $(NH_4)_2SO_4$:117.91 mg/L, $NaHCO_3$: 325mg/L, $MgSO_4.7H_2O$: 325 mg/L, $CaCl_2.2H_2O$:29.34 mg/L, KH_2PO_4: 79.09 mg/L, $FeSO_4.7H_2O$: 4.98 mg/L. Carbon source: $C_6H_{12}O_6$: 9.45 mg/L, NaAc: 5.04 mg/L, Peptone: 9.45 mg/L Trace nutrients; $MnCl_2.4H_2O$: 200mg/L, $NaMoO_4.2H_2O$: 49.62 mg/L, $CuSO_4.5H_2O$: 205.1 mg/L, $CoCl_2.6H_2O$: 2 mg/L, $ZnSO_4.7H_2O$: 59.82 mg/L.

The glucose, acetate and peptone were supplied to mimic the readily degradable carbonaceous content of wastewater at a concentration of 5 mg BOD/L under NLC. During the HLC phase of the study the ammonia, alkalinity and BOD were all proportionally doubled to maintain the same nitrogen to carbon ratio.

3.3 Constituent Analysis

Nitrogen constituent samples were collected in triplicate to encourage result accuracy and all samples were analysed by photometric methods. Prior to analysis samples were first filtered through 0.45µm Whatman glass fiber filters to reduce the effect of suspended particles on measured absorbance values. All measurements for nitrogen were carried out in accordance with the outlined methods in Standard Methods (APHA, 1989), Method 4500- NH_3 4500-NO_3^- B, and 4500-NO_2^-.

Dissolved Oxygen and pH were measured using a symphony Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario). Temperature within the reactors was measured daily using a mercury glass thermometer. All constituent statistical analyses were based on 5 samples measured in triplicate (15 measurements) with statistical significance based on t-test with a p-value of 0.05 for statistical significance.

3.4 Solids Analysis

Solids measurements were carried out in accordance with methods outlined by Standard Methods (APHA, 1989), 2540 D- Total Suspended Solids (total suspended Solids dried at 103-105˚C), 2540 E- Volatile Solids and Volatile Suspended Solids (fixed and volatile solids ignited at 550˚C) and 2540 B- Total Solids (total solids dried at 103-105˚C). Three samples were taken from each reactor for solids analysis and statistical significance was determined by the t-test with a p-value of 0.05.

3.4.1 Digital Particle Analysis

In an effort to investigate the smaller solids being produced by nitrifying reactors and observe the effects of carrier type and loading rates on size distribution and settleability a digital particle analyzer (DPA) 4100 (Brightwell Technologies Inc., Canada, Ontario) was used in low magnification mode. With this set up it was possible to observe and quantify particles between 2.25 µm and 400 µm. A filtration step was first required to remove the particles larger than 400 µm with a total solids analysis conducted on the trapped solid material.

DPA produces sizing information by passing a sample through a continuous light field, with a camera continuously recording images of the interaction. Particles appear as dark spots in the

images which are then analysed by the software to present particle counts, concentrations and circularity coefficients among other variables.

$$v_x = r_x{}^3 \times \frac{4\pi}{3} \times c \times N_x \quad \textbf{3.1}$$

$$r_x = \frac{x}{2} \quad \textbf{3.2}$$

Where v_x = volume of particle with diameter x, r_x = radius of particle , c = circularity coefficient of the particles observed, N_x = number of particle counted with diameter x.

Settlement effects on particle size distribution were investigated by analysing DPA prior to settling and after 30 minutes of settling in an imhoff 1000 ml cone. Due to the fact that the nitrification MBBR process produced a low concentration of effluent TSS, the sludge volume index (SVI) of the samples could not able to accurately measured. All solids analyses are based on 3 samples measured in triplicate (9 measurements), with statistical significance being based on t-test with a p-value of 0.05.

3.4.2 Biofilm Mass

For each loading phase three carriers were removed from each reactor. Carriers were rinsed with distilled water then cut; the K3 carriers were cut into halves, while the M and P carriers were cut into thirds. The pieces were then centrifuged for thirty minutes at 9000 rpm in a Sorvall Legend T+ centrifuge (Thermo Scientific, USA, New York). Samples were then vortexed using a vortex-genie model K-550-G for 15 minutes at maximum speed (10).Total solids and volatile solids tests were then carried out on the resultant biofilm-water mixture according to Standard Methods (APHA, 1989): 2540 B- Total Solids (total solids dried at 103-105˚C) and 2540 E-Volatile Solids (fixed and volatile solids ignited at 550˚C). Three samples were tested and

statistical significance for mass was based on t-test with a p-value of 0.05 for statistical significance.

3.5 Biofilm Thickness and Morphology

MBBR biofilm thicknesses and morphology were observed through the use of a Vega II-XMU variable pressure scanning electron microscope (VPSEM) (Tescan USA Inc., USA, Pennsylvania). Regular SEM requires destructive sample preparation which was not necessary with the VPSEM (Flemming et al., 2000). Though there is resultant shrinkage in extracellular polymeric substances (EPS) and biomass layers. VPSEM allowed for high magnification and analysis of samples many hundreds of micrometers in thickness which is not achievable with light microscopy (Flemming et al., 2000). No sample preparation was required for VPSEM were simply placed in the pressure chamber and images captured.

One carrier was selected from each reactor during each testing phase and 20 thickness measurements were taken at randomly selected points, statistical significance was determined using the t-test with a p-value of 0.05 for statistical significance.

3.6 Cell Viability

An upright confocal laser scanning microscope (CSLM) was used along with fluorescent dyes; Propium iodide and Syto 9 to observe cell viability within the biofilm. Confocal microscopy has been recognised as an established advanced tool for biofilm research (Lewandowski & Beyenal, 2007). Confocal microscopy allows optical sectioning of fully hydrated biofilm structures several hundreds of micrometers thick with no out of focus disturbance. In combination with the fore mentioned dyes a quantification of live and dead cells was possible. Propium iodide is a

membrane potential sensitive dye and so it stains all cells green (live cells); Syto 9 is a red nucleic acid stain and so it stains those cells with a damaged cell membrane (dead cells) (Flemming et al., 2000). A LSM 510/Axio imager M.1 confocal Microscope (Carl Zeiss Canada Ltd, Ontario, Canada) equipped with argon and helium- neon lasers was used to capture the images. NI Vision Assistant 7.1 (LabView 8.0- National Instruments Canada, Vaudreuil-Dorion, Canada) software was used to quantify the fluorescence. One carrier was selected from each reactor and cut into 4 sections, each section was imaged at 5 layers each 6 μm thick using the CLSM stacking function. A total of 20 images per reactor were analysed based on the methodology used by Delatolla et al. (2009). The tracer function within NI Vision software was used to measure the biofilm area separately from the pore spaces and the threshold function was then used to measure the red and green fluorescence. Exclusions of values smaller than those expected for N*itrospira*, *Nitrobacter* and *Nitrosomonas* bacteria acted as a check to ensure that only nitrifying cells were counted (Okabe et al., 2004; Off et al., 2010). These species were selected because they have been recognised as the most common nitrifying species. The area of the nitrifying bacteria cells was calculated and then correlated to the number of pixels in each image and with our image pixel size 133.6 x 133.6.

3.7 DNA characterisation of the microbial community

The bacterial community embedded in the biofilm attached to each carrier type was identified and quantified using next generation sequencing. At both HLC and NLC, five carriers were removed from each reactor and scraped to remove the attached biofilm. The DNA therein contained was extracted by a combination of cell lysis, centrifuging and homogenization using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA) in accordance with previously

developed extraction protocols (Stintzi, 2011). At the end of the extraction period Quanti-iT^{TM} dsDNA HS Assay Kits (Life Technologies, Burlington, Canada) were used to quantify the DNA which was then stored at -80˚C until all samples had been collected.

Amplification of the extracted DNA was carried out in accordance with protocol developed by Sundquist et al. (2007). The protocol includes a two-step polymerase chain reaction (PCR) and the use of a unique barcode for each sample. The extracted DNA was purified using a Montage PCR 96 Clean up kit (EMD Millipore, Billerica, MA) and inspected on 2% agarose gel. 50 ng of the amplified DNA was then collected and sent to the Centre for Applied Genomics (TCAG) at the Hospital for Sick Children in Toronto for sequencing.

Upon receipt of the sequencing results the Galaxy tool was used for quality filtering and only sequences with an exact match to the amplification primers were kept for analysis. The Greengenes database along with the Quantitative Insights into Microbial Ecology (QIME) software was used to identify and determine the relative abundance of the various bacteria present (Hoang et al., 2014).

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Chapter 4- Carrier Effects on Tertiary Nitrifying MBBRs - An Examination of Performance, Biofilm and Biologically-Produced Particles

4.1 Setting the Context

The article presented in this chapter is entitled *Carrier Effects on Tertiary Nitrifying MBBRs - An Examination of Performance, Biofilm and Biologically-Produced Particles* by Daina Forrest, Robert Delatolla and Kevin Kennedy and has been prepared for submission to Environmental Technology. It investigates the effects of three specific carrier types on ammonia removal rates, biofilm morphology, along with solids production and settleability for tertiary nitrifying MBBR systems.

4.2 Introduction

Nitrogen release is well documented as harmful to aquatic environments due to ammonia and nitrite toxicity to fish along with the potential to promote eutrophication events (Gerardi, 2002). Discharge of wastewater effluent has been recognised as a major source of ammonia release to aquatic ecosystems and as such many governments are implementing progressively stricter regulations for ammonia release (Canada Gazette, 2012; Ministère du Développement durable de l'Environnement et des Parcs, 2013; AECOM & City of Regina, 2014). It is important to note however that this isn't a newly recognized problem; in 1985, Norway was directed to reduce its nitrogen discharges to the North Sea by 50% (Ospar Commission, 1984), in response, the moving bed biofilm reactor (MBBR) was developed (Rusten et al., 1994). Over the last 30 years the MBBR technology has proven effective for chemical oxygen demand (COD) and nitrogen removal and is in operation in numerous countries worldwide (Verma et al., 2006). More

recently, the ability of the MBBR system to maintain nitrification during extensive exposure to very cold temperatures has promoted these systems as upgrade options to existing plants in the northern and colder regions (Delatolla et al., 2009; Hoang et al., 2014).

There are many marketed carrier types for MBBR systems. These carriers differ in size, shape, pore spacing and surface area. Ødegaard et al. (2000) established that removal rates in COD removal MBBR systems are not dependant on carrier type or reactor volume; rather the COD removal rates are based on surface area available for biofilm attachment. Hence, carriers with the highest specific surface area to volume ratio can improve the design efficiency of MBBR systems by reducing unit footprints as well as reducing capital and operational costs.

Studies of COD removal MBBR systems have linked carrier type and surface area loading rate (SALR) to effluent solids characteristics. Differences in produced biomass settleability have been observed with different carrier types (Ødegaard et al., 2000) and higher SALRs for COD removal MBBRs and have been shown to result in reactor effluents with more suspended solids and poor settling characteristics (Aygun et al., 2008; Ivanovic & Leiknes, 2012). Researchers have noticed differences in biofilm morphology between high and low SALRs for COD removal MBBRs (Ivanovic et al., 2006). They have observed denser biofilms under low loading conditions (Wang et al., 2005) and effluents with favourable characteristics as it relates to solids separation (Ivanovic et al., 2006). Studies investigating the effects of loading and hydraulic retention time (HRT) for COD removal MBBRs on particle size distribution have demonstrated the tendency for distribution shifts towards larger particles with increasing COD loads in activated sludge membrane bioreactors (Wisniewski et al., 2000) and with increasing HRTs in MBBRs (Melin et al., 2005). Studies have also revealed better aggregation and submicron particle reduction with low loading rates (Åhl et al., 2006).

As most previous studies investigating carrier effects on kinetics and solids have been focused on COD removal systems, the effects of ammonia loading and carrier type on biofilm morphology and effluent solids characteristics and settleability remains unclear. Hence, it is necessary to investigate whether the findings from COD removal systems translate to ammonia removal systems. As such, the goal of this study is to investigate the effects of carrier type on tertiary nitrifying MBBR systems under normal and high loading conditions. In particular, various carriers are studied with respect to their effect on ammonia removal kinetics of the systems, the morphology of the nitrifying biofilm along with the distribution and settleability of the biologically-produced solids.

4.3 Methods

4.3.1 Experiments

Four laboratory scale MBBR reactors, containing three different carriers, were fed with the same influent synthetic wastewater and operated under identical temperatures, HRTs and SALRs during two experimental phases. Phase one is a high loading condition (HLC) with a SALR of 2 g-N/m^2·d while phase 2 is the normal loading condition (NLC) with a SALR of 1 g-N/m^2·d, both phases were operated at a HRT of 3 hours. At the HLC influent COD was 10 mg/l later reduced to 5mg/l for the NLC and the COD: BOD ratio was 1:1 The C: N ratio was thus maintained at 1:5.2. This carbon was not a source of nutrients for growth of the autotrophic nitrifiers, unlike the heterotrophic organism, nitrifiers use inorganic carbon from carbon dioxide or carbonate. Two carriers were shown to become clogged at HLC and one of the carriers demonstrated a noticeably longer transition period from HLC to NLC. Hence, in addition to investigating the two main loading phases, HLC and NLC, the clogged operation of the carriers and the

transitional operation were also investigated. Table 4.1 is a summary of the operational conditions.

Operational Condition	**Phase 1- High Loading Phase (HLC) ± 95% Confidence Interval**	**Phase 2- Low Loading Phase (NLC) ± 95% Confidence Interval**
SALR ($g\text{-}N/m^2 \cdot d$)	1.89 ± 0.12	0.91 ± 0.08
Temperature (˚C)	22.0 ± 0.5	21.0 ± 0.7
Dissolved Oxygen (DO) (mg/l)	9.0 ± 0.6	9.0 ± 0.3
pH	8.0 ± 0.0	8.0 ± 0.1

Table 4.1 Average and 95% confidence intervals of operating conditions for all reactors during both experimental phases.

4.3.2 Reactors

The four laboratory reactors were operated with three different carriers. Two 2 litre cylindrical Perspex reactors containing 50% by volume of Anoxkaldnes K3 carriers were operated to study the effects of K3 carriers and to verify duplication of the experimental design. The reactors were 12 cm in diameter and 17 cm in height. The K3 carrier is 7 mm in depth with a diameter of 10 mm with an available surface area of 500 m^2/m^3 (WEF, 2011).

Two 1.2 litre cylindrical Perspex reactors with 30% by volume fill of Anoxkaldnes P carriers and 20% by volume fill of Anoxkaldnes M carriers were operated to study the effects of the P and M carriers. These reactors were 10 cm in diameter with a height of 15 cm. The P carrier is 2mm in depth with a diameter of 48 mm and has an available surface area of $900m^2/m^3$ (WEF, 2011).

The M carrier is 2 mm in depth with a diameter of 48 mm and has a surface area for biofilm attachment of 1200 m^2/m^3 (WEF, 2011).

4.3.3 Synthetic Wastewater

A synthetic wastewater was used throughout the research period to provide stable loading rates with limited variations and to prevent the introduction of solids to the reactors; hence enabling an investigation of the biologically produced solids due to the nitrification process of the reactors. As such, the biologically produced solids were not subject to interactive effects with influent solids. This allowed for carrier and loading effects to be isolated to the detached and produced solids originating from the nitrifying biofilm. The synthetic wastewater was based on a variety of sources (De Beer et al., 1993; Rusten et al., 1994; Gieseke et al., 2001; Lee et al., 2004; Rochex et al., 2008 ; Karizmeh et al., 2014) and is as follows for NLC: $(NH_4)_2SO_4$:117.91 mg/L, $NaHCO_3$: 325mg/L, $MgSO_4.7H_2O$: 325 mg/L, $CaCl_2.2H_2O$:29.34 mg/L, KH_2PO_4: 79.09 mg/L, $FeSO_4.7H_2O$: 4.98 mg/L. Carbon source: $C_6H_{12}O_6$: 9.45 mg/L, NaAc: 5.04 mg/L, Peptone: 9.45 mg/L Trace nutrients; $MnCl_2.4H_2O$: 200mg/L, $NaMoO_4.2H_2O$: 49.62 mg/L, $CuSO_4.5H_2O$: 205.1 mg/L, $CoCl_2.6H_2O$: 2 mg/L, $ZnSO_4.7H_2O$: 59.82 mg/L.

The glucose, acetate and peptone were supplied to mimic the readily degradable carbonaceous content of wastewater at a concentration of 5 mg BOD/L at NLC. During the HLC phase of the study the ammonia, alkalinity and BOD were all proportionally doubled to maintain the same nitrogen to carbon ratio.

4.3.4 Nitrogen Constituent Analysis

Nitrogen constituent samples were collected in triplicate and filtered through 0.45µm glass fiber filters to reduce the effect of suspended particles on measured absorbance values. All measurements for nitrogen were carried out in accordance with the outlined methods in Standard Methods (APHA, 1989), Method 4500- NH_3 4500-NO_3^- B, and 4500-NO_2^-.

Dissolved oxygen (DO) and pH measurements were taken using a symphony Multi Parameter Meter with an attached DO probe and pH probe (VWR, Canada, Ontario). Temperature within the reactors was measured daily using a mercury glass thermometer. All constituent statistical analyses were based on 5 samples measured in triplicate (15 measurements) with statistical significance based on t-test with a p-value of 0.05 for statistical significance.

4.3.5 Solids Analysis

Total solids (TS), total suspended solids (TSS), volatile solids (VS) and volatile suspended solids (VSS) were measured using Standard Methods (APHA, 1989), 2540 D- Total Suspended Solids (total suspended Solids dried at 103-105˚C), 2540 E- Volatile Solids and Volatile Suspended Solids (fixed and volatile solids ignited at 550˚C) and 2540 B- Total Solids (total solids dried at 103-105˚C).

In an effort to investigate the smaller solids being produced by the reactors and observe the effects of carrier type and loading rates on size distribution and settleability a digital particle analyzer- 4100 (Brightwell Technologies Inc., Canada, Ontario) was used in low magnification mode. Digital particle analysis (DPA) enabled the distribution of particles between 2.25µm and 400µm to be measured. A filtration step was first required to remove the particles larger than 400 µm with a total solids analysis conducted on the trapped solid material.

Settlement effects on particle size distribution were investigated by analysing DPA prior to settling and after 30 minutes of settling. Due to the fact that the nitrification MBBR process produced a low concentration of effluent TSS, the sludge volume index (SVI) of the samples could not able to accurately measured. All solids analyses are based on 5 samples measured in triplicate (15 measurements), with statistical significance being based on t-test with a p-value of 0.05.

4.3.6 Biofilm Morphology, Mass and Thickness Analysis

Carriers were randomly selected from each reactor at both HLC and NCL to characterize the biofilm morphology. 20 randomly selected locations were used for imaging on the sampled carriers from each reactor. Biofilm morphology images were acquired using a Vega II-XMU variable pressure scanning electron microscope (VPSEM) (Tescan USA Inc., USA, Pennsylvania). Regular SEM requires destructive sample preparation which is not necessary due to the vapour chamber of the VPSEM.

The biofilm mass and thickness of the P carriers demonstrated four distinct operational phases during the study. In particular, the P carriers transitioned from a HLC (non-clogged phase) to a HLC (clogged phase). The carriers were then exposed to a normal load and were observed at NLC (transitional phase) and subsequently were observed at NLC (acclimatized phase). For each phase 20 randomly selected locations were used for imaging on sampled P carriers and subsequently three additional carriers were sampled from the reactor to measure the mass of the biofilm on the carrier. The carriers were centrifuged in 85ml centrifuge tubes for thirty minutes at 9000 rpm in a Sorvall centrifuge (Thermo Fisher Scientific, USA, Massachusetts). Subsequently, the samples were vortexed using a vortex-genie (Fisher Scientific, USA, New York) for 15 minutes at a maximum speed of 10. The TS and VS were measured on the resultant

extracted biofilm according to Standard Methods (APHA, 1989): 2540 B- Total Solids (total solids dried at 103-105˚C) and 2540 E- Volatile Solids (fixed and volatile solids ignited at 550˚C). Statistical significance for mass and thickness values across carrier types and between the 2 loading phases were based on t-test with a p-value of 0.05 for statistical significance.

4.4 Results and discussion

4.4.1 Nitrifying Kinetics

The SARR and the percent removal of ammonia were studied at two phases, HLC and NLC, to determine the effects of carriers on the operational performance of tertiary nitrifying MBBR systems (Figure 4.1 and Table 4.2). The duplicate K3 reactors confirmed successful repeatability of the experiments. Under HLC the K3 and P carriers performed similarly for a period of 35 HRTs. Both carriers demonstrated high and stable SARR and removal efficiencies values with minimum variations shown in Table 4.2 and Figure 4.1. The K3 and P carriers produced effluent ammonia concentrations below 2 mg-N/L.

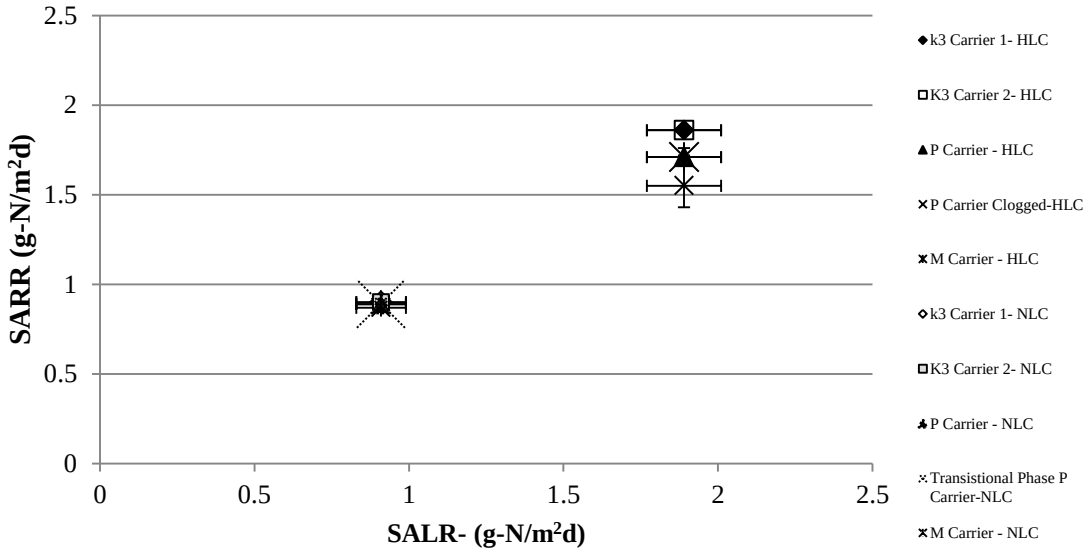


Figure 4.1 SARR versus SALR relationship for three carrier types at HLC and NLC

HLC (SALR 1.89 g-N/m^2·d)	K3 Carrier (R1)	K 3 Carrier (R2)	P Carrier	P Carrier (Clogged)	M Carrier (Clogged)
SARR (g-N/m^2·d)	1.86 ± 0.01	1.86 ± 0.00	1.86 ± 0.01	1.55 ± 0.12	1.71 ± 0.05
Min – Max SARR	1.85 – 1.88	1.85 – 1.87	1.84 – 1.88	1.39 – 1.67	1.56 – 1.81
Percent Removal efficiency (%)	98.7 ± 0.4	98.2 ± 0.4	98.5 ± 0.6	82.1 ± 5.4	90.6 ± 4.0
NLC (SALR 0.91 g-N/m^2·d)	**K3 Carrier (R1)**	**K 3 Carrier (R2)**	**P Carrier (Transitional)**	**P Carrier**	**M Carrier**
SARR (g-N/m^2·d)	0.90 ± 0.01	0.90 ± 0.01	0.89 ± 0.05	0.89 ± 0.00	0.88 ± 0.01
Min – Max SARR	0.90 – 0.92	0.90 – 0.92	0.80 – 0.89	0.89 – 0.89	0.88 – 0.89
Percent Removal efficiency (%)	98.8 ± 1.1	98.8 ± 1.1	97.2 ± 0.3	97.4 ± 0.2	95.9 ± 1.7

Table 4.2 Average kinetics and 95% confidence levels

The P carriers became clogged after 35 HRT cycles at HLC, subsequently in the clogged state the SARR and removal efficiencies were shown to be by approximately 16% lower. Further, the clogged P carriers showed a loss of operational stability as demonstrated by the observed variance in the SARR values and the percent removal shown in Table 4.2 and Figure 4.1. This loss of performance and operational instability resulted in a maximum recorded effluent ammonia concentration for the reactors. In particular, the effluent ammonia concentration increased from 1.2 mg-N/L to 17.8 mg-N/L for the unclogged P carriers as compared to the clogged P carriers. The P carriers remained clogged for the remainder of operation at HLC (an addition of approximately 80 HRTs), with no indication that the carriers would return to an

unclogged state.

The M carriers became clogged immediately during HLC operation. The M carriers hence demonstrated significantly lower SARR values and percent removals at HLC as compared to the K3 and P carriers. In addition as shown in Table 4.2 and figure 4.1, the M carriers demonstrated a loss of operational stability which was also observed in the clogged P carriers; indicated by the, SARR values, the large range in the min and max SARR and percent removal efficiencies. From an operational standpoint the performance reductions which accompany clogging events may result in an inability of treatment plants to meet strict effluent quality guidelines. At HLC the beneficial attributes of using carriers with higher specific surface areas are reduced by the occurrence of clogging events. The carrier with the highest specific surface area was shown to clog first followed by the carrier with the second highest specific surface area. Hence carrier clogging potential is of significant importance for tertiary nitrifying systems operating at or experiencing intermittent high loadings.

At NLC all carriers performed well and demonstrated high SARR values and high removal efficiencies with no statistical differences in rates or percentage removals between the various carriers. The P carriers demonstrated a transitional phase with respect to the production or detachment of solids for a period of approximately 300 HRTs. This extended transitional period of increased solids detachment observed for the P carriers did not result in a statistically significant reduction in the SARR or percent removal performance as compared to the acclimatized phase of the carriers or compared to the K3 and M carriers. As such, under normal or lower loading conditions the effects of carrier type on nitrification kinetics are shown to be similar to those observed by Ødegaard et al. (2000) for COD removal conditions; with the

nitrification kinetics being independent of carrier type and dependent upon the surface area available for biofilm attachment.

4.4.2 Solids Analysis

4.4.2.1 Total Suspended and Volatile Solids

An influent void of suspended solids was fed to all reactors throughout the experimental phase; hence the effluent solids are biologically-produced solids by the nitrifying biofilm attached to the various carriers. The general trend in TSS production of the various carriers at HLC demonstrates that there is no significant difference in the quantity of solids produced by the K3 and P carriers (Figure 4.2). There is however a significant reduction in the produced solids by the K3 and P carriers as compared to the clogged P and M carriers. The clogged carriers demonstrate a significantly lower production of solids than the non-clogged carriers with an average reduction in solids production of 29%. This reduction in TSS output is accompanied by an 18% average reduction in removal efficiency in the P carrier when compared to measured removal rates before clogging.

At NLC a similar trend is noted (figure 4.3); the measured effluent TSS values do not distinguish one carrier type from another. Here however the P carrier's transitional phase following the change from high loading to normal loading conditions is observed as having significantly greater solids production than the P carrier operation following its acclimatization to lower loading conditions. This increase in percentage of produced solids during an acclimatization period of approximately 300 HRTs is likely an effect of the clogged biofilm of the P carrier being shed from the carriers. The M carriers were also clogged during HLC but experienced a sloughing event shortly before the transition from high loading to normal loading

conditions.

This sloughing event allowed the M carriers to respond more quickly than the P carriers to the decrease in loading and as such the shedding of the M carrier biofilm was not recorded during the TSS measurements of the reactors. Without this sloughing event the P carriers required an additional 56 HRT cycles to transition to the normal loading operation. Though the SARR and percent ammonia removals are similar during the transitional period and the post transitional period (Table 4.2 and Figures 4.1), the quantity of produced solids differ significantly; suggesting that transitions from higher to lower loadings does not adversely affect ammonia removal, but may cause a temporary increase in effluent TSS.

The effluent VSS to TSS ratio for all carriers under high and normal conditions was stable at approximately 0.86 ± 0.05. A trend for reduced solids production at lower loading conditions as compared to the high loading condition is observed for the carriers with the K3 carriers (reactor 2) and the P carriers showing the greatest differences in TSS production at HLC as compared to NLC. This decrease in TSS production at lower loadings is in agreement with studies on COD removal MBBR systems which have shown decreased solids production with decreased loading (Aygun et al., 2008). A decrease in solids production is expected due to the lower ammonia removal rates (Metcalf & Eddy, 2002).

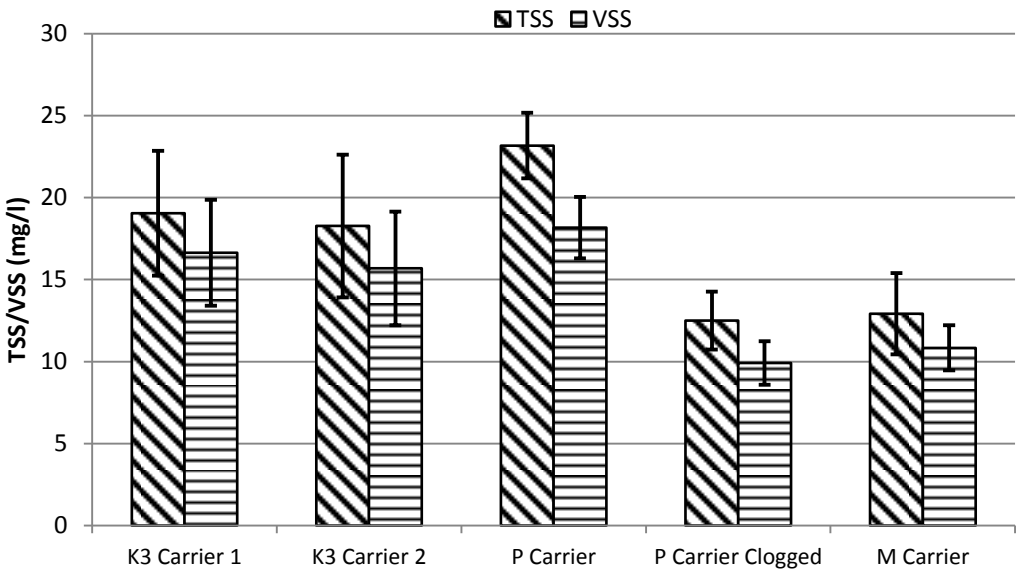


Figure 4.2 Average and 95% confidence intervals of TSS & VSS at HLC

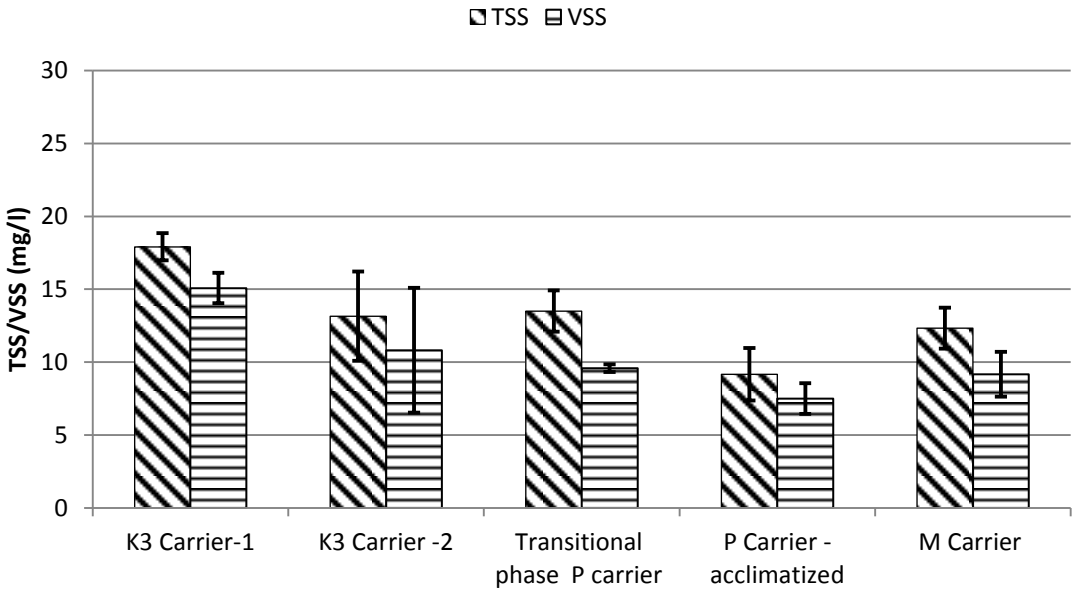
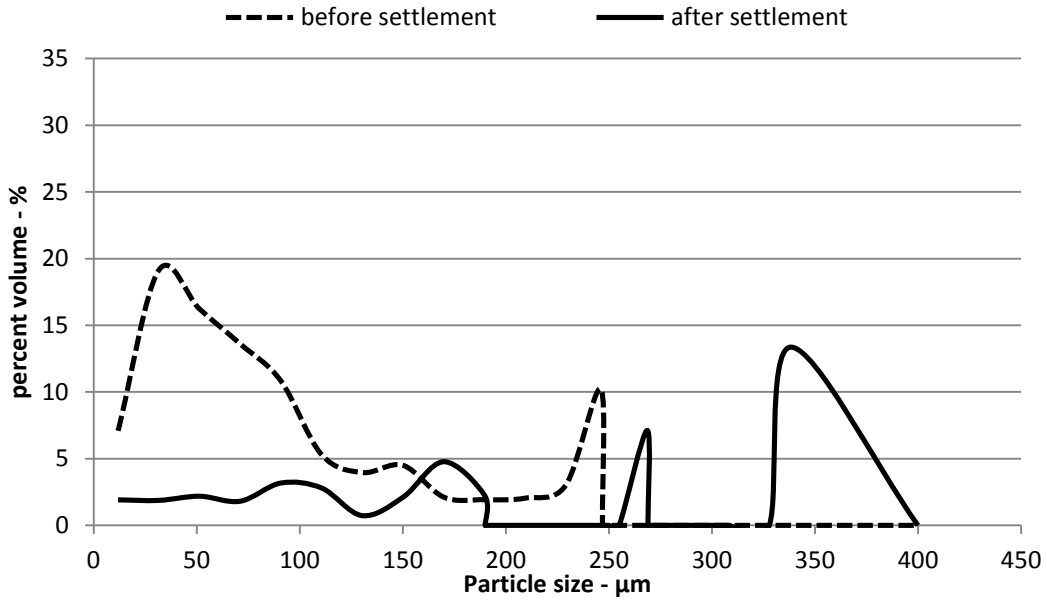


Figure 4.3 Average and 95% confidence intervals of TSS & VSS at NLC

4.4.2.2 Particle Size Distribution

The particle size distribution of the biologically produced solids was analysed for both unsettled and settled effluent to observe the effect of different carrier types on the size distribution and settleability of the effluent biologically produced solids under high and normal loading conditions. Figures 4.4 (a and b) show the K3 carrier to have a greater abundance of particles under 100 μm at the HLC than that of the P carrier and M carriers shown in in figures 4.4 c and 4.4 d, respectively. These small particles are seen to agglomerate after the 30 minute settlement period shifting the particle size distribution towards 200-400 μm particles. It is possible that these large particles are able to remain in suspension after the settling period because of their low density, it is understood that mat like agglomerations of particles have the ability to remain in suspension at larger sizes unlike denser particle agglomerates. As expected there is more efficient removal of the larger particle sizes with the 30 minutes settlement period than the smaller sizes. Under both the HLC and NLC (figures 4.4 and 4.5 respectively) particles greater than 400 μm were found to be less than 1% of the total volume percent.

The average and 95% confidence interval of the percentage of settled solids Table 4.3 are in accordance with previous research on COD removal MBBR systems that show less settleability of effluent solids under conditions of high surface area loading (Ivanovic & Leiknes, 2012). Hence higher loadings have demonstrated a trend towards higher effluent solids concentrations and lower solids settleability in tertiary nitrifying MBBR systems. This highlights to plant designers and operators an advantage to operating at lower loading conditions by designing larger reactor design or running at higher percent fills. Another option is the adoption of advanced solids separation techniques if high loading rates are unavoidable.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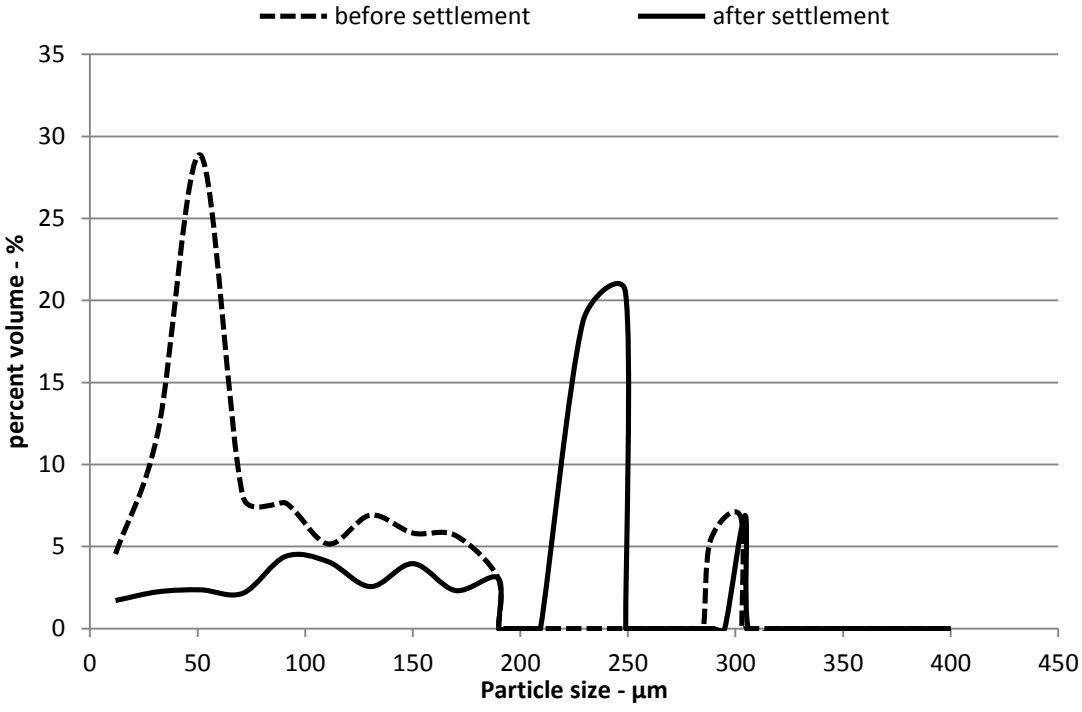
(a)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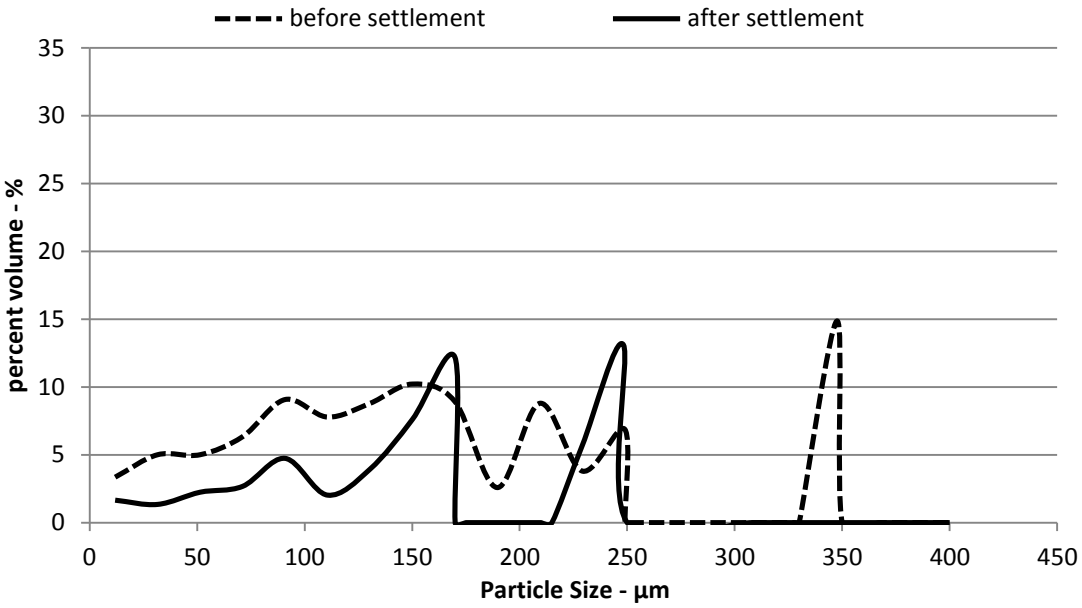

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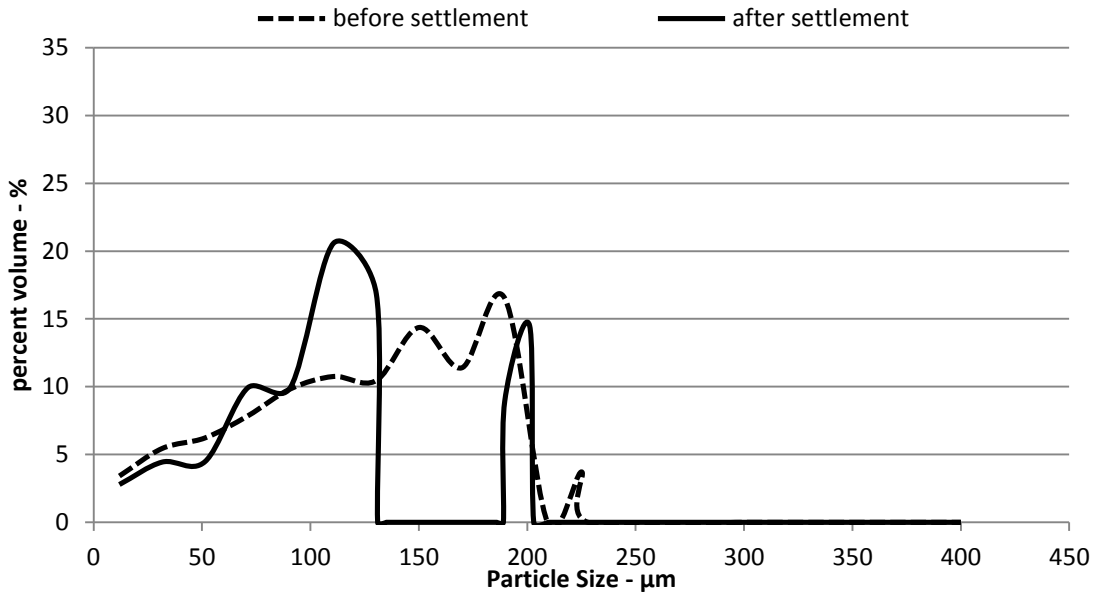
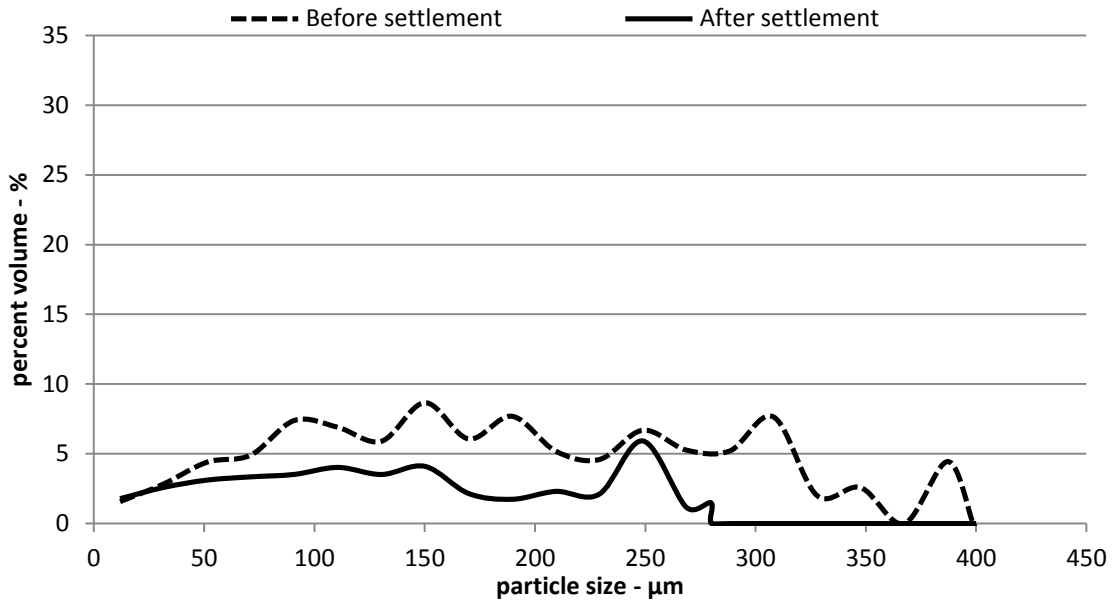


(d)

Figure 4.4 Particle size distribution at HLC (a) K3 Carrier 1, (b) K3 Carrier 2, (c) P Carrier and (d) M Carrier


Before settlement
After settlement
percent volume - %
particle size - µm
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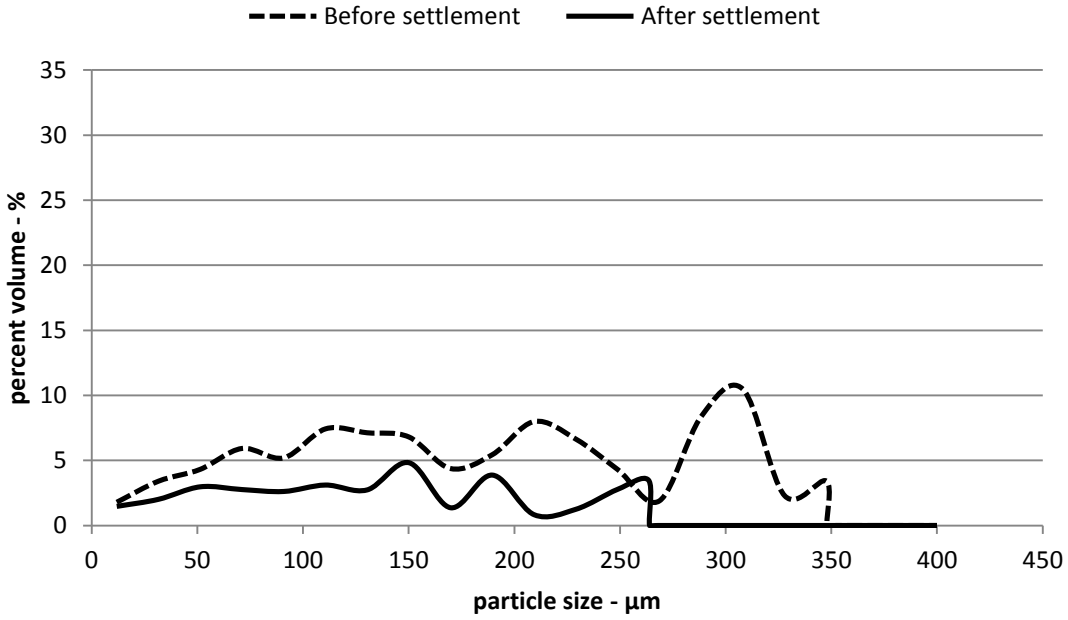
(a)


Before settlement
After settlement
percent volume - %
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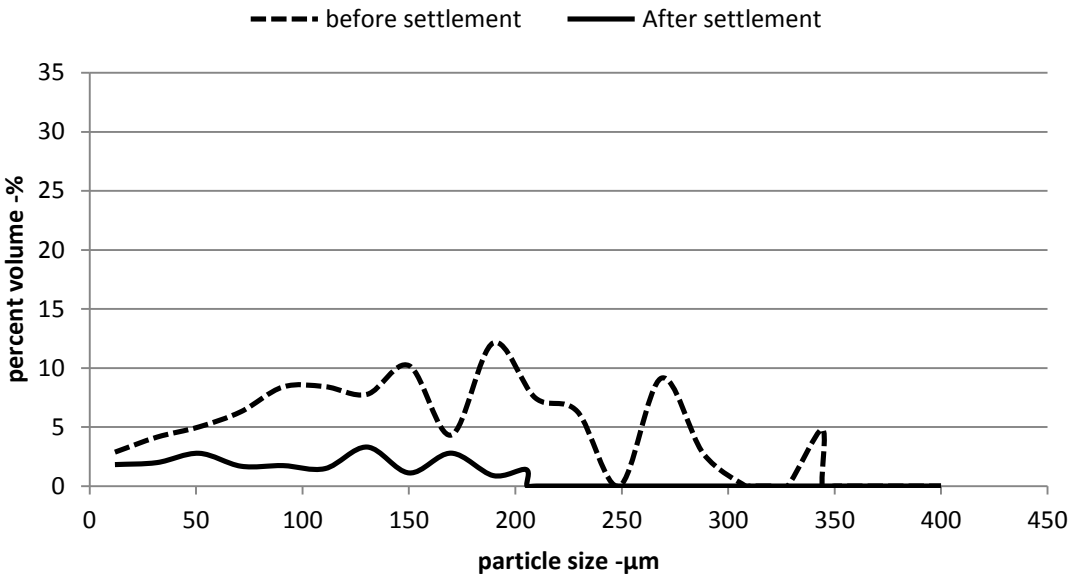
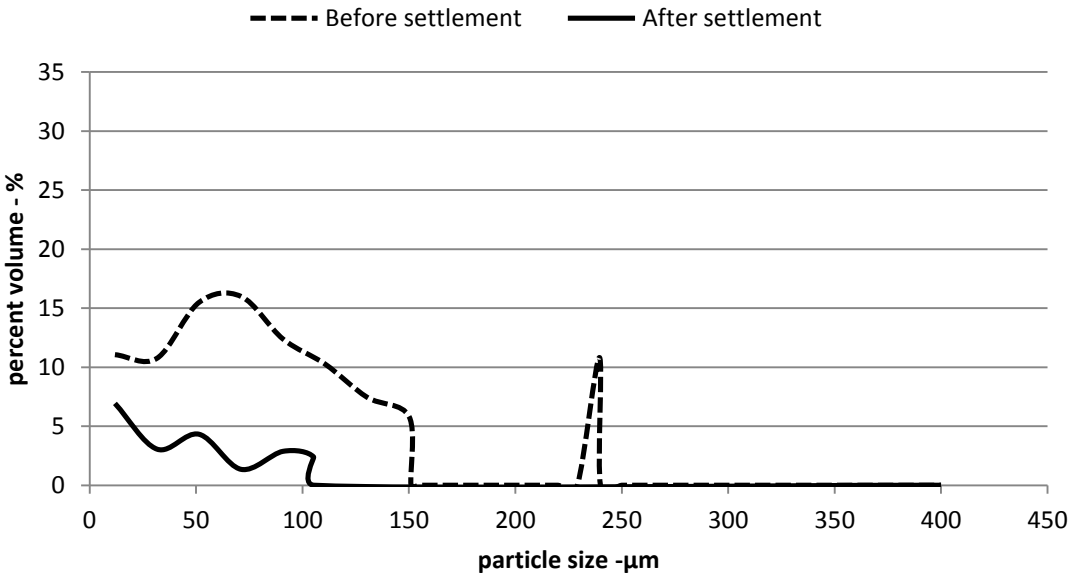
(b)



(c)



(d)

Figure 4.5 Particle size distributions at NLC (a) K3 Carrier reactor 1, (b) K3 Carrier reactor 2, (c) P Carrier and (d) M Carrier

Carrier type	Percent Settled at HLC	Percent Settled at NLC
K3 Carrier 1	56 ± 16	57 ± 11
K3 Carrier 2	55 ± 32	66 ± 6
P Carrier	59 ± 21	79 ± 3
M Carrier	11 ± 5	79 ± 4

Table 4.3 Solids removed after 30 minutes of settlement

4.4.2.3 Biofilm Mass and Thickness

The biofilm mass and thickness of the biofilm were analysed for the four distinct conditions of the P carrier (Figure 4.6). It is observed that both biofilm mass and total suspended solids are reduced from HLC to NLC. The transitional condition during the normal loading phase produced significantly more solids than its acclimatized phase. HLC saw greater biofilm mass on the carriers and this also translated to greater measured biofilm thicknesses. This is in agreement with existing literature which supports biofilm growth as increases in thickness due to restricted lateral growth on carriers (WEF, 2011).

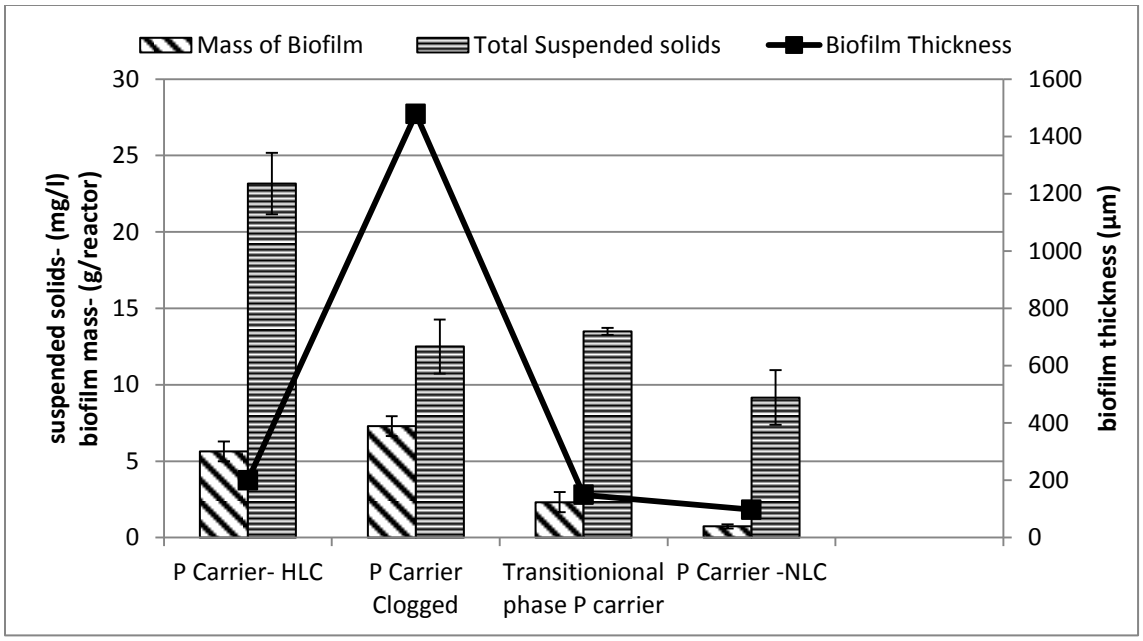


Figure 4.6 Suspended solids, reactor biofilm mass and biofilm thickness for the P Carrier.

4.5 Conclusion

This research confirms that MBBR design for tertiary nitrifying systems should be based on surface area available for biofilm attachment. It found no significant difference in removal efficiencies or solids production rates across different carrier types. It can be concluded that since larger surface area to volume carriers perform equally well and have smaller foot prints and operational cost, they may be considered a preferred selection for nitrifying MBBR systems. However the study has shown that larger surface area to volume carriers have a greater propensity to become clogged under high loading conditions with subsequent significant reductions in performance. In addition they require longer transitional periods which were observed to have higher than normal solids output. Essentially high surface area carriers do not operate as well under stressed conditions. Therefore for varying load designs and high loading conditions the lowest surface area carrier is proposed as preferential with all carriers equally

effective under low loading conditions.

The study has observed differences in produced biomass settleability between different carrier types with the P carrier showing the best results. The observation of poor settlement and higher solids production at high loading conditions further encourages low SALR operation.

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Chapter 5 Meso and Micro-scale response of tertiary nitrifying MBBR biofilm to variations in carrier type and loading

5.1 Setting the Context

The article presented in this chapter is entitled *Meso and Micro-scale response of tertiary nitrifying MBBR biofilm to variations in carrier type and loading* by Daina Forrest, Bradley Young, Robert Delatolla, Kevin Kennedy and Alain Stintzi and has been prepared for submission to the journal of Bioresource and Technology. It investigates the effects of three specific carrier types and two surface area loading rates on the biofilm thickness, morphology, microbial species as well as on the overall system ammonia removal rates and effluent solid settleability.

5.2 Introduction

The moving bed biofilm reactor (MBBR) was initially developed as a nitrogen removal system in the 1980's (Rusten et al., 1994), it quickly evolved to operate effectively as a chemical oxygen demand (COD) removal system and has been extensively studied in this regard (Ødegaard et al., 1994). The biomass and in particular the bacterial cells of MBBR systems are the functional units of attached growth systems and hence the MBBR technology. The structure of the biofilm matrix is of significant importance, as the biofilm matrix controls the rate of mass transfer of nutrients and substrates to the microbial community embedded in the biofilm. Hence, the performance of MBBR systems, measured at the macro-scale of influent and effluent concentrations, is ultimately dependent upon the meso-scale biofilm properties of the system and the micro-scale bacterial cell community.

The effect of MBBR carrier type at the macro scale has been studied for COD removal systems (Ødegaard et al., 2000). The research concluded that system performance is not dependent on carrier type but instead is a function of surface area loading rate (SALR). Despite this Ødegaard found inexplicable differences in effluent biomass settleability from the 2 different carrier types used in the study indicating the possibility of a difference in biofilm morphology between different carriers and highlighting the a potential difference in the bacterial community.

Furthermore, previous work on COD removal MBBR systems have shown increases in biofilm thickness as a response to increases in loading rates (Wang et al., 2005). More recently, Karizmeh et al. (2014) found that the greatest biofilm thickness was measured at the highest COD loading rate. The study also revealed differences in biofilm morphology, with low density mushroom like structures observed at low loading and filamentous like morphologies observed at higher loading. These differences in morphologies suggest a potential difference in the microbial communities within the biofilm.

Similar studies examining the effects of carrier type and loading effects have yet to be carried out on tertiary nitrifying MBBR systems; with tertiary nitrifying MBBR systems becoming increasingly popular as cost effective and efficient upgrade systems to current treatment plants with the implementation of more stringent regulation in many countries (Verma et al., 2006) (Canada Gazette, 2012). In particular, there is no research on biofilm thickness, morphology and microbial population community shifts in response to carrier types and surface area loading rates in tertiary nitrifying MBBR systems. Population shifts have been observed in nitrifying trickling filters, with low ammonia environments being dominated by *Nitrosospira* while high ammonia concentrations were shown to harbour *Nitrosomonas* as the dominant AOB (Zhang et al., 2013).

The study also found *Nitrobacter* to be the dominant NOB with high substrate availability and *Nitrospira* being dominant during starved conditions.

A true understanding of the meso and micro-scale effects of carrier type and loading on tertiary nitrifying MBBR units is critical to the further development and optimizing of these systems for tertiary nitrification. Therefore the aim of this study is to establish the effect of carrier type and surface area loading rate (SALR) at the meso and micro-scale within tertiary nitrifying MBBRs. It specifically focuses on biofilm thickness and morphology, bacterial community analysis and cell viability in response to carrier type and variations in SALR as well as their correlation to ammonia removal rates and effluent settleability.

5.3.1 Experimental Design

Four lab scale reactors with 3 different carrier types were operated under identical conditions at two distinct loading conditions, a high loading condition (HLC) of 1.9 g-N/m^2·d and a normal loading condition (NLC) of 0.9 g-N/m^2·d. The reactors were operated at a HRT of 3 hours, pH of 8.0 ± 0.1, a Dissolved Oxygen concentration (DO) of 9.0 ± 0.9 mg/l and were fed with synthetic wastewater. A synthetic wastewater was used throughout the research period to provide stable loading rates with limited variations based on a variety of sources (De Beer et al., 1993; Rusten et al., 1994 ; Gieseke et al., 2001; Lee et al., 2004; Rochex et al., 2008; Karizmeh et al., 2014) and is as follows for NLC: $(NH_4)_2SO_4$-117.91 mg/L, $NaHCO_3$- 325mg/L, $MgSO_4.7H_2O$- 325 mg/L, $CaCl_2.2H_2O$-29.34 mg/L, KH_2PO_4- 79.09 mg/L, $FeSO_4.7H_2O$- 4.98 mg/L. Carbon source: $C_6H_{12}O_6$- 9.45 mg/L, NaAc- 5.04 mg/L, Peptone- 9.45 mg/L Trace nutrients; $MnCl_2.4H_2O$- 200mg/L, $NaMoO_4.2H_2O$- 49.62 mg/L, $CuSO_4.5H_2O$- 205.1 mg/L, $CoCl_2.6H_2O$- 2 mg/L, $ZnSO_4.7H_2O$- 59.82 mg/L.

The glucose, acetate and peptone were supplied to mimic the readily degradable carbonaceous content of wastewater at a concentration of 5 mg BOD/L under NLC. During the HLC phase of the study the ammonia, alkalinity and BOD were all proportionally doubled to maintain the same nitrogen to carbon ratio.

Reactors 1 and 2 were 2L cylindrical reactors operating with 50% fill of K3 Anoxkaldness biofilm media. The K3 Carrier is 10 mm in diameter and 7 mm deep with an available surface area of 500 m^2/m^3. Reactor 3 and 4 were 1.2 L cylindrical reactors operating with 30% fill of the P carrier and 20% of the M carrier respectively. The available surface area of the P carrier is 900 m^2/m^3, while the surface area of the M carrier is 1200 m^2/m^3.

5.3.2 Nitrifying Kinetics

Effluent concentrations of ammonia, nitrite and nitrate were measured in accordance with the outlined methods in Standard Methods (APHA, 1989), Method 4500- NH_3 , 4500-NO_3^- B, and 4500-NO_2^-. 5 samples were collected in triplicate over a 7 day period. All collected samples were filtered through a 0.45µm filter before testing to reduce the effect of suspended solids on measured values.

5.3.3 Settleability of Biologically-Produced Solids

Due to the fact that the nitrification MBBR process produced a low concentration of effluent TSS, the sludge volume index (SVI) of the samples could not able to accurately measured. The settleability of the solids produced by the reactors were investigated using a digital particle analyzer (Brightwell Technologies Inc., Canada, Ontario). Digital particle analysis (DPA)

enabled the distribution of particles between 2.25 µm and 400 µm to be measured. Using DPA to quantify the particle distribution of the solids prior to and following 30 minutes of settling enabled the settleability of the solids to be quantified in this study. 3 effluent samples were collected from each reactor and DPA runs carried out in triplicate.

5.3.4 Biofilm Morphology and Thickness

Biofilm morphology and thickness were observed through the use of a Vega II-XMU variable pressure scanning electron microscope (VPSEM) (Tescan USA Inc., USA, Pennsylvania). During each phase of the study a carrier was randomly selected from each reactor and without any pre-treatment analysed at 40 Pa within the VPSEM chamber. Images were taken at 20 points across the carrier with magnification ranging from x20 to x5000. Though VPSEM results in some shrinkage of the biomass and EPS layers it eliminated the need for destructive sample preparation which is a necessity of traditional SEM (Flemming et al., 2000).

5.3.5 Bacterial Population

5.3.5.1 Cell Viability

One carrier was randomly selected from each reactor for analysis during both the HLC and NLC. The carrier was cut to expose the inner surfaces and stained with Propium Iodide and Syto 9 of the film Tracer^{TM} LIVE/DEAD$^{®}$ biofilm viability kit (Life Technologies, Ontario, Canada).Confocal light scanning microscopy (CLSM) has become a conventional method for the enumeration of micro-organisms on surfaces and the characterization of biofilms. CLSM allows for the non – destructive optical sectioning of the biofilm. An LSM 510/Axio imager M.1 confocal microscope (Carl Zeiss Canada Ltd, Ontario, Canada) equipped with argon and helium-neon lasers was used to image the biofilm samples in this study. A total of 20 images per reactor

or 4 stacks of approximately 30 µm thick were captured for each study phase and were analysed using NI Vision Assistant 7.1 (LabView 8.0- National Instruments Canada, Vaudreuil-Dorion, Canada). The biofilm area and the area of the fluorescent red and green illuminated cells were quantified (Delatolla et al., 2010).

5.3.5.2 DNA Extraction and Amplification

For each set of microbial community data, 0.25 g of biofilm was abraded from the media into a 1.5 mL sterilized Eppendorf tube. DNA was extracted from the biofilm using FastDNA® spin kit (MP Biomedicals, Santa Ana, CA) where the DNA was stored at -80°C until it was used for library construction.

DNA amplification utilized a two-step polymerase chain reaction (PCR) targeting the V6 hyper-variable region of the 16s rRNA was achieved using Phusion® High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA). The primers used for the first and second PCR reaction were selected from a barcoding approach previously described by Arthur et al. (2012) and Abujamel et al. (2013). The PCR amplicons were inspected by electrophoresis on a 2% agarose gel and purified with a Montage PCR96 cleanup kit (EMD Milipore, Billerica, MA). The purified amplicons were quantified using Quant-iT™ dsDNA HS Assay Kit (Life Technologies, Burlington, Canada) and pooled with 200 ng of DNA from each sample to be sequenced in one lane of an Illumina Hiseq2500 at the center for applied genomics (TCAG, Toronto, Canada). TCAG generated paired-end reads 2x100 base pairs.

5.3.5.3 DNA sequencing analysis

The entire DNA sequencing analysis was disseminated using Bio Linux operating platform. The paired-end reads were assembled using the fast length adjustment of short reads (FLASH) software (Majoc and Salzberg, 2011) and quality filtered using the fastq_quality_filter command from the Fastx toolkit with the minimum quality score of 20 over 97% of the sequences. Reads that passed the quality filter were demultiplexed and the barcodes were trimmed using Novobarcode (Goecks et al., 2010). The quantitative insights into microbial ecology (QIIME) software, version 1.8, (Majoc and Salzberg, 2011) was used to compute operational taxonomical unit (OTU) clustering with a closed reference of 97% sequence similarity. QIIME compared the OTUs with UCLUST against the Greengenes database 13.8; the singletons were removed and the relative abundance of the bacterial taxa present in the biofilm was determined (Caporoso et al., 2010). Linear discriminate analysis (LDA) was performed using Galaxy software (Goecks et al., 2010; Blankenberg et al., 2010; Giardine et al., 2005).

5.3.6 Statistical analysis

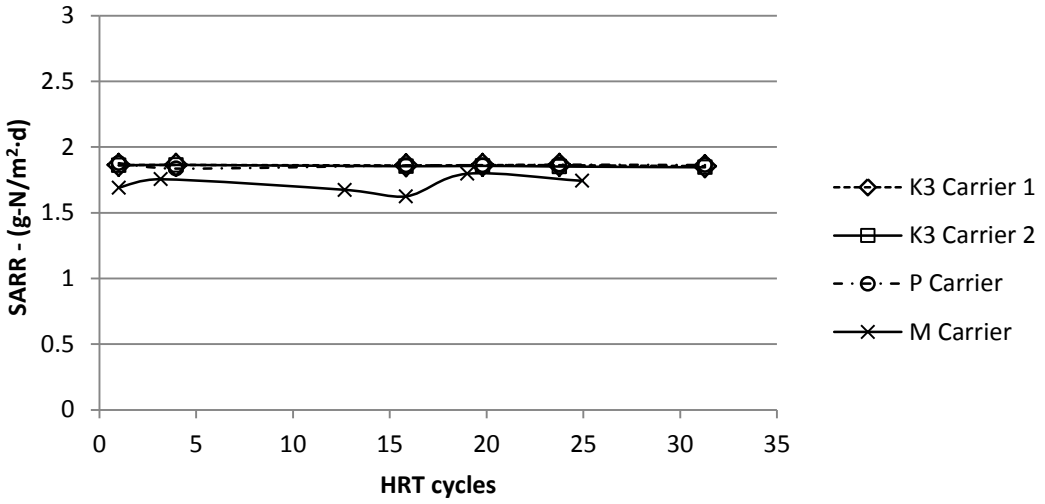
All constituent statistical analyses were based on 5 samples taken in triplicate (15 measurements); biofilm thicknesses were based on 20 measurements acquired from 5 images; cell viability analyses were based on 20 acquired images and sequencing percent abundances were based on 5 samples. Statistical significance of measured constituents, biofilm thickness, cell viability and percent abundance values across carrier types and between the two loading phases were tested by comparison of 95% confidence intervals and confirmed by the t-test with a p-value of 0.05 for statistical significance. All constituent, biofilm thickness, cell viability and percent abundance samples were acquired at steady state.

For the bacterial community shift analysis, statistical significance of the population shifts were tested using LDA analysis, which subjects the data to the Kruskal Wallis test and assigns an LDA log-score; with and LDA score of 2 or greater being considered statistically significant (Segata et al., 2011). T-test was also used for statistical significance with P values 0.01 and 0.001.

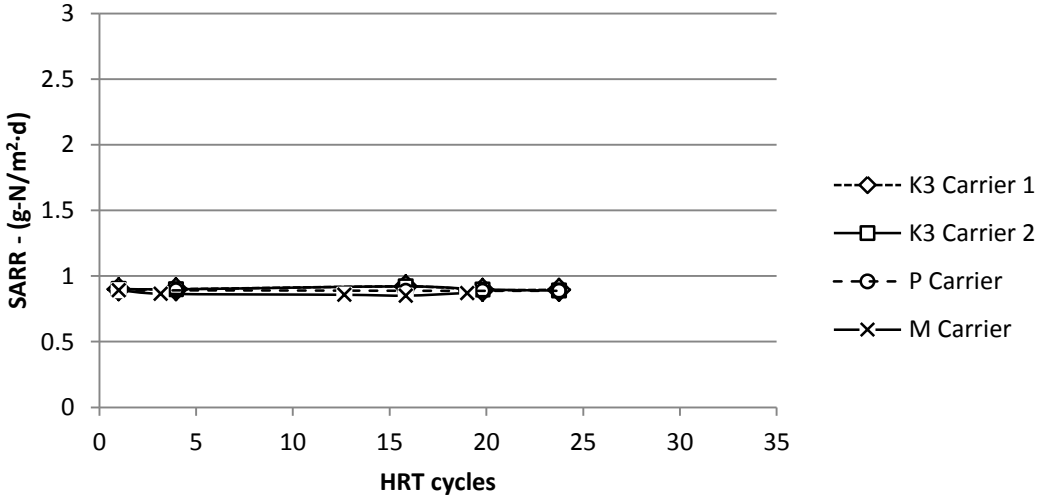
5.4 Results and Discussion

5.4.1 Nitrifying Kinetics

No significant difference in the substrate removal rates was observed for different carrier types with the exception of the M carrier at HLC (Figure 5.1 a). The lower SARR values of the M carrier at HLC are attributed to the clogging of the carrier's pore spaces due to excessive biofilm growth, leading to reduced biofilm surface area for substrate penetration. The lower SARR values of the M carrier at HLC coincided with fluctuations in the effluent ammonia concentrations. At both HLC and NLC(figure 5.1a and 5.1b) high removal efficiencies were maintained with system removal efficiencies averaging 96.5% at HLC and 97.7% at NLC with surface area removal rates (SARR) of 1.82 ± 0.01 and 0.89 ± 0.01 g-N/m^2·d, respectively . Nitrogen mass balance showed an average loss of 4% influent nitrogen possible through testing errors or volatilization of ammonia.



(a)



(b)

Figure 5.1 Kinetics at (a) HLC and (b) NLC

5.4.2 Settleability

The average removal of particles after 30 minutes of settling at HLC for all carriers was 45 ± 19%, with the P carrier (59% removal) producing effluent with slightly better settling characteristics than the K3 carrier (56% removal), the M carrier (11% removal)was observed to show the poorest solids removal with settlement. At NLC the average percentage of particles removed by settlement was 70 ± 6%, the M and P carrier demonstrated similar settling (79 %removal) and the K3 carrier (62% removal) showed the least removals. Hence, the study shows that high loading conditions caused a reduction in particle settleability. This is in agreement with previous studies on COD removal MBBR systems that showed poor biomass settleability at higher loads (Ivanovic & Leiknes, 2012; Ivanovic et al., 2006).

5.4.3 Biofilm Thickness and Morphology

VPSEM results (figure 5.2) show thicker biofilms at HLC than NLC. This observation is expected as higher substrate concentrations promote deeper substrate penetration into the biofilm as well as greater cell reproduction and hence a thicker biofilm (Gerardi, 2002). Results also offer a comparison of the biofilm growth across different carrier types and demonstrate no significant differences in biofilm thickness between the K3 and P carriers. However the biofilm thickness of the M carrier at HLC and NLC differs significantly from both the K3 and P carriers. The observed significantly thicker biofilm of the M carrier at HLC corresponds to the clogging of the M carrier. Under HLC the M carrier demonstrated lower SARR values compared to the K3 and P carriers in addition to demonstrating variance and fluctuation in the effluent ammonia concentrations (Figure 5.1 a).

Under NLC the M carrier also showed a larger biofilm thickness than the other carrier types; during this phase however its SARR value did not significantly differ from those of the K3 and P carriers. Therefore for tertiary nitrifying MBBRs biofilm thickness is not a direct indicator of system performance, however under high loading conditions excessive growth and thickness can reduce nitrifying kinetics.

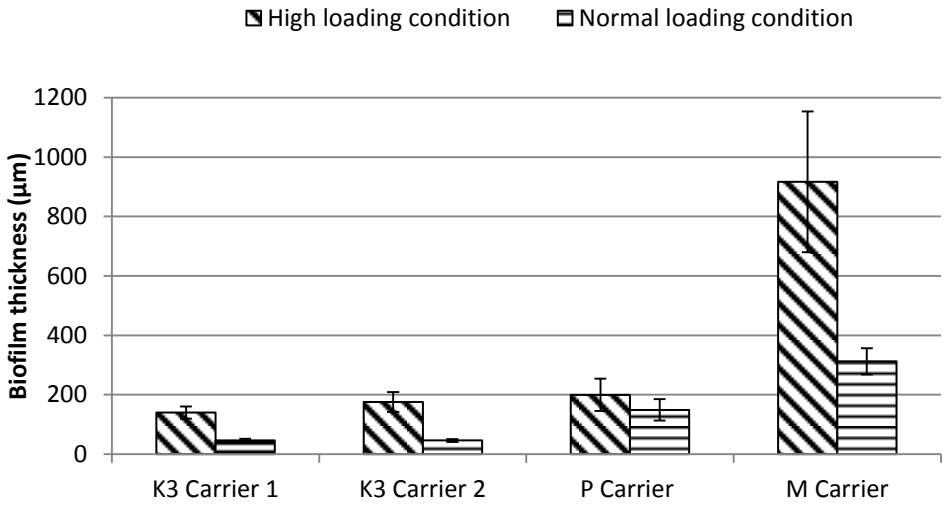


Figure 5.2 Biofilm thickness across carrier type and loading condition

Researchers studying MBBR COD removal systems were able to observe distinct differences in the biofilm morphology between different loading conditions; clear shifts from thick to fibrous films and clear changes in density (Karizmeh et al., 2014). Fibrous structural elements were not observed in the tertiary nitrifying biofilm grown of this study (Figures 5.3 and 5.4). The acquired VPSEM images do however highlight differences in the protozoa, nematodes and water mites communities across carrier type and across loading conditions. At HLC an abundance of water mites on the K3 Carrier can be noted, as well as their absence from the M and P carriers (Figure 5.3). In addition there are numerous ciliates present at the surface of the P and M carriers biofilm at HLC, which are absent from the K3 Carrier. Stalked ciliates were seen to be the predominant

feature on the P carrier while free-ciliates and nematodes tend to be more dominant on the M Carrier. It is possible that the micro-environments developed on each carrier type differ and as a result each carrier type promotes the proliferation of different species. At NLC the VPSEM images show the absence of the protozoa, nematodes and water mites seen at HLC and demonstrate distinctly thinner biofilms at NLC as compared to HLC (Figure 5.4).

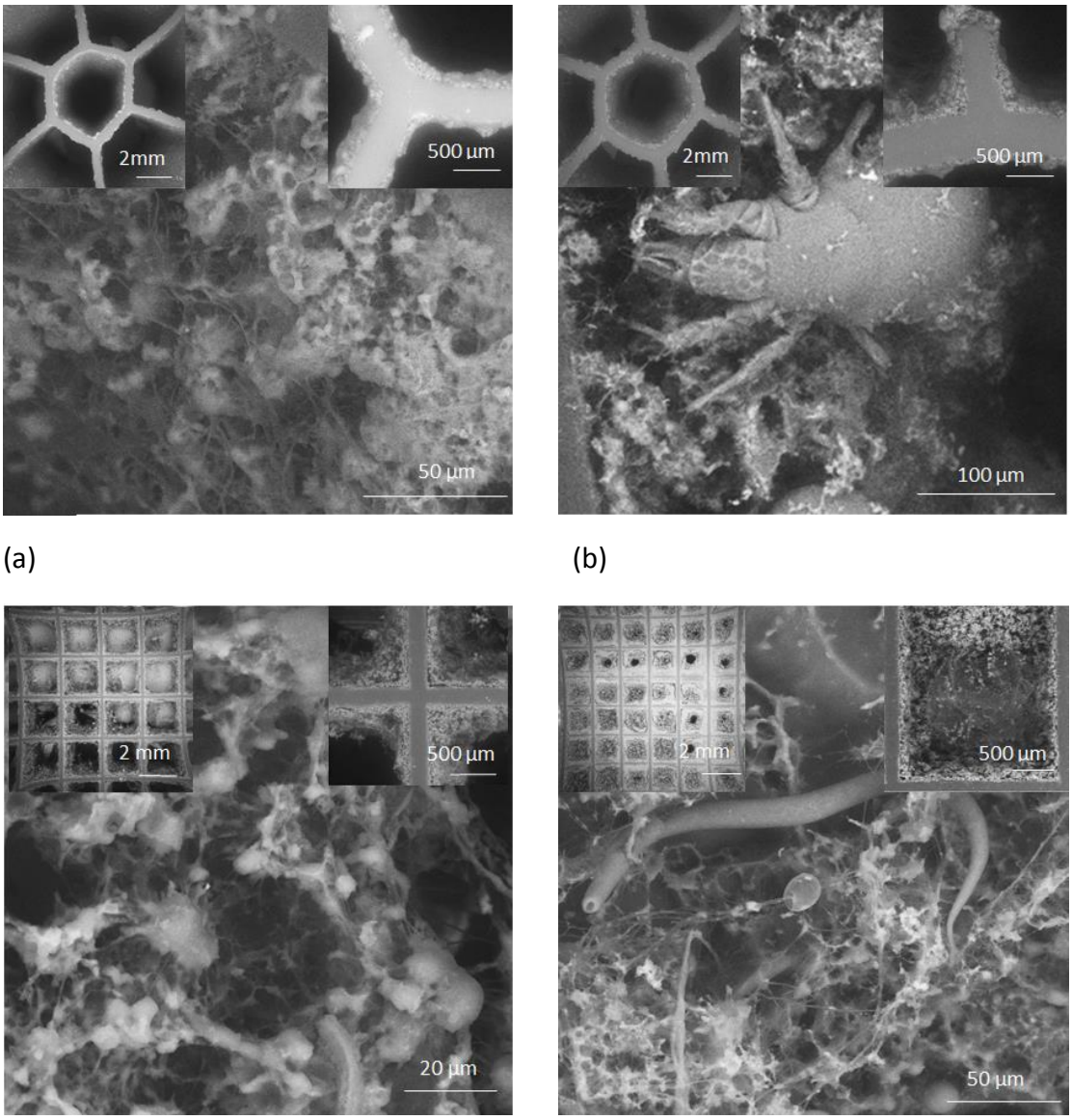


Figure 5.3 VPSEM HLC images (a) K3 Carrier 1, (b) K3 Carrier 2, (c) P Carrier and (d) M Carrier

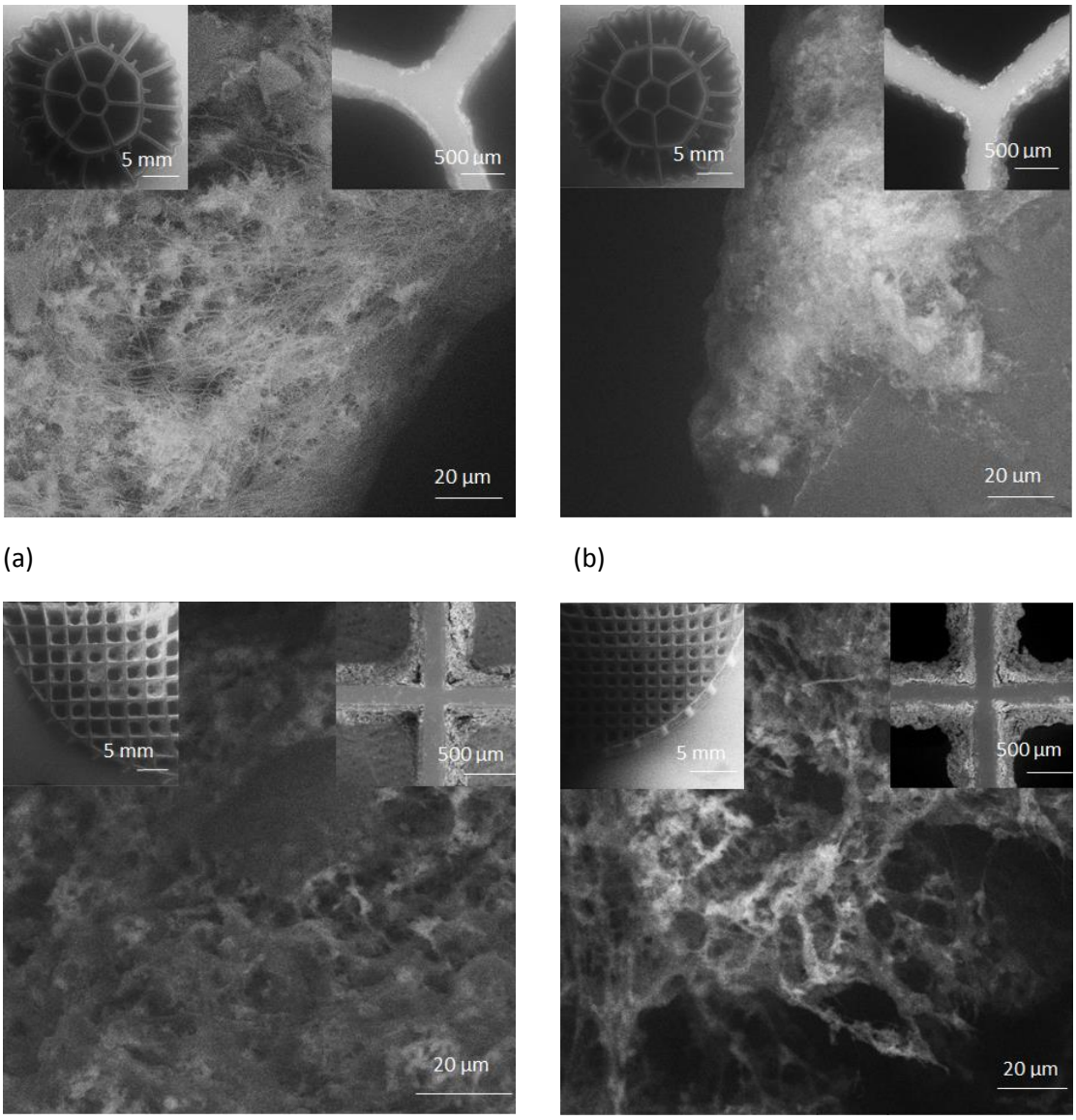


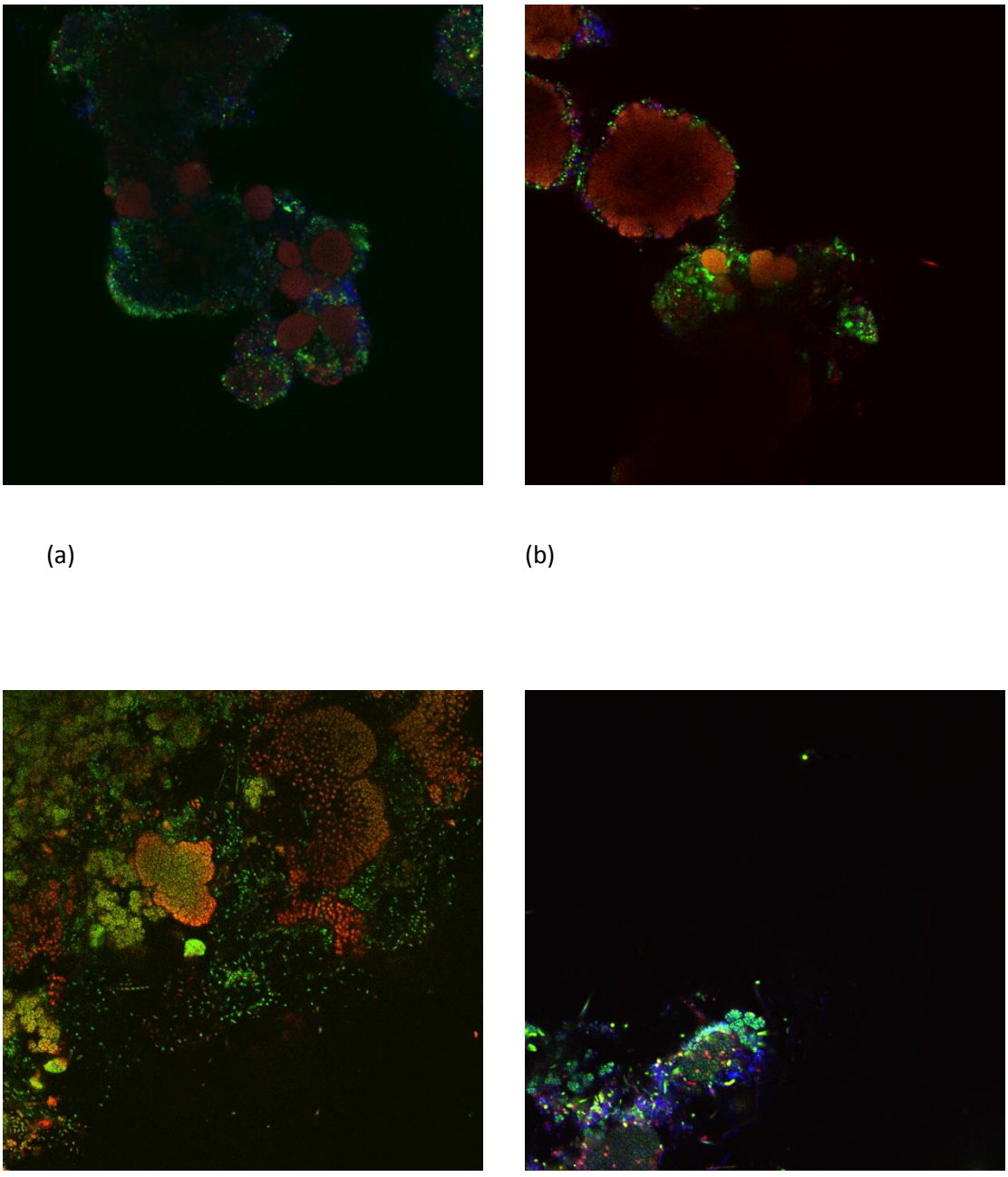
Figure 5.4 VPSEM NLC images (a) K3 Carrier 1, (b) K3 Carrier 2, (c) P Carrier and (d) M Carrier

5.4.4 Biomass Viability

The viability test illuminates live cells as green and dead cells as red in images captured at depth in the biofilm by CLSM (Figure 5.5). The percent of live and dead cells were quantified in the upper 30 µm thick biofilm section of the samples (Figure 5.6). Viability analysis was limited to a

depth of 30 μm to ensure that when analyzing locations of thinner biofilm (approximately 30 μm) the same depth and number of images acquired were consistent for all locations analyzed. Further, the upper 30 μm of the biofilm is believed to be the most active section of the biofilm.

No significant difference was observed between HLC and NLC with respect to the percent of total cells, percent of live cells or percent of dead cells present per area of biofilm in the upper 30 μm of the biofilm (Figure 5.6). As such, no difference in percentage of cells was observed at various loading conditions despite the fact that thicker biofilms were shown at HLC as compared to NLC. The percent coverage of total and live cells in the upper 30 μm of thick biofilm at higher loading conditions is consistent with the percent coverage of total and live cells in thinner biofilm at lower loading conditions. Hence, this suggests that the activity of the cells themselves and not cell coverage is linked to kinetic performance.



(a) (b)

(c) (d)

Figure 5.5 CLSM images at 6 µm depth for (a) K3 Carrier 1, (b) K3 Carrier 2, (c) P Carrier and (d) M Carrier

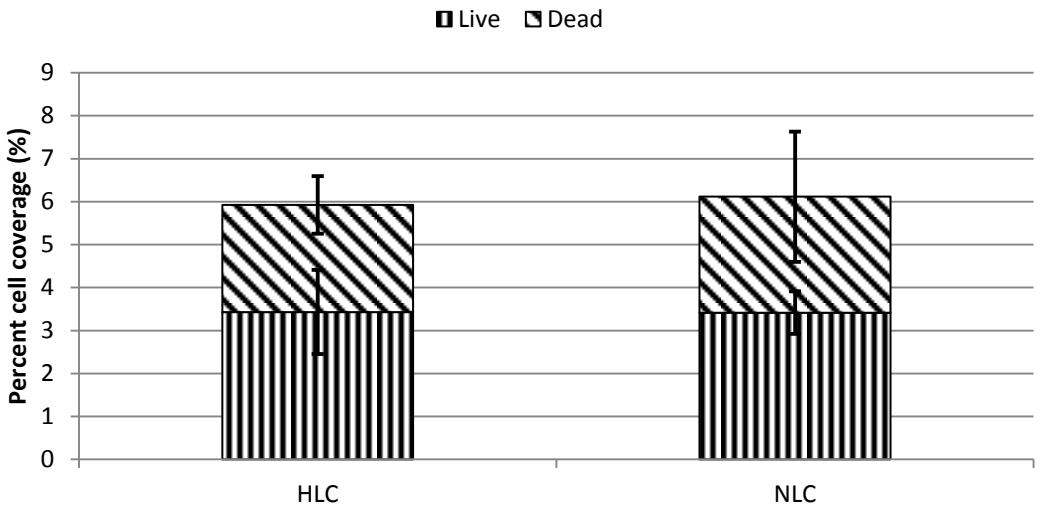


Figure 5.6 Percent coverage of total cells per biofilm area.

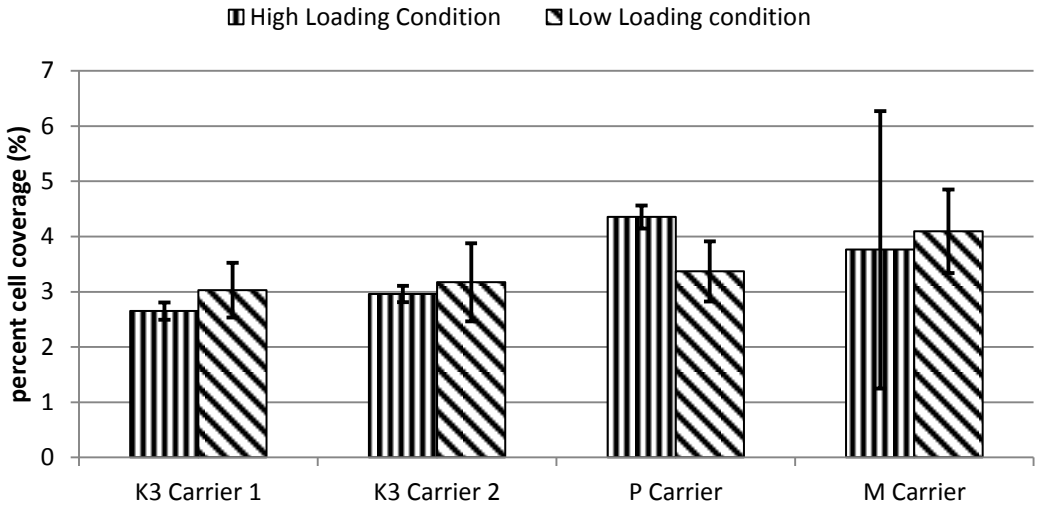


Figure 5.7 Percent coverage of Live cells per biofilm area at HLC and NLC

The research was also aimed at determining effects of carrier type on the viability of the biomass. The various carriers investigated in this study demonstrated similar percent coverage of live cells per biofilm area irrespective of carrier type at HLC and NCL (Figures 5.7). The M and P carriers, however, did demonstrate a significantly higher percent coverage of dead cells as

compared to K3 carriers at NLC. This significant difference in dead cell coverage can be due to the fact that the M carriers were consistently clogged during operation at HLC. Subsequently, during the transition from HLC to NLC a large change in biofilm thickness occurred in the M Carriers, where the thickness of the biofilm was reduced by approximately half (Figure 5.2). This sloughing of the biofilm thickness may have exposed deeper areas of biofilm that was less active due restrictive mass transferring of nutrients and substrates during the excessive overgrowth observed during the clogging condition of M carriers.

Although the biofilm thickness measurements of the P carrier do not demonstrate a large change in biofilm thickness between HLC and NLC, the P carriers are believed to have experienced excessive biofilm growth at the end of the HLC experimental phase. This excessive growth was not captured by the VPSEM imaging of the study and thus does not appear in Figure 5.3. The excessive growth period, just prior to the transition to NLC, is believed to have caused similar condition to the clogged M carriers and may be responsible for the observed significant increase in dead cell coverage of the P carriers as compared to the K3 carriers. The clogging at HLC observed in this study occurred in the carriers with the smallest pore spaces, where the M carrier (1200 m^2/m^3) clogged immediately, the P carrier (900 m^2/m^3) clogged at the end of the HLC and the K3 carrier (500 m^2/m^3) did not experience any clogging events in this study.

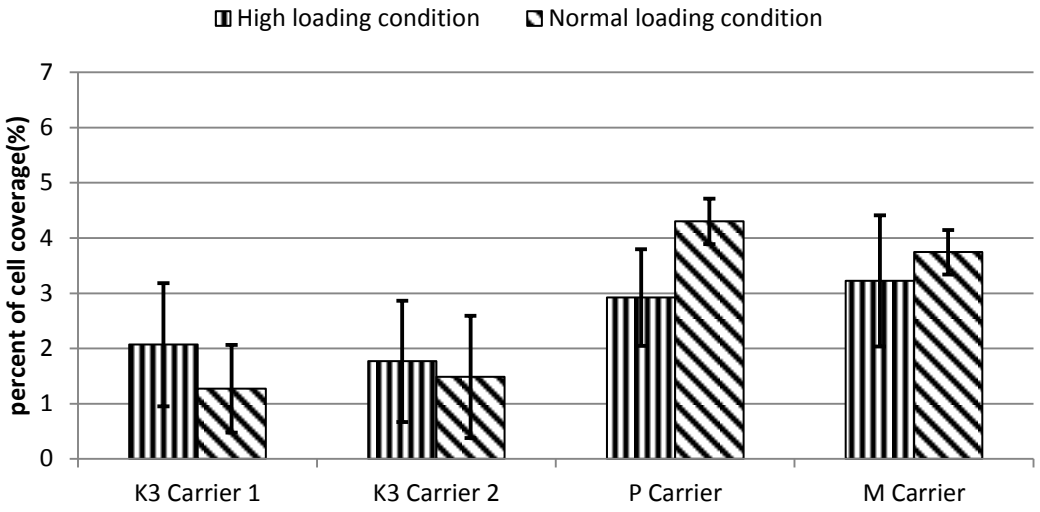


Figure 5.8 Percent coverage of dead cells per biofilm area at both HLC and NLC

The ammonia removal rate was expressed per biofilm volume by dividing the SARR by the biofilm thickness in an aim to evaluate the biofilm volume ammonia removal rate (BVRR) across carrier type and loading conditions (Hoang et al., 2014)

$$\boldsymbol{BVRR = \frac{SARR}{B_T}} \qquad \textbf{5.1}$$

Where BVRR (g-N/m^3.d) is biofilm volume ammonia removal rate, SARR (g-N/m^2.d) is surface area removal rate and B_T is biofilm thickness.

The ammonia removal rate was expressed per live cell volume by dividing the SARR by the live cell coverage of the biofilm in order to evaluate the viable cell ammonia removal rate (VCRR) across carrier type and loading conditions (Hoang et al., 2014).

$$VCRR = \frac{SARR}{B_T \times L_C} \quad \textbf{5.2}$$

Where VCRR (g-N/m^3.d.%viable cells) viable cell ammonia removal rate, SARR (g-N/m^2.d) is surface area removal rate, B_T is biofilm thickness and L_C (%) is percent live cell coverage.

At HLC the BVRR of the K3 and P carriers do not differ significantly, while the M carrier BVRR is significantly lower than both the K3 and P carriers (Table 5.1); indicating that the thicker biofilm on the M carrier at HLC is less efficient with respect to both SARR and ammonia removal kinetics relative to total attached biofilm. The VCRR of the K3 carriers at HLC are significantly higher than both the P and M carriers. Further, the VCRR of the P carrier is significantly greater than the M carrier. Hence, the cells embedded in the K3 carriers, the carriers with the lowest surface area per volume, demonstrate the highest level of cellular activity compared to the P and M carriers.

At NLC there are significant differences between the BVRR and VCRR values of the K3 carriers as compared to both the P and M carriers; with no statistical difference observed between the BVRR or VCRR values of the P and M carriers (Table 5.1). Hence, under normal loads all the carriers demonstrated similar and highly efficient SARR values; while the carriers with the lowest surface area per volume, K3, demonstrated the highest cellular activity compared to the P and M carriers.

Microscopy results (Figure 5.9) highlight no significant difference in the percent of cells within the biofilm. Suggesting that the substrate limitations associated with biofilms will do not occur in tertiary nitrifying films which are thinner than 30 μm.

HLC	BVRR $\times 10^3$ (g-N/m^3·d)	VCRR $\times 10^3$ (g-N/m^3·d·%viable cells)
K3 Carrier 1	13.3 ± 2.0	5.0 ± 0.0
K3 Carrier 2	10.6 ± 2.1	3.6 ± 0.0
P Carrier	9.3 ± 2.6	2.1 ± 0.0
M Carrier	1.9 ± 0.5	0.5 ± 0.0
NLC		
k3 Carrier 1	19.4 ± 2.4	6.4 ± 0.1
K3 Carrier 2	19.6 ± 2.2	6.2 ± 0.1
P Carrier	9.1± 3.5	2.7 ± 0.0
M Carrier	10.5 ± 5.7	2.6 ± 0.0

Table 5.1 BVRR and VCRR at HLC and NLC

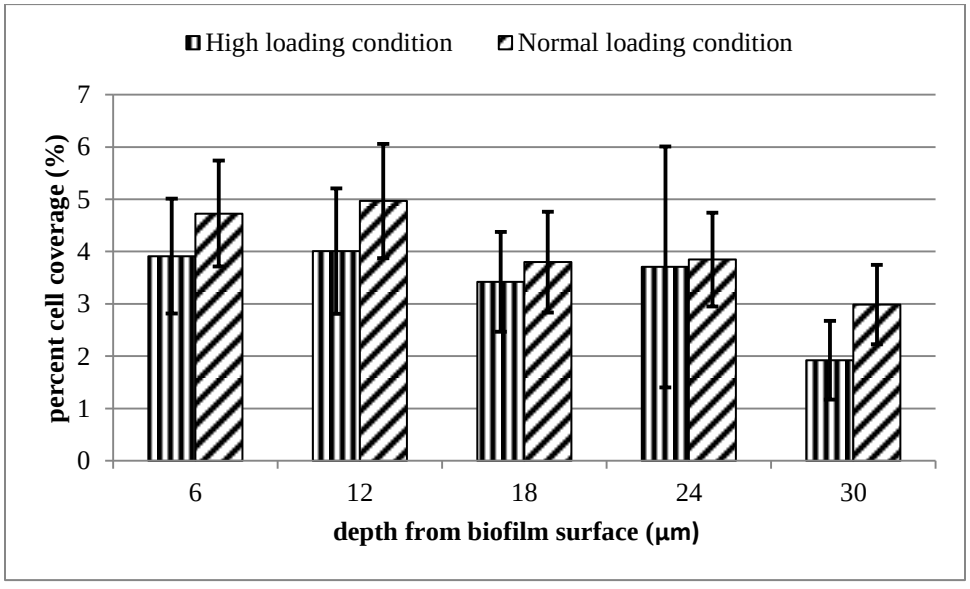


Figure 5.9 Percentage coverage of live cells per biofilm area at specific biofilm depths, for HLC and NLC

5.4.5 Bacterial Population

Figure 5.10 shows a phylogenetic tree identifying the core microbial phyla present within the biofilm samples analyzed in this study with the relative abundance of the genus of the various carriers at HLC and NLC. A dominance of *Proteobacteria* is observed in the samples, which is expected as synthetic wastewater with a low organic load was fed to the all the reactors. Low organic loadings at both HLC and NLC were used to simulate tertiary nitrification conditions following conventional secondary treatment.

The dominant AOBs were present in the *Proteobacteria* phylum, and were observed as *Nitrosomonadaceae* with *Nitrosomonas* being the highest relative abundance genus. *Nitrosospira* and *Nitrosococcus* were not detected in the samples with *Nitrosococcus*, a known wastewater AOB in nitrifying systems (Daims et al., 1999; Gieseke et al., 2001; Park et al., 2008), also not being detected. No statistically significant differences were found in the AOB phylogenic classifications and relative abundances across carrier types and loading rates, with the average relative abundance of the samples analyzed in this study being 4.6 ± 1.4% (Table 5.2).

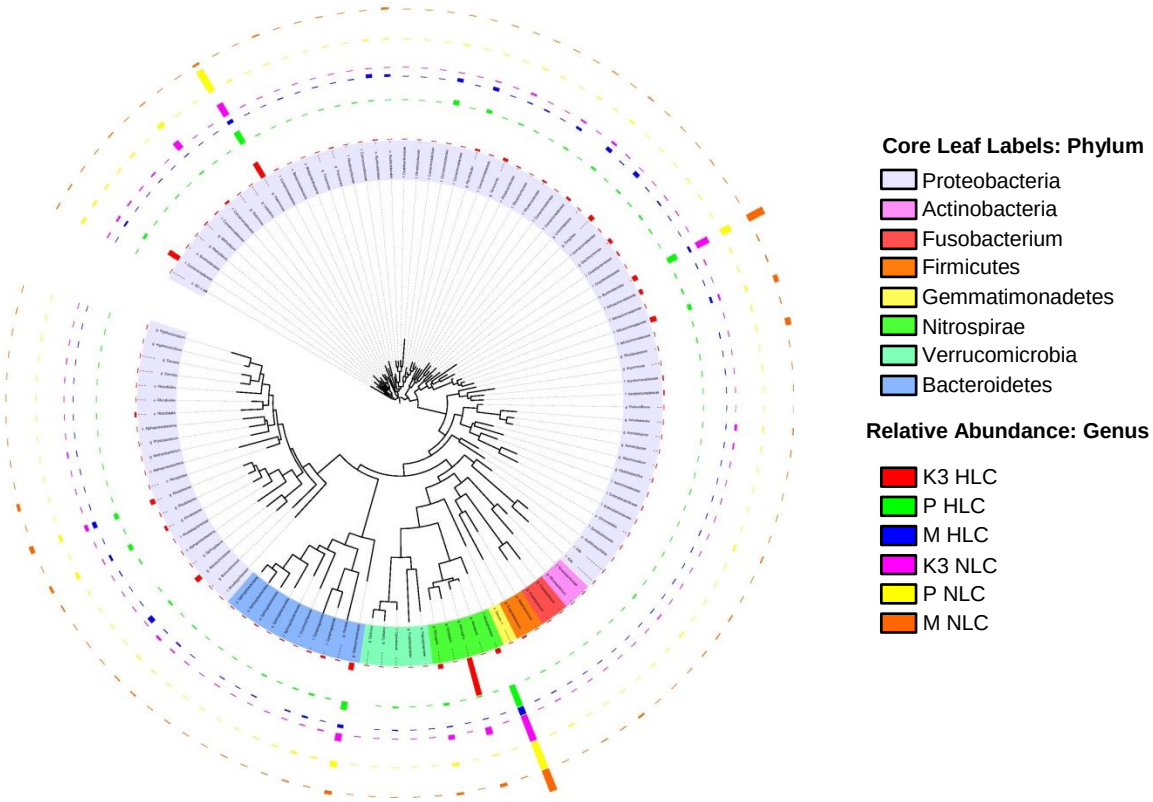


Figure 5.10 Core OTU phylogenetic tree with relative abundance of carriers at HLC and NLC

The NOBs observed were in the *Nitrospirae* phylum and were *Nitrospiraceae*, with *Nitrospira* being highest relative abundance genus. *Nitrobacter* was detected in less than half of the samples and was of negligible relative abundance in all.

Within *Nitrospiraceae* there exist 2 genera *Nitrospira* and *Nitrobacter*. Research has found Nitrospira dominance in wastewater systems and in some cases no *Nitrobacter* present (Daims, et al., 2001; Hovanec et al., 1998). This combined with the research findings lead to the assumption that the NOB population was entirely *Nitrospira*. The NOB population for the various carriers and for the two loading conditions examined in this study; no significant shifts in the relative abundance or dominant NOB was observed across the various carriers or loading rates with an average NOB abundance of all samples of 24.8 ± 3.7% (Table 5.2). Combining the

consistent relative abundance values observed for AOBs and NOBs of the various carrier types (Table 5.2) with the significantly greater VCRR observed for the K3 carrier (Table 5.1), leads to reason that the nitrifiers in the K3 carriers are at higher activities as compared to the P and M carriers.

	K3Carrier HLC	**P Carrier HLC**	**M Carrier HLC**	**K3 Carrier NLC**	**P Carrier NLC**	**M Carrier NLC**
AOB (%)	5.6 ± 2.7	3.8 ± 1.0	2.7 ± 0.4	6.7 ± 6.1	4.6 ± 3.0	7.6 ± 1.6
NOB (%)	30.3 ± 4.9	19.2 ± 2.4	12.5 ± 33.6	26.3 ± 10.1	24.6 ± 7.4	31.9 ± 5.6

Table 5.2 Relative abundance of AOB and NOB in each carrier

Table 5.2 also shows that there is a higher relative abundance of NOBs than AOBS for all carrier types in the tertiary nitrifying MBBR system. This finding is by Hoang et al. (2014) who showed that NOB relative abundance 16 % and AOB 5% for tertiary nitrifying MBBR. The observed significant relative abundance of NOBs is supported by the reactor nitrite concentrations being maintained below 2 mg/l for all reactors, even during transition to and operation at HLC which may have potentially stressed the NOBs and caused nitrite build-up.

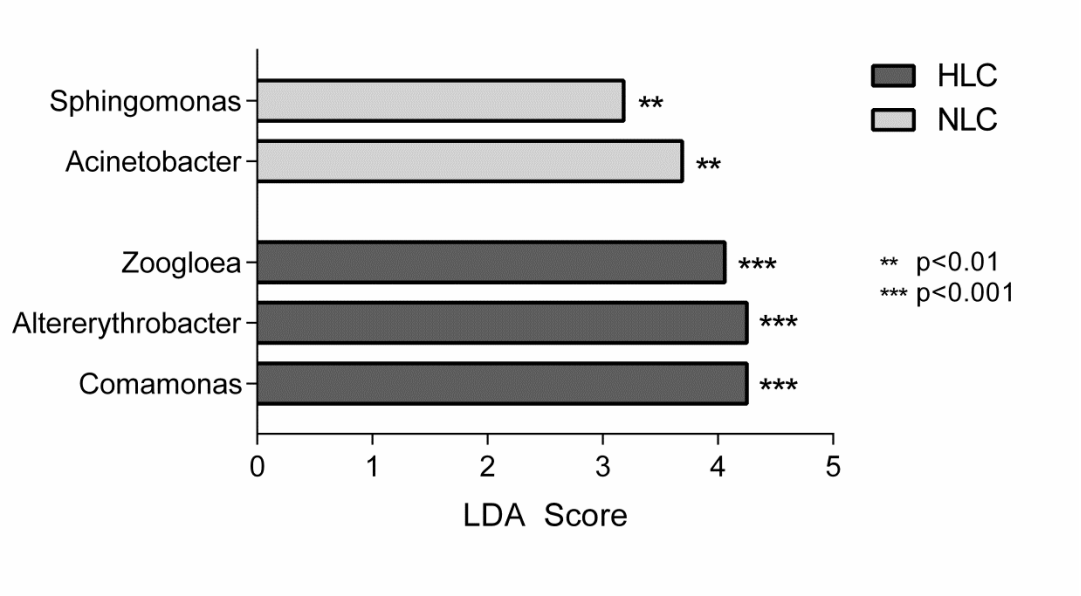


Figure 5.11 Linear discriminate analysis (LDA) of HLC vs NLC

Analyzing the entire taxa for statistical changes in bacterial abundance did not produce significant community shifts when comparing carrier types at the same loading conditions. Grouping the carriers at HLC and NLC demonstrated a significant shift in communities due to loading (Figure 5.11). The five highest LDA scores corresponded to *Comamonas, Alterethryobacter, Zoogloea, Acinetobacter* and *Sphingomonas*. *Comamonas* is statistically in higher relative abundance as HLC as compared to NLC and is a ubiquitous organism that is common is wastewater treatment environments. The enrichment of filamentous bulking organisms, identified as *Zoogloea* and *Altererythrobacter*, was also observed at HLC as compared to NLC. The presence of these organisms likely contributed to the poor settling characteristic observed at HLC in this study (Aonofriesei & Petrosanu, 2007; Xue et al., 2012). In this case a link can be made between the reduced settleability observed at HLC and the proliferation of filamentous bacteria that form matt-like flocs that remain suspended during settlement. At NLC a shift towards the increased relative abundance of aromatic degrading

organisms, *Sphingomonas* and *Acinetobacter*, was observed and may be a function of higher aromatic compound concentrations due to lower loading rates.

5.5 Conclusion

No significant difference in removal efficiencies were observed at HLC and NLC for tertiary nitrifying MBBR systems. Though no differences were seen at the macro-scale, significant differences were observed at the meso and micro-scales. It has been observed that biofilm response to increases in loading is an increase in thickness as well as the advancement of the microbial community (habitation by protozoa, nematode and mites). With this increase in thickness there is the maintenance of the relative percentage of both live and dead cells and there are no significant differences in the nitrifying species or their relative abundance.

The M carrier was found to support a consistently thicker film with lower activity rates than other carrier types. The study showed the large surface area to volume carriers (P and M carriers) to have lower cellular removal rates than the smaller surface area to volume carrier K3 carriers which also had biofilm with greater relative abundance of AOBs under both HLC and NLC. The greater biofilm thickness measured on the larger surface area carriers combined with the low activity rates resulted in removal rates indistinguishable from that of the K3 carrier.

Nitrospira was found to be the dominant NOB while *Nitrsomonnas* was the dominant AOB under both high and normal loading conditions. Filamentous species such as *Zoogloea* and *Altererythrobacter* were seen to proliferate under high loading conditions with a corresponding reduction in effluent settleability measured. As such low reactor loading is recommended in tertiary nitrifying MBBR systems to result in effluent with favourable settling characteristics

The research work suggests that reactor performance cannot be predicted solely by biofilm thickness, the number of cells present or cell activity rates. Instead it is a correlation across all 3 variables. Larger surface area carriers appear more inclined to develop and maintain thicker films with resultant low microbial activity rates which do not have an impact at the macro-scale.

5.6 References

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Chapter 6.

This research investigated the effect of MBBR carrier type and surface area loading rate (SALR) on the performance of tertiary nitrifying MBBR systems. It also investigated the meso and micro scale responses of tertiary nitrifying biofilm to changes in loading and the effect of different carrier types. The main conclusions are as follows:

- Tertiary nitrifying MBBR performance is not dependent on carrier type, but on surface area available for biofilm attachment.
- Despite this, larger surface area to volume carriers such as the M and P carrier have greater propensity than smaller surface area carriers such as the K3 to become clogged during high loading operation, thereby leading to reductions in performance.
- Larger surface area to volume carriers also take longer periods to adjust to reductions in loading, thereby producing effluent with higher than normal total suspended solids(TSS) for longer periods than smaller surface area / volume carriers, such as the K3.
- For treatment processes expected to experience high ammonia loading or great fluctuations in influent ammonia loading smaller surface area to volume carriers such as the K3 are recommended over larger surface area to volume carriers such as the M and P.
- For tertiary nitrifying MBBR systems, increases in SALR result in effluent with increased TSS and reduced solids settleability.
- This reduction in solids settleability was found to be a result of an increase in the relative abundance of filamentous bacteria such as *Zoogloea* and *Altererythrobacter* at high loading conditions. These organisms are known for their poor flocculation behavior,

instead of dense flocs which will settle with time, they form low density nets which remain in suspension.

- Increases in the thickness of tertiary nitrifying MBBR biofilms was observed as the system response to increases in SALR.
- The dominant AOB across all carrier types and loading conditions was *Nitrosomonas*. While the dominant nitrite oxidising bacteria (NOB) was *Nitrospira*.
- High surface area to volume carriers showed the lower cellular activity when compared to the small surface area carrier. Despite this they did not present differently at the macro-scale. This leads to the conclusion that system performance is not only dependent on cellular activity rates, but also number of cells present and biofilm thickness.
- At the meso and micro-scale carrier type was found to have no significant effects.
- Biofilm thickness and microbial population within tertiary nitrifying MBBRs were found to be more greatly linked to SALRs than to carrier type.

6.1 Future recommendations

1. This study was performed using synthetic wastewater, which proved beneficial for a study isolating the effects of ammonia loading on system performance, solids production and community microbiology. With this as a baseline it may prove useful to repeat the study with real wastewater from a secondary treatment plant to observe any changes at the macro, meso or micro –scale due to the introduction of suspended solids, additional substrates and new microbes.
2. A pilot scale study with the system attached as a tertiary unit for a lagoon treatment system is another recommendation.

3. Cell viability was studied through 30 μm biofilm sections. Viability analysis throughout the entirety of the biofilm is recommended to get a clear image of the operation of the microbial layers.