Microbial contaminant removal and alternative nitrogen removal pathways in denitrifying bioreactors

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Abstract

Denitrifying bioreactors, simple treatment systems consisting of a container filled with a particulate organic carbon source, are an effective and low-cost technology for the effective removal of nitrogen (N) from water by enhancing denitrification (i.e. the bacterial conversion of nitrate [NO_3^-] to N gas). To date, studies on denitrifying bioreactors have mainly focused on the ability of, and factors influencing, the removal of NO_3^- within these systems. In the research presented in this thesis, a more holistic view of denitrifying bioreactors was taken in which N removal was assessed in conjunction with the removal of faecal microbial contaminants. The objectives of this research were: (1) to assess how well denitrifying bioreactors can remove microbial contaminant loads from wastewater and (2) to assess the potential role of alternative N removal pathways, namely anaerobic ammonium oxidation (anammox) and codenitrification for N removal in denitrifying bioreactors.

The first part of this thesis focused on the removal of microbial contaminants in denitrifying bioreactors. Removal of microbial contaminants from wastewater is a key factor in wastewater treatment, since the contamination of receiving waters with inadequately treated wastewater can contribute to the transmission of infectious disease caused by waterborne pathogenic microorganisms. First, the removal of bacterial and viral indicators (*Escherichia coli* [*E. coli*] and F-specific RNA bacteriophage [FRNA bacteriophage]) was assessed along the longitudinal transect of a full-scale operating woodchip bioreactor (~114 m^3 in size with 9 sampling wells along the length of the bioreactors) loaded with nitrified septic tank effluent. In addition to significant reduction in NO_3^- loads, the bioreactor demonstrated consistent and substantial reduction of *E. coli* (2.9 $\log_{10}$ reduction) and FRNA bacteriophage (3.9 $\log_{10}$ reduction) despite receiving highly fluctuating inflow concentrations (up to 3.5×10^5 MPN/100mL and 1.1×10^5 PFU/100 mL,

respectively). In a follow-up experiment, removal of *E. coli,* total coliforms (TC) and FRNA bacteriophage was analysed in fifteen mesocosm scale bioreactors (~700 L each) filled with two different carbon sources: woodchip or coconut husk. The effect of media age on attenuation of microbial contaminants was assessed by comparing the performance of 8-year old systems with equivalent newly constructed woodchip and coconut husk bioreactors. Additionally, removal performance of these carbon substrates was compared to that of gravel, a non-carbon substrate commonly used in subsurface flow constructed wetlands. Substantial reduction of *E. coli*, TC and FRNA bacteriophage from primary treated municipal wastewater was achieved in all bioreactors. Mean annual $\log_{10}$ removal efficiencies were similar between microbial indicators ranging from 1.4 to 1.9 for TC, 1.3 to 1.8 for *E. coli* and 1.3 to 2.0 for FRNA bacteriophage. All denitrifying bioreactors showed consistent year-round performance and long-term performance that was not greatly dependent on age of carbon material. The results from both studies suggested that denitrifying bioreactors, as well as reducing N loads, can effectively reduce microbial contaminants in wastewater, providing a complimentary disinfection role.

Denitrification has generally been considered the major pathway converting NO_3^- to dinitrogen gas (N_2) in denitrifying bioreactors. In the second part of the thesis, the importance of anaerobic ammonium oxidation and codenitrification (jointly referred to as An/coD), was assessed by monitoring the removal of N species from partially nitrified municipal wastewater passing through the mesocosm scale bioreactors described above. Lab experiments using a ^{15}N isotope-pairing technique were also performed to partition production of N_2 to these different microbial processes. Results obtained from this study altered our understanding of the potential mechanisms responsible for N loss in these systems. The effective removal of both NO_3^- and ammonium (NH_4^+) and the formation of hybrid N_2 (i.e.

$^{29}N_2$) observed in bioreactors demonstrated that the An/coD pathway was an effective pathway for N removal when both NO_3^- and NH_4^+ were present. An/coD removal rates ranged from 0.6 to 3.8 g N per m^3 reactor volume per day while denitrification rates ranged from 0.7 to 2.6 g N per m^3. The contributions of An/coD to N removal was dependent on media, with An/coD becoming more dominant in bioreactors where denitrification was carbon limited.

The research presented in this thesis has important implications for the use of denitrifying bioreactors for domestic wastewater treatment, since it demonstrates that in addition to removing NO_3^-, denitrifying bioreactors can also remove microbial contaminants and NH_4^+, both commonly present in domestic wastewater. A greater understanding of factors controlling microbial contaminant removal, denitrification and An/coD activity in these systems would allow improved design of bioreactors with the capacity to treat a broader range of wastewater contaminants. The thesis opens a discussion on the potential of denitrifying bioreactors to evolve into a reliable treatment technology for different types of wastewater contaminants.

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about. Thank you for being my home. Mette, thank you for choosing me to be your mum and for showing me what is most important in life.

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Chapter 1

Introduction, Thesis Aim and Outline

1.1 Introduction

The focus of this thesis is on denitrifying bioreactors, simple technologies developed to reduce nitrate (NO_3^-) present in tile drainage and wastewater with the goal to prevent a host of problems generated by excess NO_3^- in aquatic systems, such as eutrophication, algae blooms and fish kills (Schipper et al., 2010b, Christianson et al., 2012b). Denitrifying bioreactors are engineered systems, comprising beds, walls or layers, filled with a porous, organic carbon media (often fragmented woodchip), through which water containing NO_3^- is passed (Schipper et al., 2010b). With passage of water through the bioreactor, the organic carbon creates an anoxic environment and acts as an electron donor to support the microbial conversion of NO_3^- to dinitrogen gas (N_2) by denitrification (Seitzinger et al., 2006, Schipper et al., 2010b). Their simplicity, low maintenance requirements and ability to effectively remove nitrogen (N), with removal rates generally ranging from about 2 to 11 g N per m^3 reactor volume per day, has led to accelerated adoption of denitrifying bioreactors for NO_3^- mitigation in a variety of settings over the past decade (Schipper et al., 2010b, Christianson et al., 2012b, Addy et al., 2016).

Previous studies on denitrifying bioreactors have mainly focused on the ability of, and factors influencing, the removal of NO_3^-, within these systems (e.g. Cameron and Schipper, 2010, Warneke et al., 2011a, Christianson et al., 2012a, Addy et al., 2016). The ability of these systems to remove other contaminants commonly present in wastewater has not been extensively investigated (Schipper et al., 2010b). In this thesis a more holistic view of denitrifying bioreactors was taken, in

which N removal processes were assessed in conjunction with the removal of faecal microbial contaminants. The focus on microbial contaminants was chosen because onsite wastewater treatment systems and drainage water from land receiving animal waste application, have long been implicated in being major sources of microbial contaminants to surface and ground waters (Viraraghavan, 1978, Scandura and Sobsey, 1997, Sinton et al., 1997, Charles et al., 2003, Soupir et al., 2006, Sapkota et al., 2007, Habteselassie et al., 2011). The removal of microbial contaminants from wastewater and drainage water is important from a human health perspective, since the contamination of environmental waters with inadequately treated wastewater can contribute to the potential transmission of infectious disease caused by waterborne pathogenic microorganisms (Craun, 1985, Borchardt et al., 2011). There is, therefore, a widespread need for appropriate technologies that can reduce the risk of potential transmission of infectious disease via waterborne pathogenic microorganisms, by effectively removing microbial contaminants from municipal wastewater or drainage water. Ideally, these systems would be simple and affordable to build, maintain and operate and minimise risk of human contact. The initial aim of this thesis was to assess whether denitrifying bioreactors, in addition to effectively reducing N loads, could provide a complementary role by reducing microbial contaminant loads.

The ability of bioreactors to reduce microbial contaminants has been briefly assessed by Robertson et al. (2005) and Tanner et al. (2012), who reported significant reductions (0.2 to 1.9 $\log_{10}$ reduction) of indicator bacteria *Escherichia coli* (*E. coli)* with passage through a denitrifying bioreactor. While, these studies suggested that denitrifying bioreactors can remove microbial contaminants from wastewater, monitoring data remains scarce; Robertson et al. (2005) only reported 10 data points scattered over a period of 3 years and Tanner et al. (2012) only reported annual median reduction of *E. coli*. Additionally, previous work has only

focused on the removal of *E. coli*, a faecal indicator commonly used as an indirect measure of the removal of enteric pathogens in wastewater treatment (e.g. Tanner et al., 2012, Headley et al., 2013, Wu et al., 2016), but did not investigate the removal of other pathogens commonly present in wastewater, such as viruses. To address the paucity of information in regard to microbial contaminant removal in bioreactors, in the first study of this thesis removal of bacterial indicator *E. coli*, and viral indicator F-specific RNA (FRNA) bacteriophage were assessed along a longitudinal transect of a full-scale operating woodchip bioreactor. Subsequently, seasonal removal of *E. coli*, total coliforms (TC) and FRNA bacteriophage was analysed in fifteen mesocosm scale bioreactors filled with two different types of carbon-rich porous media (woodchip and coconut husk). Using an existing set-up (Tanner et al., 2012), the performance of 8-year old mesocosm scale systems was compared with that of equivalent newly constructed woodchip and coconut husk bioreactors to determine the effect of media maturity on microbial removal. Additionally, removal performance within these different carbon substrates was compared to that of gravel, a non-carbon substrate commonly used in subsurface flow constructed wetlands.

During the second study, the unexpectant removal of both NO_3^- and ammonium (NH_4^+) was observed in the denitrifying bioreactors. It is generally assumed that heterotrophic microbial denitrification is the main mechanism responsible for N removal in denitrifying bioreactors (Greenan et al., 2006, Greenan et al., 2009, Schipper et al., 2010a, Warneke et al., 2011a, Warneke et al., 2011b). Denitrification could, however, not account for the observed removal of NH_4^+. Therefore, the focus of the thesis was broadened to include the assessment of alternative N removal pathways that would allow for the removal of both NO_3^- and NH_4^+, namely anaerobic ammonium oxidation (anammox) and codenitrification.

Anammox and codenitrification are both microbial processes which can produce N gases (N_2O and N_2) from the utilization of NH_4^+ and NO_3^- (Kuenen, 2008, Spott et al., 2011). In this thesis, the importance of the An/coD pathways was assessed by monitoring the removal of N species from partially nitrified municipal wastewater passing through fifteen mesocosm scale bioreactors (as described above). Additionally, lab experiments using a ^{15}N isotope-pairing technique were performed to partition production of N_2 to these different microbial processes.

1.2   Thesis aim objectives

In this study the broader versatility of denitrifying bioreactors for wastewater treatment was assessed. More specifically, the objectives of this thesis were (1) to assess whether denitrifying bioreactors could provide a complementary alternative for removing microbial contaminants while also removing NO_3^- and (2) to assess the potential role of alternative N removal pathways, namely anammox and codenitrification for N removal in denitrifying bioreactors. These aims are further expanded upon in the subsequent research chapters.

1.3   Thesis outline

In the following chapter a focused literature review is presented (Chapter 2) intended to provide a theoretical base for the research that was conducted and to identify shortcomings in the current understanding of microbial contaminant removal and nitrogen removal processes in denitrifying bioreactors. The subsequent chapters (3 to 5) detail the research that was conducted and specifically address each of the three studies described above. Each of these chapters is an independent manuscript, with its own introduction, methods description and relevant discussion of the results as they relate to previously published literature. Chapter 6 summarises the overall results and conclusions

obtained from this research, and provides some insight into the broader implications of, and recommendations for, future work.

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Chapter 2

Literature Review

The development and use of passive denitrifying bioreactors for nitrate (NO_3^-) removal from water has drawn increasing interest in the past two decades. As a result, considerably more is known today about how denitrifying bioreactors function compared to the introduction of the concept of using a solid organic carbon for the treatment of septic systems by Stewart et al. (1979). Not surprisingly, there are three excellent general reviews on denitrifying bioreactors (Schipper et al., 2010b, Christianson et al., 2012, Addy et al., 2016), which all focus on the ability of, and controlling factors on NO_3^- removal within these systems. A truly comprehensive review of denitrifying bioreactors for NO_3^- removal was therefore not the focus of this literature review. The main aim of this literature review was to explore the broader versatility of denitrifying bioreactors for wastewater treatment and to provide a theoretical base for the research that was conducted. First, a brief introduction into the concept of denitrifying bioreactors for NO_3^- removal is given in section 2.1. Subsequently, in section 2.2 the current knowledge on the removal of contaminants common in wastewater in denitrifying bioreactors is summarized and reviewed. The potential of denitrifying bioreactors for microbial contaminant removal is then explored in more detail in section 2.3. In section 2.4, alternative NO_3^- removal pathways and their potential in denitrifying bioreactors are discussed. The final section (2.5) summarizes the main conclusions of the literature review and discusses the shortcomings in current understanding with respect to denitrifying bioreactors.

2.1 A short introduction into denitrifying bioreactors

2.1.1 Simple engineered systems for nitrate removal

The general term "denitrifying bioreactor" was introduced by Schipper et al. (2010b) as an overarching name for all systems that use a solid carbon (C) substrate to enhance heterotrophic denitrification (i.e. the microbial conversion of NO_3^- to N_2) to reduce NO_3^- present in shallow groundwater, streams, agricultural tile drainage or wastewater. Simply stated, denitrifying bioreactors are engineered structures (generally beds, walls or layers) containing a solid, but porous, C source (commonly woodchips) through which water containing NO_3^- is passed (Schipper et al., 2010b). The C-source plays two key roles in promoting denitrification. First, it provides an anoxic environment by oxidation of the organic compounds by aerobic microorganisms, making the environment energetically favourable for the use of NO_3^- as an electron acceptor. Secondly, the C-source act as an electron donor for denitrification (Seitzinger et al., 2006, Rivett et al., 2008).

There are various denitrifying bioreactor designs which can be tailored to specific hydrological settings (Figure 2.1). They can be installed as beds (or wetlands) to enhance denitrification in wastewaters (e.g., septic tank discharges and storm water), or as trenches or walls to enhance denitrification in water derived from agricultural practices (e.g., in groundwater, tile flow, and in streams). While designs differ, bioreactors are generally considered to be simple to build, low cost systems with low maintenance requirements (Schipper et al., 2010b).

2.1.2 Removal rates and factors affecting nitrate removal

Extensive research on NO_3^- removal in denitrifying bioreactors has shown that these systems are effective at nitrogen (N) removal, with removal in woodchip
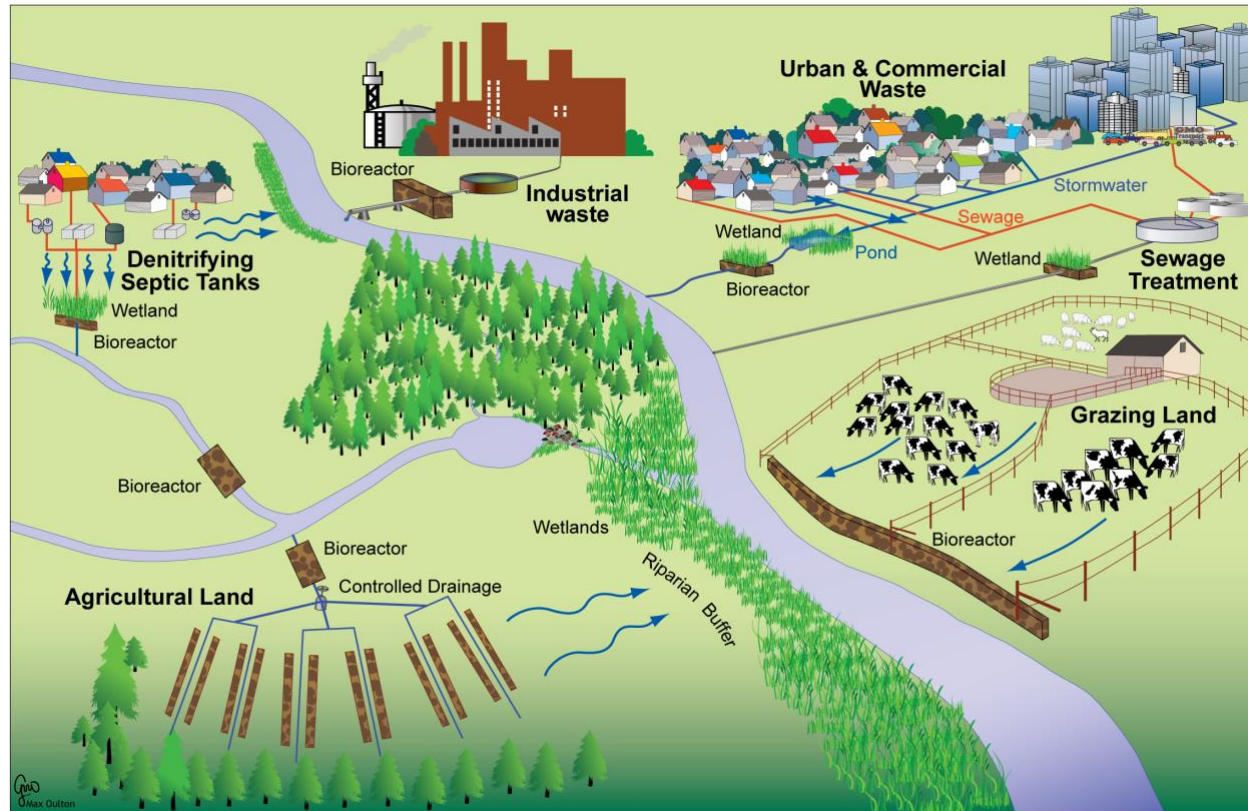
Figure 2.1 Schematic of potential sites and approaches for implementing denitrifying bioreactors for enhancing denitrification in order to remove nitrogen from water (from Schipper et al., 2010c).

bioreactors generally ranging from about 2 to 11 g N m^{-3} day^{-1} (grams of N per cubic meter of bioreactor per day) (Addy et al., 2016). However, N removal rates of up to 44 g N m^{-3} day^{-1} have been reported for woodchip bioreactors (Lepine et al., 2016). Factors influencing NO$_3^-$ removal in denitrifying bioreactors have been extensively evaluated (Schipper et al., 2010b, Addy et al., 2016). The key factors that have been identified to control the rate of denitrification are temperature, the availability of C to denitrifiers (i.e. organism responsible for denitrification) and concentrations of NO$_3^-$ and dissolved oxygen (DO) (Seitzinger et al., 2006).

1. Temperature

Temperature is considered to be the most important environmental factor controlling denitrification, with increased denitrification with increasing temperature (Cameron and Schipper, 2011, Warneke et al., 2011a, Addy et al., 2016). This is generally accepted to be the result of increased microbial activity stimulated by increasing temperature (Schipper et al., 2010, Addy et al., 2016). Operating temperatures in denitrifying bioreactors have typically been found to range from 2 to 20°C (Addy et al., 2016). At low bioreactor temperatures (2 to 5°C) reported NO$_3^-$ removal rates were generally below 3 g N m^{-3} day^{-1} (Addy et al., 2016). A high variability in NO$_3^-$ removal rates (0 to 44 g N m^{-3} day^{-1}) have been reported for denitrifying bioreactors operating at high temperatures (>16°C) (Addy et al., 2016). This large variability could potentially be attributed to differences in C and NO$_3^-$ availability between bioreactors.

2. Nitrate concentration

It is commonly assumed that denitrification is only affected by NO$_3^-$ concentrations when the bioreactors are NO$_3^-$ limited (i.e. when NO$_3^-$ concentration are < 1 mg N L^{-1}) (Addy et al., 2016). When NO$_3^-$ is present in excess, as often is the case in

agricultural drainage, the availability of a C source is commonly considered to be the limiting factor for denitrification in bioreactors.

3. The availability of carbon

Denitrifying bioreactors use solid C substrates to enhance heterotrophic denitrification. Fragmented wood is typically used as C media in bioreactors since it is readily available in New Zealand and the United States (Robertson, 2010, Schipper et al., 2010b). Higher N removal rates have, however, been found for more labile C sources such as maize cob, wheat straw and green waste media (Cameron and Schipper, 2010). While wood media has shown to deliver consistent longer term (5 to 15 years) removal of NO_3^- (Robertson et al., 2008, Robertson et al., 2009, Schipper et al., 2005, Jaynes et al., 2008), more long-term data is necessary to determine how N removal rates change in bioreactors employing more labile C sources.

4. Dissolved oxygen content

The main process that might outcompete denitrifiers for available carbon is the removal of dissolved oxygen (DO) by aerobes (Rivett et al., 2008). The time required for depleting DO in water in woodchip media has, however, found to be to relatively short (± 1 hour) (Schipper et al., 2010b). Poor NO_3^- removal due to high dissolved oxygen concentrations is therefore only considered to be a problem in systems with a short retention time.

2.1.3 Synopsis and conclusions

In summary, extensive research on denitrifying bioreactors conducted over the past three decades has led to an improved understanding of the ability and factors affecting NO_3^- by denitrification in these systems. While, further research on NO_3^- removal in denitrifying bioreactors is still warranted to advance our understanding

of bioreactor performance, especially their long-term performance (Addy et al., 2016), it has been established that denitrifying bioreactors hold promise as a low cost, simple technology for effective reduction of NO_3^- from agricultural drainage or wastewater.

2.2 Removal of other contaminants in denitrifying bioreactors

Denitrifying bioreactors can be installed to enhance NO_3^- removal in wastewaters (e.g., septic tank discharges and storm water) and water derived from agricultural practices (e.g., in groundwater, tile flow, and in streams). Previous studies on denitrifying bioreactors have mainly focused on the ability of, and factors influencing, the removal of NO_3^-, within these systems (e.g. Cameron and Schipper, 2010, Warneke et al., 2011a, Christianson et al., 2012a, Addy et al., 2016). The ability of these systems to remove other contaminants commonly present in wastewater has, however, not been extensively investigated (Schipper et al., 2010b). Wastewater and agricultural drainage can comprise a wide range of contaminants, including dissolved organic matter, suspended solids, as well as metals, trace organics, and pathogens (i.e. disease-causing microorganisms). In this section, the current knowledge on the removal of wastewater contaminants other than NO_3^- in denitrifying bioreactors is summarized and reviewed to assess the capacity of these passive treatment systems to treat a broader range of wastewater contaminants. The data discussed below is also presented in Tables 2.1 and 2.2.

2.2.3 Other nitrogen species

Nitrogen compounds are among the principal constituents of concern in wastewater because of their role in eutrophication and their toxicity to both humans and animals In water, N can exist in multiple oxidation states and chemical forms; ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and organic N. In typical settled

municipal or domestic wastewater N is mainly available in the forms of NH_4^+ (~83%) and organic N (~17%), whereas the oxidised N compounds are usually present in low quantities (0-1%) (Rossle and Pretorius, 2001).

2.2.3.1 Ammonium

Changes in concentrations of NH_4^+ have been analysed in lab and field scale denitrifying bioreactors. For denitrifying bioreactors receiving low NH_4^+ inflow concentrations (< 1 mg L^{-1}), which is common in agricultural drainage, an initial flush of NH_4^+, with concentrations elevated above the influent concentrations, has been reported after bioreactor establishment (Gibert et al., 2008, Cameron and Schipper, 2010, Warneke et al., 2011b, Healy et al., 2015, Lepine et al., 2016). The amount of leaching varied between studies, from relatively low increases in concentration (<2 mg L^{-1} increase in concentration) (Gibert et al., 2008, Cameron and Schipper, 2010) to an increase in concentration up to 16.5 mg L^{-1} (Lepine et al., 2016). While for most studies, these losses dropped considerably after the first 6 months, with NH_4^+ concentrations <1 mg L^{-1}, Lepine et al. (2016) observed NH_4^+ outflow concentrations >3 mg L^{-1} beyond 6 months. Although not systematically analysed, these increases in NH_4^+ concentration have been attributed to dissimilatory nitrate reduction to ammonium (DNRA) occurring during start-up of the bioreactors when highly reducing conditions existed (Gibert et al., 2008, Lepine et al., 2016).

In contrast, in denitrifying bioreactors receiving higher NH_4^+ inflow concentrations, reductions in NH_4^+ have been observed (Robertson et al., 2005, Schipper et al., 2010a, Warneke et al., 2011a). Observed reductions were, however, not consistent or considered statistically significant. Therefore, it is generally assumed that NH_4^+ removal in denitrifying bioreactors is negligible (Greenan et al., 2006, Greenan et al., 2009, Warneke et al., 2011a, Warneke et al., 2011b, Schipper et al., 2010a).

Table 2.1 Overview of the removal of contaminants present in wastewater in a range of denitrifying bioreactors (HLR is hydraulic loading rate, NA indicates when data was not available)

Contaminant	Scale	Media	Size (m³)	HLR (m³/d)	Concentration (g/m³) in	Concentration (g/m³) out	Reduction (%)	Reference
NH₄-N	Full	bark, sawdust and woodchips	9	~1	3.0	1.2	60	Robertson et al., 2005
	Full	bark, sawdust and woodchips	108	7	5.2	5.3	2	Robertson et al., 2005
	Full	bark, sawdust and woodchips	360	73	3.0	1.6	47	Robertson et al., 2005
	Full	bark, sawdust and woodchips	120	18	5.3	3.8	28	Robertson et al., 2005
	Lab	50% (v/v) softwood and sand	0.006	0.00043	0.29	1.8	-84	Gilbert et al. (2008)
	Lab	50% (v/v) softwood and sand	0.006	0.0016	0.29	0.5	-42	Gilbert et al. (2008)
	Pilot	softwood	0.2	0.056	<0.1	2	NA	Cameron & Schipper (2010)
	Pilot	Hardwood	0.2	0.056	<0.1	0.8	NA	Cameron & Schipper (2010)
	Full	Pine woodchip	40	~ 1-2	0.1 - 0.4	0.1 - 0.8	NA	Elgood et al. (2010)
	Full	Coarse sawdust and pine woodchips	1320	8.8	0.0 - 1.4	0.0 - 2.7	NA	Schipper et al. (2010a)
	Full	Coarse sawdust and pine woodchips	294	1.6	0- 15	0 - 12.5	NA	Schipper et al. (2010a)
	Full	Coarse sawdust and pine woodchips	77	52	0 - 82	0 - 68	NA	Schipper et al. (2010a)

Contaminant	Scale	Media	Size (m³)	HLR (m³/d)	Concentration (g/m³)		Reduction (%)	Reference
					in	out		
NH$_4$-N	Pilot	Woodchip and coconut bioreactor placed in succession	1.8	0.254	0.06	0.05	20	Tanner et al. (2012)
	Full	woodchips and sawdust	1320	145	0 - 2.2	0.0	NA	Warneke et al. (2011a)
	Pilot	Various carbon substrates	0.2	-	< 0.01	< 0.01 - 0.8	NA	Warneke et al. (2011b)
	Pilot	Woodchip	2.2	2.42	0.5 - 4	2 - 11	NA	Lepine et al. (2016)
	Pilot	Woodchip	2.2	1.22	0.5 - 4	2 - 15	NA	Lepine et al. (2016)
	Pilot	Woodchip	2.2	0.68	0.5 - 4	3 - 16.5	NA	Lepine et al. (2016)
	Pilot	Woodchip	2.2	0.52	0.5 - 4	3 - 14.5	NA	Lepine et al. (2016)
TON	Full	Coarse sawdust and pine woodchips	1320	8.8	0.0	0.0 – 27.0	NA	Schipper et al. (2010)
	Full	Coarse sawdust and pine woodchips	294	1.6	1 - 6	1 - 4	NA	Schipper et al. (2010)
	Full	Coarse sawdust and pine woodchips	77	52	3 - 25	4 - 17	NA	Schipper et al. (2010)
PO$_4$-P	Full	bark, sawdust and woodchips	120	18	4.5	3.2	29	Robertson et al., 2005

Contaminant	Scale	Media	Size (m³)	HLR (m³/d)	Concentration (g/m³)		Reduction (%)	Reference
					in	out		
PO$_4$-P	Full	woodchips and sawdust	1320	145	20 - 63	12 - 32	NA	Warneke et al. (2011a)
	Pilot	Woodchip and coconut bioreactor placed in succession	1.8	0.254	3.3	3.1	6	Tanner et al. (2012)
	Full	Woodchip	5.2	10.8	2.1	1.9	10	Choudhury et al. (2016)
TP	Full	Coarse sawdust and pine woodchips	294	1.6	5.5 - 16.5	5.0 -16.0	NA	Schipper et al. (2010a)
	Full	Coarse sawdust and pine woodchips	77	52	0 - 37	3 - 43	NA	Schipper et al. (2010)
	Pilot	Woodchip and coconut bioreactor placed in succession	1.8	0.254	4.0	3.1	23	Tanner et al. (2012)
	Full	Woodchip	5.2	10.8	4.0	2.6	54	Choudhury et al. (2016)
Veterinary antibiotics	Pilot	Woodchip	3	0 -108	NA	NA	>80	Gottschall et al. (2016)
TSS	Full	bark, sawdust and woodchips	120	18	7	9	-29	Robertson et al., (2005)
	Pilot	Woodchip	2.2	0.5 - 2.4	NA	NA	90	Lepine et al. (2016)

Contaminant	Scale	Media	Size (m³)	HLR (m³/d)	Concentration (g/m³)		Reduction (%)	Reference
					in	out		
TSS	Pilot	Woodchip and coconut bioreactor placed in succession	1.8	0.3	3.7	< 3	NA	Tanner et al. (2012)
	Full	Woodchip	5.2	10.8	410	86	79	Choudhury et al. (2016)

However, no studies were found in which changes in NH_4^+ concentrations were systematically analysed for denitrifying bioreactors receiving high (>5 mg N L^{-1}) and constant NH_4^+ concentrations.

Potential N removal mechanisms which would allow for the effective removal of NH_4^+ under anaerobic conditions are anammox and codenitrification, both microbial processes which can produce N gases (N_2O and N_2) from the utilization of NH_4^+ and NO_3^- (Spott et al., 2011, Kuypers et al., 2018). The potential role of anammox and codenitrification in bioreactors will be reviewed in more detail in section 2.4.

2.2.3.2 Organic nitrogen

The existence of organic forms of N (particulate organic nitrogen (PON) colloidal organic nitrogen (CON) and dissolved organic nitrogen (DON)) in agricultural drainage waters has been known for many years, but these have not been generally regarded as significant pools of N. Therefore, limited research is available on the presence of organic N in agricultural drainage (Kessel et al., 2009). However, a study by Kessel et al. 2009, suggested that average DON leaching losses of agricultural soils can equal to a third of the NO_3^- losses, and that DON losses should therefore be taken into account when total N budgets are constructed. Similarly, because of the low fraction of organic N in settled municipal wastewater, the removal of organic N is generally not considered to be of concern in wastewater treatment (Czerwionka et al., 2012). However, with wastewater effluent total N permit limits becoming more strict in the United States and Europe, the removal of organic N from wastewater, is potentially becoming more important as it can account for 30 to 50% of the effluent total N (Czerwionka et al., 2012).

So far, very little research has been conducted on the fate and characteristics of wastewater organic N in denitrifying bioreactors. Only one study was found in which changes in organic N were measured in denitrifying bioreactors (Schipper et al., 2010a). This study, in which organic N was calculated as total N minus NH_4^+, NO_3^- and NO_2^-, did not report a consistent reduction in organic N within three large woodchip denitrification beds. Present knowledge on the characteristics and behaviour of organic N in denitrifying bioreactors is thus very limited and insufficient to make any statements of the potential of these systems for organic N removal.

2.2.4 *Phosphorus*

Phosphorus (P) is a common constituent of agricultural fertilizers, manure, and organic wastes in sewage. While, denitrifying bioreactors are developed to reduce NO_3^- in water with the goal to prevent eutrophication (Schipper et al., 2010b, Christianson et al., 2012b), in some aquatic ecosystems, P rather than N is the nutrient considered most responsible for eutrophication (Dillon and Rigler, 1974, Schindler, 2006).

Removal of P has been analysed in number of lab and field scale denitrifying bioreactors (Robertson et al., 2005, Schipper et al., 2010a, Warneke et al., 2011a, David et al., 2016, Pluer et al., 2016). Although some studies indicated a moderate reduction in dissolved reactive P (phosphate; PO_4^-) within denitrifying bioreactors, it is commonly assumed that no significant PO_4^- removal can be expected with passage of water through a denitrifying bioreactor (Schipper et al., 2010b, Pluer et al., 2016). A study by Choudhury et al. (2016) suggested that woodchip bioreactors do have the capacity to effectively remove particulate P associated with suspended solids.

To improve PO_4^- removal the incorporation of P adsorbing compounds in bioreactors has been suggested (Schipper et al., 2010b). The addition of biochar (i.e. charcoal used as a soil amendment) to woodchip has been found to significantly increase PO_4^- removal, with an average PO_4^- removal of 65% for amended woodchip compare to 8% removal in unamended woodchip (Bock et al., 2014). Increased removal of PO_4^- (from 35 to 89% removal) was also observed when woodchip bioreactors were amended with aluminium based drinking water treatment plant residuals (Gottschall et al., 2016). The addition of ferric solids (e.g. $Fe(OH)_3$) to bioreactors has also resulted in considerable P removal from septic tank effluent, lowering influent PO_4^- concentrations of 10.2 mg L^{-1} to <0.05 mg L^{-1} (Robertson, 2000). These studies highlight the potential to expand denitrifying bioreactor functionality to include P mitigation. Further monitoring studies using biochar, aluminium and ferric solids should be undertaken to further assess their potential for P removal and to address their potential for the removal of other contaminants such as microbial contaminants, pesticides and pharmaceuticals.

2.2.5 Organic matter in the aqueous phase

The release of organic matter to environmental waters can constitute a problem since this can reduce dissolved oxygen in receiving waters and negatively affect biota. Changes in biochemical oxygen demand (BOD), which represent the amount of microbially degradable organic matter in the aqueous phase, have been assessed in a number of denitrifying bioreactors receiving drainage water with low BOD concentrations (< 10 mg L^{-1}) (Robertson and Cherry, 1995, Cameron and Schipper, 2010, Leverenz et al., 2010, Schipper et al., 2010a). In general, elevated BOD levels were generated during the first several months of bioreactor operation as organic constituents were leached from the reactive media, with BOD effluent concentrations of up to hundreds of mg L^{-1} (Robertson et al., 2005). However, in

most studies, dissipation of initial high BOD effluent concentrations has been observed after 3 to 6 months of bioreactor operation to concentrations <10 mg L^{-1}, indicating that BOD release is not a long-term concern (Schipper et al., 2010a, Robertson and Cherry, 1995, Cameron and Schipper, 2010). While, BOD release is reasonably well documented at start-up, the release of BOD in older bioreactor should be assessed to establish if BOD release after 6 months represents the long-term operational release.

Compared to agricultural drainage water, wastewater contains high levels of BOD with concentrations of up to hundreds of mg L^{-1} (Charles et al., 2005). Only one study was found in which denitrifying bioreactors were loaded with high BOD concentrations; Lepine et al. (2016) observed a reduction in COD (chemical oxygen demand; a measure of all chemicals in the water, biodegradable and non-biodegradable, that can be oxidized) in woodchip bioreactors receiving aquaculture waste with high COD (influent mean ± SD, 83 ± 21 mg COD L^{-1}). Outflow COD concentrations in the woodchip bioreactors ranged from approximately 10 to 80 mg COD L^{-1}. These findings suggested that woodchip bioreactors have the capacity to reduce organic matter in wastewater. However, more monitoring data remains limited and more systematic analysis of BOD removal in denitrifying bioreactors is recommended.

2.2.6 Suspended solids

Suspend solids are one of the most common contaminants found in wastewater. Elevated levels of solids increase turbidity and reduce the penetration of light at depth within the water column, which can limit the growth of desirable aquatic plants. Additionally, solids can also provide a medium for the accumulation, transport and storage of other pollutants including nutrients and metals.

A number of studies observed a reduction in total suspended solids (TSS) with passage through a woodchip bioreactor, with reduction ranging from 90-98% (Choudhury et al., 2016). Additionally, Tanner et al. (2012) noted a reduction in TSS with passage through a woodchip bioreactor to concentrations below detection limit (<3 mg L^{-1}). Contradictory to these findings, (Robertson et al., 2005) observed a slight increase in TSS with passage through a denitrifying bioreactor (7 to 9 mg L^{-1}). While, removal of TSS has been observed in denitrifying bioreactors, results are not conclusive.

2.2.7 Microbial contaminants

Removal of microbial contaminants from wastewater is important from a human health perspective, since the contamination of environmental waters with inadequately treated wastewater can contribute to the potential transmission of infectious disease caused by waterborne pathogenic microorganisms (Craun, 1985, Borchardt et al., 2011).

The potential of denitrifying bioreactors to remove faecal microbes has been briefly assessed in studies by Robertson et al. (2005) and Tanner et al. (2012) in which *E. coli* reductions of 0.2 to 1.9 log$_{10}$ were reported. This suggested that denitrifying bioreactors, as well as effectively reducing N loads, could reduce microbial contaminants in wastewater, providing a complimentary disinfection role. Despite the apparent success of denitrifying bioreactors in reducing microbial contaminant loads, monitoring data remains scarce and the potential of denitrifying bioreactors for microbial contaminant removal remains unclear. The potential of denitrifying bioreactors for microbial contaminant removal is discussed in more detail in section 2.3.

Table 2.2 Overview of the removal of Escherichia coli (EC), faecal coliforms (FC), total coliforms (TC), faecal streptococci (FS), MS2 bacteriophage (MS2) and Giardia (G) in a range of denitrifying bioreactors, peat filters and subsurface flow (SSF) wetlands (HLR is hydraulic loading rate, NA indicates when data was not available, BD indicates when data was below detection limit).

System type	Microbe	Scale	Media	Size (m³)	HLR (m³/d)	Log₁₀			Reference
						in	out	reduction	
Denitrifying bioreactor	EC	Full	bark, sawdust and woodchips	9	~1	NA	< 1	NA	Robertson et al., 2005
	EC	Full	bark, sawdust and woodchips	108	7	1.3 -2.9	1.0 -2.0	NA	Robertson et al., 2005
	EC	Full	bark, sawdust and woodchips	360	73	1.3 -3.0	1.0 -3.1	NA	Robertson et al., 2005
	EC	Full	bark, sawdust and woodchips	120	18	2.7	2.5	NA	Robertson et al., 2005
	TC	Full	bark, sawdust and woodchips	120	18	> 4.0	3.1	NA	Robertson et al., 2005
	EC	Pilot	woodchip	0.9	0.254	4.1	NA	1.2 - 1.9	Tanner et al., 2012
	EC	Pilot	woodchip and coconut bioreactor in succession	1.8	0.254	NA	NA	2.2	Tanner et al., 2012
Peat filter	FC	Lab	peat	0.0038	* 10 cm/d	4.8	0.9	3.9	Lens et al., 1994
	FS	Lab	peat	0.0038	* 10 cm/d	4.4	0.5	3.9	Lens et al., 1994

System type	Microbe	Scale	Media	Size (m³)	HLR (m³/d)	Log₁₀ in	out	reduction	Reference
Peat filter	FC	Pilot	blended peat	6.3	NA	5.3	3.0	2.3	Patterson et al., 2001
SSF wetland	EC	Full	unknown	280	5.9	6.1 - 6.3	3.7 - 4.2	2.2 - 2.5	Galvao et al., 2009
	EC	Full	gravel 5-10 mm	112	27.2	6.7	3.6	3.1	Masi et al., 2004
	EC	Full	gravel 5-10 mm	162	27.0	5.8	2.0	3.8	Masi et al. 2007
	EC	Full	gravel of 3-6 mm	72	6.3	6.0	3.4	2.6	Mantovi et al., 2003
	TC	Full	unknown	280	5.9	5.6 – 6.6	4.2 - 4.5	2.2 - 2.4	Galvao et al., 2009
	TC	Full	unknown	34	4	6.5	4.4	2.0	Nokes et al., 2003
	TC	Full	unknown	85	10	5.6	3.9	1.7	Nokes et al., 2003
	FC	Full	gravel 5-10 mm	112	27.2	6.8	6.9	2.9	Masi et al., 2004
	FC	Full	gravel 5-10 mm	162	27.0	6.5	2.5	4.0	Masi et al. 2007
	FC	Full	-	34	4	5.4	4.4	1.0	Nokes et al., 2003
	FC	Full	-	85	10	5.1	3.5	1.6	Nokes et al., 2003
	FC	Full	-	280	5.9	6.1- 6.4	3.8 – 4.2	2.2 - 2.5	Galvao et al., 2009
	MS2	Pilot	gravel	49	5.5	7.7	5.1	2.7	Gersberg et al., 1987

System type	Microbe	Scale	Media	Size (m³)	HLR (m³/d)	Log₁₀ in	out	reduction	Reference
SSF wetland	MS2	Pilot	gravel	49	5.5	8.7	5.2	3.5	Gersberg et al., 1987
	SC	Lab	pea gravel	0.11	5.7	5.2	3.5	1.7	Hench et al., 2003
	SC	Lab	pea gravel	0.11	5.7	5.2	4	1.2	Hench et al., 2003
	SC	Full	-	34	4	0.5	BD	NA	Nokes et al., 2003
	G	Full	-	34	4	1.4	BD	NA	Nokes et al., 2003
	G	Full	-	85	10	0.8	BD	NA	Nokes et al., 2003

2.2.8 Emerging contaminants

Emerging contaminants are contaminants which have only appeared recently, or which have been in the environment for a while but for which concerns have been raised much more recently, such as pesticides, pharmaceuticals and personal care products, hormones, nanoparticles and various trace elements. The presence of emerging contaminants has attracted increasing interest in the last decade, mainly due to their potentially negative effect on receiving environments (Enick and Moore, 2007).

The potential of denitrifying bioreactors to remove antibiotics has been briefly assessed by Gottschall et al. (2016), who observed high removal efficiencies (>80%) for a suite of veterinary antibiotics, such as tylosin, chlortetracycline, and isochlortetracycline in a woodchip bioreactor treating agricultural drainage. In addition, Ilhan et al. (2012) demonstrated strong sorption of enrofloxacin, sulfamethazine and monensin A (veterinary antibiotics) and atrazine (an herbicide) to woodchip obtained from a five-year-old denitrifying bioreactor, suggesting that woodchip bioreactors could potentially act as a potential sorbent for herbicides and antibiotics in water. These findings were in line with other studies that found fast and high sorption of pesticides and antibiotics onto wood components and other lignocellulosic materials (Bras et al., 1999, Boudesocque et al., 2008). These studies suggest that woodchip bioreactors could have the added benefit of retaining herbicides and antibiotics. However, monitoring data remains scarce and further studies to assess the potential of denitrifying bioreactors for emerging contaminant removal are recommended. These should include research on the removal of pharmaceuticals, personal care products, fragrances, hormones, nanoparticles and various trace elements.

2.2.9 Synopsis and conclusions

To date, the majority of research on denitrifying bioreactors for onsite wastewater treatment has focused on NO_3^- by denitrification in these systems. There are, however, other contaminants in domestic wastewaters that might be treated using bioreactors. These include other forms of N (NH_4^+ and organic N), P, microbial contaminants and emerging contaminants in wastewaters. Monitoring of NH_4^+ and organic N in denitrifying bioreactors is, as yet, lacking. To date, research suggests no significant PO_4^- removal can be expected with passage of water through an unamended denitrifying bioreactor. However, increased removal of PO_4^- in bioreactors amended with biochar, aluminium or ferric solids highlight the potential of amending these systems to expand bioreactor functionality to include P mitigation. Removal of *E. coli* has been observed with passage through denitrifying bioreactors. This suggested that denitrifying bioreactors, as well as effectively reducing N loads, could reduce microbial contaminants in wastewater, providing a complimentary disinfection role. Results of two studies analysing removal of antibiotics and herbicides in woodchip bioreactors indicated that these systems could have the added benefit of retaining emerging contaminants. Removal of other emerging contaminants, such as personal care products, fragrances, hormones, nanoparticles remains unassessed. Monitoring of changes in BOD in denitrifying bioreactors receiving high levels of BOD (concentrations of up to hundreds of mg L^{-1}) is, as yet, lacking. Removal of suspended solids has been observed with passage of water through denitrifying bioreactors, however, results are not conclusive.

Overall, these findings suggest that denitrifying bioreactors could have the added benefit of removing other contaminants common in domestic wastewaters. However, for all contaminants reviewed above (e.g. NH_4^+, organic N, PO_4^-, organic matter, TSS, emerging contaminants) monitoring data is, as yet, lacking and further

studies to assess the potential of denitrifying bioreactors for their removal are required. In this thesis the potential of denitrifying bioreactors for microbial contaminant and ammonium removal were explored. These topics will be discussed in more detail in the subsequent sections.

2.3 The potential of denitrifying bioreactors for microbial contaminant removal

Elevated concentrations of faecal bacteria and viruses have been detected in surface and groundwater located downstream of septic tanks, animal feeding operations, and land receiving animal waste application (Viraraghavan, 1978, Charles et al., 2003, Soupir et al., 2006, Sapkota et al., 2007). This represents a serious health concern since waterborne diseases can occur when humans ingest or come into contact with water that contains pathogens (i.e. disease-causing microbes). The possible illnesses that result from infection through consumption of water vary with the organism and vary markedly in their severity (Pedley et al., 2006). The predominant recognized illness is generalized acute gastrointestinal illness (Pedley et al., 2006). In New Zealand, the number of annual waterborne disease outbreaks varied from 19 to 62 from 2013 to 2015 (ESR, 2014, ESR, 2015, ESR, 2016). In the US, the consumption of contaminated groundwater was estimated to account for approximately 6.5 million illnesses per year (Reynolds et al., 2008). There is, therefore, a widespread need for appropriate technologies that can reduce the risk of potential transmission of infectious disease via waterborne pathogenic microorganisms, by effectively removing microbial contaminants from septic tank effluent or drainage water. Ideally, these systems would be simple and affordable to build, maintain and operate and minimise risk of human contact. Extensive research has shown that denitrifying bioreactors can be an effective, low-cost, and simple technology for reducing N from septic tank effluent and drainage water (Robertson et al., 2005; Robertson et al., 2008; Schipper et al.,

2010a; Christianson et al., 2012). The potential of denitrifying bioreactors for microbial contaminant removal is assessed below by summarizing and reviewing the current knowledge of microbial contaminant removal in these systems (section 2.3.2), comparing performance with other passive systems for wastewater treatment (section 2.3.3) and reviewing the potential mechanisms and factors that control removal of microbial contaminants in denitrifying bioreactors (section 2.3.4).

2.3.1 Microbial contaminant removal in denitrifying bioreactors

Two studies were found in which microbial contaminant removal was analysed in denitrifying bioreactors (namely Robertson et al., 2005, Tanner et al., 2012). Tanner et al. (2012) reported 1.2 to 1.9 $\log_{10}$ reduction *E. coli,* a commonly used faecal indicator bacteria, in two-year-old mesocosm scale (~1 m^3) woodchip bioreactors loaded with ~0.25 m^3 wastewater per day (see Table 2.2). Robertson et al. (2005) reported comparable removals of *E. coli* for four similar bioreactor systems (containing bark, sawdust and woodchips) receiving sand-filter pre-treated domestic wastewaters, operating at nominal HRTs of 1.7-5.4 d. While, Robertson et al. (2005) generally observed outflow concentrations below 10 CFU (100 ml)$^{-1}$, several breakthroughs with high outflow concentrations (> 10^4 CFU (100 ml)$^{-1}$) were recorded, potentially due to hydraulic short-circuiting at high flow rates.

While, these initial studies suggest that denitrifying bioreactors can effectively remove microbial contaminants from wastewater, this assumption is based on very limited data. Robertson et al. (2005) only reported 10 data points scattered over a period of 3 years and Tanner et al. (2012) only reporting annual median reduction of *E. coli*. Additionally, previous work has only focused on the removal of *E. coli*, a faecal indicator commonly used as an indirect measure of the removal of enteric pathogens in wastewater treatment (e.g. Tanner et al., 2012, Headley et al., 2013,

Wu et al., 2016) and did not address the removal of other pathogens commonly present in wastewater, such as viruses. Due to differences in size, shape and susceptibility to disinfection, *E. coli* is unlikely to be good models for the removal of viruses (Havelaar et al., 1993). Viruses are present in large numbers in wastewater (Simmons and Xagoraraki, 2011), have the ability to migrate over long distances through the subsurface (Keswick and Gerba, 1980), and have high potential to initiate waterborne infections (Craun, 1985, Leclerc et al., 2002). Removal of viruses from wastewater is therefore important from a human health perspective.

Furthermore, previous work has not addressed the effect of factors that could potentially affect removal of microbial contaminants in denitrifying bioreactors. As found for other passive treatment technologies that employ porous media for water treatment, such as constructed wetlands, the removal of microbial contaminants in denitrifying bioreactors is likely dependent on numerous different physical, biological and chemical factors, including size, surface texture and charge of porous media, temperature, hydraulic retention time and wastewater composition (Stevik et al., 2004; Wu et al., 2016). A greater understanding of factors controlling microbial contaminant removal in these systems would allow improved design of bioreactors with the capacity to effectively remove microbial contaminants. This will be discussed in more detail in section 2.3.4.

2.3.2 *Microbial contaminant removal in alternative passive water treatment technologies*

While, studies on microbial contaminant removal in denitrifying bioreactors are limited, there are a number of studies available on the use of peat, a carbon-rich filter material, for wastewater treatment (see review by Couillard, 1994). Published studies on peat filter systems indicate these systems are effective at removing

faecal indicator organisms, such as faecal coliforms (FC) (Couillard, 1994, Lens et al., 1994, Patterson et al., 2001), with $\log_{10}$ removal of bacterial coliforms ranging from 2.3 to 3.9 (Table 2.2). The high bacterial removal rate of peat filters is often attributed to the high adsorption capacity of the lignocellulosic media (Patterson et al., 2001). While, it could be deduced that high adsorption of bacteria can also be expected for other lignocellulosic media, such as woodchip, conditions between peat filters and denitrifying bioreactors vary considerably; peat filters are commonly vertical flow systems through which water flows under unsaturated conditions, denitrifying bioreactors on the other hand are saturated anaerobic systems. The precise effect of oxygen and soil moisture content on the removal of microbial contaminants in porous media still remains to be elucidated. In general, higher bacterial removal is observed in saturated compared to unsaturated porous media (Stevik et al., 2004; Headley et al., 2013; Wu et al., 2016). Removal of microbial contaminant in peat filters may therefore not be representative of microbial contaminant removal in denitrifying bioreactors.

Another alternative passive wastewater treatment technology that employs a porous media for water treatment under saturated conditions are subsurface flow (SSF) wetlands. The design of these systems is similar to that of denitrifying bioreactors; SSF wetlands commonly consist of a large basin through which water is passed under saturated conditions (Morsy et al., 2007, Kadlec and Wallace, 2009). In contrast to denitrifying bioreactors, SSF wetlands are commonly filled with gravel (Morsy et al., 2007, Kadlec and Wallace, 2009). SSF wetlands have been found to reduce microorganisms with varying but significant degrees of effectiveness (Table 2.2), with removal of faecal bacteria ranging from 1.0 to 3.8 $\log_{10}$ between systems. While data on the removal of viruses and protozoa remains scarce, studies indicate SSF wetland can also achieve significant reductions in viruses with 1.2 to 3.5 $\log_{10}$ reduction found for bacteriophage (MS2 and somatic

coliphage) (Gersberg et al., 1987, Hench et al., 2003, Nokes et al., 2003). The large variation in removal efficiencies between SSF wetlands systems is likely the result of within system type variability in design (e.g. differences in system size, filter material and vegetation) and operational parameters (e.g. difference in hydraulic loading rate, climatic conditions and wastewater composition) (Wu et al., 2016).

In summary, substantial removal of microbial contaminants has been observed in other passive wastewater treatment employing carbon-rich filter material, such as peat filters, and saturated treatment systems, such as SSF wetlands. The large variations in removal efficiency observed between and within systems types suggests that differences in design, operational and environmental conditions have a large effect on microbial contaminant removal, making it difficult to extrapolate results from these evaluations to other settings and other system designs, such as denitrifying bioreactors.

2.3.3 Microbial removal mechanism in denitrifying bioreactors

While, no research effort has been aimed at elucidating microbial removal mechanism in denitrifying bioreactors, the removal of microbial contaminants in denitrifying bioreactors are likely a combination of different physical, chemical and biological processes as reported for other passive systems for wastewater treatment employing porous media, such as SSF wetlands (Wu et al., 2016). It is generally assumed that, with passage through porous media, microbes can either be inactivated (i.e. eliminated from water) or immobilized (Schijven and Hassanizadeh, 2000, Stevik et al., 2004). Processes for inactivation of microbial contaminants include microbial predation by other microbes, bacterial and viral lysis, antibiosis, die-off due to biocide exposure and natural die-off (Yates et al., 1988, Schijven and Hassanizadeh, 2000, Stevik et al., 2004). The two mechanisms

responsible for immobilization of bacteria in wastewater moving through a porous media are straining (i.e. the physical capture in pores) and adsorption (Keswick and Gerba, 1980, Yates and Yates, 1987, Stevik et al., 2004, Wu et al., 2016). Immobilization of viruses is, however, mainly attributed to adsorption (Keswick and Gerba, 1980, Yates and Yates, 1987). Key factors identified as influencing the degree of removal of microbial contaminants in porous media are biofilm formation, water organic matter content, conductance properties of the fluid, pH, DO content, hydraulic loading rate (HLR), hydraulic residence time (HRT), filter media properties, such as grain size, surface texture and charge, and microorganism size and shape (Schijven and Hassanizadeh, 2000, Stevik et al., 2004).

While, there are several reviews available which examine the factors which govern microbial retention and elimination of bacteria and viruses with passage through porous media (Schijven and Hassanizadeh, 2000, Stevik et al., 2004, Tufenkji, 2007, Wu et al., 2016) they do not rank the importance of the various factors on removal. This is mainly because studies aimed at elucidating the various factors affecting microbial contaminant removal are often conducted in controlled laboratory experiments (Weiss et al., 1995, Stevik et al., 1999, Hijnen et al., 2005). Although these well-defined laboratory experiments have provided an improved understanding of the mechanisms governing and factors controlling microbial transport and removal in porous media, the results of these studies are not directly relevant to treatment technologies such as constructed wetlands or denitrifying bioreactors. These ecotechnologies present a highly complex environment with an inherent physical, chemical and biological heterogeneity in which a wide range of microbial immobilisation and inactivation processes may occur simultaneously that are difficult to deconvolve. A great deal more research is, therefore, required before accurate predictions about the transport and removal of microbes in passive water treatment systems can be made.

2.3.4 Synopsis and conclusions

There is a widespread need for appropriate technologies that can effectively remove microbial contaminants from wastewater or agricultural drainage. Ideally, these would be simple and affordable to build, maintain and operate. Previous studies observed effective removal of *E. coli* within denitrifying bioreactors, suggesting that these systems could provide an effective technology for the removal of microbial contaminants. However, monitoring data remains scarce and is limited to the removal of *E. coli*, the removal of other pathogens commonly present in wastewater is not addressed. Substantial removal of microbial contaminants has been observed in other passive treatment technologies employing carbon-rich filter material, such as peat filters, or operating under saturated conditions, such as SSF wetlands. However, the large variations in removal efficiency observed between and within systems types suggests that differences in design, operational and environmental conditions have a large effect on microbial contaminant removal, making it difficult to extrapolate results from evaluations to other settings and system designs. Key factors that are likely to influence the degree of removal of microbial contaminants in denitrifying bioreactors are biofilm formation, organic matter content, conductance properties of the fluid, pH, dissolved oxygen content, hydraulic loading rate, hydraulic residence time, filter media properties, such as grain size, surface texture and charge, and microorganism size and shape. A greater understanding of factors controlling microbial contaminant removal in denitrifying bioreactors would allow for improved prediction of microbial contaminant removal in these passive treatment systems. However, this is challenging to research as denitrifying bioreactors are a highly complex environment with an inherent physical, chemical and biological heterogeneity in which a wide range of microbial immobilisation and inactivation processes may occur simultaneously.

2.4 Alternative nitrogen removal processes in denitrifying bioreactors

While there are multiple microbial processes that compete for NO_3^- in the nitrogen cycle, it is generally assumed that heterotrophic microbial denitrification (i.e. the conversion of NO_3^- to dinitrogen gas) is the main mechanism responsible for N removal in denitrifying bioreactors (Schipper et al., 2010b). Other possible NO_3^- transformation processes include N assimilation into organic N (i.e. biomass), dissimilatory nitrate reduction to ammonium (DNRA, i.e. ammonification), anaerobic ammonium oxidation (anammox) and codenitrification (Spott et al., 2011, Kuypers et al., 2018). While several studies have reported that N assimilation and DRNA play only a minor role in NO_3^- removal in denitrifying bioreactors (Schipper and Vojvodić-Vuković, 1998, Robertson, 2000, Robertson et al., 2005, Robertson et al., 2007, Schipper et al., 2010a, Gibert et al., 2008), the role of anammox and codenitrification in bioreactors have not been investigated. While, anammox and codenitrification are unlikely to be a major N transformation pathway for N removal from agricultural drainage, in which N is mainly available as NO_3^-, these processes could potentially be an effective pathway for N removal from municipal and domestic wastewater in which both NO_3^- and NH_4^+ are potentially present. This section, first, provides an introduction into the concepts of anammox and codenitrification (section 2.4.1). Subsequently, the potential of both processes for N removal in denitrifying bioreactors treating domestic wastewater is discussed (section 2.4.2).

2.4.1 An introduction into anammox and codenitrification

2.4.1.1 Anammox

Anammox produces N gas by oxidizing ammonium (NH_4^+) with NO_3^- or nitrite (NO_2^-) under anoxic conditions. The process was first discovered in an anoxic wastewater treatment plant designed for denitrification by Mulder et al. (1995), in which disappearance of NH_4^+ at the expense of NO_3^- and a clear increase in N gas production was observed. The first few years of research on anammox showed that it was a bacterial process based on energy conservation from anoxic NH_4^+ oxidation with NO_2^- as the preferred electron acceptor (Eq. 1, van de Graaf et al., 1995):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad\qquad\qquad (Eq.\ 1)$$

An analysis of mass balances showed that bacteria used carbon dioxide as its C source to produce biomass and that NO_2^- also functioned as an electron donor for the reduction of carbon dioxide (Strous et al., 1997):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$

$$(Eq.\ 2)$$

$$1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15}$$
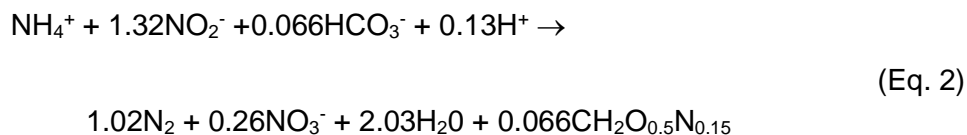
Further research identified that anammox was mediated by bacteria affiliated to order the *Brocadiales*, which are part of the phylum *Planctomycetes,* of which, five genera of anammox bacteria have been described, '*Brocadia*', '*Kuenenia*', '*Anammoxoglobus*', '*Jettenia*' and '*Scalindua*' (Kuenen, 2008, Jetten et al., 2010).

Since its discovery, anammox has been found to contribute significantly to total N turnover (4 to 92%) in a wide range of ecosystem settings including marine sediments (Thamdrup and Dalsgaard, 2002, Kuypers et al., 2003, Engström et al.,

2005) and paddy fields (Zhu et al., 2011, Yang et al., 2015). Additionally, analyses of anammox organism abundance via genomic analysis have revealed substantial anammox activity in wetland ecosystems (Humbert et al., 2012, Wang et al., 2013, Zhang et al., 2007) and treatment wetlands (Waki et al., 2015), suggesting that in these systems anammox organisms could potentially be responsible for substantial removal of N deriving from NH_4^+ and NO_3^-.

2.4.1.2 Codenitrification

Codenitrification is assumed to produce N_2O and N_2 when, during the reduction of NO_3^- by denitrification, a side reaction occurs between NO_2^- or NO^- and a nucleophile, such as NH_4^+ or other monomeric organic N sources such as amine (Spott et al., 2011, Tanimoto et al., 1992). The mechanism is assumed to be based on a microbially mediated N-nitrosation reaction; dissimilatory NO_2^- reductase catalyses a nitrosyl transfer (nitrosation) from NO_2^- to N-nucleophiles producing N_2O and N_2. The individual atoms of the N_2O or N_2 molecules formed by codenitrification are thus derived from two distinct N sources. Formation of N gas production due to codenitrification is assumed to be mediated by denitrifying microorganisms known to occur in all three domains of microorganisms (i.e. archaea, bacteria and eukarya) (Spott et al., 2011).

In contrast to anammox, there are only few studies that analysed N_2 production due to codenitrification, focusing on agricultural soils (e.g. Laughlin and Stevens, 2002, Selbie et al., 2015, Clough et al., 2017). Although the relative contribution of codenitrification varied between studies (12 to 97%), they all noted a significant contribution of codenitrification to total N_2 production in agricultural soils. To date, codenitrification has not been demonstrated to occur in aquatic environments.

2.4.1.3 Anammox and codenitrification; analogues processes?

Anammox and codenitrification are thus both microbial processes which can produce N gases from the utilization of NH_4^+ and NO_2^-. N_2 gas production by the anammox and codenitrification process thus appear to be similar. However, they are generally considered to be two different processes (Selbie et al., 2015, Long et al., 2013, Yang et al., 2015), mainly because they are mediated by different organisms; codenitrification is mediated by denitrifying organisms, while anammox is mediated by specific anammox (i.e. non-denitrifying) species of bacteria and archaea (Kuenen, 2008, Spott et al., 2011). Nevertheless, both types of organisms have exhibited the same nitrate reductase (NIR; classes of enzymes that catalyse the reduction of NO_2^-) (Strous et al., 2006, Spott et al., 2011). This suggests that N gas production by both organisms follows the same N reaction pathway and that both microbial N transformation processes could potentially be viewed as analogous (Spott et al., 2011). These findings are in line with the growing realization that microorganisms have tremendous metabolic versatility and that it therefore is very difficult to classify them according to the traditional N cycling processes (Kuypers et al., 2018). While, further work on the molecular biology, biochemistry and physiology of anammox and codenitrification is required to further elucidate to what extent they can be viewed as analogous processes of microbial N transformation, this is beyond the scope of this thesis.

2.4.2 The potential for anammox and codenitrification in denitrifying bioreactors treating domestic wastewater

As stated above, microbial denitrification is commonly assumed to be the main mechanism responsible for N removal in denitrifying bioreactors. However, in settled domestic wastewater N is mainly available as NH_4^+. Before application of domestic wastewater to a denitrifying bioreactor, a primary treatment thus needs

to be introduced to increase oxygen availability in order to promote microbial nitrification (i.e. the microbial oxidation of NH_4^+ to NO_3^-). If present in denitrifying bioreactors, anammox and/or codenitrification would be beneficial for N removal from domestic wastewater as they would, in addition to removing NO_3^-, also allow for the effective removal of residual NH_4^+. Additionally, the presence of anammox and/or codenitrification reduces the oxygen (and associated energy) requirement for preceding nitrification stages and increases the N_2 production capacity per gram of C consumed from the organic media (Van Loosdrecht et al. 2004).

The roles of anammox and codenitrification have not been extensively studied in denitrifying bioreactors. Previous studies concluded that the presence of anammox in denitrifying bioreactors is unlikely, due to the fact that no consistent decrease in NH_4^+ has been observed with passage of water through these systems (Greenan et al., 2006, Greenan et al., 2009, Warneke et al., 2011a, Warneke et al., 2011b, Schipper et al., 2010a). However, no studies were found in which partially nitrified water, with high (>5 mg N L^{-1}) and constant NO_3^- and NH_4^+ concentrations, was passed through a denitrifying bioreactor. It can thus be concluded that the role of anammox and codenitrification in bioreactors have not been systematically investigated and deserves further attention.

Below, the potential of anammox and codenitrification in denitrifying bioreactors is assessed, by reviewing the various factors controlling both processes. While, the two transformation processes could potentially be viewed as analogous (see section 2.4.1), in this section they will be discussed separately.

2.4.2.1 Considerations for anammox in denitrifying bioreactors

The anammox process is ideally suited for the treatment of N-rich wastewater streams in which NH_4^+ and NO_2^- are present. Research has explored the removal

of N in NH_4^+-rich wastewaters employing several types of reactors, commonly mechanized systems, in which anammox was the dominant N removal process (Van Dongen et al., 2001, Van der Star et al., 2007, Lotti et al., 2015, Li et al., 2016). In these studies, nitrogen removal rates varied markedly between reactors (0.1 to 20 kg N m^{-3} d^{-1}), but were generally comparable or higher than those of conventional N-removal systems employing a nitrification and denitrification step Nevertheless, widespread application of anammox for wastewater treatment remains a challenge due to the many factors that affect the anammox process and the low growth rate of anammox bacteria. The key factors that have been identified to control the rate anammox are the availability of NH_4^+ and NO_2^- , competition between anammox and denitrifying bacteria in the presence of organic compounds, temperature and dissolved oxygen content. Additionally, a number of substances commonly found in domestic wastewater have been found to inhibit anammox activity. Below, the various factors controlling anammox and their potential role in inhibiting anammox in denitrifying bioreactors treating domestic wastewater are discussed.

1. Low growth rate of anammox bacteria

The low growth rate of the anammox bacteria (doubling time of 10 to 14 days at 30 to 40 °C) is one of the main factors limiting the application of anammox in wastewater treatment (Van der Star et al., 2007, Strous et al., 1998). As a result of these low growth rates, start-up periods for reactors treating wastewater in which anammox is the dominant N removal process have reported to take up to 2 years (Van der Star et al., 2007).

2. Availability of reactants

Anammox bacteria derive their energy from the oxidation of NH_4^+ by NO_2^- (Van de Graaf et al., 1995). Consequently, the process relies on the availability of these N

species. In settled domestic wastewater, N is mainly available as NH_4^+, whereas the oxidised N compounds are usually present in low quantities (0-1%) (Rossle and Pretorius, 2001). In settled domestic wastewater anammox is thus likely to be limited by the availability of NO_2^-. In mechanized wastewater treatment reactors, NO_2^- can be produced by partial nitrification of NH_4^+ to NO_2^- through appropriate regulation of the pH, temperature and DO concentrations (Ruiz et al., 2013, Jianlong, 2004). In passive wastewater treatment systems for nitrification, such as unsaturated gravel or sand filters, NO_2^- rarely accumulates in nitrified wastewater due to the conversion of NO_2^- to NO_3^- by nitrite oxidizing bacteria (Crites and Tchobanoglous, 1998). In these situations, the NO_2^- may originate from NO_3^- reduction at anaerobic conditions, which, in turn, may be due either to common denitrifying organisms or NO_3^- reduction by the anammox bacteria in the presence of organic compounds such as formate, acetate or propionate (Kuenen, 2008). Thus, in anaerobic conditions, such as denitrifying bioreactors, NO_2^- may not be limiting for anammox as long as NO_3^- is available at sufficient concentrations.

3. Competition between denitrifying and anammox bacteria in the presence of organic compounds

If organic C and NH_4^+ are present in abundance, competition by heterotrophic denitrifying bacteria for available NO_3^- can be a factor limiting anammox activity. The yield of free energy ($\Delta G°$) controls the transformation of reactants in natural environments and thus determines the probability for certain reactions to take place. Denitrification (using formaldehyde as an electron donor) yields more energy than anammox (Table 2.3). The large difference in energy yield suggests that anammox bacteria would not compete well with heterotrophic denitrifying bacteria in the presence of a high concentration of organic C.

Table 2.3 Reactions for denitrification and anammox and their yield of free energy (ΔG°) during standard state.

Pathway	Reaction	ΔG°
Denitrification	$5/4CH_2O + NO_3^- + H^+ \rightarrow$ $5/4CO_2 + 1/2N_2 \ 7/4H_2O$	$-635 \ kJ \ mol^{-1} \ NO_3^-$
Anammox	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	$-358 \ kJ \ mol^{-1} \ NO_2^-$

While, the above argument a simplification of in situ conditions in natural environments as it is based on the net change of free energy at steady state (ΔG°), this argument is supported with studies that generally found that the presence of organic C adversely affected anammox activity (Tang et al., 2010, Van de Graaf et al., 1996, Molinuevo et al., 2009). However, the effect of organic C on anammox activity differs between studies and appears to be concentration dependent, with high concentrations of organic C (acetate concentration >25 mmol L^{-1} and COD concentrations >240 mg L^{-1}) inhibiting anammox activity (Tang et al., 2010), low concentrations (acetate concentrations of <10 mmol L^{-1}) not having a significant negative effect (Dapena-Mora et al., 2007) and even increased anammox activity at low concentrations of glucose (1 mmol L^{-1}) (Oshiki et al., 2011). The effect of organic C on anammox is not fully understood and should be investigated further.

4. Temperature

The temperature dependency of anammox rates has been studied in a number of lab scale experiments. Egli et al. (2001) and Strous et al. (1999) reported an optimum temperature range of 30 to 40 °C for anammox in wastewater treatment systems. Studies of anammox in natural systems have reported anammox activity at a large range of temperatures, between -2 (sea ice of Greenland, as reported in Rysgaard et al., 2004) and 85 °C (a ridge vent in the mid-Atlantic, as reported in

Byrne et al., 2009), suggesting that anammox organisms can adapt to a wide range of temperature conditions.

5. Dissolved oxygen

The anammox process is inhibited by high dissolved oxygen concentrations. Inhibition was found to be reversible inhibition at low oxygen concentrations (0.5-2%) (Strous et al., 1997, Egli et al., 2001) and irreversible at higher oxygen concentrations (18%) (Egli et al., 2001). In denitrifying bioreactors, the C source creates an anaerobic environment in the systems by oxidation of the organic compounds. Dissolved oxygen concentrations in denitrifying bioreactors have generally been reported to range from 0 to 1 mg DO/L, depending on hydraulic retention time (Lepine et al., 2016, Christianson et al., 2012). Under anaerobic conditions anammox could potentially be used to treat wastewater.

6. Anammox inhibition by substances commonly present in domestic wastewater

A variety of substances commonly present in municipal wastewater, such as toxic organic compounds (e.g. alcohol, aldehydes and antibiotics), ammonia (NH_3), phosphate (PO_4^-) and hydrogen sulphide (H_2S) have been found to inhibit anammox activity (Jin et al., 2012). Alcohol, aldehydes and antibiotics, at levels found in sewage treatment plants, have been found to inhibit anammox by microbial poisoning or enzyme inactivation (Jin et al., 2012). High levels of NH_3 have also been found to suppress anammox activity. However, the threshold concentration for the inhibition of anammox by NH_3 is unknown with concentrations varying from 1.7 to 38 mg L^{-1} between studies (Jin et al., 2012). Observations on anammox inhibition due to PO_4^- are limited to a few lab scale studies and the extent to which PO_4^- affected anammox differs between studies, with some studies reporting no inhibition at concentrations of 20 mmol L^{-1} while other report inhibition

at concentrations <2 mm L^{-1} (Jin et al., 2012). Anammox inhibition can also be caused by H_2S generated from SO_4^- reduction under anaerobic conditions. Again, observations on inhibition due to H_2S are limited and observed effects varied markedly between studies (Jin et al., 2012). It can be concluded that a number of substances in domestic wastewater have been found to inhibit anammox activity, but that the extent to which they affect anammox is largely unknown.

2.4.2.2 Considerations for codenitrification in denitrifying bioreactors

To date, codenitrification has not been demonstrated to occur in water treatment systems or other aquatic environments. The few studies that analysed N_2 production due to codenitrification, focused on agricultural soils (e.g. Laughlin and Stevens, 2002, Selbie et al., 2015, Clough et al., 2017). Unsurprisingly, compared to the anammox process, less is known about the factors potentially affecting codenitrification in aquatic environments. It is assumed that the process of codenitrification is controlled by the same factors that control denitrification: temperature, the availability of C and concentrations of dissolved oxygen (Spott et al., 2011, see section 2.1.2). It could therefore be deduced that conditions in a denitrifying bioreactor could be considered favourable for codenitrification to occur.

2.4.3 Synopsis and conclusions

It is assumed that heterotrophic microbial denitrification (i.e. the conversion of NO_3^- to dinitrogen gas) is the main mechanism responsible for N removal in denitrifying bioreactors. However, the role of anammox and codenitrification in bioreactors have not been systematically investigated. Anammox and codenitrification are both microbial processes which can produce N gases (N_2O and N_2) from the utilization of NH_4^+ and NO_3^-. While they are considered to be two different processes, it has been suggested that they could potentially be viewed as analogous. If present in

denitrifying bioreactors, anammox and/or codenitrification would be beneficial for N removal from domestic wastewater as they would, in addition to removing NO_3^-, also allow for the effective removal of residual NH_4^+. Data on the removal of NH_4^+ in denitrifying is, as yet, lacking. Overall, it can be concluded that anaerobic and temperature conditions commonly observed within denitrifying bioreactors are favourable for anammox and codenitrification to occur. The main factor potentially inhibiting anammox activity in denitrifying bioreactors is the presence of denitrifying bacteria which can compete with anammox microorganisms for oxidized N, especially in the presence of high concentrations of organic C. The role of anammox in denitrifying bioreactors could also be restricted due to low growth rates and the presence of compounds in wastewater that can inhibit the anammox process.

2.5 Overall summary and conclusions

Extensive research has shown that denitrifying bioreactors can be an effective, low-cost, and simple technology for removing excess NO_3^- from wastewater. Although giant strides have been made in the last two decades on the use of denitrifying bioreactors for NO_3^- removal by denitrification, the efficacy of denitrifying bioreactors to remove other contaminants common in domestic wastewater, such as NH_4^+, organic N, PO_4^-, organic matter, TSS, microbial contaminants and emerging contaminants, remains largely unassessed and deserves further exploration. The objectives of this thesis were (1) to assess whether denitrifying bioreactors could provide a complementary alternative for removing microbial contaminants⁻ and (2) to assess the potential role of alternative N removal pathways, namely anammox and codenitrification for N removal in denitrifying bioreactors.

Removal of microbial contaminants from wastewater is considered a key issue for wastewater treatment, since elevated microbial concentrations in ground or surface water present a serious public health concern due to the potential outbreak of waterborne diseases when humans ingest, or come into contact with, water that contains pathogenic organisms. Denitrifying bioreactors could potentially provide an appropriate solution for the removal of microbial contaminants from wastewater due to their robust operation and low maintenance requirements. Findings by two studies suggest that denitrifying bioreactors can reduce microbial contaminants. However, monitoring data remains scarce and is limited to the removal of *E. coli*. The removal of other pathogens commonly present in wastewater remains unaddressed. While, no research effort has been aimed at elucidating microbial removal mechanism in denitrifying bioreactors, the removal of microbial contaminants in these systems are likely a combination of different physical, chemical and biological processes. A greater understanding of the mechanisms and factors controlling microbial contaminant removal in denitrifying bioreactors would allow for improved prediction of microbial contaminant removal in these systems. However, this is challenging to research as denitrifying bioreactors present a highly complex environment in which a wide range of microbial immobilisation and inactivation processes may simultaneously occur, making it difficult to identify the most important processes.

It is generally assumed that heterotrophic microbial denitrification (i.e. the conversion of NO_3^- to dinitrogen gas) is the main mechanism responsible for N removal in denitrifying bioreactors. In typical settled municipal or domestic wastewater, N is mainly available in the forms of NH_4^+. Therefore, a primary treatment needs to be introduced to increase oxygen availability in order to promote microbial nitrification (i.e. the microbial oxidation of NH_4^+ to NO_3^-) before application of domestic wastewater to a denitrifying bioreactor. Anammox and

codenitrification are both microbial processes which can produce N gases (N_2O and N_2) from the utilization of NH_4^+ and NO_3^-. If present in denitrifying bioreactors, anammox and codenitrification would be beneficial for N removal from domestic wastewater as they would, in addition to removing NO_3^-, also allow for the effective removal of residual NH_4^+. To date, the role of anammox and codenitrification in denitrifying bioreactors have not been systematically investigated. Overall, anaerobic and temperature conditions commonly observed within denitrifying bioreactors are favourable for anammox and/or codenitrification to occur. The main factor potentially inhibiting anammox activity in denitrifying bioreactors is the presence of denitrifying bacteria which can compete with anammox microorganism for oxidized N.

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Chapter 3

Faecal Bacteria, Bacteriophage, and Nutrient Reductions in a Full-Scale Denitrifying Woodchip Bioreactor

3.1   Abstract

Denitrifying bioreactors using woodchips or other slow-release carbon sources can be an effective method for removing nitrate (NO_3^-) from wastewater and tile drainage. However, the ability of these systems to remove faecal microbes from wastewater has been largely uninvestigated. In this study, reductions in faecal indicator bacteria (*Escherichia coli* [*E. coli*]) and viruses (F-specific RNA bacteriophage [FRNA bacteriophage]) were analysed by monthly sampling along a longitudinal transect within a full-scale denitrifying woodchip bioreactor receiving secondary-treated septic tank effluent. Nitrogen, phosphorus, 5-d carbonaceous biochemical oxygen demand (CBOD5), and total suspended solids (TSS) reduction were also assessed. The bioreactor demonstrated consistent and substantial reduction of *E. coli* (2.9 log_{10} reduction) and FRNA bacteriophage (3.9 log_{10} reduction) despite receiving highly fluctuating inflow concentrations (up to 3.5 $\times 10^5$ MPN (100 mL)$^{-1}$ and 1.1 $\times 10^5$ plaque-forming units (100 mL)$^{-1}$, respectively). Most of the removal of faecal microbial contaminants occurred within the first meter of the system (1.4 log_{10} reduction for *E. coli*; 1.8 log_{10} reduction for FRNA

bacteriophage). The system was also efficient at removing NO_3^- (>99.9% reduction) and TSS (89% reduction). There was no evidence of consistent removal of ammonium, organic nitrogen, or phosphorus. Leaching of CBOD5 occurred during initial operation but decreased and stabilized at lower values (14 g O_2 m^{-3}) after 9 months. We present strong evidence for reliable microbial contaminant removal in denitrifying bioreactors, demonstrating their broader versatility for wastewater treatment. Research on the removal mechanisms of microbial contaminants in these systems, together with the assessment of longevity of removal, is warranted.

3.2 Introduction

Extensive research has shown that denitrifying bioreactors can be an effective, low-cost, and simple technology for reducing nitrogen (N) from septic tank effluent and drainage water (Robertson et al., 2005, Robertson et al., 2008, Schipper et al., 2010a, Christianson et al., 2012). They generally comprise beds, walls, or layers of porous, carbon-rich media (commonly woodchips) through which nitrified effluent or agricultural drainage water is passed (Schipper et al., 2010b). During passage through the carbon-rich media, nitrate (NO_3^-) is converted into nitrogen gas (N_2) by microbial denitrification (Robertson, 2000; Greenan et al., 2006; Gibert et al., 2008; Schipper et al., 2010b). In a comparative study, Oakley et al. (2010) concluded that denitrifying bioreactors, preceded by a sand filter, performed better than any other onsite wastewater treatment technology in reducing N loads. To date these systems have been designed to target a single contaminant - NO_3^-- but their efficacy in removing other wastewater contaminants such as faecal microbes has been largely uninvestigated.

Removal of microbial contaminants from septic tank effluent and tile drainage is important from a health perspective because the disposal of poorly treated septic

tank effluent or tile drainage can result in the potential transmission of infectious disease via waterborne pathogenic microorganisms (Craun, 1985, Gerba and Smith, 2005, Asano et al., 2007). Elevated concentrations of faecal bacteria and viruses have been detected in surface and groundwater located downstream of septic tanks, animal feeding operations, and land receiving animal waste application (Viraraghavan, 1978, Charles et al., 2003, Soupir et al., 2006, Sapkota et al., 2007). Because drinking and irrigation water is frequently sourced from waterbodies that receive upstream inputs of human or animal waste, these elevated concentrations present a serious public health concern. Therefore, there is a widespread need for appropriate on-site technologies that can reduce the risk of faecal pathogen contamination.

The ability of bioreactors to reduce microbial contaminants has been briefly assessed by Robertson et al. (2005) and Tanner et al. (2012), who reported 0.2 to 1.9 $\log_{10}$ reductions in *E. coli* with passage through a denitrifying bioreactor. This indicated that these systems can reduce microbiological contaminant loads. However, the datasets reported were limited, with only 10 data points scattered over a period of 3 years (Robertson et al., 2005) or only annual median reduction of *E. coli* reported (Tanner et al., 2012). Additionally, systems were solely analysed in terms of their inlet and outlet concentrations. Consequently, there was little information about the distance over which *E. coli* was removed, which is critical if bioreactors are to be designed to remove microbial contaminants. Furthermore, both studies solely measured changes in indicator bacteria and did not consider viruses. Viruses, however, pose an important health risk because they are present in large numbers in wastewater (Yates, 1985, Simmons and Xagoraraki, 2011), have the ability to migrate over long distances through the subsurface (Keswick and Gerba, 1980), and have high potential to initiate waterborne infections at low concentrations (Craun, 1985; Leclerc et al., 2002). Consequently, enteric viruses

have been recognized as a significant cause of waterborne disease outbreaks, with Norwalk-like viruses as one of the major causes of waterborne illnesses worldwide (Leclerc et al., 2002; Hrudey and Hrudey, 2007). Therefore, determining the ability of denitrifying bioreactors to remove viruses is important for assessing their capacity to reduce waterborne disease risks.

Due to differences in size, shape, survival characteristics, and susceptibility to disinfection, *E. coli* is unlikely to be a good model for the removal of viruses (Leclerc et al., 2000). Bacteriophage (viruses that infect bacteria) are commonly used to assess human enteric virus removal because direct detection and enumeration of pathogenic viruses is costly and time consuming. A specific group of bacteriophages that have particularly attractive features as models of human enteric viruses are F-specific RNA bacteriophages (FRNA bacteriophages). FRNA bacteriophages are commonly excreted in human faeces, and their physical structure, composition, and morphology closely resemble those of many human enteric viruses (Leclerc et al., 2000; Grabow, 2001). They have therefore been widely used in studies on wastewater virus transport and removal (Sinton et al., 2002; Hijnen et al., 2005; Zhang and Farahbakhsh, 2007; Aronino et al., 2009; Marti et al., 2011; De Luca et al., 2013) and are widely accepted as a model organism for viruses.

To address the paucity of information in relation to faecal microbial removal within bioreactors, we studied an operational full-scale denitrifying bioreactor receiving secondary-treated septic tank effluent initially established in 2013 for NO_3^- removal. We extended the performance evaluation to include an investigation into the removal of bacterial and viral faecal microbial contaminants, *E. coli*, and FRNA bacteriophage. Information about the distance over which *E. coli* and FRNA bacteriophage were removed was acquired by sampling along a longitudinal

transect within the bioreactor. Additionally, reduction in the major constituents of typical domestic wastewater, such as nutrients (nitrogen, phosphorus) and organic load (total suspended solids [TSS] and 5-d carbonaceous biochemical oxygen demand [CBOD5]), were quantified. This study allows us to assess the potential complementary use of denitrifying bioreactors for microbial contaminant and nutrient removal as well as organic load reduction in onsite wastewater treatment systems.

3.3 Materials and methods

3.3.1 Study site

In this study, we made use of a full-scale denitrifying bioreactor constructed in May 2013 at the Livestock Improvement Corporation, Newstead, New Zealand. The bioreactor consisted of a trapezoidal bed (20 m top length, 7 m top width, side slope of ~1:1 [width/height], 1.0 m depth, and zero bottom slope) lined with polyethylene and filled with woodchips (*Pinus radiata* D. Don, 10–30 mm in size) (Figure 3.1). A 150-mm-deep layer of planting media consisting of sand and coconut peat was placed over the top of a geotextile mesh overlaying the woodchip and was planted with *Carex virgata* Sol. Ex. Boott and *Cyperus ustulatus* A Rich. The roots of the plants did not penetrate the geotextile mesh and therefore remained restricted to the surficial layer of growth media.

The bed received effluent from a research station consisting of wastewater from laboratories and ablution blocks serving approximately 500 people during the majority of the year. The system was designed and sized based on required NO_3^- removal taking into account an anticipated increase in flow rate into the system as a result of an expected increase in occupancy. Before discharge into the bed, the
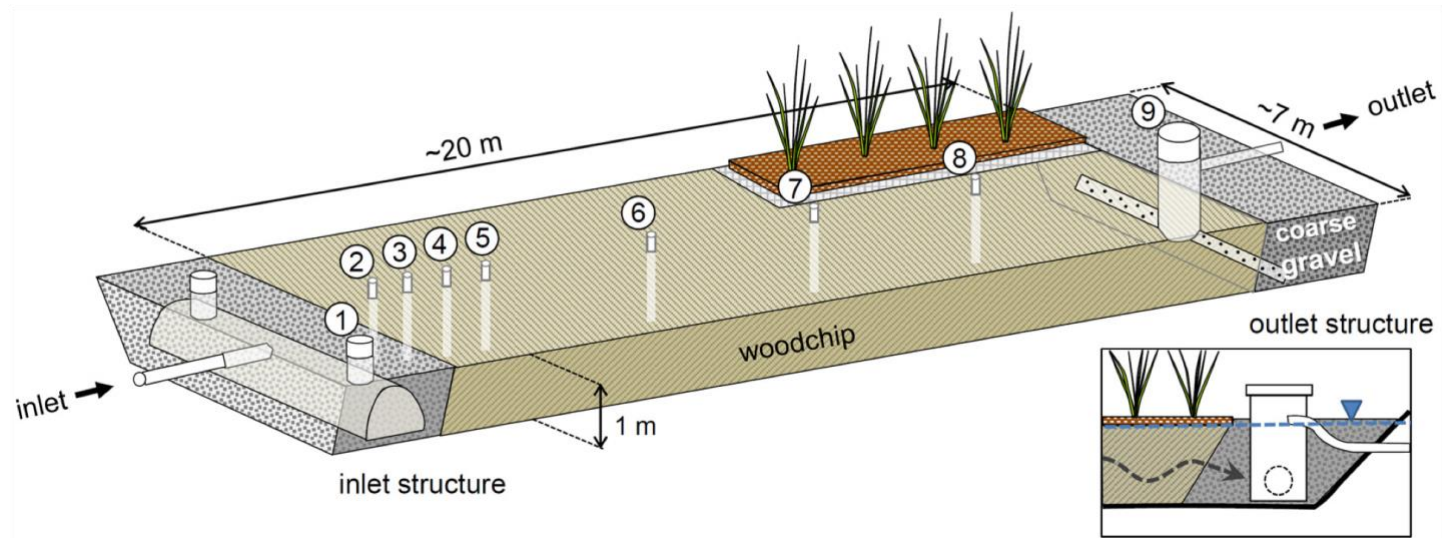
Figure 3.1 Schematic of the denitrifying bioreactor, indicating Sampling Points 1 through 9, the inlet structure (a slotted plastic arch vault with inspection risers set in coarse gravel), and the outlet structure (a slotted collection pipe set in coarse gravel connected to a sump with a standpipe). The overlaying planted coconut peat and sand layer is only partially shown.

effluent was pre-treated by passage through a septic tank and a recirculating textile filter (AdvanTex AX100, Orenco Systems Inc.). Effluent entered the denitrification bed through a slotted plastic arch vault with inspection risers at each end and exited through a slotted collection pipe connected to an outlet sump at the other end of the bed. The water level in the bed was controlled by a standpipe in the outlet sump keeping the water level in the system at 1 m above the bottom of the trench, near the surface of the woodchip media. After construction of the trench, four PVC sampling wells (50 mm diameter, 900 mm length) were installed at even intervals (of ~4 m) along the length of the bed pipe to allow for sampling along the longitudinal transect. In February of 2015, three additional PVC sampling wells (30 mm diameter, 900 mm length) were installed at even intervals (of ~1 m) between the inlet and first sampling well.

3.3.2 *Sampling and analysis*

From August 2013 to June 2015, bimonthly sample collections were made, each consisting of two grab samples from inspection risers at the inlet (Sampling Point 1 in Figure 3.1) and the outlet sump (Sampling Point 9). Samples were immediately placed on ice for transport for subsequent analysis. All samples were analysed for *E. coli* (most probable number [MPN] count in EC MUG Broth), total suspended solids (TSS; filtration, gravimetric), CBOD5 (incubation for 5 d at 20°C, dissolved oxygen meter), total Kjeldahl nitrogen (phenyl/hypochlorite colorimetry discrete analyser), ammoniacal nitrogen (NH4-N; phenyl/hypochlorite colorimetry by flow injection analyser), total phosphorus (TP; ascorbic acid colorimetry), and total oxidized nitrogen (NO_x-N; automated cadmium reduction by flow injection analyser) using standard methods (APHA, 2012). The inlet and outlet analysis were extended in June 2014 to February 2015 to include sulphate (SO_4^{2-}; filtered sample, ion chromatography).

Additional monthly grab samples were taken at Sampling Wells 4 through 8, located along the longitudinal transect of the denitrifying bioreactor, and analysed for NO_x-N. Because nitrite (NO_2^-) levels are often much lower than nitrate (NO_3^-), NO_3^-, and NO_x-N (the sum of NO_3^- and NO_2^-) were considered to be approximately equivalent for the purposes of this assessment.

From February to June 2015, sampling was extended to include analysis of *E. coli* and FRNA bacteriophage along the longitudinal transect of the denitrifying bioreactor. On a monthly basis, grab samples were collected from the inlet riser (Sampling Point 1), intermediate sampling wells (Sampling Points 2-8), and outlet sump (Sampling Point 9). Samples for *E. coli* and FRNA bacteriophage were collected on separate days. Samples were analysed for *E. coli* (MMO–MUG test using Colilert; IDEXX Laboratories), FRNA bacteriophage (double-layer agar technique), and NO_x-N (automated cadmium reduction by flow injection analyser) using standard methods (APHA, 2012). The FRNA methods were adapted to improve the level of detection in low concentration samples [<100 plaque-forming units (PFU) $(100\ mL)^{-1}$] by increasing the sample volume to 50 mL and adding this to 50 mL of top agar, which was then distributed over six plates lowering the detection limit to 2 PFU $(100\ mL)^{-1}$.

3.3.3 Flow rate, theoretical hydraulic residence time, and temperature

Total daily flow rate was measured before the inlet of the system using an electromagnetic flow meter (MagMaster, ABB Limited). Nominal (or theoretical) hydraulic retention time (nHRT) in the bed was calculated as nHRT = $(V_s\ n)/Q$, where V_s is the saturated volume of the bed, Q is the flow rate, and n is the primary porosity of the woodchip media. The primary porosity of the woodchip media was assumed to be 0.7 (Schipper et al., 2010b). Spot measurements of temperature

within the bioreactor were measured on a monthly basis using a calibrated meter (model WP81, TPS Pty.). As a result of a change in sampling protocol, no temperature measurements were conducted from January 2014 to August 2014.

3.3.4 Statistical analysis

As a result of the sampling frequency used (i.e., periodic sampling of all sampling wells occurred on the same day), outlet concentrations did not necessarily correspond to the inlet concentrations sampled on the same day. It was, therefore, not possible to precisely calculate contaminant reduction for each month. Reduction was consequently calculated as the difference between the average inlet and outlet concentration throughout the complete period of monitoring. When the data were non-normal, reduction was calculated on a median basis. Microbial removal was calculated as median log_{10} reduction. The values for the detection limits were used for censored data when concentrations were below detection limit. The Shapiro–Wilk's W test of normality was conducted to test if a distribution could be considered to be normal (Statistica version 12, StatSoft Inc.). Subsequently, differences between the concentrations of the microbiological and physiochemical parameters at the inlet and those at the outlet were tested for significance by ANOVA (for normal distributions) or Mann-Whitney test (for nonparametric distributions) (Statistica version 12, StatSoft Inc.). P values of <0.05 were considered significant.

3.4   Results

3.4.1 Flow rate and temperature

Flow rate through the denitrifying bioreactor varied with weekly and seasonal work patterns and subsequent laboratory and ablution block usage. Daily inflows varied between 0 and 29.9 m^3 from August 2013 to June 2015, with an average influent
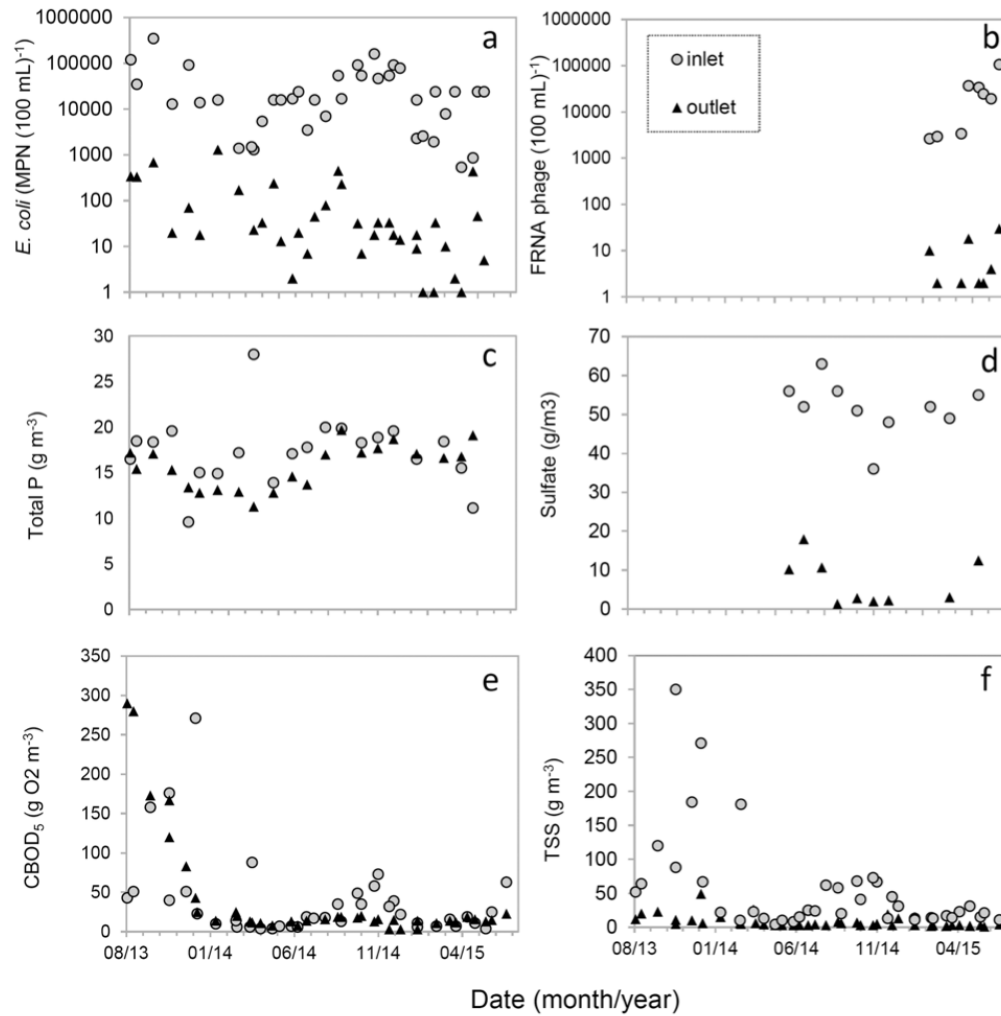
Figure 3.2 Inlet and outlet concentrations for (a) Escherichia coli (E. coli), (b) F-specific RNA bacteriophage (FRNA phage), (c) total phosphorus, (d) sulphate, (e) 5-d carbonaceous biochemical oxygen demand (CBOD5), and (f) total suspended solids (TSS) between August 2013 and June 2015. For E. coli and FRNA phage, the y axis is a log_{10} scale.

flow rate of 10.0 m^3 d^{-1} (SD, 6.8 m^3 d^{-1}), which approximately equals the use of 10 households (five persons, 1000 L d^{-1}). The average hydraulic residence time (HRT) in the denitrifying bioreactor was calculated to be ~8 d.

The average water temperature within the bioreactor ranged between 13 and 23°C, with the highest temperatures recorded in summer (February and March) and the lowest temperatures in winter (July and August).

3.4.2 Microbial contaminant reduction

The denitrifying bioreactor achieved a significant reduction in *E. coli* between the inlet and outlet, resulting in a median reduction of 2.9 $\log_{10}$ (P < 0.01) (Table 3.1). Although inlet concentrations of *E. coli* varied greatly through time [from ~500 to 3.5 × 10^5 MPN (100 mL) $^{-1}$] (Figure 3.2), reduction of *E. coli* was consistent over the 2-year period with 90% of all *E. coli* concentrations in the outlet being <350 MPN (100 mL)$^{-1}$ and a median outflow concentration of 20 MPN (100 mL)$^{-1}$ (Table 3.1). However, on two occasions concentrations above 500 MPN (100 mL)$^{-1}$ were recorded (Figure 3.2). The longitudinal survey of *E. coli* revealed that most of the removal occurred within 1 m from the inlet (Sampling Point 1) (Figure 3.3), with a median reduction of 1.4 $\log_{10}$ reduction. The average hydraulic retention time of the wastewater at this distance was approximately six hours (SD, 12 hours). Annual median *E. coli* reduction was 2.7 $\log_{10}$ (P < 0.01) in the first year of operation (August 2013 to July 2014) and 3.1 $\log_{10}$ (P < 0.01) in the second year of operation (August 2014 to June 2015).

FRNA bacteriophage inlet concentrations fluctuated from 2.7 × 10^3 to 1.1 × 10^5 PFU (100 mL)$^{-1}$ (Figure 3.2). The median inlet concentration was 2.2 × 10^3 PFU (100 mL)$^{-1}$ (Table 3.1). Overall, the denitrifying bioreactor achieved a 3.9 median $\log_{10}$ reduction in FRNA bacteriophage. Median outlet concentrations were very
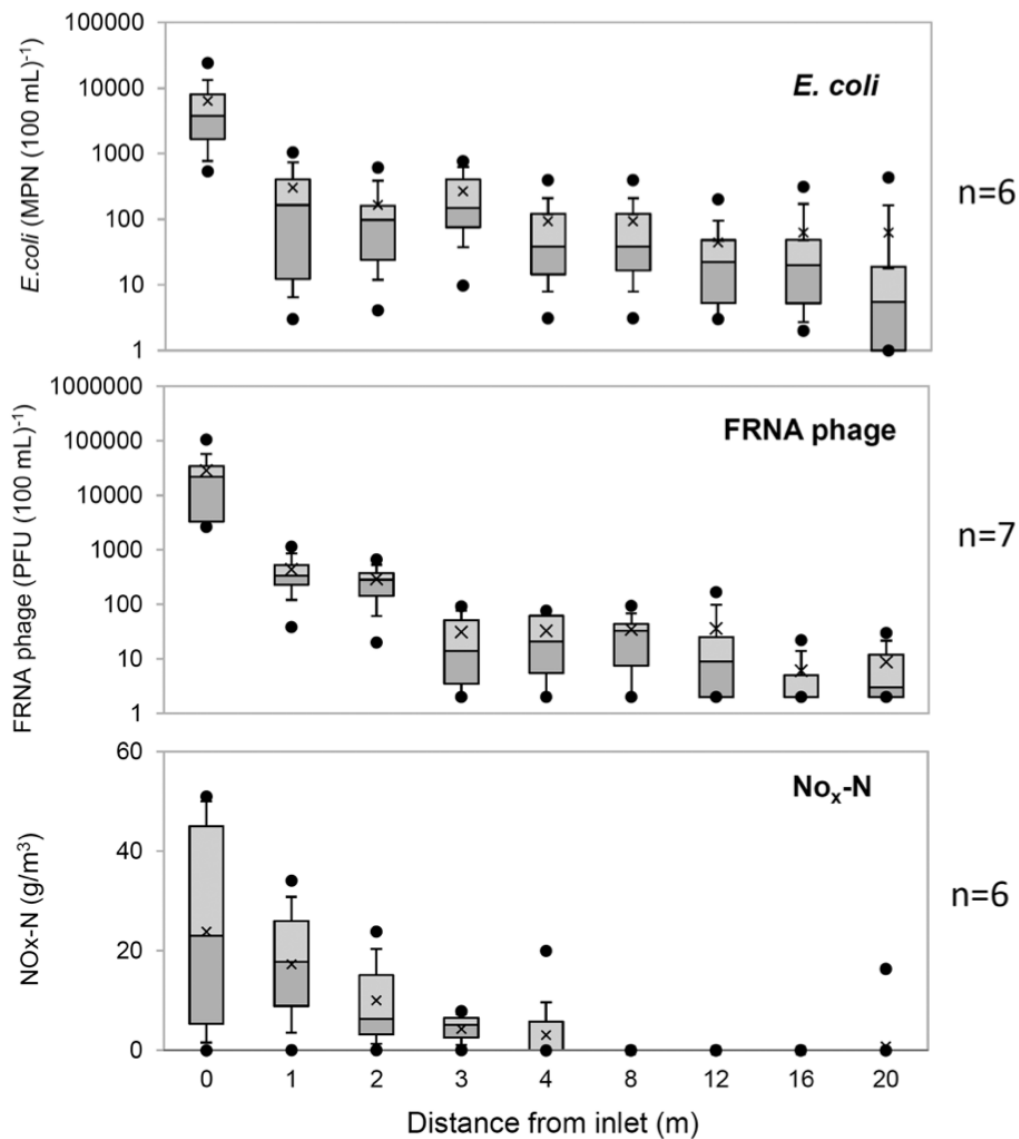
Figure 3.3 Box and whisker plot of Escherichia coli (E. coli), F-specific RNA bacteriophage (FRNA bacteriophage), and total oxidized nitrogen (NO_x-N) concentrations along the longitudinal transect of the denitrifying bioreactor measured between January 2015 and June 2015. Lines within the boxes are median values, the bottom and top of the boxes are the 25th and 75th percentiles, and error bars are the 10th and 90th percentiles. Dots represent the minimum and maximum values of the data, crosses represent the mean concentrations, and n refers to the sample size for each sampling well. For E. coli and FRNA bacteriophage, the y axis is a log10 scale.

low at 3 PFU $(100 \text{ mL})^{-1}$). Figure 3.3 shows that, similar to *E. coli*, most of the removal (1.8 median $\log_{10}$ reduction) FRNA bacteriophage occurred by the first sampling well (~1 m from the inlet). Near complete removal (3.2 median $\log_{10}$ reduction) was achieved before well 4 (at ~3 m from the inlet), which represents an average hydraulic retention time of about 1 day (SD, 2 days).

3.4.3 Nutrients and organic load reduction

Concentrations of N species in the inlet and outlet of the denitrifying bioreactors are given in Figure 3.4. Total N loads entering the bioreactor varied with time, ranging from 42 to 134 g N m^{-3}. Additionally, composition of inlet N loads varied with time, with NO_x-N inlet concentrations varying from 0.002 to 74 g NO_x-N m^{-3} (Figure 3.4). Nitrate was the major form of N removed from the effluents passing through the bed (Figure 3.2 and 3.4), with outlet concentrations generally below 0.02 g m^{-3} (with the exception of two outliers) and a median reduction of over 99.9% (Table 3.1). Average nitrate mass removal rate, calculated from the difference between the mass of NO_x-N at the inlet and Sampling Well 4 (at ~4 m from the inlet) divided by the volume of bioreactor up to this sampling well, was ~14 g N m^{-3} d^{-1}. The system received substantial NH_4^+ and organic N at the inlet. For these N species, the mean reduction was calculated to be 12 and 39%, respectively, but this was not a statistically significant reduction (Table 3.1). During the first 17 months of measurements, a significant reduction in phosphorus concentration (~14%) was observed as effluent passed through the denitrifying bioreactor (Figure 3.2). After this period, phosphorus outlet concentrations increased, resulting in an overall mean reduction of 7% for the entire monitoring period ($P = 0.06$). A substantial decrease in SO_4^{2-} concentration (94% reduction) was obtained between inlet and outlet wells (Figure 3.2). The denitrifying bioreactor

Table 3.1 Summary of contaminant concentrations for the inlet and outlet of the denitrifying bioreactor from August 2013 to June 2015.

Contaminant†	Inlet			Outlet			Reduction	P value‡
	n	Mean or median	SD or 90th percentile	n	Mean or median	SD or 90th percentile		
Escherichia coli, MPN (100 mL)$^{-1}$	42	**1.6×10^4**§	**9.2×10^4**	41	**20**	**350**	2.9 log$_{10}$¶	**<0.01**
F-RNA phage, MPN (100 mL)$^{-1}$	8	**2.2×10^4**	**3.4×10^4**	8	**3**	**23**	3.9 log$_{10}$¶	**<0.01**
TN, g m^{-3}	35	95.2	27.2	40	59.7	21.7	37.3%	<0.01
NO$_x$–N, g m^{-3}	35	31.2	24.5	40	0.0	0.0	99.9%	<0.01
NH$_4$–N, g m^{-3}	35	57.1	18.2	38	50.3	16.8	11.8%	0.11
Organic N, g m^{-3}	33	8.5	15.2	36	5.0	13.4	41.0%	0.34
TP, g m^{-3}	23	16.8	2.8	23	15.7	2.4	6.8%	0.06
SO$_4^{2-}$, g m^{-3}	10	15.6	3.1	10	15.0	2.4	4.2%	<0.01
TSS, g m^{-3}	40	51.7	8.4	42	6.7	6.3	87.0%	<0.01
CBOD$_5$, g m^{-3}	42	**54.8**	**8.0**	40	**8.0**	**8.9**	85.3%	**0.81**

† CBOD$_5$, 5-d carbonaceous biochemical oxygen demand; FRNA phage, F-specific RNA bacteriophage; MPN, most probable number; TN, total nitrogen; TP, total phosphorus; TSS, total suspended solids.

‡ Obtained with ANOVA or Mann–Whitney test as appropriate.

§ Bold values indicate situations where median, 90th percentile, and P values obtained with a Mann–Whitney test are given.

¶ Reduction efficiencies for *E. coli* and F-RNA phage are expressed as log$_{10}$ removals.

effluent had high CBOD5 (>100 g O_2 m^{-3}) during the first 9 months after start-up (Figure 3.2). Subsequently, CBOD5 decreased and stabilized at much lower values (mean outlet concentration, 14 g O_2 m^{-3}). After stabilization, the system achieved a significant reduction in CBOD5 load of 40% ($P = 0.04$). The system was able to substantially reduce TSS (87%), with a 90th percentile value of 18 g m^{-3} at the outlet ($P < 0.01$) (Table 3.1, Figure 3.2).

3.5 Discussion

3.5.1 Microbial contaminant reduction

This study demonstrated that a significant reduction of *E. coli* of around three orders of magnitude can be achieved by passing secondary-treated, nitrified effluent through a denitrifying bioreactor. These findings are supported by studies by Tanner et al. (2012) and Robertson et al. (2005), who also reported substantial reductions in *E. coli* within denitrifying bioreactors. However, quantitative comparison of *E. coli* reduction between these studies is challenging due to differences in experimental conditions (e.g. system size, nominal hydraulic retention time, and inlet concentration). Nevertheless, Tanner et al. (2012) reported slightly higher median outlet concentrations (70–1250 CFU [100 mL] $^{-1}$) and lower median $\log_{10}$ reductions (1.2–1.9 $\log_{10}$) for smaller bioreactor systems (1.8 m^3) with nominal retention times of 7 and 10 days, respectively. In the work by Robertson et al. (2005), the majority (79%) of all denitrifying bioreactor outlet samples had no detectable *E. coli* (<10 CFU [100 mL] $^{-1}$). These systems, however, received relatively low *E. coli* loads (up to 2000 CFU [100 mL]$^{-1}$). In our full-scale system, most of the reduction in *E. coli* in the denitrifying bioreactor occurred within the first meter from the inlet (at Sampling Well 2). It is therefore likely that this bioreactor has the capacity to manage substantially higher loads (i.e., higher concentrations or shorter hydraulic retention times).
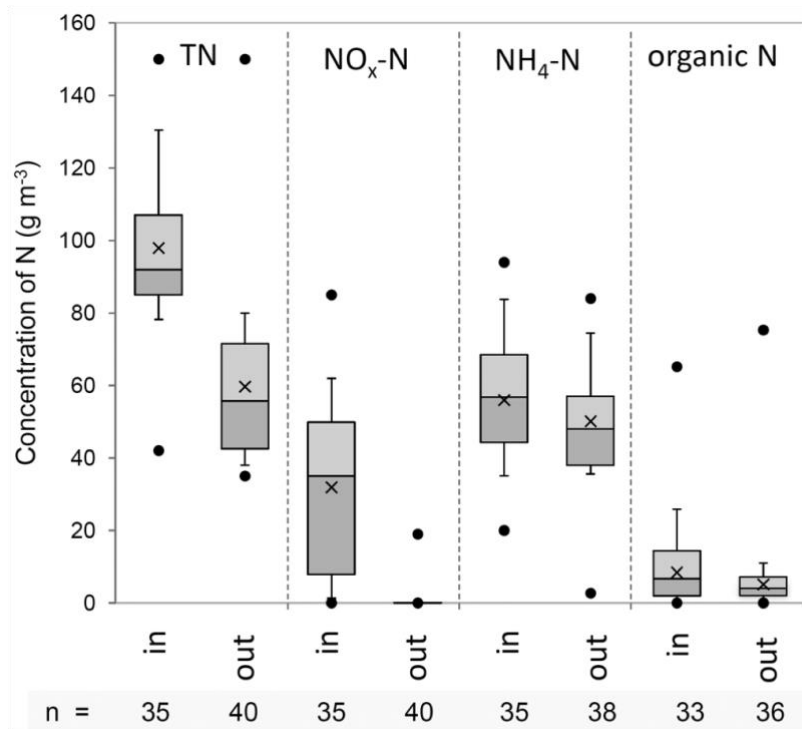
Figure 3.4 Box and whisker plot of inlet and outlet concentrations for different nitrogen species. Lines within the boxes are median values, the bottom and top of the boxes are the 25th and 75th percentiles, and error bars are the 10th and 90th percentiles. The dots represent the minimum and maximum values of the data, the crosses represent the mean concentrations, and n refers to the sample size. TN, total nitrogen; NO_x-N, total oxidized nitrogen; NH_4-N, ammoniacal nitrogen.

The removal performance of the bioreactor compared favourably with other passive technologies for wastewater treatment that are suggested as appropriate solutions for reducing pathogen loads from wastewater, such as treatment wetlands. Subsurface flow wetlands have been found to reduce microbial populations with varying but significant degrees of effectiveness. In general, reduction of *E. coli* achieved by this full-scale denitrifying bioreactor was at the upper end of the range (1.3-3.1 $\log_{10}$ reduction) reported in literature for horizontal subsurface flow (HSSF) wetlands (Green et al., 1997, Ottová et al., 1997, Decamp and Warren, 2000, Mantovi et al., 2003, Molleda et al., 2008). It is likely that there is a greater capacity for removal in the bioreactor system under investigation as outlet concentrations generally remained low and steady despite fluctuating inflow concentrations, with median and 90th percentile concentrations for *E. coli* of 20 and 350 MPN $(100 \text{ mL})^{-1}$, respectively, in the final effluent. This demonstrates the resilience of these systems for microbial contaminant removal.

In contrast to findings by Robertson et al. (2005), the denitrifying bioreactor in this study was not able to consistently reduce *E. coli* concentrations to near zero (i.e. below detection limit). The observed background concentration could be the result of the production of faecal indicator bacteria by animals that frequent the treatment system (Kadlec and Wallace, 2009) or the result of regrowth of *E. coli*, which has been observed in aquatic environments (Gerba, 2000, Ishii et al., 2006). Effluent concentrations from the bioreactor would generally be suitable for subsurface irrigation. To achieve concentrations for safe reuse within gardens or homes, where there is potential for human contact, effluent would require a greater degree of disinfection (WHO, 2006).

Due to fluctuations in inflow concentration, no pronounced seasonality effects for *E. coli* removal could be detected. Some HSSF wetlands display seasonal effects

for faecal coliform removal, with lower efficiencies at lower water temperatures (Rivera et al., 1995). The effect of seasonality on microbial reduction efficiency in denitrifying bioreactors should be investigated further under more controlled conditions.

Although some evidence for *E. coli* removal has previously been documented, there are no data available on the removal of viruses within denitrifying bioreactors. This study demonstrated that denitrifying bioreactors can also achieve significant and consistent reduction in FRNA bacteriophage. Because enteric viruses can behave similarly to FRNA bacteriophages in wastewater treatment processes (Grabow, 2001), the results of this study indicate that denitrifying bioreactors could also remove enteric viruses from wastewater.

Compared with *E. coli*, there is very limited information on the removal of FRNA bacteriophage in onsite treatment systems such as HSSF wetlands. Compared to FRNA bacteriophage removal reported in this study, in the literature, poorer removal rates for FRNA bacteriophage are generally reported for HSSF wetlands, with the degree of effectiveness between systems varying widely from -0.1 to 3.5 $\log_{10}$ reduction (Gersberg et al., 1987, Barret et al., 2001). Therefore, reductions in FRNA bacteriophage achieved by the full-scale denitrifying bioreactor in the present study exceeded the upper limit found in literature for HSSF wetlands. Because near complete reduction (3.2 median $\log_{10}$ reduction) in FRNA bacteriophage occurred by the fourth sampling well (at ~3 m from the inlet), the system is expected to be able to cope with higher loads.

Nitrate removal in denitrifying bioreactors has been shown to decline with time (Robertson et al., 2008, Moorman et al., 2010). Extended studies are required to determine if microbial contaminant removal decreases as the bioreactor matures. In the current study there was no obvious decline in removal rate of *E. coli* or FRNA

bacteriophage with time during the period of monitoring. In contrast, Tanner et al. (2012) observed an apparent decrease in *E. coli* removal performance with maturation of denitrifying bioreactors over 1 year. The long-term ability of denitrifying bioreactors to remove microbial contaminants from wastewater will depend on the main removal mechanisms. An understanding of these processes is needed to improve prediction of microbial contaminant removal in denitrifying bioreactors and to define standards for effective design of denitrifying bioreactors for microbial contaminant removal. For nitrate removal, a supply of carbon to denitrifying bacteria from woodchip is essential (Schipper et al., 2010b). Microbial contaminant removal mechanisms could include a variety physical, chemical, and biological processes, such as predation, adsorption, filtration, and die-off (Schijven and Hassanizadeh, 2000, Stevik et al., 2004). Further research on removal mechanisms of bacteria and viruses, how long these will remain active, and how they are affected by factors such as seasonality, loading rate, and inflow concentration is warranted.

3.5.2 *Nutrient and organic load reduction*

As expected, the denitrifying bioreactor was effective in removing NO_3^- from wastewater. The mass removal rate of 14 g N m^{-3} d^{-1} is at the high end of removal rates recorded for denitrifying bioreactors (Schipper et al., 2010b). The removal rate is expected to decrease as carbon depletes with maturation of the system (Schipper et al., 2005, Robertson et al., 2008, Moorman et al., 2010). However, throughout the period of monitoring, denitrification in the bioreactor was likely nitrate limited rather than C limited. The observed removal of SO_4^{2-} was in keeping with complete NO_3^- removal, which allowed SO_4^{2-} reduction (Schipper et al., 2010b). Robertson et al. (2005), Schipper et al. (2010a), and Tanner et al. (2012) also reported no significant removal of NH_4^+ or organic N with passage through the

denitrifying bioreactor. In contrast to findings by Schipper et al. (2010a), a small but significant reduction (~14%) in phosphorus concentration was observed as effluent passed through the denitrifying bioreactor. This reduction, however, only occurred in the first 17 months. This could be due to initial immobilization of phosphorus in microbial biomass or adsorption to the woodchip media during the first 17 months, followed by subsequent saturation of, or phosphorus release from the woodchip or microbial biomass. To improve phosphorus removal, the incorporation of phosphorus-adsorbing compounds in denitrifying bioreactors should be assessed. The high outlet CBOD5 during the first 9 months of operation of the denitrifying bioreactor was likely the result of leaching of soluble organic constituents from the woodchips, which may result in undesirable oxygen consumption in receiving waters (Robertson et al., 2005, Schipper et al., 2010a). The gradual decrease and stabilization of CBOD5 in the outlet over time indicate that CBOD5 loss is likely to be a temporary concern. The reduction and subsequent low TSS and CBOD5 concentrations at the outlet make the effluent readily amenable to disinfection via chlorination or ultraviolet lamps (Leverenz et al., 2006).

3.6 Conclusions

This study demonstrated that, in addition to significant reduction in NO_3^- loads, denitrifying bioreactors are effective at reducing bacterial and viral concentrations of secondary-treated, nitrified septic tank effluent. Substantial reductions in TSS were also achieved. Leaching of CBOD5 out of denitrifying bioreactors should be expected during the first months of operation; however, this is a short-term concern. Although the hydraulic loads entering the bioreactor varied substantially and influent bacterial and viral concentrations were often quite high and variable over time, the outlet concentrations generally remained low and stable but would require further disinfection for safe reuse of wastewater where there is potential for

human contact. The low TSS and CBOD5 outlet concentrations make the effluent readily amenable to further disinfection via chlorination or ultraviolet lamps. Despite high levels of NO_3^- removal, there was no evidence of removal of NH_4^+ or organic N during passage through the bioreactor. Although removal of phosphorus was observed, the overall reduction was relatively small and decreased with time. Overall, we present strong evidence for microbial contaminant removal in denitrifying bioreactors. To improve prediction of microbial contaminant removal in denitrifying bioreactors and to support the development of effective design criteria of denitrifying bioreactors for microbial contaminant removal, longer-term studies under well-controlled conditions are needed to identify the dominant microbial removal mechanisms, the longevity of removal and the influence of seasonality, loading rate, and inflow concentration on removal.

3.7 Acknowledgments

3.8 References

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Chapter 4

Bacteria and Virus Removal in Denitrifying Bioreactors: Effects of Media Type and Age

4.1 Abstract

Denitrifying bioreactors are simple, low-cost technologies designed to reduce nitrate (NO_3^-) present in septic tank effluent and drainage water. Recent studies indicate that, in addition to significant reduction in NO_3^- loads, these systems are also able to remove microbial contaminants from municipal wastewater. However, the removal of microbial contaminants in denitrifying bioreactors remains poorly characterised and factors that control removal in denitrifying bioreactors remain unexplored. In this study, the removal efficiency of faecal indicator bacteria *Escherichia coli* (E. *coli)* and total coliforms (TC) as a model for bacterial pathogens and F-specific RNA bacteriophage (FRNA bacteriophage) as a model for viruses was assessed for mesocosm-scale (~700 L) bioreactors receiving municipal wastewater. Systems were filled with two different slow-release carbon sources: woodchip and coconut husk. The effect of media age on attenuation of microbial contaminants was assessed by comparing the performance of 8-year old systems with equivalent newly constructed woodchip and coconut husk bioreactors. Additionally, removal performance of these carbon substrates was compared to that of gravel, a non-carbon substrate commonly used in subsurface flow (SSF) constructed wetlands. Substantial reduction of *E. coli* , TC and FRNA bacteriophage from primary treated municipal wastewater was achieved in all bioreactors. Mean annual $\log_{10}$ removal efficiencies were similar between microbial indicators ranging from 1.4 to 1.9 for TC, 1.3 to 1.8 for *E. coli* and 1.3 to 2.0 for FRNA bacteriophage. All denitrifying bioreactors showed consistent year-round

performance and long-term performance which did not markedly change in the ninth year of operation. The woodchip or coconut husk bioreactors achieved microbial effluent quality within the same range of $\log_{10}$ removal rates achieved in gravel-based systems. This suggests that denitrifying bioreactors, as well as reducing N loads, can effectively reduce microbial contaminants in wastewater, providing a complimentary disinfection role. Further research is needed to increase understanding of factors affecting removal of microbial contaminants in denitrifying bioreactors to support design of these systems for microbial contaminant removal.

4.2 Introduction

Extensive research has shown that denitrifying bioreactors can be an effective, low-cost, and simple treatment technology for reducing nitrogen (N) from septic tank effluent and agricultural drainage water (Schipper et al., 2010, Addy et al., 2016). Simply stated, denitrifying bioreactors are engineered structures, generally comprising beds, walls or layers, containing a porous carbon-rich media, commonly woodchips, through which water containing nitrate (NO_3^-) is passed (Schipper et al., 2010). During passage, the carbon media serves as an electron donor, and creates the anaerobic conditions needed to stimulate denitrification, the conversion of NO_3^- to N gas (Seitzinger et al., 2006).

To date, the majority of research on denitrifying bioreactors for onsite wastewater treatment has focused on NO_3^- removal from septic tank effluent (Robertson et al., 2005, Lopez-Ponnada et al., 2017). A recent study of a full-scale denitrifying bioreactor treating nitrified septic tank effluent provided strong evidence that these systems are also able to consistently reduce microbial contaminants (Rambags et al., 2016). Removal of microbial contaminants from wastewater is important from a human health perspective, since the contamination of environmental waters with inadequately treated wastewater can contribute to the potential transmission of

infectious disease caused by waterborne pathogenic microorganisms (Craun, 1985, Borchardt et al., 2011). Rambags et al. (2016) demonstrated a significant reduction of *both Escherichia coli* (*E. coli*; a bacterial indicator for enteric pathogenic bacteria) and F-specific RNA bacteriophage (FRNA bacteriophage; an indicator for human enteric viruses) of 2.9 and 3.9 $\log_{10}$ respectively after passing secondary-treated, nitrified effluent through a full-scale bioreactor filled with woodchip. The potential of denitrifying bioreactors to remove faecal microbes is supported by earlier studies by Robertson et al. (2005) and Tanner et al. (2012) in which reductions of *E. coli* up to 2.2 $\log_{10}$, were reported for woodchip bioreactors. This suggests that denitrifying bioreactors, as well as effectively reducing N loads, can reduce microbial contaminants in wastewater, providing a complimentary disinfection role.

Despite the apparent success of denitrifying bioreactors in reducing microbial contaminant loads, monitoring data remains scarce and factors that could potentially affect removal, such as type of filter material and media age remain unassessed. Variability in size, surface texture and charge between different bioreactor media is likely to affect removal of faecal microbes, as reported for other wastewater filter systems (Stevik et al., 2004, Wu et al., 2016). While wood media has shown an ability to deliver consistent NO_3^- removal over a longer term (5 to 15 years) (Robertson et al., 2008, Robertson et al., 2009, Schipper et al., 2005, Jaynes et al., 2008), the physical properties of the carbon media has been found to change over time (Robertson, 2010, Warneke et al., 2011), potentially affecting the longevity of these systems for microbial contaminant removal. Knowledge of the influence of filter media type and age on microbial contaminant removal will enable improved design of denitrifying bioreactors with the capacity to remove microbial contaminants.

A diverse range of pathogens are present in wastewaters. The faecal indicator bacteria *E.coli*, and to a lesser extent total coliforms (TC), are commonly used as an indirect measure of the removal of enteric pathogens in wastewater treatment (e.g. Tanner et al., 2012, Headley et al., 2013, Wu et al., 2016). Due to differences in size, shape, survival characteristics, and susceptibility to disinfection, these faecal indicator bacteria (FIB) are unlikely to be good models for the removal of viruses (Havelaar et al., 1993). FRNA bacteriophages, which are commonly excreted in human faeces, have a physical structure, composition, and morphology closely resembling those of many human enteric viruses (Leclerc et al., 2000, Grabow, 2001). They are, therefore, widely accepted as a model organism for human enteric viruses and are commonly used in studies on the transport and removal virus during soil passage and wastewater treatment (e.g. Gersberg et al., 1987, Barret et al., 2001, Quiñónez-Dìaz et al., 2001).

The current study addresses the paucity of information regarding removal of faecal microbes in denitrifying bioreactors by characterizing and comparing the removal of *E. coli*, TC and FRNA bacteriophage for two different types of carbon-rich porous media (woodchip and coconut husk) over the period of one year. Wood-particle media is typically the most commonly used material in field trials (Addy et al., 2016). Coconut husk is potentially an effective alternative for woodchip in low resource settings, as it is a low-cost material widely available throughout the tropics (Sato et al., 2017). Coconut husk has been found to deliver effective removal of NO_3^- (Tanner et al., 2012) as well as dyes, phenolic pollutants and inorganic anions from water (Bhatnagar et al., 2010). The ability of coconut husk for the removal of microbial contaminants, however, has not been investigated which limits understanding of its performance capabilities and application in the design of bioreactors for wastewater treatment. Using an existing set-up (Tanner et al., 2012), the performance of 8-year old mesocosm scale systems was compared with

that of equivalent newly constructed woodchip and coconut husk bioreactors to determine the effect of media maturity on microbial removal. In addition, the performance of equivalent gravel bioreactors was used to compare disinfection of wastewaters in the absence of organic lignocellulosic substrates.

An experimental facility, located at a wastewater treatment plant in Hamilton, New Zealand, enabled side-by-side comparisons and allowed for temporal effects (e.g. seasonality) on microbial contaminant removal to be explored in denitrifying bioreactors receiving the same wastewater at a controlled loading rate. To further characterise and compare systems, hydraulic retention time and a range of physio-chemical parameters for water quality were measured in inflow and outflow wastewater samples. Concurrent information on removal of different forms of N in denitrifying bioreactors will be reported separately in Chapter 5.

4.3 Materials and methods

4.3.1 Experimental set-up

The experimental set-up to test the performance of denitrifying bioreactors was constructed at the Pukete Wastewater Treatment Plant which serves the city of Hamilton in the North Island of New Zealand (population ~160,000). The location is characterised by a temperate climate with air temperatures ranging from -3.1 to 29.8°C throughout the period of monitoring (January 2016 to December 2016). The basic configuration of the experimental set-up is illustrated in Figure 4.1. Primary screened and settled wastewater (PSWW) from the municipal treatment plant (similar to septic tank effluent) was intermittently dosed half hourly onto two unsaturated vertical-flow gravel filters (VGF) to promote nitrification before application to the bioreactors. The nitrified wastewater was subsequently collected
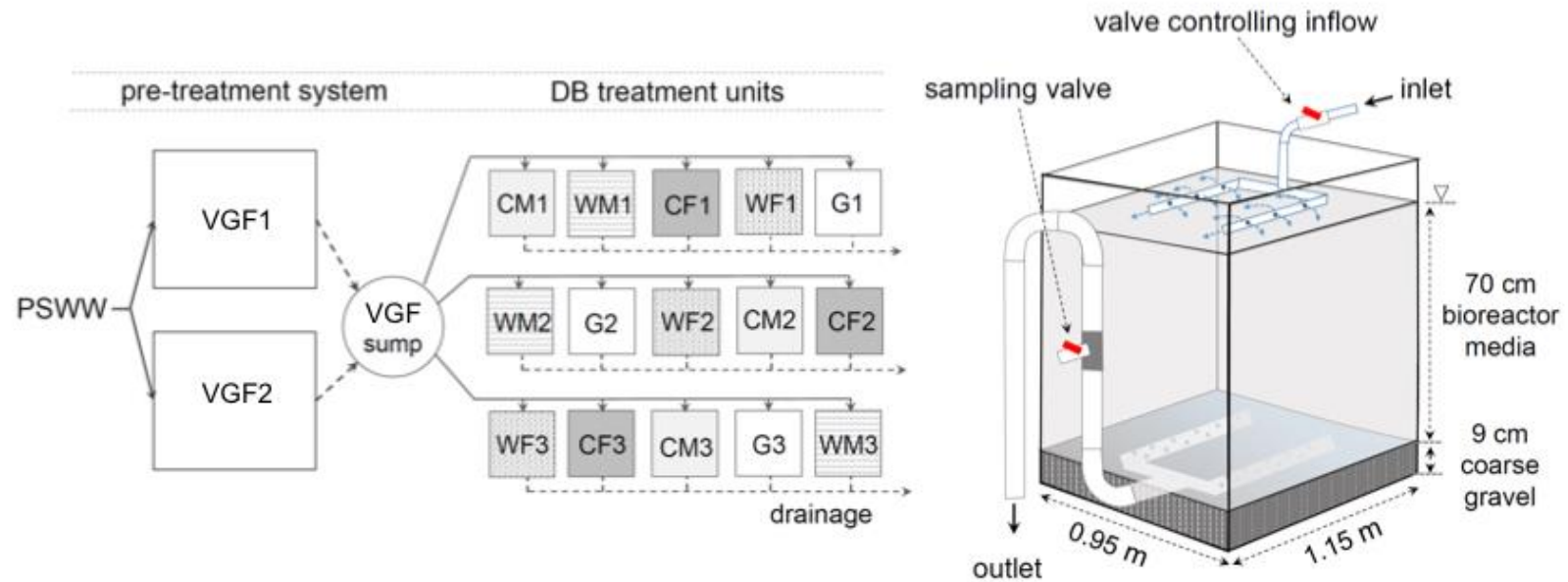
Figure 4.1 Conceptual flow diagram for experimental set-up, indicating the location of the pre-treatment systems (vertical gravel filter 1 (VGF1) and vertical gravel filters 2 (VGF2) which receive primary settled wastewater, and the denitrifying bioreactor units (DB) (left) and a schematic of a bioreactor unit (right). The bioreactor units comprised of bioreactor media: mature woodchips (WM), fresh woodchips (WF), mature coconut husk (CM), fresh coconut husk (CF) or gravel (G).

in a common sump from which three pumps each dosed wastewater via a manifold into a set of five discrete tanks. Each of the five tanks in a set was filled with a different media type (see below for treatment description). Treatment system specifications are summarised in Table 4.1.

The bioreactor mesocosms consisted of fifteen 1.15 x 0.95 m (1.1 m^2) x 1 m deep high-density polyethylene (HDPE) tanks (Figure 4.1). The tanks were constructed by cutting the top off food-grade intermediate bulk containers (IBCs, Schütz GmbH & Co., Victoria, Australia). The tanks were each filled with either (1) mature woodchip (WM: 20–50 mm wood chips, mixed *Pinus radiata* and *Pseudotsuga menziesii*, (2) fresh woodchip (WF: 20–50 mm wood chips, *Pinus radiata*), (3) mature coconut husk (CM: 10–20 mm chopped coir fibre pith from the mesocarp of *Cocos nucifera*, (4) fresh coconut husk (CF: 10-18 mm) or (5) gravel (G: 10-15 mm greywacke-derived river gravel), with three replicates for each media type. The mature woodchip and coconut husk bioreactors were from a previous trial reported by Tanner et al. (2012) and had received nitrified wastewater continuously for a period of 8 years before the start of this experiment. The fresh woodchip and coconut husk bioreactors were in operation for two months prior to the start of the present trial to enable stable operation after the initial flush of organic carbon from the carbonaceous media (Schipper et al., 2010). The bioreactors operated in saturated down-flow mode. The water level was maintained near the surface of the media using an exterior stand-pipe. Each bioreactor received hourly doses (24 per day) of ~6 L pre-treated wastewater (~146 L d^{-1}), which is just below a 1-person flow equivalent (165 L person^{-1} d^{-1}; based on AS/NZS1547 (2012)).

Table 4.1 Summary of treatment system specifications and inflows

Attribute*	Bioreactor treatments				
	Woodchip		Coconut husk		Gravel
	Mature	Fresh	Mature	Fresh	
System area (m^2)	1.1	1.1	1.1	1.1	1.1
System volume (m^3)	0.8	0.8	0.8	0.8	0.8
Mean inflow ± SD (L d^{-1})	144 ± 9	143 ± 10	144 ± 10	144 ± 10	144 ± 9
HLR ± SD (mm/d)	131 ± 9	130 ± 9	131 ± 9	131 ± 9	131 ± 8
drainable porosity	0.40	0.55	0.40	0.62	0.37
nHRT (d)	2.1	3.0	2.1	3.3	2.0

*SD is standard deviation, HLR is hydraulic loading rate, nHRT is nominal hydraulic retention time

4.3.2 Measurements, sampling and analysis

Wastewater treatment performance was compared over an annual period (January 2016 to December 2016) by taking monthly grab samples from the inflow point (i.e. the VGF sump) and outflow points of each bioreactor. Samples were analysed

within 24 h for TC and *E. coli* (Colilert, IDEXX Laboratories, Maine, USA), FRNA bacteriophage (double-layer agar technique), total suspended solids (TSS) (filtration, gravimetric) and five-day carbonaceous biochemical oxygen demand (CBOD5; incubation for 5 d at 20 °C) using standard methods (APHA, 2012).

Dissolved oxygen, pH, and electric conductivity were measured directly in the field monthly using calibrated meters (TPSTM models WP81 and WP82Y, TPS Pty., Queensland, Australia). The oxidation reduction potential (ORP) was measured monthly in the VGF sump using a platinum ORP electrode (model 96-78-00, Orion Research, Inc., Florida, USA) and also at 5 different depths within each bioreactor (5, 15, 25, 35 and 45 cm below surface) using welded platinum electrodes (Faulkner et al., 1989) inserted into the bioreactor and a double junction Ag/AgCl reference electrode (Model 90-02, Orion Research, Inc., Florida, USA) connected to an ISE meter (model 290A, Orion Research, Inc., Florida, USA). Temperature was recorded on a half hourly basis using 9 HOBO® pendant temperature data loggers (HOBO Data Loggers, Australia) in the nitrifying bioreactor sump (VGF) and at approximately 20 cm below surface in the bioreactors.

4.3.3 Data analysis

TC, *E. coli* and FRNA bacteriophage removal was determined as the $\log_{10}$ reduction between the mean measured inlet and outlet concentration for each treatment type (WM, WF, CM, CF or G) throughout the complete period of monitoring. To assess any seasonal effect on removal efficiency data points were
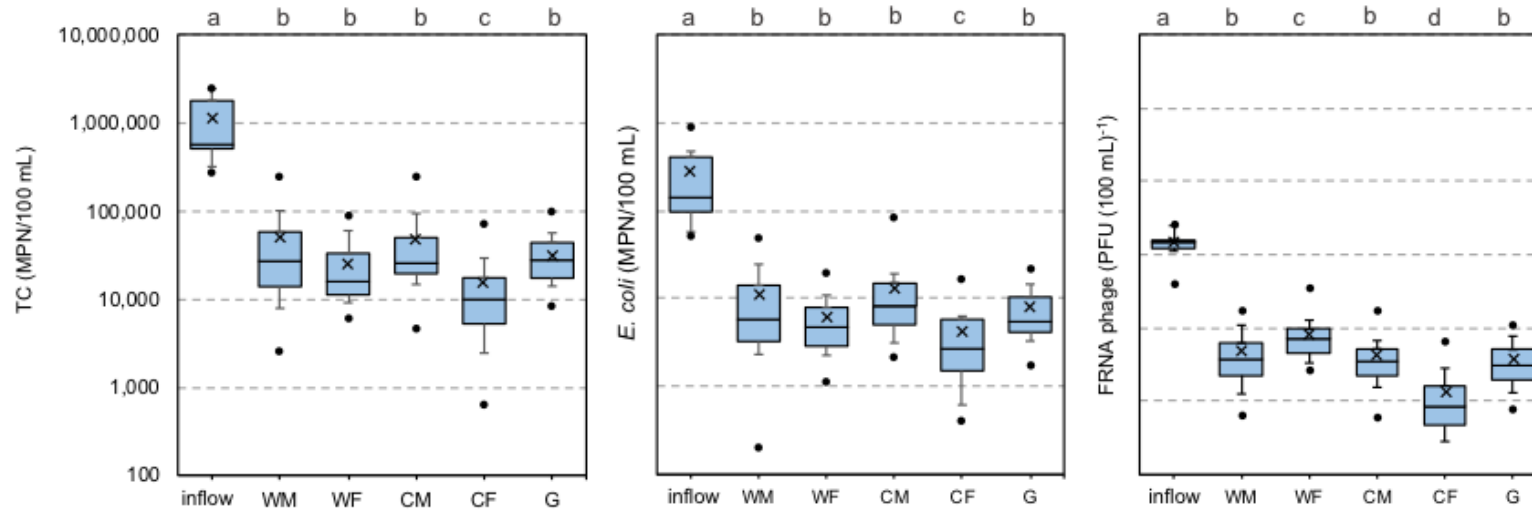
Figure 4.2 Box and whisker plots of inflow and outflow concentrations of the different treatment systems for total coliforms (TC), Escherichia coli (E. coli) and FRNA bacteriophage (FRNA bacteriophage). Lines within the boxes are median values, the bottom and top of the boxes are the 25th and 75th percentiles, and error bars are the 10th and 90th percentiles. The dots represent the minimum and maximum values of the data, the crosses represent the mean concentrations. The letters above the graph represent statistically homogeneous groups.

split into two groups: samples collected between the colder months of the year (May to October) and the warmer months (November to April). Microbial data was $\log_{10}$ transformed to meet data assumptions of normality for parametric analysis. Outflow concentrations of microbial contaminants in different treatment types were compared using an analysis of variance (ANOVA) assuming a randomised block design. Posthoc Newman-Keuls tests were then carried out on ANOVA results to identify treatment types that were significantly different from one another (p values < 0.05). Statistical analyses were conducted using the Genstat statistical software package for Windows 10th Edition. Simple linear regression analyses were conducted to examine the relationship between various physiochemical characteristics (pH, EC, DO, ORP and TSS and CBOD5 outflow concentration) and microbial contaminant reduction. Mean values for the different characteristics over the complete period of monitoring were used as the independent variables and mean $\log_{10}$ reduction over the complete period of monitoring per treatment unit for each microbial contaminant were used as dependent variables. Regression analyses were conducted in Statistica version 12, Statsoft, Inc.

4.4 Results

4.4.1 Reduction of TC and E. coli

All bioreactors effectively reduced FIB with overall $\log_{10}$ reductions in TC and *E. coli* concentrations ranging from 1.4 to 1.9 and 1.3 to 1.8 respectively (Table 4.2). Greatest reductions in TC and *E. coli* were recorded for the bioreactors filled with fresh coconut husk (CF). The CF bioreactors demonstrated consistently lower outflow concentrations for TC and *E. coli* compared to the other bioreactors with 0.2 to 0.5 $\log_{10}$ units greater removal of both indicator bacteria (Figure 4.2, Table 4.2). ANOVA and subsequent Newman-Keuls post hoc tests revealed that only CF bioreactor outflow concentrations differed significantly from the outflow

concentrations of the other bioreactors at p < 0.05; whereas WM, WF, CM and G bioreactor outflow concentrations were not significantly different (Figure 4.2).

Slight temporal variation in inflow concentrations of TC and *E. coli* were observed with levels of TC ranging over an order of magnitude from 2.76×10^5 to 2.42×10^6 MPN/100 mL and *E.coli* ranging from 4.33×10^5 to 5.83×10^5 MPN/100 mL. All three media types of bioreactors were able to consistently reduce TC and *E. coli* outflow concentrations to levels below 2.5×10^5 MPN/100 mL and 3.5×10^4 MPN/100 mL respectively, throughout the entire period of monitoring (Figure 4.2).

4.4.2 Reduction of FRNA bacteriophage

As well as effectively reducing bacterial loads, all bioreactors were able to significantly reduce concentrations of viral indicator FRNA bacteriophage with $\log_{10}$ reduction ranging from 1.3 to 2.0 (CF) and mean outflow concentrations ranging from 1.37×10^3 to 8.33×10^3 PFU/100 mL. The best performance was recorded in the CF bioreactors ($2.0 \log_{10}$ reduction), with intermediate performance in the WM, CM and G bioreactors (1.5 to 1.6 $\log_{10}$ reduction) and poorest performance recorded for the WF bioreactors ($1.3 \log_{10}$ reduction). ANOVA and subsequent post hoc Newman-Keuls revealed outflow concentrations for these three groups differed significantly at p < 0.05 (Figure 4.2)

4.4.3 Effects of seasonality

Small seasonal differences in microbial reduction were observed for the bioreactors with fresh media only with slightly greater removal observed in warmer months (November to April) of 0.3-0.4 $\log_{10}$ compared to the colder months (May to October); no seasonal difference was observed for the mature bioreactors (Table 4.2). Overall, ANOVA and subsequent posthoc Newman-Keuls tests did not

show any significant seasonality effect on bioreactor outflow concentration for *E. coli*, TC or FRNA bacteriophage between the warmer and colder months.

4.4.4 System characteristics and microbial contaminant reduction

Little variation was observed in pH, DO content and EC between bioreactor outflows (Table 4.3). Throughout the period of monitoring, all bioreactor outflows showed low oxygen concentrations (mean concentration of 0.3 g O_2 m^{-3} for all bioreactors), neutral pH levels (mean values ranged from 6.5 to 7.5) and stable EC values (mean values ranged from 531 to 591 μS cm^{-1}). With passage through the bioreactors TSS concentrations were substantially reduced (>77% reduction) in all systems to mean outflow concentrations below 3.1 g m^{-3}. Nominal HRT varied between bioreactor types (ranging from 2.0 to 3.3 days), resulting from differences in porosity (Table 4.1).

Mean CBOD5, an indicator of the amount of microbially degradable organic matter in the aqueous phase, in the outflow of the bioreactors ranged from 1.4 to 15.0 g O_2 m^{-3} (Table 4.3). Higher CBOD5 outflow concentrations were observed in outflow of the woodchip compared to the coconut husk bioreactors, suggesting higher release, or lower containment, of biodegradable organic compounds in the woodchip compared to coconut husk media.

While a wide range of ORP values were recorded for each measuring point throughout the year, in general lowest ORP (i.e. most reducing conditions) were observed in the woodchip bioreactors (mean ORP of -117 and -119 mV for WM and WF respectively). Less reducing conditions were observed in the coconut husk bioreactors (mean ORP of 187 and 162 mV for CM and CF respectively) and least reducing conditions were observed in the gravel bioreactors (mean ORP of 285

Table 4.2 Summary statistics for microbial contaminant concentrations in the in- and outflows of the different treatment systems. Removal efficiencies are reported as log$_{10}$ reductions (n is sample size, SD is standard deviation).

	Inflow	Outflow				
		Woodchip		**Coconut husk**		**Gravel**
		Mature	**Fresh**	**Mature**	**Fresh**	
TC (MPN/100 mL)						
n	11	30	30	28	31	29
Mean	1.11×10^6	4.92×10^4	2.53×10^4	4.87×10^4	1.52×10^4	3.17×10^4
SD	8.40×10^5	6.29×10^4	2.16×10^4	6.04×10^4	1.68×10^4	2.03×10^4
Removal Overall	1.2	1.4	1.6	1.4 (2.5)	1.9	1.5
Warmer months	-	1.4	1.4	1.3	1.7	1.4
Colder months	-	1.3	1.8	1.3	2.0	1.6
***E. coli* (MPN/100 mL)**						
n	11	33	33	33	33	33
Mean	2.75×10^5	1.10×10^4	6.01×10^3	1.28×10^4	4.15×10^3	7.83×10^3
SD	2.50×10^5	1.20×10^4	4.49×10^3	1.50×10^4	3.73×10^3	5.11×10^3
Removal Overall	1.2	1.4	1.7	1.3	1.8	1.5
Warmer months	1.5	1.4	1.8	1.3	2.0	1.4
Colder months	1.1	1.4	1.5	1.3	1.6	1.6
FRNA bacteriophage (PFU/100 mL)						
n	11	33	33	33	33	33
Mean	1.50×10^5	5.06×10^3	8.33×10^3	4.39×10^3	1.37×10^2	3.89×10^3
SD	6.10×10^4	4.17×10^3	6.38×10^3	3.51×10^3	1.49×10^3	2.70×10^3
Removal Overall	0.5	1.5	1.3	1.5	2.0	1.6
Warmer months	0.5	1.5	1.2	1.5	1.9	1.5
Colder months	0.3	1.5	1.4	1.6	2.1	1.7

mV). The wide variation in ORP values for each measuring point, however, indicate it was difficult to obtain accurate, reproducible and comparable measurements.

While pH, EC, DO content and TSS concentration were not correlated to microbial removal, simple linear regression to predict log_{10} removal based on nHRT provided a marginally good fit to the bacterial indicator data with around 60% of the variation in FIB mean removal explained by the retention time (R^2 = 0.58, p < 0.05 for TC; R^2 = 0.59, p =0.05 for *E. coli*). No significant correlation was found between mean log_{10} effluent reductions of FRNA bacteriophage and nHRT (R^2 = 0.17, p =0.2). Values for CBOD5 and ORP also did not appear to markedly influence microbial contaminant removal. No significant correlation was found between mean log_{10} effluent reductions of TC, *E. coli* or FRNA bacteriophage and CBOD5 outflow concentration (R^2 = 0.02, p = 0.82 for TC; R^2 = 0.06, p = 0.69 for E. coli; R^2 = 0.38, p = 0.27 for FRNA bacteriophage). Similarly, microbial removal did not correlate with ORP values (R^2 = 0.01, p = 0.87 for TC; R^2, 0.01, p > 0.99 for *E. coli*; R^2 = 0.29, p = 0.35 for FRNA bacteriophage).

4.5 Discussion

The removal of TC, *E. coli* and FRNA bacteriophage observed in this study suggests that, in addition to their known potential for removing NO_3^-, woodchip or coconut husk bioreactors can also effectively remove microbial contaminants from wastewater. Bioreactor systems achieved good microbial reduction even after 8 years of operation with levels of treatment and outflow quality comparable to that achieved in bioreactors filled with gravel. Although variability was observed in microbial removal performance between the denitrifying bioreactor types, differences in performance between bioreactors were relatively small. In general, annual mean removal differed by 0.5 to 0.7 log_{10} for bacterial or virus indicators. Overall, fresh coconut husk bioreactors consistently performed slightly better than

the other systems, typically by 60% (0.4 $\log_{10}$). The lower microbial removal efficiency found for the mature relative to fresh coconut husk bioreactors (by 68% as an annual average), however, indicated that the benefits of using coconut husk media may not persist with maturation. All denitrifying bioreactors showed robust year-round performance, with no significant difference in performance between warmer and colder months. The removal of microbial contaminants in porous media is known to be influenced by a variety of physicochemical factors (Schijven and Hassanizadeh, 2000, Stevik et al., 2004, Wu et al., 2016). Regression analyses suggested that differences in FIB reduction between bioreactors could partially be attributed to measured differences in media porosity and related hydraulic residence times within the systems. Variations in pH, EC, DO content and TSS concentrations observed between bioreactor outflows were likely too small to have a major influence on differences in microbial removal between the bioreactor types. Additionally, differences in dissolved organic matter content between bioreactor outflow did not appear to result in differences in microbial removal between bioreactor types.

4.5.1 Reduction of TC and E. coli

Significant reductions of TC and *E. coli* was achieved by passing secondary-treated, nitrified effluent through a denitrifying bioreactor filled with either woodchip or coconut husk. The 1.8 and 1.4 annual average $\log_{10}$ reductions for *E. coli* in fresh and 8-year old woodchip bioreactors respectively in this study were comparable to those found by Tanner et al. (2012) of 1.2 to 1.9 $\log_{10}$ units in 2-year-old woodchip bioreactors of the same size and with a similar mean hydraulic loading rate ($\pm$ 167 mm d^{-1}). Rambags et al. (2016) also reported comparable reductions for *E. coli* (1.4 $\log_{10}$ units) in the first metre of a 1-year old woodchip bioreactor, although the hydraulic residence time at this distance was much shorter (about 10 hours).

Table 4.3 Summary of mean (± standard deviation) pH, electrical conductivity (EC), dissolved oxygen content (DO), oxidation reduction potential (ORP) and concentration of total suspended solids (TSS) and 5-day carbonaceous biochemical oxygen demand (CBOD5) in the outflows of the different treatment system.

	Inflow	Outflow				
		Woodchip		Coconut husk		Gravel
		mature	fresh	mature	fresh	
pH	6.9 ± 0.2	6.8 ± 0.2	6.9 ± 0.3	6.8 ± 0.2	6.8 ± 0.2	7.0 ± 0.2
EC (μS cm^{-1})	653 ± 81	557 ± 48	580 ± 44	531 ± 42	546 ± 51	591 ± 55
DO (g O$_2$ m^{-3})	1.9 ± 0.6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
TSS (g m^{-3})	13.4 ± 6.2	2.9 ± 2.1	2.9 ± 1.7	3.1 ± 2.7	2.9 ± 2.1	1.7 ± 1.0
CBOD5 (gO$_2$ m^{-3})	13.5 ± 6.2	9.4 ± 4.5	15.0 ± 7.0	1.4 ± 0.8	4.2 ± 4.6	1.4 ± 0.6
ORP (mV)*	224 ± 104	-117 ± 57	-119 ± 40	187 ± 88	162 ± 102	285 ± 49

The relative greater reduction of *E. coli* in the fresh coconut husk bioreactors observed in this study supports previous findings by Tanner et al. (2012) who noted more effective removal of *E. coli* in coconut husk bioreactors compared to woodchip bioreactors during the first year of operation. The removal performance of the coconut and woodchip bioreactors compared favourably with the performance of the gravel bioreactors. Overall, removal of TC and *E. coli* in the woodchip and coconut husk bioreactors (1.3 to 1.9 $\log_{10}$) were in the same range (0.5 to 2.0 $\log_{10}$ for TC and 1.3 to 1.5 $\log_{10}$ for *E. coli*) as reported for pilot and full scale SSF gravel wetlands operating at similar hydraulic retention times of 2-3 d (Headley et al., 2013, Wu et al., 2016). This substantiates previous findings by Rambags et al. (2016) that denitrifying bioreactors can achieve similar treatment levels as gravel-based SSF wetlands and that they could therefore be considered as appropriate solutions for reducing bacterial loads from wastewater.

While FIB inflow concentrations did not fluctuate highly throughout the period of study, concentrations were elevated by at least an order of magnitude above that observed at peak flows for bioreactors treating agricultural drainage water during storm events of 5×10^4 *E. coli* per 100 mL (Tomer et al., 2010). Removal rates of the bioreactors were not altered by the fluctuating inflows and *E. coli* outflow concentrations from all bioreactors generally ranged from 10^3 to 10^4 MPN/100 mL. These outflow concentrations may be considered marginally acceptable for unrestricted irrigation (WHO, 2006) and acceptable for restricted irrigation reuse on crops that are not eaten raw (Blumenthal et al., 2000). Near zero concentrations of *E. coli* (i.e. outflow concentrations <20 MPN/100 mL) have previously been achieved in full scale bioreactors (Robertson et al., 2005, Rambags et al., 2016) suggesting that lower outlet concentrations can be achieved by increasing system size relative to inflow rate (i.e. increasing residence time).

4.5.2 Reduction of FRNA bacteriophage

FRNA bacteriophage were effectively removed with concentration reductions of at least 95% ($\geq 1.3 \log_{10}$) in denitrifying woodchip or coconut husk bioreactors receiving secondary-treated, nitrified effluent. Overall, annual average FRNA bacteriophage reductions achieved in the woodchip and coconut husk bioreactors (1.3 to 2.0 $\log_{10}$), were comparable to reductions found by Rambags et al. (2016) who reported 1.7 $\log_{10}$ reduction in the first metre of a woodchip bioreactor during the second year of operation. Both studies also found slightly higher FRNA bacteriophage removal (0.1 to 0.4 $\log_{10}$) than that observed for *E.coli* despite the much smaller size (by at least 2 orders of magnitude) of FRNA bacteriophage. Similar observations of greater FRNA removal than *E.coli* have also been reported for other treatment systems such as storm water biofilters (Li et al., 2012).

In contrast to *E. coli* removal, the mature woodchip bioreactors consistently achieved higher FRNA bacteriophage reductions (1.5 $\log_{10}$) compared to the fresh woodchip (1.3 $\log_{10}$), suggesting a positive effect of maturation on virus reduction for woodchip media. In contrast, a negative effect of maturation on FRNA bacteriophage reduction was observed for the coconut husk bioreactors, with higher removal in the fresh (2.0 $\log_{10}$) compared to the mature coconut husk bioreactors (1.5 $\log_{10}$). While differences in virus reduction were observed between woodchip and coconut husk bioreactors in the first year of operation, the lack of difference in FRNA bacteriophage outflow concentrations between the mature woodchip and coconut husk bioreactors indicated that denitrifying bioreactors filled with woodchip or coconut husk could be expected to achieve similar effluent quality with maturation. Factors potentially controlling removal are discussed further below, but processes such as biofilm formation, adsorption and straining could

have contributed to differences in microbial removal observed between woodchip and coconut husk.

In this study, the removal performance of the mature coconut and woodchip bioreactors compared favourably with the performance of the gravel bioreactor, which is analogous to an unvegetated SSF constructed wetland. Limited information exists for the fate of viral indicators in SSF wetlands, however, all $\log_{10}$ reductions for FRNA bacteriophage recorded in our bioreactors (1.2 to 2.1 $\log_{10}$) were within the wide range (−0.1 to 4.3 $\log_{10}$ reduction) reported in the literature (Gersberg et al., 1987, Quiñónez-Dìaz et al., 2001, Barret et al., 2001). These findings suggest that woodchip or coconut husk denitrifying bioreactors can effectively reduce virus concentrations for more than 8 years within the same range of treatment levels achieved in SSF gravel wetlands.

4.5.3 Seasonality

The removal of microbes should ideally be consistent throughout the year. This study provided no evidence for an effect of temperature on microbial removal in denitrifying bioreactors. This is in line with findings by Kadlec and Wallace (2009), who found insufficient evidence as to the effect of temperature on FIB removal in a review of different SSF constructed wetlands. However, a recent study by Soupir et al. (2018) reported significantly greater removal of *E.coli* (0.6 $\log_{10}$) in a laboratory scale woodchip bioreactor operating at 21.5 °C compared to 10°C, indicating that temperature significantly increased bacterial removal. Further research on the effect of temperature on microbial contaminant removal in denitrifying bioreactors is therefore recommended.

4.5.4 System characteristics and microbial contaminant reduction

The removal of microbial contaminants in denitrifying bioreactors are likely a combination of different physical, chemical and biological processes as reported for other passive systems for wastewater treatment such as SSF wetlands (Wu et al., 2016). It is generally assumed that, with passage through saturated porous media, microbes can either be inactivated (i.e. killed or rendered unculturable) or immobilized (Schijven and Hassanizadeh, 2000, Stevik et al., 2004). Processes for inactivation of microbial contaminants include predation by other microbes, bacterial and viral lysis, antibiosis, die-off due to biocide exposure and natural die-off (Yates et al., 1988, Schijven and Hassanizadeh, 2000, Stevik et al., 2004). The two mechanisms responsible for immobilization of bacteria in wastewater moving through a porous media are straining (i.e. the physical blocking of movement through pores) and adsorption (Keswick and Gerba, 1980, Yates and Yates, 1987, Stevik et al., 2004, Wu et al., 2016). Key factors identified as influencing removal of microbial contaminants are biofilm formation, water organic matter content, conductance properties of the fluid, pH, DO content, hydraulic loading rate, HRT, grain size of the porous media, filter media properties and microorganism size and shape (Schijven and Hassanizadeh, 2000, Stevik et al., 2004).

Retention time is known to be a key factor influencing bacteria removal in denitrifying bioreactors and constructed wetlands (Vymazal, 2005, Headley et al., 2013, Wu et al., 2016, Soupir et al., 2018). In this study, porosity and residence time of both the woodchip and coconut husk bioreactors decreased with maturity, most likely due to media decomposition and repacking. Enhanced hydraulic retention time is assumed to increase microbial removal due to increased exposure to inactivation processes and increased likelihood for microbial adsorption (Wu et al., 2016). In our study, linear regression to predict $\log_{10}$ removal based on nHRT

provided a marginally good fit to the bacterial data, indicating that differences in bacterial reduction between bioreactors could partially be the result of the differences in hydraulic residence times within each system (mainly a function of media porosity).

While microbial removal in porous media is influenced by a variety of physicochemical factors, as described above, in this study, pH, EC, DO content, TSS concentrations and CBOD5 in the bioreactor outflow were not correlated to microbial removal. The small variations in EC (ranging from 531 to 591 μS cm^{-1}), pH (ranging from 6.8 to 7.0) and DO (0.25 to 0.30 g O$_2$ m^{-3} for all bioreactor types) observed between bioreactor types were likely too small to have a major influence on differences in microbial removal between the bioreactor systems (Stevik et al. 2004). A large part of *E. coli* removal in the bioreactors could be due to attachment to wastewater particles which in turn can be removed by settling or straining (Walters et al., 2014, Boutiliera et al., 2009). However, in this study, all bioreactor systems were able to effectively reduce TSS loads and differences in TSS outflow concentrations could not be correlated to microbial removal. Dissolved organic matter has both enhancing and attenuating effects on microbial removal; it can enhance bacterial survival and decrease microbial adsorption by competing for the same binding sites but can also provide binding sites for bacteria and viruses (Schijven and Hassanizadeh, 2000; Stevik et al., 2004). Variations in CBOD5 between the bioreactor outflows, ranging from 1.4 to 15.0 g O$_2$ m^{-3}, suggested higher release, or lower containment, of biodegradable organic compounds in the woodchip compared to coconut husk and gravel media. However, no correlation between CBOD5 and microbial removal was found, suggesting that differences in dissolved organic matter content did not result in marked differences in microbial removal between bioreactor types.

The variability in size, surface texture and charge of porous media can greatly influence the adsorption of microbial contaminants (Stevik et al., 2004). The ability of coconut husk to remove dyes, phenolic pollutants and inorganic anions is commonly attributed to the relatively large surface area per unit volume of coconut husk media (Bhatnagar et al., 2010). The high removal efficiencies found for fresh coconut husk in this study could potentially be attributed to its relatively large surface area, providing more adhesion sites for adsorption of bacteria and viruses (Stevik et al., 2004).The lower microbial removal efficiency found for the mature coconut husk bioreactors, however, indicated that the benefits of using coconut husk media may not persist with maturation, potentially due to changes in the physical properties of the coconut husk media over time. Alternatively, changes in removal efficiency with maturation could potentially be attributed to the release of antimicrobial compounds by the coconut husk. When soaked in water, coconut husk has been found to release extracts that negatively affect biofilm formation and adhesion of bacteria onto surfaces (Viju et al., 2013). Additionally, coconut husk extracts have demonstrated effective antimicrobial activity against a variety of bacterial indicators and pathogens (Akinpelu et al., 2015). Future work should, therefore, assess the longevity of coconut husk alongside its disinfection. This is particularly important for the application of coconut husk in wastewater treatment systems in low resource settings where coconut husks offer a sustainable solution as a natural support media for wastewater treatment (Sato et al., 2017).

The removal of faecal microbial contaminants in bioreactors could be influenced by biofilm formation on surfaces leading to increased attachment (Stott and Tanner, 2005). Studies on the role of biofilm formation for wastewater treatment in porous media suggest that biofilm formation increases the total surface area and water retention time, which is considered favourable for the retention and inactivation of bacteria and viruses (Schijven and Hassanizadeh, 2000, Stevik et

al., 2004). The positive effect of maturation of woodchip on FRNA removal might be attributed to enhanced biofilm formation on media surfaces. However, little research has been done on biofilm formation in denitrifying bioreactors.

The ORP of flooded soils and sediments is commonly used as a semi-quantitative measure of the degree of anaerobiosis, and an indicator of the dominant microbial transformations occurring (e.g. denitrification at ~250 mV) (Mitsch and Grosselink, 2015). ORP is also a key physiochemical factor affecting the growth and functioning of microbes (Breznak and Costilow, 2007) and has been widely used as a control parameter for process control of bacterial inactivation processes (Goncharuk et al., 2010). In the present study, we found substantial differences in mean ORP values between the bioreactor types ranging from -119 to 285 mV, but this did not show any significant correlation with microbial removal rates.

Denitrifying bioreactors present a highly complex environment in which a wide range of microbial immobilisation and inactivation processes may simultaneously occur, making it difficult to identify the most important processes. While our analyses provide some insight into the effect of system characteristics on microbial contaminant removal in bioreactors, more systematic analyses on the removal processes and factors which govern microbial removal are required to enhance mechanistic understanding and improve prediction of faecal microbial contaminant in denitrifying bioreactors. This should include work assessing the effect of challenging operational conditions such as fluctuating flows and peak inflow concentrations in systems treating intermittent wastewater and diffuse pollution sourced inflows (Stott et al., 2018).

4.6   Conclusions

Removal of TC, E. *coli* and FRNA bacteriophage was assessed for coconut and woodchip bioreactors to evaluate the potential complementary use of denitrifying bioreactors for faecal microbial contaminant removal in on-site wastewater treatment. This study demonstrated that:

- Effective reduction of TC, *E. coli* and FRNA bacteriophage can be achieved by passing secondary treated wastewater through a woodchip or coconut husk denitrifying bioreactor with levels of treatment and outflow quality comparable to that achieved in similar gravel-based systems,

- All denitrifying bioreactors showed consistent long-term performance which did not markedly change in the ninth year of operation,

- While enhanced removal of microbes was observed for fresh coconut husk bioreactors, the lower microbial removal efficiency found for the mature coconut husk bioreactors indicated that these benefits may not persist with maturation,

- Climatic conditions, within the range experienced in this study, made little difference to FIB treatment efficiency, with all bioreactors showing robust year-round performance,

- Small differences in FIB reduction between bioreactors could partially be the result of the measured differences in media porosity and related hydraulic residence times within the systems,

- Variations in pH, EC, DO content and TSS concentrations observed between bioreactor types were likely too small to have a major influence on differences in microbial removal between the bioreactor systems,

- Differences in dissolved organic matter content or oxidation-reduction potential did not appear to result in differences in microbial removal between bioreactor types.

4.7 Acknowledgements

4.8 References

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Chapter 5

Nitrate and Ammonium Removal in Woodchip and Coconut Husk Bioreactors: Denitrification, Anammox and/or Codenitrification?

5.1 Abstract

Denitrification has been considered the major pathway converting nitrate (NO_3^-) to dinitrogen gas (N_2) in denitrifying bioreactors. Here, the importance of anaerobic ammonium oxidation and codenitrification (jointly referred to as An/coD), was assessed by monitoring the removal of N species from partially nitrified municipal wastewater passing through fifteen mesocosm scale (~700 L) bioreactors containing woodchip, coconut husk or gravel during their initial and eighth year of operation. Lab experiments using a ^{15}N isotope-pairing technique were performed to partition production of N_2 to these different microbial processes. The effective removal of both NO_3^- and ammonium (NH_4^+) observed in the field and the formation of hybrid N_2 (i.e. $^{29}N_2$) demonstrated that the An/coD, along with denitrification, was an effective pathway for N removal when both NO_3^- and NH_4^+ were present. An/coD removal rates ranged from 0.6 to 3.8 g N per m^3 reactor volume per day, while denitrification rates ranged from 0.7 to 2.6 g N per m^3. The contributions of An/coD to N removal was dependent on media, with An/coD becoming more dominant in bioreactors where denitrification was carbon limited. Designing denitrifying bioreactors to support both denitrification and An/coD expands the utility of these passive approaches for improving treatment of wastewater.

5.2 Introduction

Denitrifying bioreactors are simple, low-cost technologies developed to reduce nitrate (NO_3^-) present in septic tank effluent and drainage water by enhancing heterotrophic denitrification (Schipper et al., 2010b, Christianson et al., 2012). Simply stated, they are engineered structures containing a porous, carbon-rich media (commonly woodchips) through which NO_3^--rich water is passed. The organic carbon creates an anoxic environment and acts as an electron donor to support conversion NO_3^- to dinitrogen gas (N_2) by denitrification (Seitzinger et al., 2006, Schipper et al., 2010b). Their simplicity, low maintenance requirements and ability to effectively remove nitrogen (N), with removal rates generally ranging from about 2 to 11 g N per m^3 reactor volume per day, has led to accelerated adoption of denitrifying bioreactors for NO_3^- mitigation in a variety of settings over the past decade (Schipper et al., 2010b, Christianson et al., 2012, Addy et al., 2016).

While there are multiple microbial processes that compete for NO_3^- in the nitrogen cycle it is generally assumed that heterotrophic microbial denitrification is the main mechanism responsible for N removal in denitrifying bioreactors (Schipper et al., 2010b). Other possible NO_3^- transformation processes include N assimilation into organic N (i.e. biomass), dissimilatory nitrate reduction to ammonium (DNRA, i.e. ammonification), anaerobic ammonium oxidation (anammox and codenitrification (Spott et al., 2011, Kuypers et al., 2018). While several studies have reported that N assimilation and DRNA play only a minor role in NO_3^- removal in denitrifying bioreactors (Schipper and Vojvodić-Vuković, 1998, Robertson, 2000, Robertson et al., 2005, Robertson et al., 2007, Schipper et al., 2010a, Gibert et al., 2008), the role of anammox and codenitrification in bioreactors has not been explored.

Anammox and codenitrification are both microbial processes which can produce N gases (N_2O and N_2) from the utilization of NH_4^+ and NO_3^-. Anammox produces N

gas by oxidizing ammonium (NH_4^+) with NO_3^- or nitrite (NO_2^-) under strictly anoxic conditions (Kuenen, 2008). Codenitrification is assumed to produce N_2O and N_2 molecules when, during the reduction of NO_3^- by denitrification, a side reaction occurs between NO_2^- or NO^- and a nucleophile, such as NH_4^+ or other monomeric organic N sources such as amines (Spott et al., 2011). While, in general, anammox and codenitrification are considered to be two different processes (Selbie et al., 2015, Long et al., 2013, Yang et al., 2015), studies on the metabolism of annamox and codenitrification bacteria suggest that N gas production by both processes follows the same N reaction pathway (Spott et al., 2011). It has, therefore, been argued that these processes could perhaps be viewed as analogous (Spott et al., 2011). Because these two processes are difficult to distinguish from one another, in this study, we do not differentiate between them.

If present in denitrifying bioreactors, anammox and/or codenitrification (An/coD) would be beneficial for N removal from domestic wastewater as it would, in addition to removing NO_3^-, also allow for the effective removal of residual NH_4^+. When considering the overall treatment process for N removal from wastewaters, An/coD capability reduces the oxygen (and associated energy) requirement for preceding nitrification stages and increases the N_2 production capacity per gram of carbon consumed from the organic media (Van Loosdrecht et al. 2004). Anammox and/or codenitrification have been found to contribute significantly to N_2 production in a wide range of ecosystem settings (4-92%), including marine sediments (Thamdrup and Dalsgaard, 2002, Kuypers et al., 2003, Engström et al., 2005), paddy fields (Zhu et al., 2011) and grassland and agricultural soils (Long et al., 2013, Selbie et al., 2015, Clough et al., 2017). The contribution of An/coD to N removal in denitrifying bioreactors, has generally been considered negligible, mainly because no consistent decrease in NH_4^+ has previously been observed with passage of water through denitrifying bioreactors (e.g. Robertson et al., 2005, Warneke et al.,

2011, Schipper et al., 2010a, Lepine et al., 2016). However, no studies were found in which partially nitrified water, with high (>5 mg N L^{-1}) and constant NO$_3^-$ and NH$_4^+$ concentrations, was passed through a denitrifying bioreactor.

Here, we assessed the importance of An/coD as alternative N removal pathways in denitrifying bioreactors. We characterized and compared the removal of different N-species [NO$_3^-$, NO$_2^-$, NH$_4^+$, total nitrogen (TN) and total organic nitrogen (TON)] in fifteen mesocosm-scale denitrifying bioreactors receiving partially nitrified wastewater (NH$_4^+$:NO$_3$/NO$_2^-$ ratio of 100:78) over a period of one year. The performance of different types of carbon-rich porous media, woodchip and coconut husk, were compared. A pre-existing set-up of 8-year old mesocosm scale systems (Tanner et al., 2012) was used to compare performance with equivalent newly constructed woodchip and coconut husk bioreactors. Additionally, equivalent gravel bioreactors were constructed and used as an analogous inorganic media to compare performance in the absence of organic lignocellulosic substrates. In addition to N balance measurements in the field, lab experiments using ^{15}N isotope-pairing technique experiments (Risgaard-Petersen et al., 2004) were performed to assess the importance of An/coD pathways for N$_2$ production in denitrifying bioreactors.

5.3 Materials and methods

5.3.1 Study site

This study made use of an experimental set-up constructed at Pukete Wastewater Treatment Plant which serves the city of Hamilton ($\pm$ 160,000 persons), Te-Ika-a Maui, Aotearoa (North Island, New Zealand), which has an oceanic climate. In short, the experimental set-up consisted of fifteen mesocosm-scale denitrifying bioreactors (five types, three replicates) receiving primary settle wastewater that

was first passed through two unsaturated dose-loaded vertical gravel filters to promote partial nitrification and subsequently collected in a common sump. The denitrifying bioreactors consisted of 1.15 x 0.95 m x 1 m deep high-density polyethylene tanks, operated in saturated down-flow mode with the influent entering the top of the tanks. The tanks were filled with 70 cm of either mature woodchip (WM; 20–50 mm wood chips, mixed *Pinus radiata* and *Pseudotsuga menziesii* that had receive denitrified wastewater for 8 years, see Tanner et al. (2012) for details), (2) fresh woodchip (WF; 20–50 mm wood chips, *Pinus radiata*), (3) mature coconut husk (CM; 10–20 mm, chopped coir fibre pith from the mesocarp of *Cocos nucifera,* see Tanner et al., 2012), fresh coconut husk (CF; 10-18 mm chopped coir fibre pith from the mesocarp of *Cocos nucifera*) or gravel (G; 10-15 mm, greywacke-derived river gravel). Each bioreactor received hourly doses (24 per day) of ~6 L pre-treated wastewater (~146 L/day), which is just below a 1 person equivalents of flow (165 L/person/d; based on AS/NZS1547 (2012)).

5.3.2 Monthly water quality assessment in the field

5.3.2.1 Assessing the removal of N-species

Monthly grab samples (1 L) were taken at the inflow (i.e. a sump receiving partially nitrified wastewater) and outflow points of each denitrifying bioreactor over an annual period (January 2016 - December 2016). Samples were returned directly to the laboratory in a cool box and analysed for ammoniacal nitrogen (NH_4–N; phenyl/hypochlorite colorimetry), total oxidized nitrogen (NO_x-N; colorimetric method), nitrate nitrogen (NO_3-N; automated cadmium reduction), and total nitrogen (TN; persulfate digestion) by flow injection analyser using standard methods (APHA, 2012). TON was calculated as TN minus NH_4 and NO_x.

Nitrogen removal rates, expressed as g N removed per m^3 reactor volume per day ($g\,N\,m^{-3}\,d^{-1}$), were calculated as the difference between inputs and N outputs using mean values over the period of monitoring, assuming no N sequestration in, or release of N by the porous media (i.e. woodchip, coconut husk or gravel). In contrast to the vast majority of denitrifying bioreactor studies, both NH_4^+ and NO_3^- removal was observed in this study. From this, mass removal was calculated for denitrification and anammox and/or codenitrification (An/coD). The contribution of An/coD was calculated assuming that reduction in NH_4^+ was due to An/coD and that N_2 produced from these processes consisted of one nitrogen atom from NO_3^- or NO_2^- and one from NH_4^+. Surplus NO_3^- removal was assumed to be the result of denitrification. While organic N has been identified as a potential source of N for the formation of N_2 by codenitrification (see introduction), it was not taken into account in calculations of An/coD rate. This decision was made because changes in organic N were not directly assessed (i.e. TON was calculated as TN minus NH_4^+) and because the amount of bioavailable organic N was unknown. The rate of An/coD as calculated in this study could thus be considered conservative.

5.3.2.2 *Physiochemical characteristics*

In addition to the analysis of nitrogen species, five-day carbonaceous biochemical oxygen demand (CBOD5; incubation for 5 d at 20 °C) and total suspended solids (TSS; gravimetric method) were analysed using standard methods (APHA, 2012). Dissolved oxygen, pH, and electric conductivity were measured monthly using calibrated meters (TPSTM models WP81 and WP82Y, TPS Pty., Queensland, Australia). The oxidation reduction potential (ORP) was measured monthly in the inflow sump using a platinum ORP electrode (model 96-78-00, Orion Research, Inc.) and at five different depths within each bioreactor (5, 5, 25, 35 and 45 cm below the bioreactor surface) using platinum electrodes (Faulkner et al., 1989) inserted into the bioreactor and a double junction Ag/AgCl reference electrode

(Model 90-02, Orion Research, Inc.) connected to an ISE meter (model 290A, Orion Research, Inc.).

5.3.2.3 Data analysis

Comparisons between the concentrations of contaminants in different treatment types were carried out using an analysis of variance (ANOVA) assuming randomized block design. Whenever a significant difference was revealed by ANOVA, posthoc Newman-Keuls tests were conducted to identify treatment types that were significantly different from one another. P values of <0.05 were considered significant. Statistical analyses were conducted using the Genstat statistical software package for Windows 10th Edition.

Simple linear regression analyses were conducted to examine the relationship between various physiochemical characteristics (pH, EC, DO, ORP and TSS and CBOD5 effluent concentration) and denitrification and An/coD rate within the carbon media bioreactors (i.e. the woodchip and coconut husk bioreactors). Mean values for the different characteristics over the complete period of monitoring were used as the independent variables and mean N removal rates (expressed as g N removed per m^3 reactor volume per day) per treatment unit over the complete period of monitoring for denitrification and An/coD were used as dependent variables. Regression analyses were conducted in Statistica version 12, Statsoft, Inc.

5.3.3 *Isotope pairing experiment with 15N-labeled nitrate in the lab*

Denitrification and An/coD rates were assessed in lab experiments, only for the mature woodchip and mature coconut media with a ^{15}N isotope-pairing technique as described in Yang et al. (2015) with some modifications. Measurements were not conducted for all treatments because of experimental complexity and resource

limitations. The objective was to demonstrate in principal whether denitrification and An/coD occurred in bioreactors and to compare rates of N_2 production with the observed removal of NO_3^- and NH_4^+ in the field study.

Media and pore water were collected in July of 2017 from a mature woodchip and mature coconut husk bioreactor by inserting a sampling sleeve in the middle of the bioreactor, removing the top 25 cm of media from within the sleeve while keeping the water level constant and filling 1 L jars with media from 25-45 cm below the surface, and closing the jars (with rubber seal) under water to minimize air contamination, taking five replicates for each media. The resulting jars were preincubated overnight at 20°C to remove residual NO_2^- and NO_3^- according to N removal rates as calculated in preliminary experiments. Water samples were collected and analysed for total oxidized nitrogen (using methods described above) to confirm complete removal. Subsequently, stock solutions of $^{15}NO_3^-$ (^{15}N-KNO$_3$ at 98%) and unlabelled NH_4 (NH_4SO_4) were injected through the stopper of each jar, resulting in final concentrations of approximately 10 mg NO_3-N and 15 mg NH_4-N L^{-1} and gently shaken for 5 minutes (80 rpm). Water samples containing dissolved gas were collected before amendment (t0) and at half-hourly intervals for 2 hours (t1 to t4) by extracting water samples from the centre of the jar using a syringe inserted through a rubber stopper, while simultaneously displacing the extracted water sample with nitrogen gas (>99.998% nitrogen) at the top of the jar. Water samples were injected into pre-evacuated exetainers (evacuated to < 50 mTorr). Microbial activity in the samples was stopped by adding 150 μL 50% (m/v) ZnCl. The exetainers were then stored in water filled PP tubes (50 ml). Jar headspace gas samples were taken and analysed for isotopic composition at the end of the experiment to assess any potential exchange between gas dissolved in liquid with the gas phase surrounding the liquid. Dissolved gas was extracted in the lab by injecting 4mL helium while simultaneously extracting 4 mL of sample as described

by Dalsgaard et al. (2000). The N_2 concentration and isotopic composition of N_2 (i.e. measured isotope-ratios of N_2, 29:28 (^{29}R) and 30:28 N_2 (^{30}R)) in the helium headspace were analysed by mass spectrometry following gas-chromatographic separation at the stable isotope facility at NIWA Wellington (GasBench II coupled to a Delta V Isotope ratio mass spectrometer). To account for interferences at m/z 30 arising from NO^+ ions formed in the ion source of the isotope ratio mass spectrometer corrections are applied to the raw ^{30}R data as suggested by Lewicka-Szczebak et al. (2013).

Denitrification was assumed to create N_2 from the utilization of NO_3^-, while An/coD was assumed to generate hybrid N_2 molecules produced from the utilization of NH_4^+ and NO_3^-. Taking into account that the pore water was amended with stock solutions of $^{15}NO_3^-$ (98.0% ^{15}N-KNO_3), production of N_2 by denitrification and An/coD (in $\mu mol\ L^{-1}$) was calculated for each measurement assuming that $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were produced through random isotope pairing (Thamdrup and Dalsgaard, 2002, Risgaard-Petersen et al., 2004). Subsequently, denitrification and An/coD production rates, expressed as g N produced per m^3 reactor volume per day (g N m^{-3} d^{-1}), were calculated from the increase in N_2 production by denitrification and An/coD assuming zero-order kinetics for both processes, and a media porosity of 40% (based on drainable porosity measurements).

5.4 Results

5.4.1 The removal of N-species in the field

5.4.1.1 Nitrogen species in the in- and outflows

Total N concentrations entering the bioreactors (i.e. measured in the VGF sump) remained relatively stable over time ranging from 26.3 to 43.1 mg L^{-1}. Composition
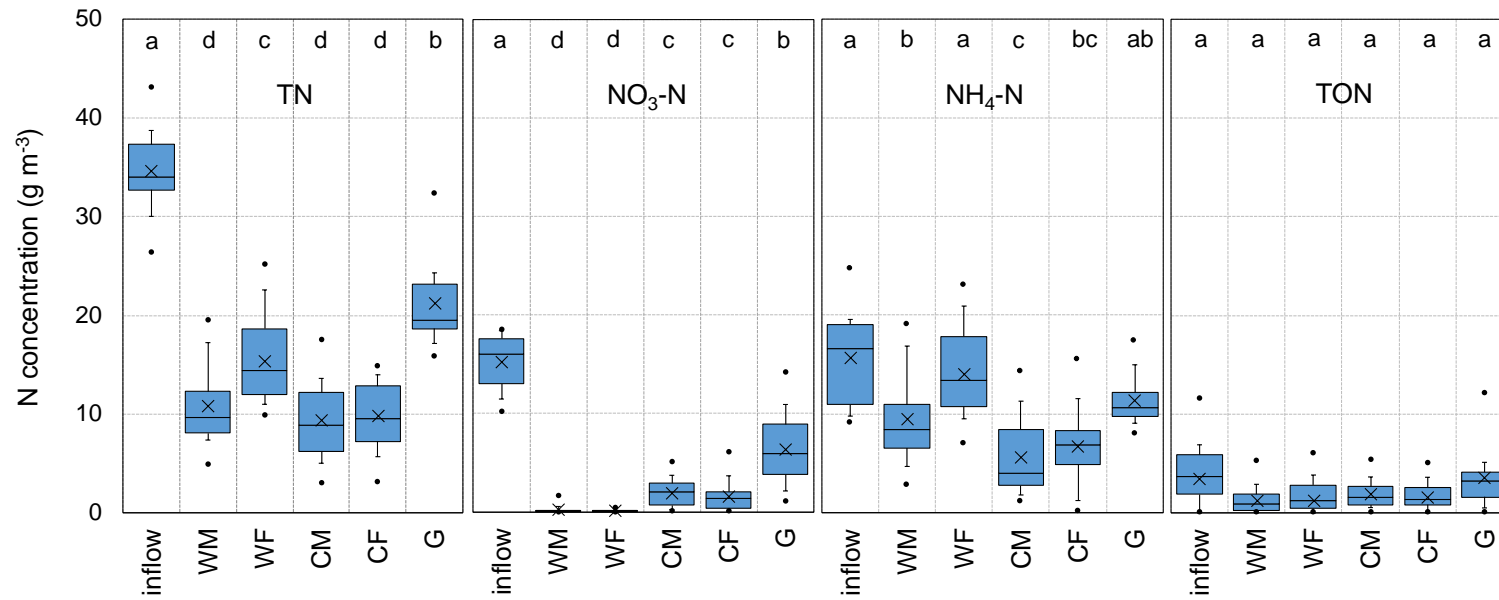
Figure 5.1 Box and whisker plots of inlet and outlet concentrations for the different nitrogen species. Lines within the boxes are median values, the bottom and top of the boxes are the 25th and 75th percentiles, and error bars are the 10th and 90th percentiles. The dots represent the minimum and maximum values of the data, the crosses represent the mean concentrations. The letters above the graphs represent statistically homogeneous groups.

Of the inflow varied with time, but contained substantial amounts of NO_3-N, NH_4-N and TON throughout the period of study with mean concentrations ($\pm$ standard deviation) of 15.3 ± 2.9, 15.7 ± 5.0, 3.4 ± 3.7 mg L^{-1} respectively. Nitrate and NH_4^+ were the major forms of N removed from the effluent with passing through the bioreactors (Figure 5.1). In both woodchip bioreactors (WM and WF) 90-percentile NO_3^- outflow concentrations were below 0.6 mg L^{-1}. The smell of hydrogen sulphide (H_2S) was observed in the outflow of the fresh and mature woodchip bioreactors throughout the period of monitoring, indicating sulphate (SO_4^{2-}) reduction within the systems. Compared to the woodchip bioreactors, nitrate concentrations exiting the coconut and gravel bioreactors were generally higher with mean values of 1.9, 1.6 and 6.3 mg L^{-1} for bioreactors CM, CF and G respectively. Substantial removal of NH_4^+ was also achieved in all bioreactors. Ammonium outflow concentrations were lowest in the CM and CF bioreactors with mean NH_4^+ effluent concentrations of 5.6 and 6.7 mg N L^{-1} for the CM and CF bioreactors respectively. Ammonium outflow concentrations were higher in the WM, WF and G bioreactors, with mean outflow concentrations of 9.5 and 14.0 mg L^{-1} and 11.4 mg L^{-1} for the WM, WF and G bioreactor respectively. Although mean TON removal was observed in the woodchip and coconut husk bioreactors, with mean outflow concentrations ranging from 1.2 to 1.9 mg L^{-1}, these were not statistically different from the inflow concentration. Nitrite was consistently analysed in the in- and outflows of the bioreactors and was consistently low (<0.4 mg L^{-1}) at all points.

5.4.1.2 Nitrogen removal rates

Overall bioreactors were able to significantly decrease N concentrations at mean mass loadings of 2.2 g NO_3-N d^{-1}, <0.1 g NO_2-N d^{-1} and 2.3 g NH_4-N d^{-1}. Highest inorganic N (i.e. NO_3^-, NO_2^- and NH_4^+) removal rates were observed for the WM, CM and CF bioreactors (4.0, 4.4 and 4.3 g N m^{-3} d^{-1} respectively), intermediate N

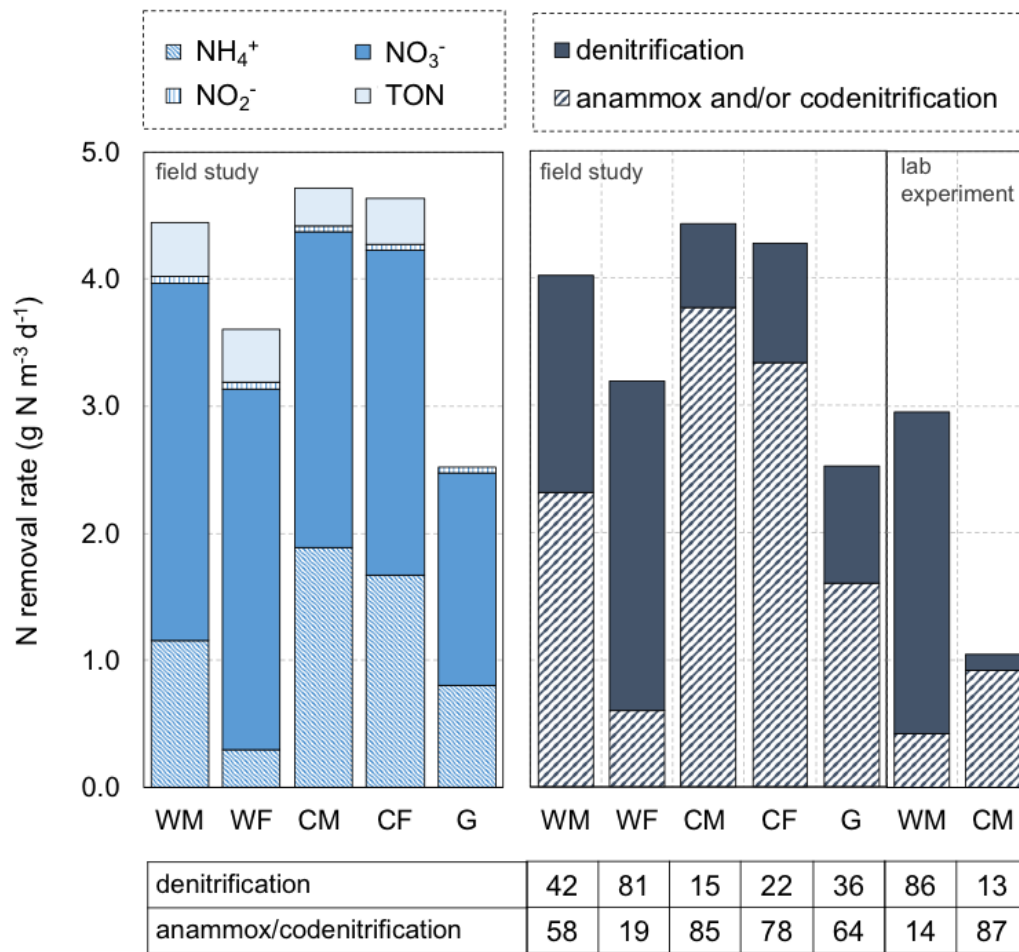*Figure 5.2 Nitrogen removal rates expressed as g N removed per m³ reactor volume per day calculated for each N species using data collected in the field (left) and for denitrification and An/coD using N species data collected in the field and the isotope data collected in the lab (right). The table provides the apparent relative contributions (in %) of denitrification and An/coD to total inorganic N removal.*

removal rates were calculated for the fresh woodchip bioreactor (3.2 g N m^{-3} d^{-1}) and lowest inorganic N removal rates for the gravel bioreactor (2.5 g N m^{-3} d^{-1}) (Figure 5.2). Calculated denitrification and An/coD removal rates, based on NO$_3^-$, NO$_2^-$ and NH$_4^+$ removal, indicated a significant contribution of An/coD for N removal in all bioreactors (Figure 5.2). An/coD was calculated to be the major removal process in the WM, CM, CF and G bioreactors, accounting for 58, 85, 78 and 64% of inorganic nitrogen removal in the systems respectively. Compared to the An/coD rates, apparent mean denitrification rates were markedly lower t in the CM, CF and G bioreactors (0.7, 0.9 and 0.9 g N m^{-3} d^{-1} respectively) and slightly lower in the WM bioreactor (1.7 g N m^{-3} d^{-1}). In contrast, denitrification was found to be the main N removal process in the WF bioreactor with a removal rate of 2.6 g N m^{-3} d^{-1} (~ 81% of inorganic N removal), while An/coD accounted a removal rate of 0.6 g N m^{-3} d^{-1} (~ 19% of inorganic N removal).

5.4.1.3 Physiochemical characteristics and nitrogen removal rates

There was little variation in pH, DO content, EC and TSS concentration in between bioreactor effluents (Table 5.1). Throughout the study, all bioreactor effluents showed low oxygen concentrations (<0.5 g O$_2$ m^{-3}), had neutral pH levels (6.5 to 7.5) and stable EC values (531 to 591 μS cm^{-1}). Additionally, all systems were able to substantially remove TSS to mean concentrations below 3.1 g m^{-3}. Values for CBOD5 in the bioreactor effluent and ORP profiles within the bioreactors varied between treatments (Table 5.1 and Figure 5.3). Mean values for CBOD5, an indicator of the amount of microbially degradable organic matter in the aqueous phase, varied between treatments (ranging from 1.4 to 15.0 g O$_2$ m^{-3}), with highest CBOD5 values in the WF bioreactor effluent. While a wide range of ORP values were recorded for each measuring point throughout the year (Figure 5.3), in general lowest ORP (i.e. most reducing conditions) were observed for the
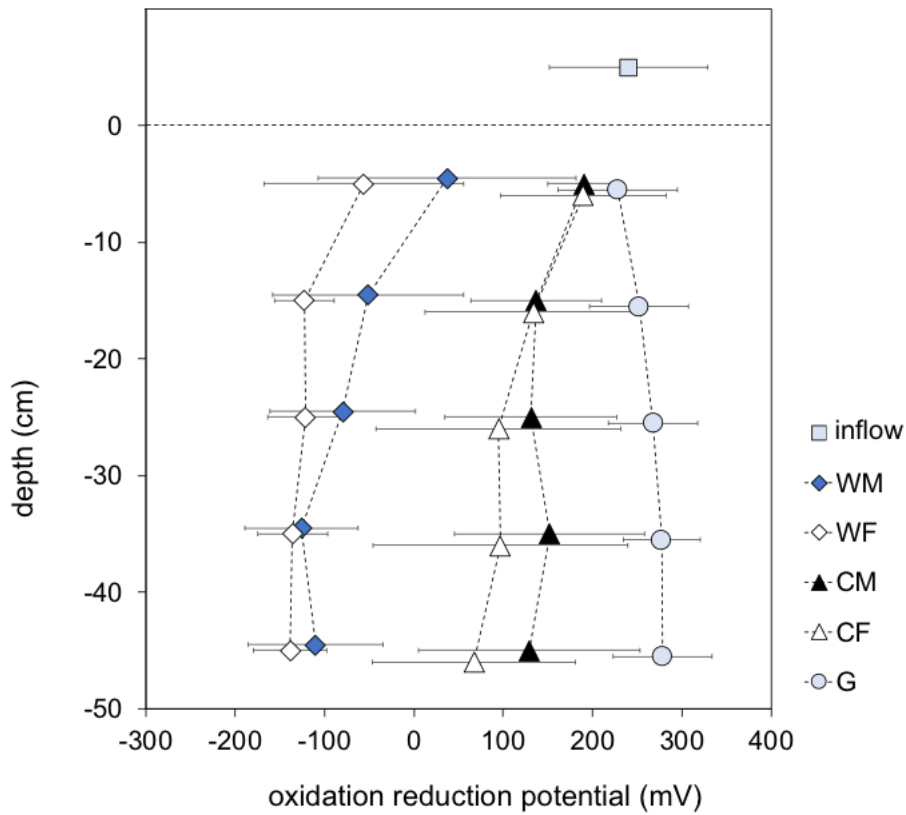
Figure 5.3 Oxidation reduction potential in the bioreactor inflow and at different depths in the mature woodchips (WM), fresh woodchips (WF), mature coconut husk (CM), fresh coconut husk (CF) and gravel (G) bioreactors. Markers are mean values over the period of monitoring, error bars are standard deviations.

woodchip bioreactors (mean ORP values of -110 $\pm$ 86 and -139 $\pm$ 31 mV at depth

of 45 cm for bioreactors WM and WF respectively). Less reducing conditions were

observed in the coconut husk bioreactors (mean ORP values of 129 $\pm$ 124 and 67

$\pm$ 115 mV at 45 cm depths for bioreactor CM and CF respectively) and least

reducing conditions were observed in the gravel bioreactors (mean ORP values of

278 $\pm$ 54 mV at 45 cm depth).

While pH, EC, DO content and TSS concentration were not correlated to N removal

rate, simple linear regression to predict denitrification and An/coD rate based on

CBOD5 effluent values exiting the mesocosm revealed significant correlations with

an R^2 of 0.995 ($F_{(1,2)}$= 408.94, p<0.01) for denitrification and with an R^2 of 0.98

($F_{(1,2)}$= 100.42, p<0.01) for An/coD. A linear increase in denitrification rate was

observed with increasing CBOD5 outlet values (y= 0.388 + 0.144x, where y =

denitrification rate in g N m^{-3} d^{-1} and x = CBOD5 value in g O_2 m^{-3}) (Figure 5.4). In

contrast a linear decrease in An/coD rate was observed with increasing CBOD5

values (y= 4.252 - 0.233x, where y = An/coD rate in g N m^{-3} d^{-1} and x = CBOD5

value in g O_2 m^{-3}). Overall, a linear decrease in the removal rate of inorganic N (i.e.

the denitrification rate + the An/coD rate) was observed with increasing CBOD5

values ($F_{(1,2)}$= 75.177, p=0.04, R^2=0.93, y= 4.641 - 0.088x, where y = total

inorganic N removal rate in g N m^{-3} d^{-1} and x = CBOD5 value in g O_2 m^{-3}).

Additionally, simple linear regression to predict denitrification and An/coD rate

based on ORP values (using mean ORP values as calculated using data collected

at depths of 25, 35 and 45 cm where the ORP remained relatively stable) provided

a marginally good fit to the data ($F_{(1,2)}$= 14.919), p=0.06 with an R^2=0.88 for

denitrification and ($F_{(1,2)}$= 9.007), p=0.10 with an R^2=0.82 for An/coD. A linear

increase in denitrification rate was observed with decreasing ORP (y= 1.451 -

6.038·10^{-3} x, where y = denitrification rate in g N m^{-3} d^{-1} and x = ORP in mV). A

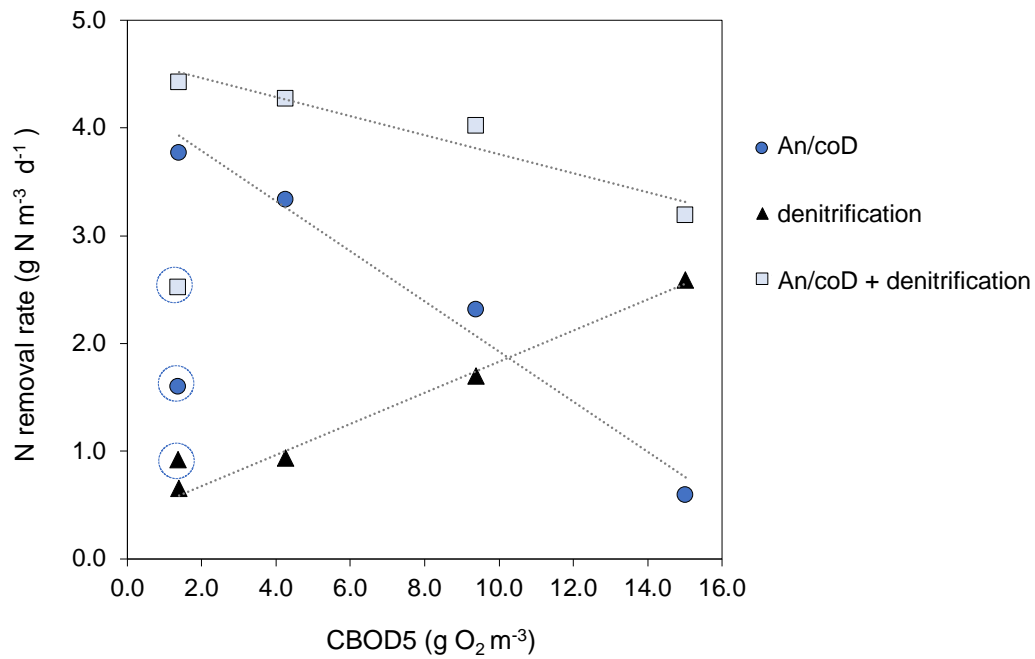*Figure 5.4 Graph depicting the relationship between CBOD5 in the bioreactor effluent and nitrogen removal rate in the bioreactor estimated for An/coD, denitrification and both together. Data points are mean values over the period of monitoring for each treatment type. The dotted lines depict a linear trend in the data. Circled data points are values for the gravel bioreactors and were excluded from the regression analysis.*

Table 5.1 Summary of mean (± standard deviation) values for pH, electrical conductivity (EC), dissolved oxygen content (DO) and concentrations of total suspended solids (TSS) and 5-day carbonaceous biochemical oxygen demand (CBOD5) in the in- and outflows of the different bioreactor treatments.

	Bioreactor inflow	Bioreactor treatments				
		Woodchip		Coconut husk		Gravel
		mature	fresh	mature	fresh	
pH	6.9 ± 0.2	6.8 ± 0.2	6.9 ± 0.3	6.8 ± 0.2	6.8 ± 0.2	7.0 ± 0.2
EC (μS cm^{-1})	653 ± 81	557 ± 48	580 ± 44	531 ± 42	546 ± 51	591 ± 55
DO (g O$_2$ m^{-3})	1.9 ± 0.6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
TSS (g m^{-3})	13.4 ± 6.2	2.9 ± 2.1	2.9 ± 1.7	3.1 ± 2.7	2.9 ± 2.1	1.7 ± 1.0
CBOD5 (g O$_2$ m^{-3})	13.5 ± 6.2	9.4 ± 4.5	15.0 ± 7.0	1.4 ± 0.8	4.2 ± 4.6	1.4 ± 0.6

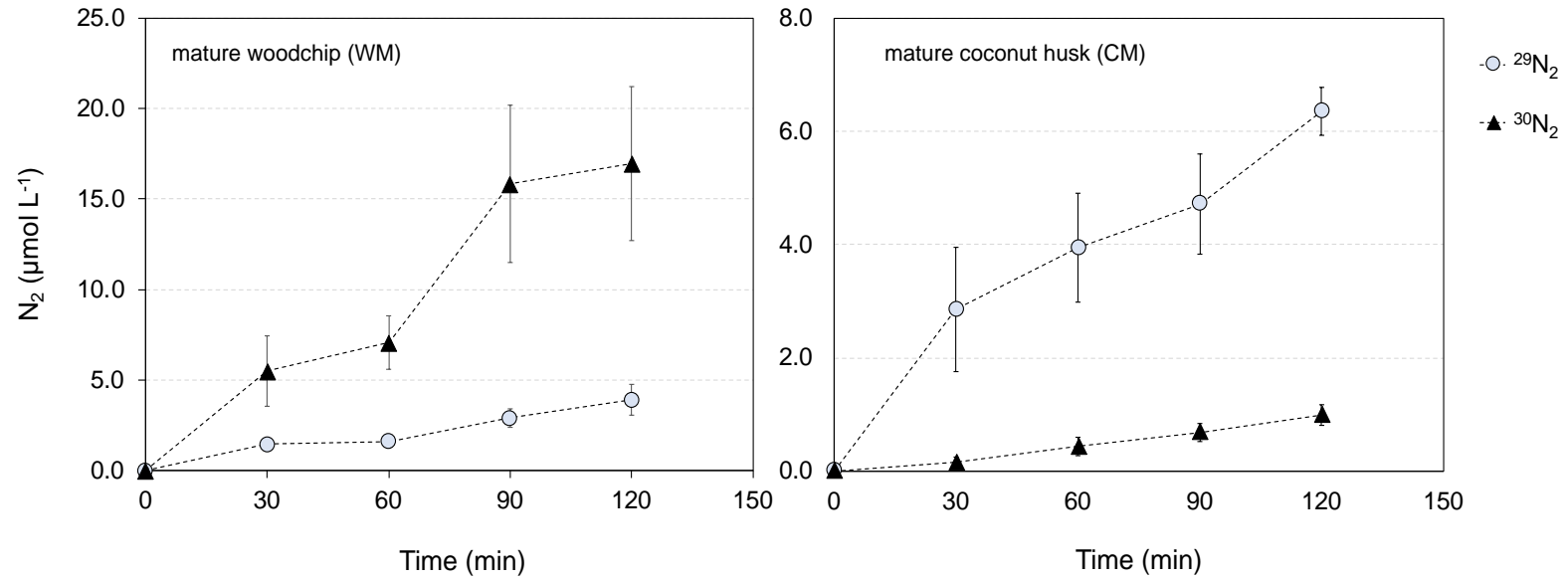*Figure 5.5 Production patterns of $^{29}N_2$ and $^{30}N_2$ in the pore water during closed incubations following additions of 10 mg N L^{-1} $^{15}NO_3^-$ (^{15}N-KNO$_3$ at 98%) and 15 mg N L^{-1} unlabelled NH_4^+ (NH_4SO_4) to mature woodchip (left) and mature coconut husk (right). The markers represent mean values of the five replicates and the error bars represent standard deviations.*

linear increase in An/coD rate was observed with increasing ORP (y= 2.539 - 9.450·10^{-3} x, where y = An/coD rate in g N m^{-3} d^{-1} and x = ORP in mV).

5.4.2 *Production of N2 with 15N-labeled nitrate in the lab*

In the laboratory experiments an increase in $^{29}N_2$ and $^{30}N_2$ concentration was observed with time in both the WM and CM jars (Figure 5.5). For the WM, the increase in N_2 gas concentration due to denitrification and An/coD with time can be described as y = 0.15x + 0.23, R² = 0.95, and y = 0.03x + 0.11, R² = 0.96, respectively, in which y is N_2 gas concentration in μmol L^{-1} and x is time in min. For the mature coconut husk, the increase in N_2 gas concentration due to denitrification and An/coD with time can be described as y = 0.009x - 0.05, R² = 0.99, and y = 0.05x + 0.68, R² = 0.94 respectively. Assuming a porosity of 40% for both WM and CM, denitrification and An/coD rates (expressed as g N produced per m^3 reactor volume per day) were calculated. Denitrification was found to be the main N_2 producing process in WM bioreactor with a production rate of 2.5 $\pm$ 0.5 g N m^{-3} d^{-1} accounting for 86 $\pm$ 19 % of total N_2 production in the systems. The An/coD rate in the WM bioreactors was calculated to be 0.4 $\pm$ 0.1 g N m^{-3} d^{-1}, accounting for 14 $\pm$ 3%. In contrast, anammox and/or codenitrification were calculated to be the major N_2 producing processes in CM bioreactors, with a production rate of 0.8 $\pm$ 0.1 g N m^{-3} d^{-1}, accounting for 87 $\pm$ 8 % of total N_2 production in the systems, while the denitrification rate was 0.1 $\pm$ 0.03 (13 $\pm$ 3%).

5.5 Discussion

The effective removal of both NO_3^- and NH_4^+ in the mesocosms and the formation of hybrid N_2 (i.e. $^{29}N_2$) observed in the laboratory trials alter our understanding of the potential mechanisms responsible for N loss in denitrifying bioreactors. To date N removal in denitrifying bioreactors has almost solely been attributed to

denitrification. Our results strongly demonstrate that, in addition to denitrification, a significant amount of N can be removed via the An/coD pathway. The wide range of An/coD removal rates (0.6 to 3.8 g N per m^3 reactor volume per day) and relative contributions to N removal and N_2 production (19 to 87%), suggest that An/coD activity was variable between systems and was dependent on media type and age.

5.5.1 The removal of N species in the field

The results from the N species analyses indicated that a substantial reduction of both NO_3^- and NH_4^+ can be achieved by passing partially nitrified wastewater through a denitrifying bioreactor. Previous studies on the use of anammox in wastewater treatment have found the anammox process to be extremely sensitive to variations in operating conditions and N loading (Trigo et al., 2006, Van der Star et al., 2007). This might explain why removal of NH_4^+ and anammox have not been previously reported in denitrifying bioreactors where NO_3^- and NH_4^+ inputs have been much more variable (e.g. Robertson et al., 2005, Warneke et al., 2011, Schipper et al., 2010a, Lepine et al., 2016). While further work is needed, our data suggested that stable influent conditions (particularly NO_3^- and NH_4^+ concentrations), might be needed to support a stable anammox or codenitrification community.

The calculated denitrification and An/coD rates (0.7 to 2.6 g N m^{-3} d^{-1} and 0.6 to 3.8 g N per m^{-3} d^{-1} respectively) indicate that both N removal pathways can co-exist, and that An/coD is an effective option for N removal in denitrifying bioreactors when both NO_3^- and NH_4^+ are present. Total N mass removal rates calculated from the removal of NO_3^-, NO_2^- and NH_4^+ (2.5 to 4.4 g N m^{-3} d^{-1}), were within the range generally recorded for denitrifying bioreactors for which only NO_3^- removal was observed (2 to 11 g N m^{-3} d^{-1}; Schipper et al., 2010b, Addy et al., 2016). These findings suggest that bioreactors that support both denitrification and An/coD can

effectively reduce N loads to the same extent as denitrifying bioreactors that only support denitrification. It should be noted that, while total inorganic N removal rates were slightly lower for the woodchip (4.0 and 3.2 g N m^{-3} d^{-1} for the mature and fresh woodchip bioreactors respectively) compared to the coconut husk bioreactors (4.4 and 4.3 g N m^{-3} d^{-1} for the mature and fresh coconut husk bioreactors respectively), removal of N in the woodchip systems was likely NO_3^- limited. The low NO_3^- effluent concentrations (90-percentile concentration <0.6 mg L^{-1}) and the observed smell of H_2S in the bioreactor outflow of the woodchip bioreactors was consistent with complete NO_3^- removal, allowing for SO_4^{2-} reduction (Schipper et al., 2010b). It is therefore likely that the woodchip bioreactors had additional capacity to remove higher NO_3^- loads than provided in this study.

The results of the present study suggest that An/coD and denitrification activity was dependent on media used. It is generally assumed that denitrification is in principle dependent on organic carbon respiration, with a decrease in denitrification rate as carbon depletes (Schipper et al., 2010b). Availability of carbon in denitrifying bioreactors, in this study, was a combination of carbon in incoming wastewater and slow degradation of solids phase carbon. We used CBOD5 leaving each bioreactor as a metric of total available carbon for each reactor. Differences in CBOD5 values leaving the bioreactors in this study suggested higher release, or lower containment, of biodegradable organic carbon in the woodchip compared to coconut husk media. Results from a simple linear regression analysis to predict denitrification rate based on CBOD5 effluent values, indicated an increase in denitrification rate with increasing organic carbon. In contrast, An/coD rates were found to increase with decreasing organic carbon content and consequently decreasing denitrification rates. It can thus be hypothesized that An/coD activity became more dominant in the bioreactors in which denitrification was carbon

limited. This hypothesis is in accordance with studies on the importance of anammox in ecosystems, which have generally found that, under high concentration of organic matter, competition by denitrifying was a major factor inhibiting anammox activity (Jin et al., 2012). While it is generally assumed that codenitrification is dependent on organic carbon respiration, it has been demonstrated that codenitrification can also occur in the absence of a strong organic electron donor (Spott et al., 2011).

While there was little variation in pH, DO content and EC between bioreactors, ORP profiles varied between bioreactor treatments. ORP measurements have widely been used as a parameter for process control of nitrification and denitrification in wastewater treatment systems (Kishida et al., 2003, Lo et al., 1994). Simple linear regression analyses signified a negative correlation between ORP and denitrification rate and a positive correlation between ORP and An/coD rate, suggesting that that ORP could potentially be used as a control parameter to establish whether denitrification or An/coD was the dominant mechanism for N removal in a denitrifying bioreactor. The wide variation in ORP values for each measuring point, however, indicated it was difficult to obtain accurate, reproducible and comparable measurements. High variability is common for field measurements of ORP, reflecting spatial and temporal variability and disequilibrium, electrode poisoning, and other limitations (Patrick et al., 1996). Further investigation on the use of ORP measurements as a control parameter for denitrification and An/coD activity is therefore required.

The gravel bioreactors, which are analogous to unplanted saturated subsurface-flow constructed wetlands, also achieved substantial removal of NO_3^- and NH_4^+. Total inorganic N removal was, however, markedly lower in the gravel (2.5 g N per m^{-3} d^{-1}) compared to the woodchip and coconut husk bioreactors (3.2 to 4.4 g N

per m^{-3} d^{-1}) . The calculated denitrification and An/coD rates (0.9 and 1.6 g N per m^{-3} d^{-1} respectively) indicate that both pathways were responsible for N removal in the gravel systems. While, N removal in subsurface flow wetlands has conventionally, solely, been attributed to denitrification (Tanner et al., 2002, Vymazal, 2005), these findings are in line with more recent studies in which that anammox activity has been observed in constructed wetlands (Dong and Sun, 2007). Since heterotrophic denitrification is controlled by availability of C (Seitzinger et al., 2006), which in the gravel bioreactors was solely derived from the incoming wastewater, it can be speculated that denitrification in the gravel systems was carbon limited. While, differences in An/coD activity between bioreactor types observed in this study were likely the result of the different substrates used, further studies are required to determine the key factors controlling An/coD activity in these systems.

5.5.2 *Isotope pairing experiment in the lab*

In addition to the analysis of N species in the field, isotope experiments were conducted to demonstrate that denitrification and An/coD occurred in denitrifying bioreactors and to compare rates of N_2 production with the observed removal of NO_3^- and NH_4^+ in the field study. Concentration patterns and the stoichiometry of $^{29}N_2$ and $^{30}N_2$ in the isotope pairing experiments demonstrated N_2 formation through denitrification as well as An/coD in both the mature woodchip and mature coconut husk media. The relative contributions of An/coD to total inorganic N removal in the mature woodchip (14%) and coconut husk bioreactor (87%) demonstrated that, when both NO_3^- and NH_4^+ are present, a substantial amount of N removal in denitrifying bioreactors can occur via the An/coD pathway, with a higher contribution of An/coD in coconut husk compared to woodchip. While denitrification rates for mature woodchip and coconut husk (2.5 and 0.1 g N m^{-3} d$^-$

[1] respectively) were roughly comparable to rates found using the N balance method, apparent An/coD rates were much lower (0.4 and 0.9 g N m^{-3} d^{-1}). This may have been the result of disturbance of bacteria during media and pore water collection, which could be a factor limiting An/coD activity in the lab experiment. Anammox bacteria, in particular, have been found to be very sensitive to changing environmental conditions (e.g. changes in temperature, DO content and pH) (Jin et al., 2012). While, in this study, we attributed the formation of hybrid N_2 to biologically mediated processes of anammox and codenitrification, recent studies using isotope pairing techniques have found abiotic production of hybrid N_2 under anoxic conditions (Phillips et al., 2016). It is recommended to include assessment of abiotic N_2 production in future studies.

Overall, the lab experiments, using a ^{15}N isotope-pairing technique, confirm findings from the N-species analysis field study by demonstrating that denitrifying bioreactors, in addition to supporting denitrification, can support the removal of N by An/coD when both NO_3^- and NH_4^+ are present. This has important implications for the use of denitrifying bioreactors for domestic wastewater treatment, since it demonstrates that in addition to removing NO_3^-, denitrifying bioreactors can also allow for the effective removal of undesired NH_4^+ commonly present in domestic wastewater. Further research is recommended to determine effectiveness and controlling factors of An/coD in denitrifying bioreactors, particularly the suitability of alternative C sources, the effect of various temperature and loading regimes, and effects on nitrous oxide emissions. It is recommended that future work is underpinned by studies on the microbial ecology of these systems, which may lead to approaches for enhancement of An/coD in these systems.

5.6 Acknowledgements

5.7 References

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Chapter 6

Summary, Conclusions and Recommendations

The focus of this thesis was on denitrifying bioreactors, simple engineered systems containing a porous carbon source, designed to remove nitrate (NO_3^-) from water (Schipper et al., 2010b). Previous studies have mainly focused on the ability of, and factors influencing, the removal of NO_3^-, within these systems (e.g. Cameron and Schipper, 2010, Warneke et al., 2011a, Christianson et al., 2012, Addy et al., 2016). In this thesis, a more holistic view of denitrifying bioreactors was taken, in which nitrogen (N) removal was assessed in conjunction with the removal of faecal microbial contaminants. Removal of microbial contaminants from wastewater is considered a key issue for wastewater treatment, since elevated concentrations in ground or surface water can present a serious public health concern due to the potential outbreak of waterborne diseases (Craun, 1985, Borchardt et al., 2011). The potential of denitrifying bioreactors to remove faecal microbes had been briefly assessed in earlier studies by Robertson et al. (2005) and Tanner et al. (2012) in which *Escherichia* (*E. coli*) reductions of 0.2 to 1.9 $\log_{10}$ were reported. These findings suggested that denitrifying bioreactors could reduce microbial contaminants in wastewater. However, monitoring data remained scarce and the potential of denitrifying bioreactors for microbial contaminant removal remained unclear. Initially, to assess the feasibility of denitrifying bioreactors for microbial contaminant removal, the removal of bacteria and virus indicators were assessed in a full-scale operating woodchip bioreactor. After effective removal of microbial contaminants was observed, a follow-up study was conducted. In this study the effects of media type, system age and seasonality on performance was assessed

by monitoring the removal of bacteria and virus indicators in fifteen mesocosm scale bioreactors receiving partially nitrified municipal wastewater. During this study, the unexpectant removal of both NO_3^- and ammonium (NH_4^+) was observed in the bioreactors. Denitrification, commonly considered the only significant process removing N in denitrifying bioreactors (Greenan et al., 2006, Greenan et al., 2009, Warneke et al., 2011a, Warneke et al., 2011b, Schipper et al., 2010a), could not account for the observed removal of NH_4^+. The focus of this thesis was therefore broadened to include the assessment of alternative N removal pathways that would allow for the removal of both NO_3^- and NH_4^+, through anaerobic ammonium oxidation (anammox) and codenitrification.

The aim of this thesis was thus twofold: (1) to assess whether denitrifying bioreactors could provide a complementary alternative for removing microbial contaminants while also removing NO_3^- and (2) to assess the potential role of alternative N removal pathways, namely anammox and codenitrification (jointly referred to as An/coD) for N removal in denitrifying bioreactors.

6.1   Microbial contaminant removal in denitrifying bioreactors

6.1.1   Summary and conclusions

In the first study, the feasibility of denitrifying bioreactors for microbial contaminant removal was assessed by monitoring the removal of *E. coli* and FRNA bacteriophage along the longitudinal transect of an operational full-scale woodchip denitrifying bioreactor (~114 m^3 in size with 9 sampling wells along the length of the bioreactor) receiving secondary-treated septic tank effluent. This study demonstrated that, in addition to significant reduction in NO_3^- loads, woodchip bioreactors can achieve consistent and substantial reduction of *E. coli* (log_{10} reduction of 2.9) and FRNA bacteriophage (log_{10} reduction of 3.9) despite receiving

highly fluctuating inflow concentrations (up to 3.5×10^5 MPN/100 mL and 1.1×10^5 plaque-forming units/100 mL , respectively).

Subsequently, a second study was conducted in which seasonal removal of *E. coli*, total coliforms (TC) and FRNA bacteriophage was analysed in fifteen mesocosm scale bioreactors (~700 L each) receiving secondary treated municipal wastewater at a controlled loading rate. Systems were filled with a slow-release carbon source: woodchip or coconut husk. The effect of media age on fate of microbial contaminants was assesed by comparing performance of 8-year old systems with equivalent newly constructed bioreactors. Additionally, performance of these carbon substrates was compared to performance of gravel, a non-carbon substrate media commonly used in subsurface flow wetlands. Substantial reduction of *E. coli*, TC and FRNA bacteriophage from primary treated municipal wastewater was achieved in all bioreactors. Mean annual $\log_{10}$ removal efficiencies were similar between microbial indicators ranging from 1.4 to 1.9 for TC, 1.3 to 1.8 for *E. coli* and 1.3 to 2.0 for FRNA bacteriophage. Climatic conditions, within the range experienced in this study, made little difference to treatment efficiency with all bioreactors showing robust year-round performance. Additionally, long-term performance which did not markedly change in the ninth year of operation. Performance of the mature coconut and woodchip bioreactors compared favourably with the performance of the gravel bioreactors suggesting that these systems are able to achieve microbial effluent quality for more than 8 years within the same range of treatment levels achieved in similar gravel-based systems, such as subsurface flow wetlands.

Overall, these studies demonstrated that:

- In addition to the effective removal of N, significant removal of faecal bacteria and viruses can be achieved by passing secondary-treated, effluent through a woodchip or coconut husk denitrifying bioreactor;

- Denitrifying bioreactors are robust, achieving consistently low and steady microbial loads in the outflow, despite varying climatic conditions and receiving fluctuating inflow concentrations;

- Woodchip and coconut husk bioreactors were able to achieve microbial effluent quality for more than 8 years within the same range of treatment levels achieved in similar gravel-based systems.

There is a widespread need for appropriate technologies that can effectively remove microbial contaminants from septic tank effluent or drainage water. Extensive research has shown that denitrifying bioreactors are an effective, low-cost, and simple technology for reducing N from water (Robertson et al., 2005, Robertson et al., 2008, Schipper et al., 2010a, Christianson et al., 2012). The research presented in this thesis provides strong evidence that denitrifying bioreactors can also effectively reduce microbial contaminants in wastewater providing a complementary alternative for the removal of microbial contaminants from wastewater.

6.1.2 Recommendation for future work

While this thesis clearly demonstrates that denitrifying bioreactors are effective at reducing bacterial and viral concentrations in domestic wastewater, a number of questions remain regarding the practical application of denitrifying bioreactors for microbial contaminant removal. The following section briefly outlines some pertinent questions and suggests how they may be addressed.

<u>What is the effect of variable loadings on microbial contaminant removal in denitrifying bioreactors?</u>

To prevent transmission of infectious disease via waterborne pathogenic microorganisms, denitrifying bioreactor outflow concentrations should ideally be consistently low. While, not observed in this study, breakthroughs with high *E. coli* outflow concentrations have been recorded in denitrifying bioreactors at high flow rates (Robertson et al., 2005). If treating wastewater from individual households, small communities or tile drainage, denitrifying bioreactors would commonly receive variable loadings both diurnally and from day-to-day (Masi et al., 2007, Zapater et al., 2011). More systematic studies are recommended in which effects of variations in loading rate, water composition, shock loadings, and intermittent usage on microbial contaminant removal are assessed.

<u>What is the effect of temperature on microbial contaminant removal in denitrifying bioreactors?</u>

While not observed in this study, temperature effects for faecal coliform removal have been displayed in column-scale bioreactors studies, with lower efficiencies at lower water temperatures (Soupir et al., 2018). It is therefore recommended that further research is conducted to systematically assess the effect of temperature on microbial contaminant removal in denitrifying bioreactors. Laboratory column studies are warranted for comparative assessment of microbial contaminant removal under a range of temperatures and temperature fluctuations. Full-scale based bed studies are needed to determine effects of different climatic setting on performance.

How well can denitrifying bioreactors remove protozoa?

In this study, TC, *E. coli* and FRNA bacteriophage were used as models for bacterial and viral pathogens. Protozoa, like *Cryptosporidium* and *Giardia*, are increasingly recognized as a significant cause of waterborne disease outbreaks (MacKenzie et al., 1994). Therefore, determining the ability of denitrifying bioreactors to remove protozoa is important for assessing their capacity to reduce waterborne disease risks. It is recommended that future analysis of microbial contaminant removal in denitrifying bioreactors is expanded to include analysis of protozoa.

Can simple, passive and maintenance free modifications be made to enhance microbial contaminant removal in denitrifying bioreactors?

It would be beneficial to amend denitrifying bioreactors in such a way that will enhance removal of microbial contaminants from wastewater in these systems. Increased removal of NO_3^- and phosphate (PO_4^-) has already been observed in denitrifying bioreactors amended with biochar (Bock et al., 2016). Amending sand filters with biochar has been found to also increase *E. coli* removal via filtration (Mohanty and Boehm, 2014). Recent developments in biochar engineering using chemical and biological modifications have also yielded so called "hybrid-chars", such as zero-valent iron modified-bamboo biochar, which has been found to restrict the growth of *E. coli* (Zhou et al., 2014). These findings suggest that, in addition to enhanced removal of NO_3^- and PO_4^-, biochar has the potential to enhance microbial contaminant removal in denitrifying bioreactors by increasing filtration and inactivation of microbes. Further studies should be undertaken to assess the potential of biochar for enhancing microbial contaminant removal in denitrifying bioreactors.

6.2 The role of anammox and codenitrification in denitrifying bioreactors

6.2.1 Summary and main conclusions

In the third study, the focus of the research was broadened to include the assessment of alternative N removal pathways that would allow for the removal of both NO_3^- and NH_4^+ in denitrifying bioreactors, namely anammox and codenitrification (jointly referred to as An/coD). The importance of An/coD was assessed by monitoring the reduction of N species with passage of partially nitrified municipal wastewater through the fifteen mesocosm scale bioreactors used in the second study. Additionally, lab experiments using a ^{15}N isotope-pairing technique were performed to assess production of N_2 in woodchip and coconut husk filled mesocosms. The effective removal of both NO_3^- and NH_4^+ observed in the field and the formation of hybrid N_2 (i.e. $^{29}N_2$) in the laboratory studies demonstrated that the An/coD pathway was an effective pathway for N removal when both NO_3^- and NH_4^+ were present. Apparent An/coD removal rates ranged from 0.6 to 3.8 g N per m^3 reactor volume per day while denitrification rates ranged from 0.7 to 2.6 g N per m^3 per day. The contributions of An/coD to N removal was dependent on media, with An/coD becoming more dominant in bioreactors where denitrification was carbon limited.

The findings of this study alter our understanding of the potential mechanisms responsible for N loss in denitrifying bioreactors, as it strongly demonstrated that denitrifying bioreactors can support removal of N through the An/coD pathway. This has important implications for the use of these passive approaches for wastewater treatment since it establishes that in addition to removing NO_3^-, denitrifying bioreactors can also allow for the effective removal of undesired NH_4^+ commonly present in domestic wastewater. In bioreactors receiving agricultural

drainage, in which N is mainly available as NO_3^-, An/coD activity is unlikely to be important for N removal.

6.2.2 Recommendations for future work

Designing denitrifying bioreactors to support both denitrification and An/coD would expand the utility of these systems for improving treatment of wastewater. While this thesis clearly demonstrates that in woodchip and coconut husk bioreactors a significant amount of N can be removed via the An/coD pathway, more monitoring data of N species removal and N_2 production in denitrifying bioreactors receiving NH_4^+ and NO_3^- rich wastewater is required to enhance our understanding of the effects of operational and environmental conditions (e.g. seasonal temperature variations, and variations in loading rate and water composition) on An/coD activity in these systems. It is recommended that future work is underpinned by studies on the microbial ecology of bioreactors. This could include investigation of the biodiversity of organisms responsible for N removal in denitrifying bioreactors and quantification of the abundance of anammox and denitrifying bacteria in denitrifying bioreactor as described by Yang et al. (2015).

Additionally, more research should be conducted to increase our understanding of the N removal processes in denitrifying bioreactors, which in turn may lead to enhancement of An/coD in these systems. One of the main factors potentially inhibiting An/coD activity in denitrifying bioreactors is the presence of denitrifying bacteria which can compete with An/coD microorganism for oxidized N, especially in the presence of high concentrations of organic carbon (Güven et al., 2005, Lackner et al., 2008). Results from this thesis suggested that An/coD activity became more dominant in bioreactors in which denitrification was carbon limited. The effect of organic carbon availability on An/coD activity is, however, not fully

understood and should be investigated further. A major question regarding the practical application of An/coD in denitrifying bioreactors, therefore, remains:

<u>How do anammox organisms interact with denitrifiers in denitrifying bioreactors and what is the role of organic carbon availability in this process?</u>

This question could potentially be addressed by analysing and comparing N species removal, N_2 production and abundance of anammox and denitrifying organisms across a number of small or mesocosm scale denitrifying bioreactors filled with different carbon sources (e.g. woodchip, coconut husk, maize cob or wheat straw), while also monitoring carbon availability. Alternatively, availability of carbon in denitrifying bioreactors could be adjusted by injecting organic compounds (e.g. cellulose) into the bioreactor water inflow and measuring its effect on N species removal and N_2 production.

Additional sub questions that could be addressed in this research are:

<u>1. What is the role of NO_3^- and NH_4^+ concentrations in this process?</u>

Concentrations of reactants provide the fundamental base for the energy balance of reactions, since microorganisms utilise energy from reactions for growth and maintenance. Anammox bacteria derive their energy from the oxidation of NH_4^+ by NO_2^- (Van de Graaf et al., 1995). Consequently, the process relies on the availability of these N species. Further research should address the effects of reactant availability on An/coD activity in denitrifying bioreactors.

2. What is the effect of temperature in these processes?

Temperature is considered is an important environmental factor controlling denitrification, codenitrification and anammox activity (Kuenen, 2008, Seitzinger et al., 2006). Differences in temperature response have been observed for anammox and denitrifying bacteria in in Artic fjord sediments, with a lower temperature optimum for anammox compared to denitrification (Canion et al., 2014). Future work should, therefore, include assessment of temperature effects on denitrification, codenitrification and anammox activity in denitrifying bioreactors and how this affects the removal of NO_3^- and NH_4^+.

6.3   Overall conclusions

This thesis demonstrated the broader versatility of denitrifying bioreactors for wastewater treatment by establishing that, in addition to removing NO_3^-, denitrifying bioreactors can effectively remove microbial contaminants and NH_4^+. Designing denitrifying bioreactors to include removal of microbial contaminants and NH_4^+ would expand the utility of these systems. However, a number of questions still remain regarding the practical application of denitrifying bioreactors for microbial contaminant removal of nitrogen by An/coD. Further research is recommended to increase understanding of factors controlling microbial contaminant removal and N removal processes in these systems, which in turn may lead to improved design of bioreactors for effective microbial contaminant and NH_4^+ removal. Overall, it is recommended that in future research a more holistic approach to denitrifying bioreactors is taken in which NO_3^- is analysed in conjunction with other types of wastewater contaminants (e.g. phosphorus, microbial contaminants, emerging contaminants, suspended solids) to establish whether denitrifying bioreactor can further evolve into simple, low-cost, low-maintenance and reliable technologies for the removal of a broader range of wastewater contaminants.

6.4 References

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Appendix A

Co-authorship forms

Co-Authorship Form

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Te Whare Wānanga o Waikato

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in your appendices for all the copies of your thesis submitted for examination and library deposit (including digital deposit).

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3 has been published in the Journal of Environmental Quality. The title of the chapter is: Faecal bacteria, bacteriophage, and nutrient reductions in a full-scale denitrifying woodchip bioreactors

Nature of contribution by PhD candidate	Collaboration on project conception, data collection, analysis and interpretation and writing of manuscript/chapter
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

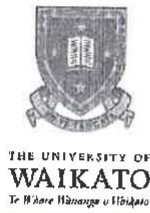| Name	Nature of Contribution
Chris C. Tanner	Collaboration on project conception and data analysis and interpretation, provided comments on manuscript versions
Rebecca Stott	Collaboartion on data analysis and interpretation, provided comments on the manuscript
Louis A. Schipper	Collaboration on project conception and data analysis and interpretation, provided comments on manuscript versions

Certification by Co-Authors

The undersigned hereby certify that:
* the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Chris C. Tanner		25/10/18
Rebecca Stott		28/10/18
Louis A. Schipper	LA Schipper	17/10/18

July 2015

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Co-Authorship Form

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

As it stands in the submitted thesis, Chapter 4 (Bacteria and virus removal in denitrifying bioreactors: effects of media type and age) is being prepared for submission to a suitable journal.

Nature of contribution by PhD candidate	Collaboration on project conception, data collection, analysis and interpretation and writing of manuscript/chapter
Extent of contribution by PhD candidate (%)	85

CO-AUTHORS

Name	Nature of Contribution
Chris C. Tanner	Collaboration on project conception and data analysis and interpretation, provided comments on manuscript versions
Rebecca Stott	Collaboartion on data analysis and interpretation, provided substantial comments on the manuscript
Louis A. Schipper	Collaboration on project conception and date analysis and interpretation, provided comments on manuscript versions

Certification by Co-Authors

The undersigned hereby certify that:
❖ the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Chris C. Tanner		25/10/18
Rebecca Stott		25/10/18
Louis A. Schipper		17/10/18

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Co-Authorship Form

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

As it stands in the submitted thesis, Chapter 6 ("Nitrate and ammonium removal in woodchip and coconut husk bioreactors: denitrification, anammox and/or codenitrification") is being prepared for submission to a suitable journal.

Nature of contribution by PhD candidate	Collaboration on project conception, data collection, analysis and interpretation and writing of manuscript/chapter
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Chris C. Tanner	Collaboration on project conception and data analysis and interpretation, provided comments on manuscript versions
Louis A. Schipper	Collaboration on project conception and data analysis and interpretation, provided comments on manuscript versions

Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Chris C. Tanner	*[signature]*	25/10/18
Louis A. Schipper	*[signature]*	17/10/18

July 2015