Diversity of Ammonia Oxidising Microorganisms in Constructed Wetlands Fed with Landfill Leachate

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Abstract

Every year five million tonnes of ammonia-containing landfill leachate is generated from closed landfills in England. Ammonia is toxic to the environment and requires costly and environmentally unsustainable treatment at municipal wastewater treatment plants. Norfolk County Council are pursuing an alternative, more cost effective and environmentally friendly leachate treatment using the microbially driven anammox (anaerobic ammonia oxidation) reaction in vertical flow constructed wetlands trials. Conversion rates of 70-100% NH_3 has typically been observed but the underpinning microbiology remains unknown, severely restricting process optimisation. The aim of this MSc thesis was to develop the molecular tools to determine if anammox microorganisms and other essential nitrogen-cycling microorganisms were present and to investigate their diversity. It was hypothesised that such major players in NH_3 transformation would be detected within the constructed wetland trials. It was further hypothesised that differences would be observed in the nitrogen-cycling community between unsaturated and saturated layers of the wetlands due to moisture content disparity and between unvegetated and vegetated wetlands. The trials tested were an unvegetated wetland and a vegetated wetland planted with the common reed (*Phragmites australis*), often found in the salt marsh environment which these trials replicate. Primers were optimised for the annamox functional marker genes hydrazine synthase (*hzsA*) and hydrazine oxidoreductase (*hzo*). Additionally, aerobic ammonia oxidising bacteria (AOB) and archaea (AOA), which are important in supplying anammox with nitrite, were studied by targeting the ammonia monooxygenase (*amoA*). Anammox bacteria, AOB and AOA were detected in both constructed wetland trials tested. The diversity of anammox bacteria, AOB and AOA was surprisingly consistent across the unsaturated and saturated regions of the wetlands and the presence of *Phragmites* reeds had little effect on anammox diversity. Detection of anammox microorganisms by PCR in these trials supports future use of this approach which has great scope for optimisation and scale-up.

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1. Introduction

1.1 Landfill leachate

1.1.1 Landfill leachate: why do we need to treat it?

Landfill sites generate 5 million tonnes of landfill leachate annually in England alone, the treatment of which is costly and detrimental to the environment due to high ammonia content (NCC, 2017). Ammonia is toxic to animals, this is due to the toxic build-up in internal tissues and blood, which occurs at relatively low concentrations (NCC, 2017; Kadlec and Wallace, 2009; Water Framework Directive, 2015). To combat this, a novel yet simple solution for the treatment of ammonium is needed. There are recently implemented specialist wastewater treatment systems which utilise the microbially-driven anaerobic ammonia oxidation (anammox) reaction. This reaction oxidises ammonium with nitrite into NO_2 gas via the intermediate hydrazine and is performed by anammox bacteria. These treatment systems aim to enhance growth of microorganisms that perform anammox and their activity by employing specific environmental conditions which favour the anammox bacteria (Annavajhala *et al*., 2018). The aim of the NCC project is to apply the microbial anammox reaction in the treatment of ammonium from landfill leachate by using constructed wetlands (CW).

1.1.2 Generation of landfill leachate

Landfill leachate is generated by rainfall falling onto the landfill cap and by percolation of groundwater up into the landfill (**Fig. 1.1**). This water subsequently mixes with the inorganic and organic degraded waste picking up various toxic compounds and generates the leachate. Without adequate systems in place, the water will leach out into the surrounding area, known as surficial drainage, which accumulates around the base of the landfill or discharges into the groundwater. The leachate can also run off into natural rivers and waterways causing eutrophication and toxicity to aquatic life (Kadlec and Wallace, 2009). This is a huge environmental problem, as

ammonium, one of the molecules produced in landfill leachate through the anaerobic hydrolysis of organic nitrogen (Morling, 2007), is highly toxic to animals. In natural unpolluted waters, small amounts of ammonia at <0.02 mg/L in natural waterways to 0.39 mg/L in drinking water are usually observed. However, the amount of ammonia depends on multiple environmental factors, including temperature, pH and salinity. These parameters, in particular the pH, determine the ammonia to ammonium ratio and therefore the toxicity of the waters, as ammonia is highly toxic in its unionised form (NH_3) (Kadlec and Wallace, 2009). The Water Framework Directive (2015) requires ammonium levels to not exceed 5 mg/L for direct discharge to a watercourse, based on a previous evaluation at the Norfolk County Council (NCC) for this project (NCC, 2013; Water Framework Directive, 2015). Other chemicals, and BOD and COD levels are assumed to be at acceptable levels when ammonium is reduced to a concentration accepted for discharge into natural waterways (NCC, 2017; Kadlec and Wallace, 2009).

1.1.3 The composition of the leachate

The composition of landfill leachate changes throughout the lifecycle of a landfill site. During the first few days to weeks after a landfill has been capped, the landfill environment is highly aerobic, before turning anaerobic. The anaerobic phase lasts for the next several months to years as methanogenic (methane producing) conditions are established (Morling, 2007).

In the early stages of the anaerobic phase, the landfill leachate generates high concentrations of soluble degradable organic compounds and often maintains an acidic pH, as well as increasing ammonium and heavy metal concentrations.

Later on, the pH will increase and become slightly basic, and heavy metal concentration and Chemical Oxygen Demand (COD) will decrease but ammonium levels can remain relatively concentrated (Kadlec and Wallace,

2009; NCC, 2013; Morling, 2007). This is the stage that the Mayton Wood Landfill site is at currently (**Fig. 1.1**).

Figure 1.1 *Schematic of a typical closed landfill site. Displayed in the middle is the overall schematic displaying the landfill hole containing the waste and the pipes for extracting gases, leachate and the monitoring systems for landfill leachate, gas and groundwater. To the top left, the landfill cap and its components are indicated and to the bottom left, the collection pipe for landfill gases and the components beneath the waste and the drainage pipe for leachate are shown (Wastenotnc.org, 2019).*

1.2 Treatment Site and Treatment Methodology

1.2.1 Mayton Wood Closed Landfill site and the feasibility of ammonia removal

Mayton Wood is an older landfill site (**Fig. 1.2**) and at this stage most of the biodegradable carbon has been degraded into biogas and dissolved organic carbon (DOC) and heavy metal concentrations have been reduced however

ammoniacal nitrogen levels remain relatively high at 1.2 g L^{-1} (70.4 mM) (Morling, 2007; Kadlec and Wallace, 2009). Concentrations of ammonium at this level are much higher than that usually treated by constructed wetlands alone, Kadlec and colleagues (2009) state that wetlands are usually utilised to treat sewage (mostly nitrifying) at influent concentrations of up to 200 mg L^{-1} (0.2 g L^{-1}). At higher concentrations treatment usually consists of an initial clean-up step (e.g. reverse osmosis), followed by constructed wetlands as a final polishing step (Kadlec and Wallace, 2009; NCC, 2013; NCC, 2017).

Figure 1.2 *Mayton Wood Landfill site, Norwich (google maps, 2020).*

Previously, when constructed wetlands have been utilised as a singular step in the treatment of ammonium-polluted waters, ammonium concentrations have been relatively low. However, a previous study by Fannin and colleagues (2009) utilised constructed wetlands as a singular treatment method for landfill leachate containing similarly concentrated levels of NH_4^+ at 1.5 g L^{-1} (Kadlec and Wallace, 2009). Based on this promising result, the Norfolk County Council (NCC) are looking to apply the same single-step constructed wetland technology to treat landfill leachate at their other sites,

Costessey and Edgefield, which receive on average ammonium loads of up to 2 and 3 g L^{-1}, respectively (NCC, 2017).

In typical constructed wetlands with low ammonia loads, the nitrogen turnover is mostly performed by vegetative uptake. However, at higher concentrations of ammonium (>120 g N m^{-2} yr^{-1}), the nitrogen turnover in constructed wetlands becomes microbially-driven (Kadlec and Wallace, 2009). Given the high ammonia loading at the Mayton Wood closed landfill site (at lowest 1,576 g N m^{-2} yr^{-1}) (Kadlec and Wallace, 2009), it is imperative to understand the microbiology underpinning the nitrogen removal in these constructed wetlands.

The NCC currently send the leachate, pumped up from the bottom of the landfill site, into temporary storage which is transferred by tanker trucks to the Whitlingham wastewater treatment plant. This is costly and incurs a surcharge from the disposal contractor when ammonium concentrations are above 1.2 g L^{-1}. This means that attention is still required on site and the NCC are looking for a passive treatment technology that when left unattended will continue to remove ammonium without extra input.

A feasibility report produced by the NCC (NCC, 2013) indicated a two-stage process, due to the high levels of ammonium in the leachate, would be the most beneficial. The process would consist of a first step of reverse osmosis, followed by constructed wetlands, utilising the anaerobic ammonium oxidation (anammox) reaction as a final polishing step. The anammox reaction utilises ammonium and nitrite, condensing these to produce dinitrogen gas through hydrazine intermediates. Wetlands utilising this reaction are a relatively novel approach in the treatment of ammonium and the mechanisms with which ammonium is removed is little understood. The NCC implemented three trial constructed wetlands, later followed by a further six at the Mayton Wood closed landfill site for testing as outlined later in this section (**pg. 31-35 '1.5 Constructed Wetland Trials**'). Constructed wetlands have been utilised in the treatment of wastewater from various sources, with varying pollutants, including pharmaceuticals, laboratory wastewater, institutional wastewater, municipal wastewater, landfill leachate and more

(UN-HABITAT, 2008). Constructed wetlands were chosen as the most effective treatment option for Mayton Wood, due to the cost-effectiveness and efficiency of this technology with regards to its operation.

1.2.2 Why are constructed wetland systems advantageous in the treatment of ammonia?

Constructed wetlands are cost effective in that they are relatively simple and do not require a high level of expensive technological input, such as that usually required for aeration. The energy consumed in the aeration of traditional nitrification/denitrification (biological nitrogen removal (BNR)) wastewater treatment systems is that of 6.5 kW h^{-1} kg^{-1} N (Gonzalez-Martinez *et al*., 2018). In comparison, the energy consumption of a constructed wetland is very favourable and typically less than 0.1 kW h^{-1} m^{-3} (Kadlec and Wallace, 2009). Whereas an ammonium removal system utilising the anammox process termed the DEamMONification (DEMON) System, requires only 1.16 kW h^{-1} kg^{-1} N for aeration, which is higher than in constructed wetlands, but much less than that required in traditional BNR systems achieving 63% savings in comparison (Gonzalez-Martinez *et al.*, 2018). For constructed wetlands, oxygen is provided by the roots of reeds, examples of the genera utilised prior include: *Zizania*, *Phragmites* and *Spartina*, which grow in flooded marshes or ponds where oxygen cannot be extracted from the soil as the immediate environment is anaerobic. To deal with this these plants have evolved an elongated aerenchyma system, the aerenchyma is a soft, spongy plant tissue forming an abundance of gas conducting spaces (Kacprzyk *et al.*, 2011). This system transports oxygen from the atmosphere through their shoots and to their roots, which is then diffused into the rhizosphere creating miniature oxic-zones, this would provide oxygen at a rate sufficient to support aerobic nitrification (Yamasaki, 1984; Chen *et al*., 2008; Koop-Jakobsen and Giblin, 2009). However, this might not be the case with the reeds utilised in the constructed wetland set-up at Mayton Wood. *Phragmites australis* (otherwise known as the common reed) releases oxygen from the roots at a concentration that is said to be insufficient to maintain a micro-oxic zone in close proximity to the root, any

oxygen that is released by the roots is quickly utilised by aerobic microbiota (Yamasaki, 1984; Chen *et al*., 2008). Oxygen is also provided for the aerobic genera in the design of the constructed wetland itself. A vertical flow wetland, such as the wetland trials at Mayton Wood (**Fig. 1.3** and **Fig. 1.9**), consist of an unsaturated (dry) aerobic layer within the first 30 cm of the treatment matrix, the level of which is controlled by the level of the outflow pipe; below is an aerobic layer within the saturated (wet) treatment medium, O_2 levels are dependent on association with the roots of the reeds and the very bottom of the treatment medium is the saturated anaerobic zone, formed by the distance from the oxidised soil that is closer to the roots (Kadlec and Wallace, 2009; Fannin et al., 2009; NCC, 2017).

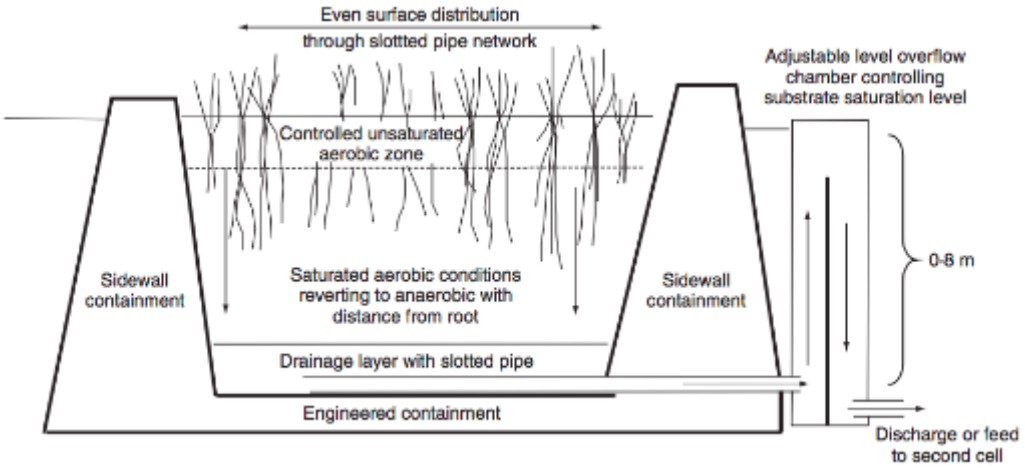

Figure 1.3 *Schematic of the constructed wetland tanks designed by Fannin et al. (2009). This set-up is based on a vertical flow (VF) wetland design that creates stratified layers from an aerobic unsaturated layer, to aerobic saturated and lastly anaerobic saturated, with O2 levels dropping with distance from the reed roots. Also shown at the bottom of the wetland is the drainage layer and outflow pipe. The level of the outflow pipe controls the saturation level, defining the height of the interface between the unsaturated and saturated levels (Fannin et al., 2009; NCC, 2017).*

There are three basic different designs of wetland: Free-Water Surface (FWS), Horizontal Sub-Surface Flow (HSSF) and Vertical Flow (VF), of which vertical flow wetlands are employed in the NCC trials (**Fig. 1.3** and **1.9**). At times, different types of wetlands are used in a sequence, e.g. HSSF wetlands can be coupled with VF wetlands to remove organics and suspended solids in the initial stage of treatment (Kadlec and Wallace, 2009). However, the NCC trials consist of vertical flow wetlands alone as this

set-up was very promising for treating high ammonia loads in a previous study (Fannin *et al*., 2009).

Constructed wetlands are cost effective, necessitate little maintenance and require no external carbon inputs (which promotes growth of specialist microorganisms, such as anammox bacteria) (Kadlec and Wallace, 2009). The reeds themselves provide small amount of carbon inputs for heterotrophs from lost rhizome material during the winter months (Graneli *et al.*, 1992). Roots and the surrounding microbial community also produce CO_2 for chemolithotrophs, e.g. anammox bacteria and aerobic ammonia oxidising microorganisms (AOMs) (Gonzalez-Martinez *et al*., 2018; Chen *et al*., 2008). Constructed wetlands are considered more environmentally sound than their more engineered counterparts as the technology is based on the natural processes found in saltmarshes and the system requires no chemical inputs. The wetlands are also a passive treatment, requiring fewer operating hours and should require none whatsoever when fully implemented. Treatment is carried out on site, meaning that there are no extra costs for waste removal and treatment (Fannin *et al*., 2009; NCC, 2017).

The constructed wetlands are designed to favour the activity of the microbially-driven anammox process. Anammox bacteria do not require carbon inputs for energy as anammox cells derive their energy from inorganic nitrogen and carbon from CO_2 leading to further reduction in costs (Gonzalez-Martinez, *et al*., 2018). The anammox process is also inherently better for the environment, when compared with denitrification as anammox bacteria does not emit nitrous oxide, a greenhouse gas (making up to 5% of the greenhouse effect) that is also responsible for destroying stratospheric ozone (Aronson and Allison., 2012), as a part of its metabolism. Instead, the anammox process only emits dinitrogen gas as the end-product, which is already plentiful in the atmosphere and not a greenhouse gas (Kartal *et al*., 2011; Annavajhala *et al*., 2018). Constructed wetlands have mostly been utilised as a final polishing step in the treatment of leachate prior to this but have been coupled together with other technologies to achieve greater savings and ammonium removal (Kadlec and Wallace, 2009; NCC, 2013).

With the exception of the research by Fannin and colleagues (2009) which did not examine the microbial communities, there have been no other studies using constructed wetlands as the primary treatment of landfill leachate (**Fig. 1.3** and **1.9**). The NCC based their constructed wetland trials on work by Fannin and colleagues (2009) which demonstrated ammonium removal rates ranging between 69-95% from leachate containing ammonium concentrations of 1.5 g L^{-1}, but neither the processes nor the microorganisms responsible for ammonium removal were investigated in this paper. This current study is therefore important and novel in gaining insights into the function and microbiology of constructed wetlands as a single-step treatment.

1.3 Microbiology and functional marker genes of ammonia oxidation and anammox

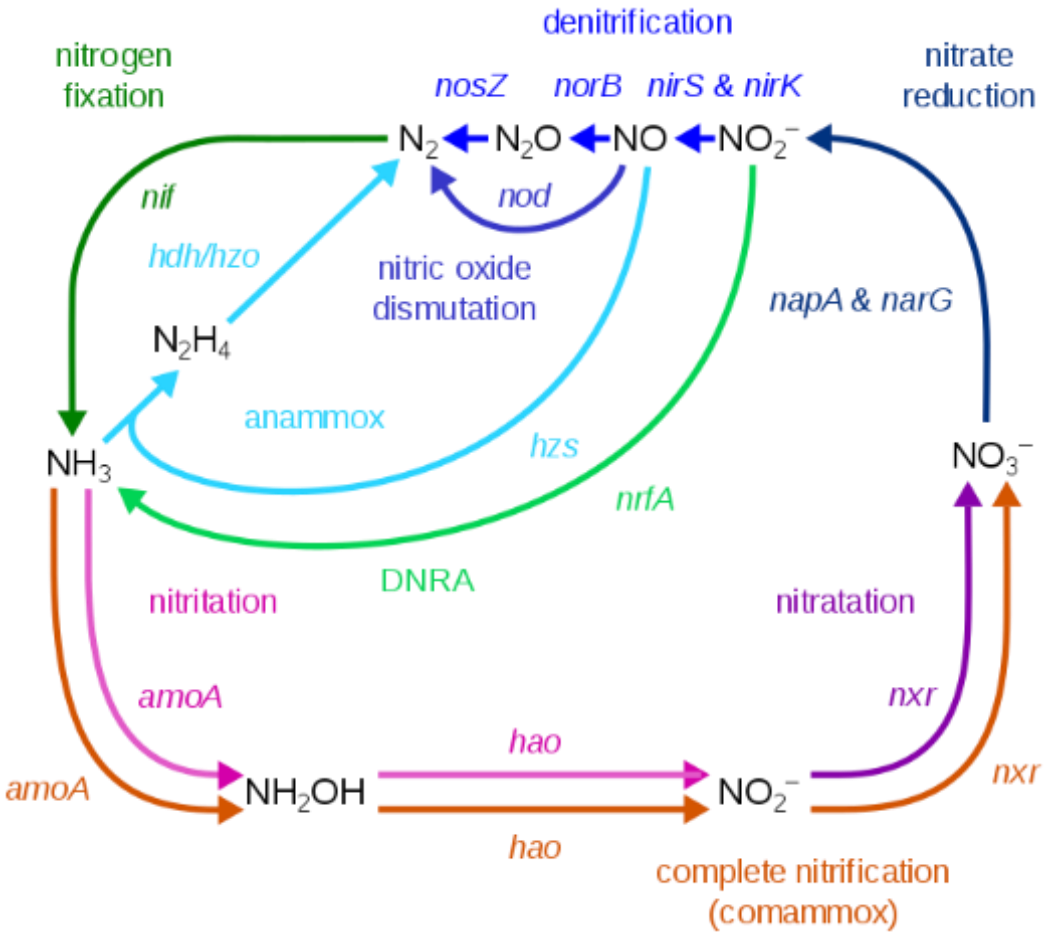

Figure 1.4 *The Nitrogen Cycle. The processes by which various nitrogenous compounds are transformed from one form to another (Sparacino-Watkins et al., 2014).*

1.3.1 The nitrogen cycle

Nitrogen is essential for all life on Earth as it is required by all living organisms for amino acids, nucleic acids and other nitrogenous compounds. Nitrogen exists in several different forms in the environment, these forms can be converted from one to another in microbially-mediated reactions, which together constitute to the global nitrogen cycle (**Fig. 1.4**). The reactions within the nitrogen cycle are performed by specific microorganisms. Microbial nitrogen transformations include: biological nitrogen fixation of gaseous

nitrogen into soil, mineralisation from soil organic matter into ammonium, nitrification which transforms ammonia to nitrite and nitrite to nitrate and denitrification and anammox which return nitrogen back into atmospheric forms such as nitrous oxide and dinitrogen gas (Robertson and Groffman, 2015; Lehtovirta-Morley *et al*., 2016; Zhang *et al*., 2018; Kuenen, 2008). In constructed wetlands, nitrogen turnover consists of six processes whereby nitrogen is taken up or lost. First is nitrification, coupled with secondly, denitrification or anammox which releases nitrogen in atmospheric forms N_2O and N_2. Thirdly, unionised ammonia is relatively volatile and can be lost to the atmosphere by a process called volatilisation, particularly when environmental pH is above 9.3. Fourthly, adsorption, whereby the ionised form of ammonium adheres to the matrix of the constructed wetland and as nitrification proceeds, the equilibrium will be maintained by adsorbed ammonium being desorped. The fifth is by plant uptake, which drives nitrogen uptake when the ammonia level is below 120 g N m^{-2} yr^{-1} (Kadlec and Wallace, 2009). A sixth step is ammonification i.e. the mineralisation of organic nitrogen to ammonia (Kadlec and Wallace, 2009; Vymazal, 2007). Sometimes nitrogen can become buried, this is where organic nitrogen is incorporated into the detritus of the wetland, becoming unavailable for additional nutrient cycling through the process of peat formation and burial (Vymazal, 2007).

Within the microbial nitrogen cycle, ammonia can be transformed by two processes: anammox and aerobic ammonia oxidation, which is the first and rate-limiting step in nitrification. **The microorganisms responsible for these processes are the main focus of this study.**

1.3.2 Functional marker genes of ammonia oxidation and anammox

During nitrification, ammonia is first oxidised to nitrite (ammonia oxidation) and nitrite is subsequently oxidised nitrate (nitrite oxidation). There are three groups of microorganisms which carry out ammonia oxidation: canonical ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA) and comammox *Nitrospira*. AOB and AOA oxidise ammonia to nitrite, whereas

comammox microorganisms oxidise ammonia to nitrate. Since nitrite is an essential substrate for the anammox process and AOA and AOB produce nitrite to feed into anammox, this study focused on studying AOB and AOA rather than comammox.

AOB and AOA perform the first step in nitrification, the oxidation of ammonium into nitrite:

$$NH_3 + 1½O_2 \rightarrow NO_2^- + H^+ + H_2O. \quad \textbf{(Eq. 1)}$$

The first step of ammonia oxidation is the oxidation of ammonium to the intermediate hydroxylamine:

$$NH_3^+ + 2H^+ + O_2 + 2e^- \xrightarrow{\text{ammonia mono-oxygenase}} NH_2OH + H_2O. \quad \textbf{(Eq. 2)}$$

This is catalysed by the membrane-bound enzyme ammonia monooxygenase (**AMO**). After this step, this is further oxidised to nitrite, which is then utilised in the anammox reaction. The oxidation of hydroxylamine to nitrite produces four electrons, two of which are recycled and utilised in the oxidation of ammonium in ammonia oxidation (**equation 2**) and the other two are utilised to generate energy. Since AMO is a key enzyme in aerobic ammonia oxidation, it was selected as the first functional marker gene in this study to examine the presence and diversity of AOA and AOB. Detection of AMO and thus the presence of aerobic ammonia oxidisers would indicate that nitrification was occurring, which in turn would suggest that it is possible for the anammox process to take place (Lehtovirta-Morley *et al*., 2016; Robertson and Groffman, 2015).

Anammox (anaerobic ammonia oxidation) is carried out by a distinct group of Planctomycetes termed anammox bacteria which convert ammonia and nitrite into dinitrogen gas via nitric oxide and hydrazine intermediates under anoxic conditions (**fig. 1.4**). Notably, anammox does not produce the greenhouse gas nitrous oxide. This is in contrast to denitrification (sequential reduction of nitrate into dinitrogen via nitrite, nitric oxide and nitrous oxide),

which has made anammox an attractive choice for ammonia waste treatment (Robertson and Groffman, 2015).

The anammox reaction occurs in three steps (with (**4**) being the overall reaction):

$$NO_2^- + 2H^+ + e^- = NO + H_2O \ (E_0' = +0.38\ V) \quad (1)$$

$$NO + NH_4^+ + 2H^+ + 3e^- = N_2H_4 + H_2O \ (E_0' = +0.06\ V) \quad (2)$$

$$N_2H_4 = N_2 + 4H^+ + 4e^- \ (E_0' = -0.75\ V) \quad (3)$$

$$NH_4^+ + NO_2^- = N_2 + 2H_2O \ (\Delta G^{o\prime} = -357\ kJ\ mol^{-1}) \quad (4)$$

The first reaction is the condensation reaction of nitrite to nitric oxide, which is then utilised in the second reaction, where the simultaneous condensation of nitric oxide with ammonium to produce hydrazine occurs. This is performed by hydrazine synthase (**HZS**) (reaction (**2**)). The third step is the last, in which hydrazine is oxidised to dinitrogen gas by the second marker gene, hydrazine oxidoreductase (**HZO**) (reaction (**3**)) also quoted in the literature as hydrazine dehydrogenase (HDH). The fourth equation is the overall anammox reaction (Kartal *et al*., 2011; Kuenen, 2008; Van Niftrik and Jetten, 2012). In this study, two functional marker genes for anammox were used: hydrazine synthase (*hzs*) and hydrazine oxidoreductase (*hzo*).

1.3.3 Discovery, cellular features and enzymology of anammox bacteria

While the nitrogen cycle has been studied for over a century, the anammox process was a recent discovery of 20 years ago. Despite the recent discovery, anammox is hugely important to the nitrogen cycle and has been estimated to account for up to 50% of nitrogen turnover in marine environments, estimated recently to be closer to 23-30%, with a lesser role in groundwater (18-36%); in paddy soils (4-37%); and in lakes (9-15%) (Van Dongen *et al*., 2001; Sonthiphand *et al.*, 2014). At the time that the anammox process was discovered, it was believed that ammonium was chemically inert and only the AMO enzyme of AOMs were capable of oxidising this compound. However, in 1992 a group from Gist Brocades Fermentation Company found that the disappearance of ammonium was taking place at

the expense of nitrate with a clear increase in dinitrogen production and the process was termed anaerobic ammonium oxidation or “anammox”. These researchers, however, were unable to enrich, grow and identify the organisms responsible. In 1995 a flow-through system that was comprised of a fluidised bed reactor, continuously fed with mineral medium containing ammonium and nitrite (instead of nitrate) was used to produce enrichments containing the bacteria responsible for anammox. Efforts into cultivating and purifying these organisms has been difficult, this is due to anammox bacteria having extremely slow growth rates, which are of approximately two weeks doubling time. Further to this, PCR amplification revealed several mismatches between the 16S rRNA universal primers and the anammox 16S rRNA sequence. Phylogenetic research confirmed this species as a member of the Planctomycetes, a phylum known for its unusual membrane-bound sub-cellular compartments (Kuenen, 2008).

Anammox bacteria, like other planctomycetes, have membrane-bound, sub-cellular compartments. However, anammox cells contain an anammox-specific, membrane-bound compartment termed the “anammoxosome”, where the anammox reaction occurs. This contains hydrazine oxidoreductase which is loosely membrane-bound as well as hydrazine synthase (**Fig. 1.5**). The anammoxosome is composed of concatenated cyclobutane rings called ladderane lipids and is important for containing and compartmentalising the toxic intermediates of the anammox reaction (Van Niftrik *et al*., 2004; Van Niftrik and Jetten, 2012).

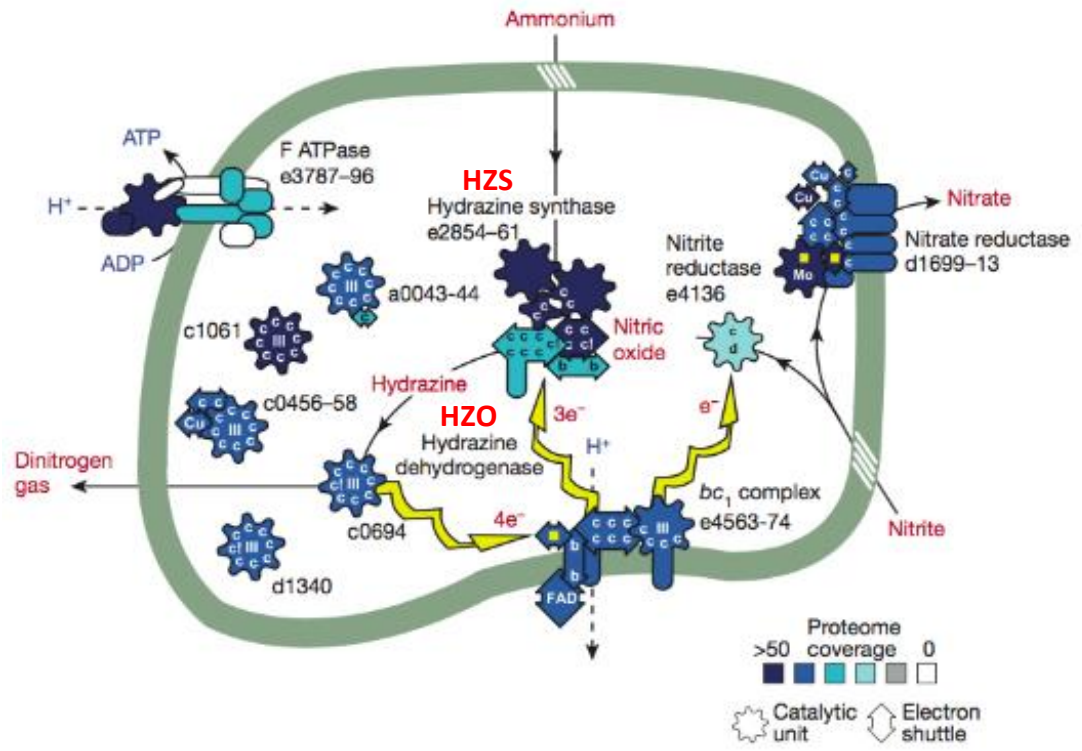

Figure 1.5 *Anammoxosome, the powerhouse of the cell where ammonium and nitric oxide are oxidised in various steps to dinitrogen gas generating protons for ATP production (Kartal et al., 2011).*

1.3.4 HZS and HZO enzymes – functional marker genes for anammox

The anammox reaction occurs in three steps, firstly nitrite (formed by the AOB/AOA in aerobic ammonia oxidation) is taken by nitrite reductase (NIR) to form nitric oxide. Secondly, the first of the enzymes targeted by key marker gene primers in this study, HZS, completes the second step with the simultaneous condensation of nitric oxide with ammonium to produce hydrazine. Lastly, the second enzyme targeted by key marker gene primers in this study, HZO, takes hydrazine and oxidises it to dinitrogen gas, a little bit of nitrate is also formed as a part of their metabolism.

The first marker gene utilised in this study in the search for the bacteria that perform anammox, Hydrazine synthase, (**Fig. 1.6**) consists of a dimer each containing a heterotrimer of alpha, beta and gamma subunits. Each heterotrimer contains four haems, two in both the alpha and gamma subunits and one zinc ion as well as several calcium ions (**Fig. 1.7**). In two half

reactions (shown in reaction (**2**)) HZS combines nitric oxide (+II oxidation number) and ammonium (-III oxidation number). It does so by reducing nitric oxide to hydroxylamine (-I oxidation number) within the gamma subunit (utilising 3 electrons from a redox partner), this enables the oxidation number of -II of the final product hydrazine to be reached. In the second part of the reaction, hydroxylamine diffuses from the gamma subunit into the alpha subunit active site, ammonia from ammonium performs a nucleophilic attack on the nitrogen molecule of hydroxylamine to form hydrazine (Kartal *et al*., 2011; Dietl *et al*., 2015).

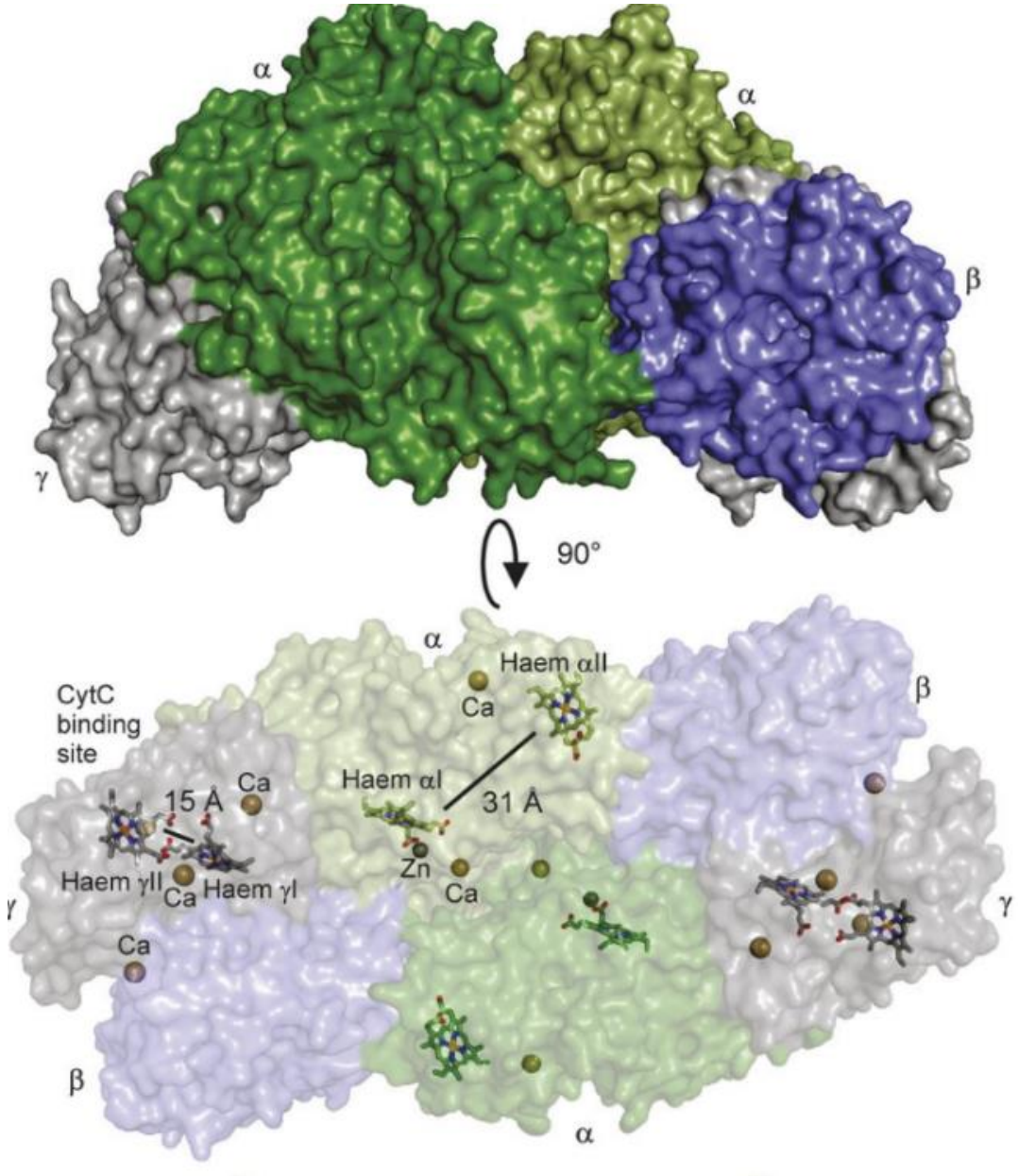

Figure 1.6 *Surface view of hydrazine synthase, which consists of two heterotrimers of alpha, beta and gamma subunits in a crescent shape (Dietl et al., 2015).*

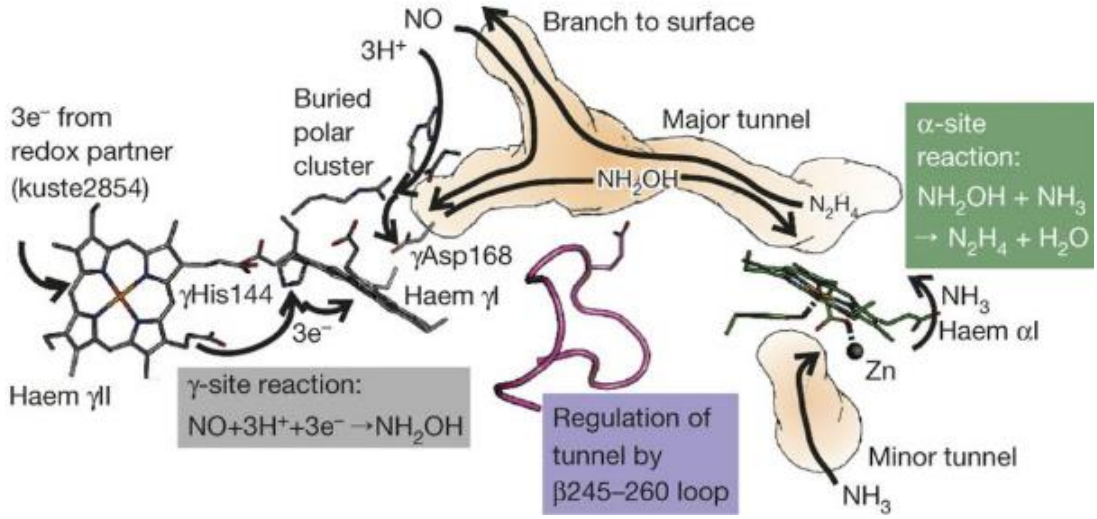

Figure 1.7 *Schematic diagram indicating the main residues involved in the catalytic function of HZS. The gamma subunit contains the active site for the first half reaction, transforming nitric oxide to hydroxylamine and the alpha subunit contains the catalytic site where hydroxylamine and ammonia are condensed to form hydrazine (Dietl et al., 2015).*

The second marker gene utilised in the search for the presence of anammox bacteria, hydrazine oxidoreductase, is a member of the octaheme cytochrome *c* hydroxylamine oxidoreductase protein family. It deviates from other proteins in this family in that it oxidises hydrazine only and is unable to oxidise hydroxylamine, whereas both hydroxylamine oxidoreductases from *Kuenenia stuttgartiensis* and *Nitrosomonas europaea* can oxidise both hydroxylamine and hydrazine. HZO performs reaction (**3**) producing 4 electrons and 4 protons in the oxidation of hydrazine, the most powerful reductant in nature, to dinitrogen gas (Maalcke *et al*., 2016). These four electrons then go on to drive the reduction reactions (**1**) and (**2**) and the protons are utilised in the production of ATP (Kartal *et al.,* 2011). The proposed catalytic cycle of HZO involves two steps: the first step is the oxidation of hydrazine to a diazene derivative, this reaction releases two electrons; the second step is the further oxidation of diazene to dinitrogen gas, again yielding two electrons (**Fig. 1.8**). Hydroxylamine is a side-product of this reaction that can inhibit HZO (Maalcke *et al.,* 2016).

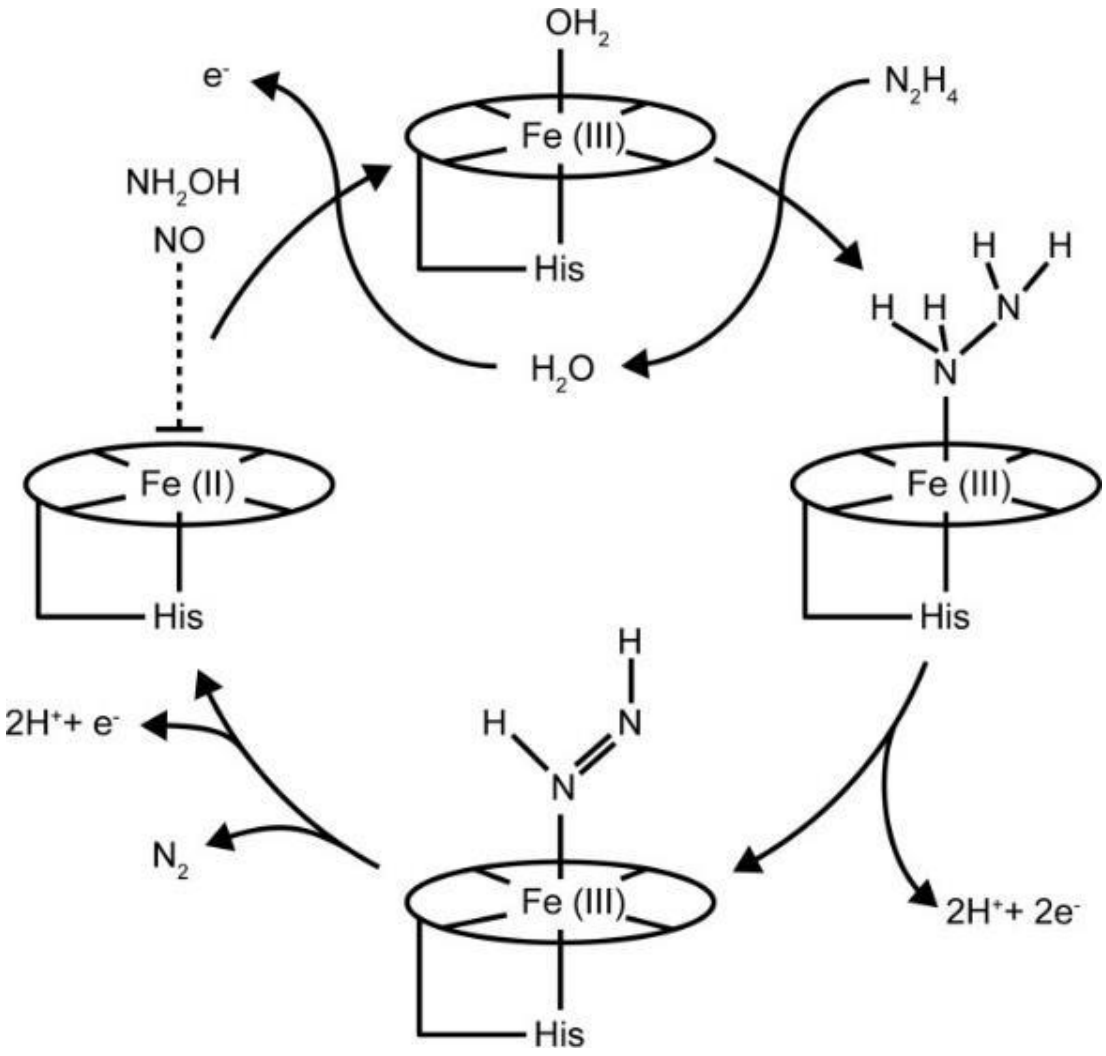

Figure 1.8 *Proposed schematic of the catalytic activity of HZO (Maalclke et al., 2016).*

1.4 Diversity and distribution of anammox bacteria and aerobic ammonia oxidisers

Understanding how environmental parameters shape the ammonia oxidising microbial communities is crucial for optimising and enhancing the landfill leachate treatment process in constructed wetlands. There is a strong body of evidence to suggest that salinity, pH and ammonia concentration are some of the major environmental drivers determining the community diversity, abundance and activity of both aerobic ammonia oxidisers and anammox microorganisms (Yang *et al.*, 2014; Pommerning-Roser and

Koops, 2005; Humbert *et al.*2010, Sonthiphand *et al.*, 2014; Koop-Jakobsen and Giblin, 2009).

Of the anammox microorganisms, representatives of genus *Scalindua* are typically found in marine habitats (Sonthiphand *et al.*, 2014). Previously characterised constructed wetlands contained anammox bacteria belonging to genera *Brocadia*, *Jettenia* and *Anammoxoglobus* (Zhu *et al.,* 2011), which have also been repeatedly found in wastewater treatment plants alongside genus *Kuenenia* and *Brocadia* (Sonthiphand *et al.*, 2014). Salinity appears to be a key driver of anammox microbial communities (Sonthiphand *et al.*, 2014; Koop-Jakobsen and Giblin, 2009). The constructed wetland technology (**Fig. 1.3** and **1.9**) is a system designed to replicate and enhance the natural occurring microbiological processes in salt-marsh systems as the conditions in this environment and mature landfill leachates are highly comparable. However, landfill leachate is less saline than in salt marshes, measured at 9,542 ppm and <35,000 ppm (just below seawater), respectively (Koop-Jakobsen and Giblin, 2009; Boorman, 2003). The stratified layers of a constructed wetland are expected to enhance anammox activity as it has been reported that in meromictic rivers, where sediments do not mix, there are a greater abundance of anammox bacteria. Further to this it is found in agricultural soils, where soils are consistently disturbed and aerated, there is little to no anammox activity found (Fannin *et al*., 2009; Kadlec and Wallace, 2009; Long *et al*., 2012; Sonthiphand *et al.*, 2014; Humbert *et al*. 2010). Globally across wetlands, drylands, groundwater aquifers and snow it has been indicated that *Candidatus Brocadia* is the dominant genus on a global scale accounting for 80.0% to 99.9% of the retrieved sequences in different habitats (Wang *et al.,* 2019). The genus *Candidatus Jettenia* has been found to be the second most abundant group detected, accounting for no more than 19.9% in the same environments (Wang *et al.*, 2019). Drylands, wetlands and groundwater aquifers indicated similar profiles in community diversity and composition, with snow showing the most difference.

Supply of nitrite has been implicated as the limiting factor for anammox in aquatic sediments (anammox microbes can form a symbiotic relationship with some nitrate reducing/sulphur oxidising bacteria that can perform denitrification, producing nitrite). Association between nitrifiers and anammox bacteria may be weak in low permeability sediments (clays, estuarine mud), this is because much of the nitrite produced in the upper few millimetres of the bed will mostly diffuse into the overlying water or be fully oxidised to nitrate before reaching the sub-oxic layer where the anammox bacteria reside (Lansdown *et al*., 2016).

AOB communities in soil ecosystems normally consist exclusively of β-proteobacterial genera *Nitrosospira* and *Nitrosomonas*, of which in general *Nitrosospira* normally predominates over *Nitrosomonas* (Pommerning-Roser and Koops, 2005). Whereas γ-proteobacterial AOB of the genus *Nitrosococcus* are found in highly saline habitats such as marine environments (Pommerening-Roser and Koops, 2005). Some *Nitrosomonas* strains are exceptionally well adapted to high ammonia concentrations, and these microorganisms are abundant members of nitrifying communities in wastewater treatment plants (Pommerning-Roser and Koops, 2005; Du *et al.*, 2016; Yamamoto *et al.*, 2010). In comparison AOA have been usually considered to be adapted to low ammonia concentrations (Sauder *et al.*, 2017; Schleper and Nicol, 2010; Yin *et al.*, 2018) however recently there have been reports of AOA genera that are adapted to high ammonia concentrations (Lehtovirta-Morley *et al*., 2016; Sauder *et al.*, 2017). The *Nitrosocosmicus* genus which has been isolated from soils and wastewater treatment systems and other AOA genera have also been detected in multiple industrial and municipal wastewater treatment plants which are high in ammonia (Mussmann *et al.*, 2011). Furthermore, AOA have been reported in paddy rice wetlands planted with a species closely related to *Phragmites australis* (Chen *et al*., 2008). Soil AOA communities are typically dominated by the genus *Nitrososphaera* in neutral pH and the genus *Nitrosotalea* in acidic pH soils (Gubry-Rangin *et al.*, 2011). AOA affiliated with genus *Nitrosopumilus* are found in marine habitats (Alves *et al.*, 2018).

1.5 Constructed wetland trials

Norfolk County Council initiated the anammox constructed wetland trials at Mayton Wood in 2013 (**Fig. 1.9**), the set-up of which is indicated in **Fig. 1.10**. Each of the constructed wetlands measures 1 m^3 (soil matrix: 1 m (height) x 1 m (width) x 77 cm (depth of treatment medium)). Each trial has an artificially maintained water table at the depth of approximately 30 cm to create an aerobic and anaerobic layer. The wetlands receive leachate from the top. Constructed wetlands contain a treatment medium of sand and compost providing a natural seed population through the compost and providing a large surface area for contact between leachate and treatment medium. The Compost was sourced from a surface water treatment system at a recycling centre and sand to aid in water flow, sourced from a local quarry. In Constructed wetlands 1-3, matrices consist of a 1:1 ratio of sand to compost. Ponding and clogging problems were observed in the first constructed wetland trials and bromide tracer experiments were performed indicating that 30% of influent leachate would take preferential pathways through gaps created by the piezometers and down the sides of the tank. Therefore, in the rest of the constructed wetland trials, the sand to compost ratio was changed to 2:1 respectively to provide the correct hydraulic properties to improve influent leachate and treatment medium contact. At the very bottom of the constructed wetlands is a basal drainage layer consisting of gravel, along with an outflow pipe, the height of which determines the saturation level in which the VF wetland system depends upon.

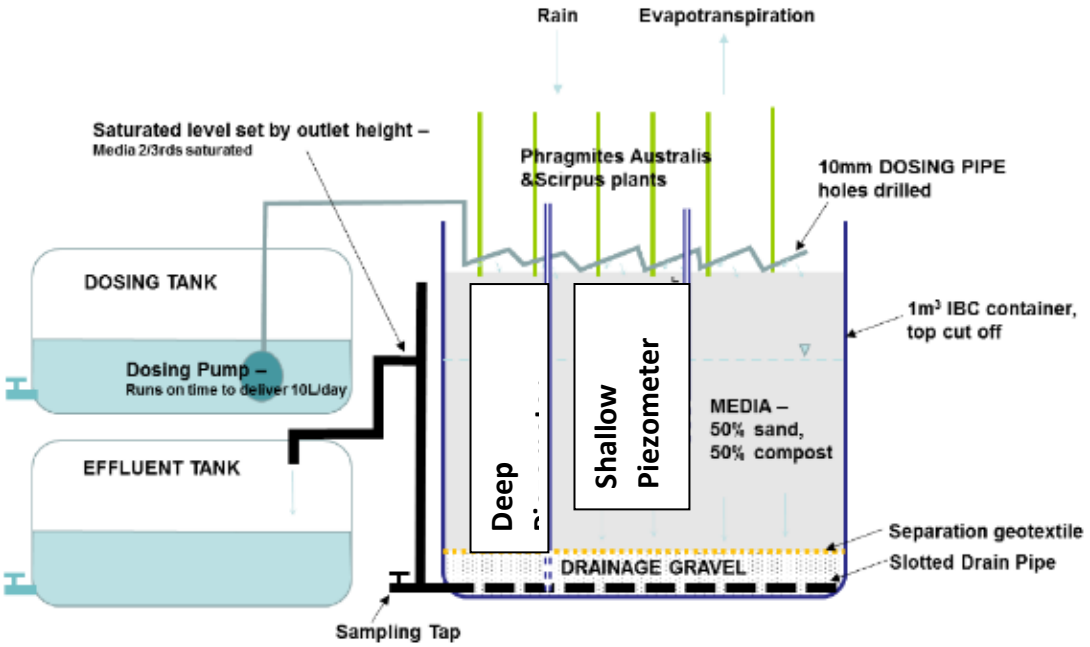

Figure 1.9 *Schematic of the Constructed Wetland trials implemented by the NCC at Mayton Wood, based off the constructed wetland trials in Fannin and colleagues (2009). There are 8 Constructed Wetlands in total (1 m in container height and width and 77 mm in treatment medium depth), each receiving different leachate loads and volume (NCC, 2017). IBC – Intermediate Bulk Container.*

Figure 1.10 *Constructed Wetland trials – trials imaged in August of 2019, polytunnel added at this time (top of image) – (**A**) Reed Beds 1-8 and Biobed 12 is out of frame. (**B**) All Reed Beds (1-8) and Biobed 12 are in view (Biobed 12 being closest on the right) and pump (at bottom) for drawing out effluent.*

Trials (termed Reed Beds) receive different volumes and concentrations of landfill leachate to identify optimal conditions for leachate loading, the concentration is calculated by varying ratios of clean water to leachate water. Trials are also testing the suitability of a two-stage treatment, where effluent from one wetland is fed into another wetland. One of the trials is unplanted (termed Biobed) to test the effect of vegetation on ammonium removal, the others are planted with the reed *Phragmites australis*. Ponding (i.e. a situation where the leachate can no longer pass through the wetland and accumulates on top) has occurred on multiple occasions but the reason for this is currently unknown. When sampling from the Constructed Wetland trials in January, the NCC were measuring a number of parameters onsite. Firstly, temperature was measured by probe to the middle of each constructed wetland trial; conductivity and chloride ion content was measured directly from landfill leachate and dosing tanks/effluent tanks by conductivity/chloride water test meter and pH and ammonium/nitrate by colorimetric strips. Landfill leachate (100%) was tested weekly at a

laboratory, here they tested for ammoniacal nitrogen NH_4^+-N, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Chloride ion content (Cl^-), conductivity (µS/cm), nitrite, pH, Total Organic Carbon (TOC), Total Oxidised Nitrogen (TON). A further extended suite of all hazardous and non-hazardous pollutants present in the leachate are carried out every six months. Currently, nitrogen species are measured weekly in the laboratory and further measurements of BOD and COD are measured monthly in the same laboratory (**Table 5.1** and **5.2**, in supplementary info indicate these measurements in Reed Bed 6 and Biobed 12 at time of sampling) and an extended suite of other chemicals is still carried out bi-annually (not shown). Onsite measurements consist of analysing conductivity by conductivity meter and dosing volumes are measured to ensure dilutions are correct. Trials have been reasonably successful in ammonium removal, particularly since the addition of the poly-tunnel, percentage of removal is estimated in the period from July to end of September 2019, as summarised in **Table 1.1**.

Table 1.1 *Key features of the constructed wetland trials by NCC (correct on December 2019)*

*RB = Reed Bed (vegetated constructed wetland), Bb = Biobed (unvegetated constructed wetland), **Decommissioned, ***Matrix of this wetland is made of gravel to reduce the risk of clogging, ND = Not determined or data not available, **** initially RB6 was loaded with 30 L day^{-1} and RB7 20 L day^{-1}, this was reduced to 8 L day^{-1} after clogging was observed.

Identifier*	**Loading volume of influent (L day^{-1})**	**g N day^{-1}**	**Ammonium removal (%) (July 2019 – September 2019)**	**Clogging**	**Vegetation (*Phragmites australis*)**	**2-stage**
RB1**	10	10	ND	Yes	Yes	No
RB2	10	4-6	100	No	Yes	No
RB3	10	1	99	No	Yes	Yes, fed from RB2 and RB4
RB4	10	5-9	100	No	Yes	No
RB5	ND	2-3	100	No	Yes	Yes, fed from RB6
RB6	8 ****	6-10	73	Yes	Yes	No
RB7**	8 ****	0	ND	Yes	Yes	No
RB8**	10	0	ND	No	Yes	Yes, fed 50% by its own effluent
RB11**/***	10	10	ND	No	Yes	No
Bb12	10	10	70	No	No	No

1.6 Project objectives

The project aims to characterise the microbial community responsible for the cycling of nitrogen in the constructed wetland trials. Although the previous study by Fannin and colleagues (2009) demonstrated successful removal of ammonia with the set-up used in the NCC trials, the work did not examine the microbiology of the ammonium removal process. This project will address this major knowledge gap in landfill leachate treatment in constructed wetlands. Given the high ammonium production and loading at our study site, ammonium removal will be driven microbially, as opposed to by uptake from the reeds, or by evapotranspiration (Kadlec and Wallace, 2009). This highlights the importance of understanding the microbes involved in nitrogen cycling, particularly those in the process of removing ammonium and their activity.

It is hypothesised that microorganisms responsible for the process of ammonium removal: ammonia oxidising archaea/bacteria and anammox bacteria will be detected within the constructed wetland trials.

It was further hypothesised that given the difference in moisture content between the unsaturated and saturated layers these sections would likely be distinct in the microbiology community they hosted. The same would be difference would be observed between the vegetated and unvegetated wetlands.

The aim of this study:

1. **Optimisation of molecular tools for studying nitrogen cycling microbial communities by utilising key marker genes, *amo*, *hzs*, and *hzo*.** The enzymes targeted by the marker gene primers are involved in the major steps of the nitrogen cycle related to anammox, the presence of HZS and HZO indicate that anammox bacteria are present as these genes are directly involved in their metabolism. The

genes that are involved in aerobic and anaerobic ammonia oxidation are indicative of the presence of these microorganisms.

2. **Investigate the presence of anammox organisms and aerobic ammonia oxidizing microorganisms in the constructed wetland trials using the newly optimised molecular tools.**
3. **Determine the diversity of the key nitrogen cycling microorganisms in the trials by sequencing and DNA fingerprinting**.

2. Materials and Methods

2.1 Description of the study site

Soil was sampled from two constructed wetland trials: Reed Bed 6 (RB6) (22/01/19) (**Fig. 2.1**) and again on the 12/3/19 along with Biobed 12 (Bb12).

Reed Bed 6 (RB6) was chosen as it was ponding at the time of sampling and was due to be decommissioned. Ponding occurs when the influent landfill leachate cannot drain through the matrix and consequent additions of influent landfill leachate build up on the matrix surface, leaving some parts of the matrix dry beneath. As RB6 was due to be decommissioned (at this time) and the method of sampling was yet to be perfected, it was decided to attempt sampling from here. A core of 5 cm width, to a depth of 45 cm was retrieved, the soil corer could not reach deeper as the corer reached a dry patch where the constructed wetland matrix had compacted.

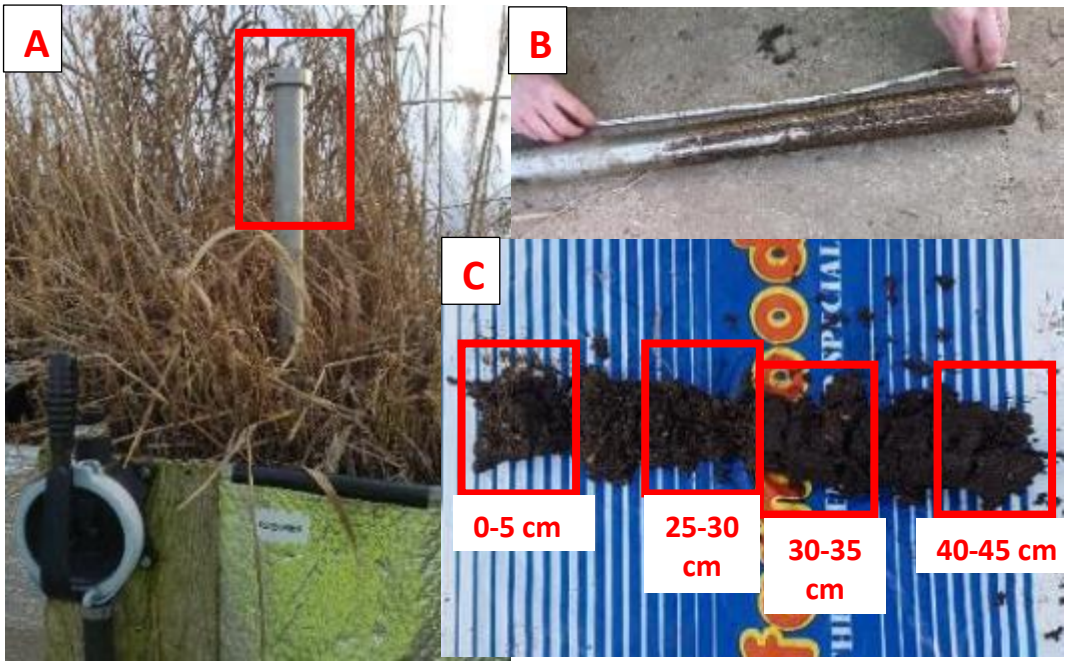

Figure 2.1 *Soil core sample taken from RB6. (**A**) Constructed wetland (RB6) set-up with soil corer imaged (highlighted) in the center. (**B**) the soil corer was measured to have reached a depth of 45 cm. (**C**) Outlined from left-to-right are the layers from top-to-bottom that were sampled.*

In sampling, a single core was taken (**Fig. 2.1 (A)**), retrieving DNA from 4 different depths (**Fig. 2.1 (C)**): top unsaturated aerobic (0-30 cm), interface zone top, interface zone bottom and bottom saturated aerobic (below 30 cm) – most likely to be aerobic as the corer made it 15 cm into the saturated zone and did not pass the roots. In choosing soil from the corer, it was decided to take soil only from the points that were clearly defined by the soil texture (**Fig. 2.1 (C)**). The top aerobic unsaturated soil was dry and crumbling, the saturated aerobic soil was wet and stuck together with bits of root. The interface was sampled from the soil between these two distinctly different textures.

At the time of sampling (22/1/19), RB6 dosing of landfill leachate had been reduced to an estimated 5 g day^{-1} of NH_3 at 8 L day^{-1} of landfill leachate (previously an estimated 18.75 g day^{-1} of NH_3 at 30 L day^{-1} of landfill leachate) and ammonia removal had improved from removing half or less NH_3 to removing 4 g day^{-1} converting 82% of all influent NH_3 and removing 50% of total nitrogen (averaged over 3 months, between October-December 2018). Ammonia removal is measured by an outside laboratory (Envirolab currently, previously National Laboratory Service) measured in the influent and effluent water – unsure of method utilised.

Constructed wetland trials were further investigated by obtaining more soil samples on the 12/3/19. RB6 was selected again as it was no longer ponding also to further test how seasonal changes may affect the microbiology of the constructed wetland. At time of sampling (and currently) dosing levels of landfill leachate in RB6 were of an estimated 6-10 g day^{-1} of NH_3 (8 L day^{-1} of landfill leachate) the trial was converting 81 % of all influent NH_3 and removing 64% of total nitrogen. It was also a first step reed bed, in which its effluent was fed into another constructed wetland (RB5) for further NH_3 treatment. Biobed 12 (Bb12) was also chosen as this trial did not contain vegetation (*Phragmites australis*) and was built with only the same sand to compost matrix of 60% to 30%, respectively, as the other trials. This trial would thus provide insights into the effect of plants in establishing and maintaining the microbial community. At this time Bb12 was converting only

32% of all influent NH_3 and removing 20% of all influent Nitrogen, Bb12 was also being dosed an estimated 10 g day^{-1} of NH_3 or 100% leachate at 10 L day^{-1} of landfill leachate. Influent and effluent water samples were taken, first to determine if the leachate contained the same community of microbes as determined in the soil matrix, this could indicate that the influent leachate was perhaps seeding the constructed wetlands. The effluent was tested to determine if these microbes would continue to convert NH_3. Influent and effluent samples were taken from RB6 and Bb12 to gain understanding to seeding and wash-off of microorganisms in these treatments and RB8 was chosen as this trial was fed half leachate, half its own effluent. The aim was to determine if the AOB and AOA diversity within RB6 and Bb12 soil samples would be similar to those found in the influent and effluent samples, and lastly if RB8 was seeding itself with its own effluent.

2.2 Soil sampling and processing

Different methods of sampling were tested to improve sampling efficacy. The aims of testing were to, firstly, diminish disturbance of the constructed wetland matrix/vegetation and secondly in finding the best method to accurately sample each section of the constructed wetland. Soil was sampled from the top of the constructed wetland (RB6) using a corer 5 cm in diameter (January 22/1/19) and in March (12/3/19) RB6 and Bb12 were sampled from the top by a smaller 3 cm open-faced soil auger – utilising a smaller diameter corer for less disturbance of soil matrix. Although the constructed wetlands were 77 cm deep, due to the roots and the compaction of the soil matrix, it was only possible to sample the top-most 45-50 cm in both sampling attempts. These contained two visually distinct sections (as described in section '**2.1 Description of Study Site**' and visualised in **Fig. 2.1**), which were separated, mixed in a plastic zip-lock bag by crushing, and stored at -4˚C until analysis – within a week prior to extraction. For influent and effluent water, 1 L was sampled and concentrated by centrifugation at 3,000 rpm for 30 mins. Supernatant was discarded and the resultant pellet and stored at -4ºC until used for molecular analysis – within a week prior to extraction.

For further sampling, ports have been introduced at the side of constructed wetland at each of the four layers as described above in '**1.5 Constructed Wetlands**' (**Fig 1.9**, **pg. 25**). Each port enables sampling by a 2.5 cm diameter open-faced auger, which is pushed in from the side, meaning a subsection of each layer across the width of the constructed wetland is taken from a specific height. This makes it easier to determine which section is being sampled (aerobic unsaturated above the water table; aerobic saturated below the water table in close proximity to roots; interface between these two sections; lastly the anaerobic saturated layer below the water table but not in association with the roots). The first port hole for sampling the aerobic unsaturated layer is placed above the interface (water table), determined by the outlet height as shown by in '**1.5 Constructed Wetlands**' **Fig. 1.9 (pg. 25)**. The next port hole is placed exactly at the level where this same outlet is and samples the interface level. The third port hole is placed below this in the saturated aerobic zone and the last port hole is placed at the very bottom of the constructed wetland, where it is expected the saturated soil will be anaerobic.

2.3 DNA extraction

2.3.1 Soil and water DNA extraction

Two types of DNA extraction procedures were used and compared in this work. The first DNA extraction method was carried out as described by Griffiths and colleagues (Griffiths *et al.,* 2000). Briefly, this procedure consisted of bead-beating a 0.5 g soil sample in the presence of CTAB-containing buffer (10% (w/v) CTAB, 0.7 M NaCl and 240 mM potassium phosphate buffer (pH 8.0)) and phenol:chloroform:isoamyl alcohol (25:24:1) (pH 8.0). Aqueous layer containing the DNA was further cleaned by chloroform:isoamyl (24:1) treatment. DNA was precipitated using 1.6 M NaCl, 30% (w/v) polyethylene glycol (PEG_{6000}) and 70% (v/v) ethanol. The DNA pellet was air-dried for 5 mins and resuspended in ddH_2O (Thermofisher Scientific nuclease-free water (not DEPC treated)). The second DNA extraction method, utilised due to presence of PCR inhibitors in

the soil, was performed using FastDNA™ Spin Kit for Soil (MP Biomedicals) according to manufacturer's instructions. Briefly, 0.2-0.5 g (wet weight) soil sample was bead-beaten in the presence of the lysis buffers supplied with the DNA extraction kit in the FastPrep® Bead-beater. DNA in the supernatant was bound to spin-columns, washed and eluted as recommended by the manufacturer.

PCR inhibitors, suspected to be humic and/or fulvic acids, were observed in the DNA extractions. This was first determined by serial dilutions performed using anammox primers, amplification either diminished or disappeared completely with increasing concentration of DNA and this also occurred in the top unsaturated layer of the 12/3/19 extractions from RB6 and Bb12 at the lowest DNA concentration with all marker gene primers (AOB *AmoA*-**1F/2R (**Rotthauwe *et al.*, 1997), AOA crena*moA-23F/616R (*Tourna *et al.*, 2008), *hzsA*_526F/1857R (Harhangi *et al.*, 2012) and *hzo*qPCR1F/qPCR1R (Schmid *et al.*, 2008) all primers are shown in **Table 2.1**). The DNA extractions were quality checked first with 16S primers (16S-27F/-1497R (Lane, 1991) as shown in **Table 2.1**) and running DNA on 1% (w/v) agarose gel (1 x TBE buffer) alone, these indicated no issues with DNA, indicating issues mentioned prior were due to PCR inhibitors. PCR inhibitors were removed from the DNA extraction by the *OneStep*™ PCR Inhibitor Removal Kit, utilizing the Zymo-Spin™ III-HRC Column, the matrix of which is utilised in the removal of polyphenolic compounds, humic/fulvic acids, etc. The Zymo-Spin™ III-HRC Column was prepared and utilised on sample DNA according to manufacturer's instructions. Serial dilutions performed after utilising Zymo-Spin™ III-HRC Columns improved amplification.

The quality and quantity of the extracted DNA was determined using 1% (w/v) agarose gel (1 x TBE buffer) electrophoresis, nanodrop (Nanodrop™ 2000) and the Qubit fluorometer (Qubit 2.0 Fluorometer). DNA extracts (5 µL) were visualised on 1% (w/v) agarose (1 x TBE buffer) gels. Qubit and nanodrop were used according to manufacturer's instructions.

2.3.2 Root DNA extraction

Sterilised workspace and tools with 70% (v/v) ethanol, removed loose soil from roots by shaking and root removed from stem by aseptically cutting with scalpel. Took a predetermined weight/length of root (1 g). Washing process started with removing rhizosphere, roots were washed in 25 mL of sterile silwett-amended phosphate buffer solution (PBS) (6.33 g $NaH_2PO_4.H_2O$; 16.5 g $Na_2HPO_4.H_2O$ dissolved together in 1 L distilled H_2O and autoclaved, addition of 200 µL Silwett L-77 after autoclaving) left in a shaker for 30 mins at 180 rpm. This step is repeated until roots are cleaned. Solution of rhizosphere and PBS from first wash step was centrifuged to form a solid pellet (3,200 x g for 15 mins) and the pellet removed from solution then snap frozen in lysis matrix E tubes and stored at -80^{o}C ready for extraction. After washing, 1 g of root tissue was placed in 25 mL of 70% (v/v) ethanol for 30 secs, then washed with sterile water (autoclaved distilled water) followed by soaking in 3% of NaOCl for 5 mins and then washed again thoroughly with sterile water (repeated 5x). Next roots were placed in fresh PBS/water and sonicated for 15-20 mins in a water bath. Roots were separated into 2 samples at approx. 500 mg each and snap frozen in an Eppendorf and stored at -80^{o}C until ready for extraction. Prior to extraction roots were ground in a sterilised mortar and pestle using liquid nitrogen until the roots resembled a fine powder. With both rhizosphere and root samples, DNA extraction process follows the process as detailed in '**2.3.1 soil and water DNA extraction**" section.

2.4 PCR

2.4.1 PCR protocols for marker gene primers and 16S primers

PCR reactions were carried out in 20 µL volume using PCRBIO Taq Mix Red (PCR Biosystems). Unless otherwise stated for specific PCR assays, the reactions contained 10 µL 2x PCRBIO Taq Mix Red, between 400-1000 nM of each primer, 1 µL of 3.5% (v/v) bovine serum albumin (BSA) and 10 ng (1 µL) DNA template. The PCRBIO Taq Mix Red contains $MgCl_2$, dNTPs and

Taq polymerase, the final concentrations of which were 6 mM, 2 mM and 5 U/5 µL, respectively. Primers used in this study are detailed in **Table 2.1**. PCR products were visualised on agarose gels as described for DNA extracts.

In optimising anammox primers *hzsA*_1597F/_1857R, *hzsA*_526F/_1857R and *hzo*1F/1R the process began with calculating the optimal annealing temperature of each primer set using the Thermofisher melting temperature (T_m) calculator (Thermofisher annealing temperature calculator). The calculated primer T_m is then included in a gradient (with the protocol temperature used as the midpoint) to determine the best temperature for annealing with each primer set. *HzsA*_1597F/_1857R optimal annealing temperature increased to 60ºC (originally 55ºC) (Harhangi *et al.,* 2012*)*; *hzsA*_526F/_1857R optimal annealing temperature remained the same (54ºC) (Harhangi *et al.*, 2012); *hzo-1F/1R* optimal annealing temperature also remained the same (60ºC) (Schmid *et al.*, 2008). Changed the length of time for each part of the cycle (denaturing, annealing and extension), either shortening or increasing the time from the primer protocol to that required by the polymerase utilised in each reaction (Taq DNA Polymerase PCR Biosystems). As per the manufacturer's instructions: initial and final steps were (3-10 mins), 30 secs denaturation, 30 secs annealing and 1 min denaturation step (PB10.13 PCRBIO Taq Mix Red Manual). Number of cycles chosen were dependent on published protocol unless unspecific binding or dim bands were observed, the number of cycles were increased/decreased accordingly. Primer concentration was increased with both *hzsA* primer sets and the *hzo* primer set. Both *hzsA* primer sets' concentration was increased from 500 nM to 1,000 nM and *hzo* primer concentration increased from 400 nM to 500 nM as this was found to work better on environmental samples. This could be due to lack of DNA template in the environmental samples however increasing of template concentration always resulted in loss of amplification in environmental samples due to PCR inhibitors.

2.4.2 amoA AOB AmoA-1F/2R

Final primer concentration used was 500 nM for each primer. Final cycling conditions were as follows: 94ºC for 5 mins initial denaturation, followed by 36 cycles of 94ºC for 60 sec denaturation, 90ºC for 30 sec annealing, 72ºC for 90 sec extension, followed by a final extension step of 72ºC for 10 mins.

2.4.3 *amoA* AOA *crenamoA*-23F/616R

Final primer concentration used was 500 nM for each primer. Final cycling conditions were as follows: 95ºC for 5 mins initial denaturation, followed by 35 cycles of 95ºC for 30 sec denaturation, 55ºC for 30 sec annealing, 72ºC for 1 min extension, followed by a final extension step of 72ºC for 10 mins.

2.4.4 Universal primer bacterial 16S 27F/1492R

Final primer concentration used was 400 nM for each primer. Final cycling conditions were as follows: 95ºC for 3 mins initial denaturation, followed by 30 cycles of 95ºC for 20 sec denaturation, 55ºC for 20 sec annealing, 72ºC for 40 sec extension, followed by a final extension step of 72ºC for 5 mins.

2.4.5 hzsA_526F/1857R

Final primer concentration used was 1 µM for each primer. Final cycling conditions were as follows: 96ºC for 5 mins initial denaturation, followed by 30 cycles of 96ºC for 30 sec denaturation, 54ºC for 30 sec annealing, 72ºC for 1 min extension, followed by a final extension step of 72ºC for 5 mins.

2.4.6 *hzo*qPCR1F/qPCR1R

Final primer concentration used was 500 nM for each primer. Final cycling conditions were as follows: 95ºC for 5 mins initial denaturation, followed by 40 cycles of 95ºC for 10 sec denaturation, 60ºC for 30 sec annealing, 72ºC for 10 sec extension, followed by a final extension step of 72ºC for 5 mins.

Table 2.1 *PCR primers used in the study.*

Primer	Position (bp)	Insert size (bp)	Sequence	T_m	Reference	Used in this study
AmoA-1F	332-349	491	5'-GGGGTTTCTACTGGTGGT-3'	60°c	Rotthauwe *et al.* (1997)	Yes
AmoA-2R	802-822		5'-CCCCTCKGSAAAGCCTTCTTC-3'			Yes
*CrenamoA*23F	23-39	594	5'-ATGGTCTGGCTWAGACG-3'	55°C	Tourna *et al.* (2008)	Yes
*CrenamoA*616R	597-616		5'-GCCATCCATCTGTATGTCCA-3'			Yes
*Amx*694F	694-713	286	5'-GGGGAGAGTGGAACTTCGG-3'	60°C	Hu *et al.* (2010)	No
*Amx*960R	960-979		5'-GCTCGCACAAGCGGTGGAGC-3'			No
*hzsA*_1597F	1597-1615	261	5'-WTYGGKTATCARTATGTAG-3'	55°C	Harhangi *et al.* (2012)	No
*hzsA*_1857R	1838-1857		5'-AAABGGYGAATCATARTGGC-3'			Yes
*hzsA*_526F	526-543	1331	5'-TAYTTTGAAGGDGACTGG-3'	54°C		Yes
*hzoq*PCR1F	742-763	244	5'-AAGACNTGYCAYTGGGGWAAA-3'	60°C	Schmid *et al.* (2008)	Yes
*hzoq*PCR1R	960-985		5'-GACATACCCATACTKGTRTANACNGT-3'			Yes
16S-27F	27-46	1466	5'-AGAGTTTGATCCTGGCTCAG-3'	55°C	Lane (1991)	Yes
16S-1492R	1476-1492		5'-TACCTTGTTACGACTT-3'			Yes

2.5 Cloning and sequencing

PCR products produced using archaeal and bacterial *amoA* and anammox *hszA* and *hzo* primer sets were purified using MP Biomedicals, LLC GeneClean® Turbo Kit and later Roche High Pure PCR Product Purification Kit according to manufacturer's instructions prior to cloning. Purified PCR products were quantified and visualised on agarose gels as described above. PCR products were ligated into pGEM-T Easy vector using pGEM®-T Easy Vector Systems Kit Promega Corporation and transformed into Top 10 competent *E. coli* by heat shock. Top 10 competent *E. coli* cells protocol consists of producing seed stocks: Top10 cells streaked on SOB plates and grown at 23^{o}C for 36 hrs, single colonies were picked into 2 mL of SOB medium and shook overnight at 23^{o}C. Glycerol was added to 15% (w/v) and 1 mL was aliquoted into cyrotubes and frozen in liquid nitrogen and stored at -80^{o}C. Seed stock (1 mL) was inoculated in 250 mL of SOB medium (Tryptone 5 g, yeast extract 1.25 g, NaCl 0.125 dissolved in 2.5 mL KCl and made up to 250 mL with water (250 mM stock, pH 7.0) just before addition to seed stocks $MgCL_2$ and $MgSO_4$ are added to a final concentration of 10 mM) and grown at 30^{o}C to an OD600 nm of 0.3-0.4**.** After growth period cells were centrifuged 2000 x g at 4^{o}C for 10 mins in a 500 mL bottle. Cells were gently resuspended in 80 mL of ice cold CCMB80 buffer (10 mM KOAc pH 7.0; 80 mM $CaCl_2.2H_2O$; 20 mM $MnCL_2$ $4H_2O$; 10 mM $MgCL_2$ $6H_2O$; 10% (w/v) glycerol (pH 6.4)) and incubated on ice for 20 mins. Centrifuged at 4^{o}C and resuspend in 10 mL of ice cold CCMB80 buffer. Added CCMB80 buffer until the cells reached an OD of 5-7.5 and incubated on ice for 20 mins. Aliquoted 200 µL and frozen with liquid nitrogen and stored at -80^{o}C.

The transformation method works by blue/white screening of colonies based on the disruption of *lacZ* gene on the vector and the presence of X-gal (40 mg/mL stock concentration) on the agar plates with the antibiotic ampicillin (100 mg/mL stock concentration). White colonies were picked and screened by M13F/M13R PCR. Clones containing the insert of the expected size were randomly selected for Sanger sequencing which was performed by Eurofins genomics. Dependent on the size of the insert, sequences were either

sequenced unidirectionally or bidirectionally –for both AOA and AOB *amoA* sequences, marker gene primers AOB *AmoA-1F/2R*; AOA *crenamoA-23F/616R* were used in a unidirectional manner and for *hzo* sequences marker gene primer pair *hzo-1F/1R* was utilised in a unidirectional manner too. Whereas for *hzsA* sequences, marker gene primer pair *hzsA-526F/1857R* produced an insert of 1331-bp, SP6 (ATTTAGGTGACACTATAG) and T7 (TAATACGACTCACTATAGGG) primers were utilised to target the corresponding sites on the pGEM®-T Easy Vector bidirectionally. Sequences were quality trimmed by utilising the ABI file in sequence alignment software, Bioedit, to remove reads of a weak signal which could therefore be incorrect. The sequence was then initially analysed by BLASTp homology-based engine search (NCBI: BLASTp) against the NCBI database. Sequences that were most similar, both cultured and uncultured, were retrieved.

2.6 Phylogenetic analysis

Environmental *amoA*, *hzsA* and *hzo* sequences as well as suitable reference sequences obtained from NCBI were aligned using ClustalW implemented within Bioedit except for RB6 environmental *hzsA* sequences which were aligned by Muscle in MEGA 5.2 (Hall, 1999). Phylogenetic analysis was performed using MEGA 5.2 software (Kumar *et al.,* 2016). Neighbour-joining trees were constructed in MEGA 5.2 with 1,000 bootstrap replicates.

2.7 Restriction fragment length polymorphism (RFLP)

In addition to Sanger sequencing, the diversity of *amoA*, *hzsA* and *hzo* sequences in the clone libraries was examined by RFLP. Prior to the digest, the suitability of the restriction enzymes was assessed *in silico* utilising text editor program Notepad++. This was achieved by inputting sequences retrieved from sanger sequencing and known sequences from NCBI taxonomy (NCBI: Taxonomy) for reference. The sequences were then examined for the frequency with which the restriction enzyme recognition sequences appeared and the location of this sequence. PCR products from

clones were purified as described above and digested using 4-bp cutter restriction enzymes *MspI* and *RsaI*. These enzymes were used as a 4-bp restriction enzyme cleaves a sequence more frequently than restriction enzymes with higher bp recognition site. *EcoRI* is utilised as it removes the pGEM®-T Easy Vector ends (left by M13 primer amplification).

Followed RFLP protocol mix from manufacturer (ThermoScientific) instructions for multiple enzymes, with a few adjustments. Added in order as stated in guide: ddH_2O 5.6 µL; ThermoScientific 10X Fast Digest™ Buffer 1 µL; DNA template (either PCR product or cleaned-up PCR product) 3 µL; FastDigest restriction enzyme 1: 0.2 µL; FastDigest restriction enzyme 2: 0.2 µL. Followed manufacturer instructions for protocol, except increased incubation time at 37^{o}C to 1 hr/1.5 hr (originally 5 mins) (Molecular Biology, FastDigest Restriction enzymes, LabAid).

Digested DNA fragments were analysed on 2% (w/v) agarose gel (1 x TBE buffer) with a 50-bp ladder (GeneRuler ThermoScientific).

3. Results and Discussion

3.1 Ammonia oxidising bacteria and archaea

3.1.1 Validating the molecular tools developed to study AOA and AOB

Aerobic ammonia oxidizing microorganisms (AOM) perform the first step in the removal of NH_3 and provide the NO_2- for the anammox reaction. Due to this and the fact that the PCR assays for these organisms had been previously developed, the first step was to look for aerobic AOMs, AOB and AOA. The primers target the gene encoding for a key enzyme of the ammonia oxidation pathway in these organisms, ammonia monooxygenase (AMO), specifically targeting the gene encoding the alpha subunit (a*moA*) of the ammonia monooxygenase. The primers (*amoA* of AOB -1F/-2R) – amplifying a region of 491-bp of the *amoA* AOB gene – (Rotthauwe *et al.*, 1997) and *amoA of* AOA *crenamoA*23F/616R – amplifying a region of 594-bp of the *amoA* AOA gene (Tourna *et al.*, 2008; Lehtovirta-Morley *et al.*, 2016) were used. PCR was performed using control DNA from the pure cultures of *Nitrosocosmicus franklandus* (AOA) and *Nitrosomonas europaea* (AOB) as a template (**Fig. 3.5** panels **A** and **C**). The PCR amplicons were clear and bright when visualised on the agarose gel and of the correct size, the protocol needed no optimisation. Primers targeting these microorganisms were next used on DNA extracted from constructed wetlands at the NCC trials in Mayton Wood.

3.1.2 Detecting presence of ammonia oxidising archaea (AOA) and bacteria (AOB) in reed bed 6 (RB6)

AmoA primers targeting both AOAs and AOBs, were used to determine the presence of aerobic AOMs. This is a robust initial indicator to determine if anammox is occurring within the constructed wetland trials as these microorganisms produce nitrite which is essential for the anammox process. It was observed that both AOB and AOA were present in each layer of the constructed wetland soil matrix, even those presumed to be anaerobic and

there were no obvious differences in the brightness of the PCR amplicons from different depths when visualised on agarose gels (**Figs: 3.1** and **3.2**). Not shown, is that both these primers were utilised on the influent and effluent samples from RB6, RB8 (to be decommissioned), and Bb12 (100% leachate), indicated that AOM were present – did not send for sequencing.

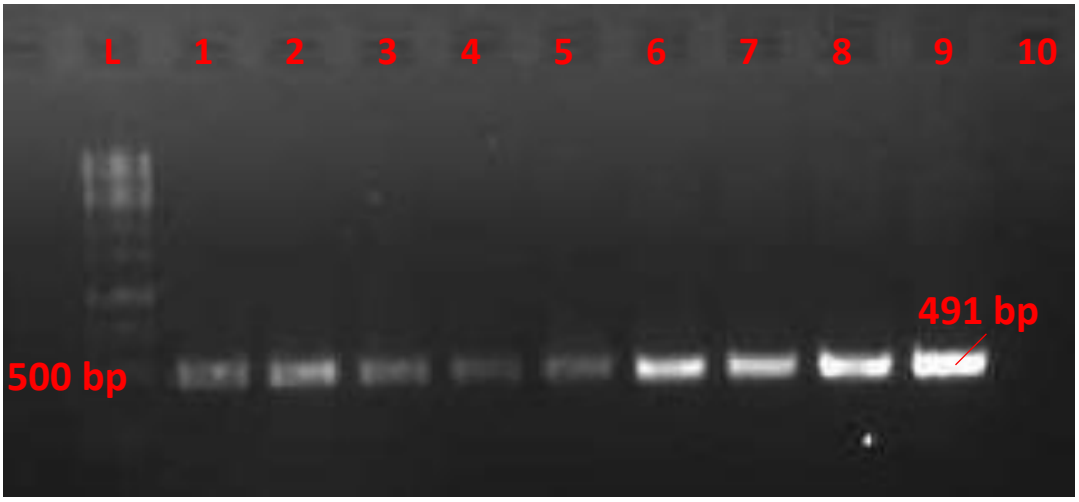

Figure 3.1 *AmoA AOB primers on DNA extracted from RB6 soil matrix, band as shown at 491-bp. As numbered: (**1-2**) unsaturated aerobic layer; (**3-4**) top interface; (**5-6**) bottom interface; (**7-8**) saturated aerobic layer; (**9**) +ve control (Nitrosomonas europaea DNA) and (**10**) –ve control of PCR master mix with 1 µL of nuclease-free water added in place of DNA. Ladder (**L**): Generuler 1kb DNA ladder*

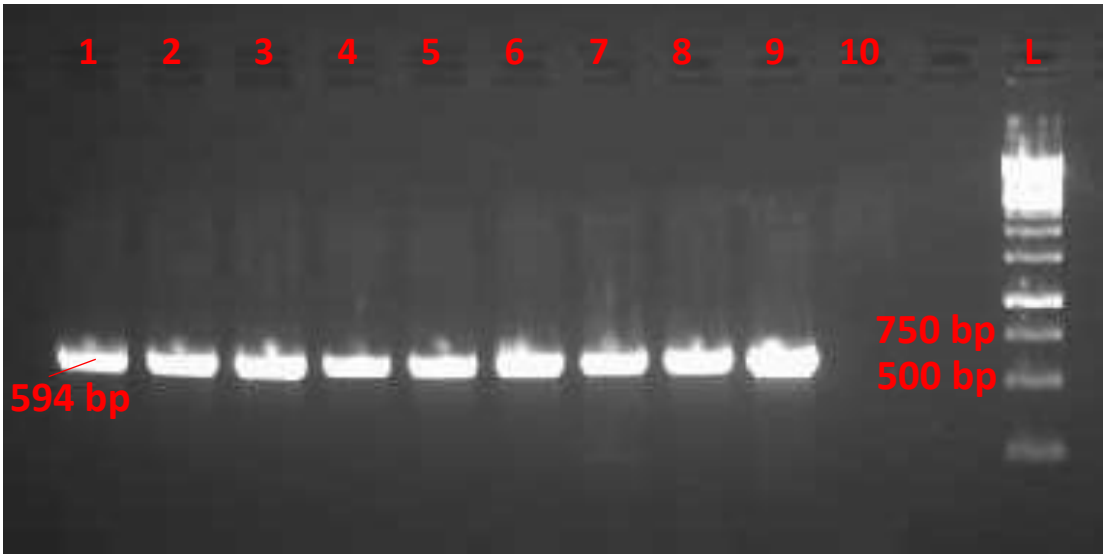

Figure 3.2 *AmoA AOA primers on DNA extracted from RB6 soil matrix, band as shown at 594-bp. As numbered: (**1-2**) unsaturated aerobic layer; (**3-4**) top interface; (**5-6**) bottom interface; (**7-8**) saturated aerobic layer; (**9**) +ve control (Nitrosococsmicus franklandus DNA) and (**10**) –ve control of PCR mastermix with 1 µL of nuclease-free water added in place of DNA. Ladder (**L**): Generuler 1kb DNA ladder.*

3.1.3 Diversity of ammonia oxidising archaea (AOA) and bacteria (AOB) in reed bed 6 (RB6)

To study the diversity of aerobic ammonia oxidisers, clone libraries were produced from these PCR amplicons, five clones were picked from the top (unsaturated aerobic) and bottom (saturated aerobic) layers. These layers were chosen as the method of sampling was still being perfected, it was unlikely that I had properly sampled the interface/ saturated anaerobic layer. Clones were sent for Sanger sequencing, and **Tables: 3.1** and **3.2** indicate the closest related genera from BLAST results (NCBI: BLASTp) and their phylogenetic affiliations (**Figs: 3.3** and **3.4**). It was observed that surprisingly the same genera of AOA and AOB were found in both the unsaturated aerobic and saturated aerobic layers – originally assumed to be the saturated anaerobic layer. This was unexpected as the saturated layer was thought to be mostly oxygen-limited and these microorganisms are considered aerobic. However as stated by Chen and colleagues (2008) it was found that the roots from rice (which have a continuous arenchmytous system similar to that found in *Phragmites australis* used in the constructed wetlands) will provide oxygen to water-logged soils as the roots of *Phragmites australis* will similarly provide oxygen to the lower layers of the constructed wetland. With the core first taken from RB6 in January (where this DNA is sampled from for AOA/AOB sequencing), the lower anaerobic layers were perhaps not sampled. With both AOB and AOA primer sets, only sequences typically found in soil environments were observed, with no marine species present however the primers used were designed to cover soil AOBs only (Rotthauwe *et al.*, 1997). It is true however the salinity of the system is likely to be too low for marine species at a concentration of 154 mM at its highest as measured in 100% leachate – in RB6 this will be diluted. *Nitrosomonas europaea* can tolerate a concentration of up to 400 mM NaCl; a halophile such as *Nitrosomonas halophila* will require a salinity higher than this and can tolerate salinity levels up to 900 mM (Yamamoto *et al*., 2010).

AmoA amino acid sequences and NCBI BLAST-based homology search were used to investigate the most highly related species (**Table 3.1)**. AOB

clones were split between the *Nitrosomonas* and *Nitrosospira* genera, with *Nitrosospira* predominating with 8 clones of this genus. Half of the Nitrosospira clones were derived from the unsaturated aerobic level and the other half from the saturated anaerobic layer. Two clones affiliated with the *Nitrosomonas* genus were recovered from each layer. *Nitrosomonas* is often found in wastewater treatment systems, eutrophic freshwater and brackish water, all of which contain high levels of NH_3 (Pommerning-Roser and Koops, 2005; Du *et al.*, 2016). This is consistent with this genus being able to tolerate up to >400 mM of ammonia before ammonia becomes inhibitory (Yamamoto *et al.,* 2010). The average concentration of ammonia, in 100% of landfill leachate influent at Mayton Wood Landfill site, is considered high at 70.4 mM. The highest salinity measured in the landfill leachate was 154 mM (in 100% landfill leachate) and some representatives of genus *Nitrosomonas* cells can withstand salinity levels of up to 400 mM (Yamamoto *et al.,* 2010). Previously characterised strains of *Nitrosomonas* can grow within a pH range of 6.0-9.0 which is consistent with the pH measured in 100% landfill leachate at pH 7.66. With RB6 the dilution was 60% landfill leachate to 30% water, thus the salinity and NH_3 concentration were reduced and the pH closer to neutral. Representatives of *Nitrosospira* genus are found over a wider pH range than *Nitrosomonas* in the environment and typically predominate over *Nitrosomonas* in soil environments including those of neutral pH. Within the small subset of clones that were sequenced, more *Nitrosospira* clones were found. Although it is quite usual to find higher abundances of *Nitrosospira*-like species in wetlands with emergent plants and in soil habitats in general, with higher substrate and oxygen concentrations it is often found *Nitrosomonas* will outcompete *Nitrosospira* (Chen *et al.,* 2008). To support the results found thus far a larger sample size would need to be sequenced by amplicon sequencing targeting *amoA* in AOB and AOA and activity studies by RT-qPCR would determine the most active of these species in nitrogen cycling. This is expanded upon in the '**4.5 Limitations of the work'** section and '**4.6 Future work**' in the **4. Overall Discussion**.

Table 3.1 *Closest protein BLAST hits of the AmoA AOB clones to the cultivated representatives of AOB.*
** unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic); saturated aerobic = bottommost layer below water table (interface) of wet soil next to roots (aerobic).*
*** colony*

RB6 sample: Clone #	**Blast Result: Highest Match Known Species**	**Query Cover**	**Per Identity**
Unsaturated Aerobic*:	*Nitrosomonas eutropha*		
Col 1**		100%	100%
Unsaturated Aerobic:	*Nitrosospira sp. LT2Fb*		
Col 2		100%	99.31%
Col 4		100%	97.24%
Unsaturated Aerobic:	*Nitrosospira multiformis*		
Col 3		100%	97.24%
Saturated Aerobic*:			
Col 15		100%	97.24%
Col 16		100%	98.62%
Unsaturated Aerobic:	*Nitrosospira* sp. Nsp22		
Col 6		100%	95.86%
Saturated Aerobic:			
Col 20		100%	100%
Saturated Aerobic:	*Nitrosospira briensis*		
Col 18		100%	99.31%

Key:

- **Clone retrieved from top layer**
- **Clone retrieved from bottom layer**
- Uncultured clone

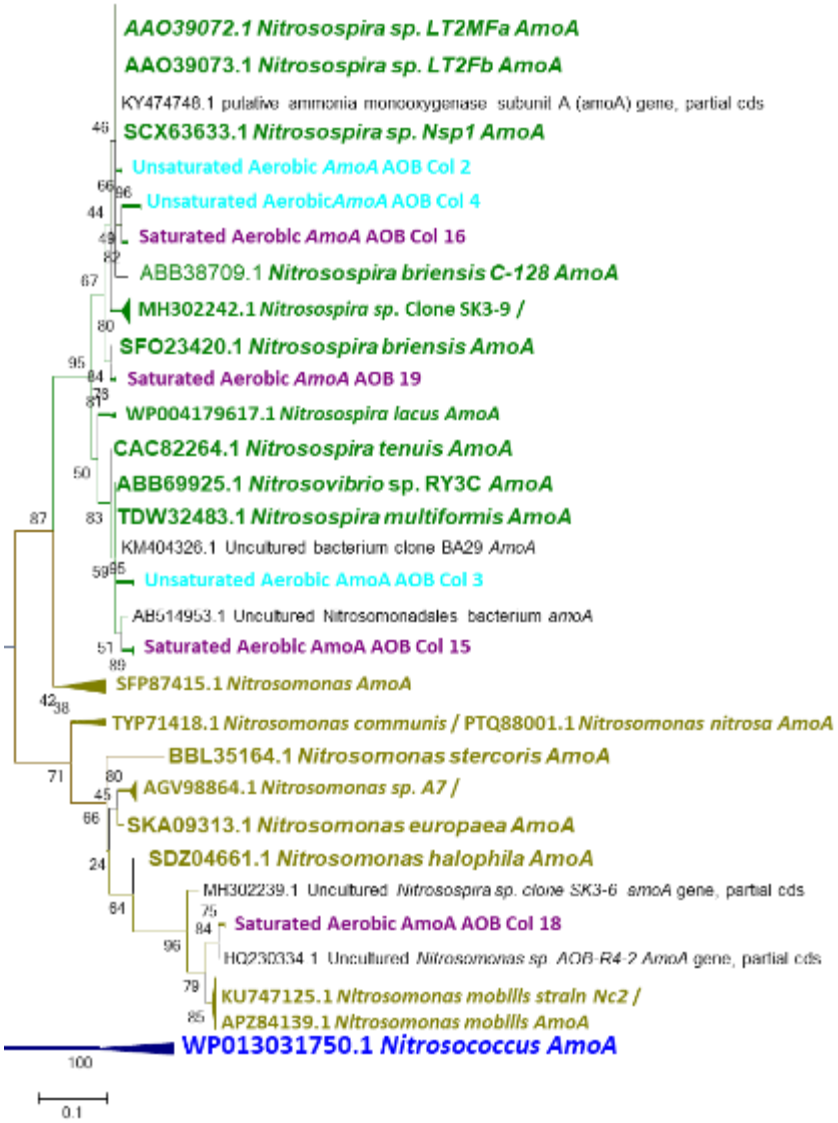

Figure 3.3 *Phylogenetic Tree of the amoA AOB amino acid sequences from RB6. Clones retrieved from the unsaturated aerobic layer were taken from soil matrix above the water table in dry soil (highlighted in blue). Clones retrieved from the saturated aerobic layer were taken from soil matrix below the water table next to Phragmites australis roots (highlighted in purple). Length of scale bar represents a difference of 10% between different sequences.*

Two main clades of AOA were detected in RB6, *Nitrososphaera* and *Nitrosocosmicus*. *Nitrosocosmicus* sequences were separated between two species *Nitrosocosmicus franklandus* and *Nitrosocosmicus arcticus*, whereas

Nitrososphaera gargensis was the only species found within the *Nitrososphaera* genus. *Nitrosocosmicus* are often found in neutral to slightly basic pH soils and wastewater, which matches the pH composition of the landfill leachate. As with the AOB sequences, the genera found are known to be able to withstand a higher concentration of ammonium than most other previously characterised AOA genera. The highest ammonium concentration *Nitrosocosmicus* can tolerate is 100 mM which is much higher than that of other genera of archaea. Lastly, they can handle a slightly higher level of salinity.

Table 3.2 *Closest protein BLAST hits of the amoA AOA clones to the cultivated representatives of AOA.*
** unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic); saturated aerobic = bottommost layer below water table (interface) of wet soil next to roots (aerobic).*
*** colony*

RB6 sample: Clone #	**Blast Result: Highest Match Known Species**	**Query Cover**	**Per Ident**
Unsaturated Aerobic*:	*Nitrososphaera gargensis*		
Col 8**		100%	94.68%
Col 10		100%	98.38%
Col 11		100%	97.91%
Saturated Aerobic*:			
Col 23		100%	98.68%
Col 27		100%	98.95%
Unsaturated Aerobic:	*Nitrosocosmicus franklandus*		
Col 9		100%	99.48%
Col 12		100%	100%
Saturated Aerobic:			
Col 22		100%	100%
Col 24		100%	98.44%
Col 25		100%	98.44%

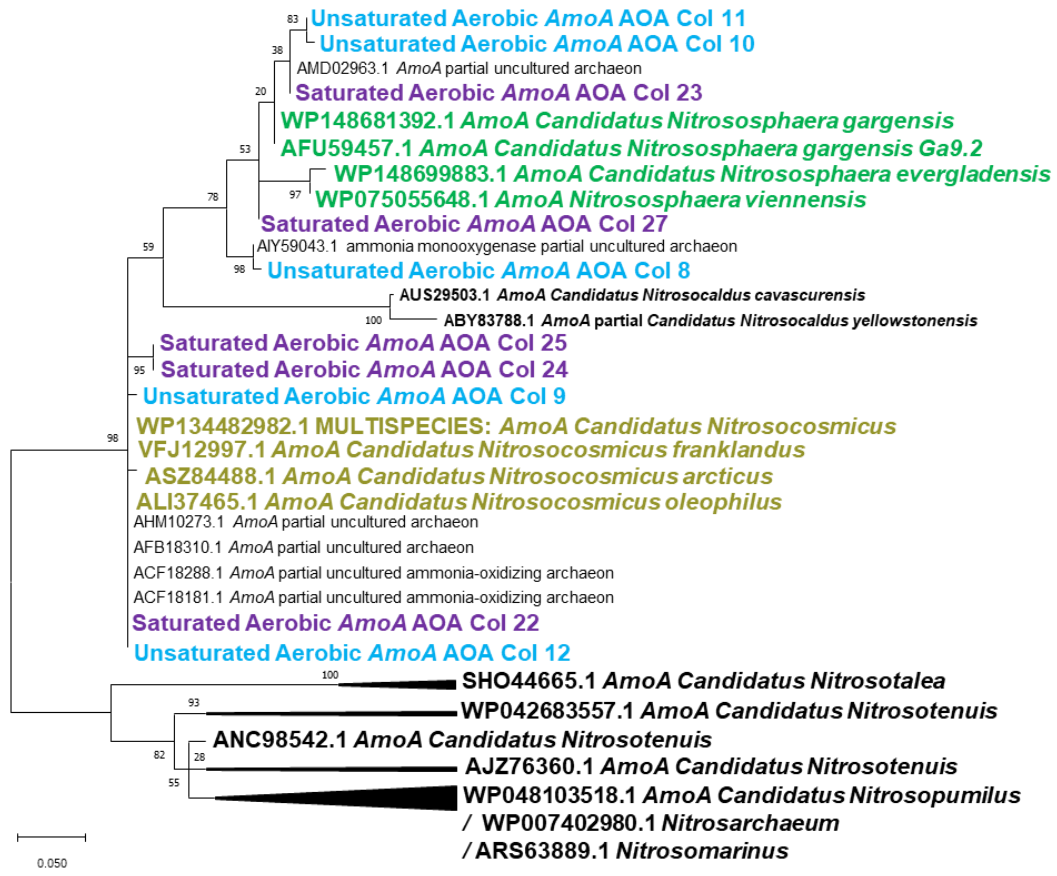

Figure 3.4 *Phylogenetic Tree of the amoA AOA amino acid sequences from RB6. Clones retrieved from the unsaturated aerobic layer were taken from soil matrix above the water table in dry soil (highlighted in blue). Clones retrieved from the saturated aerobic layer were taken from soil matrix below the water table next to Phragmites australis roots (highlighted in purple). Length of scale bar represents a difference of 2% between different sequences.*

3.2 RFLP

3.2.1 RFLP analyses

To streamline the process of identifying organisms from the CW trials in future, restriction fragment Length polymorphism (RFLP) analysis was employed to identify differences between sequences of the different OTUs. This would shorten the time to identify sequence identity and therefore determine if the process has captured the diversity within the CW trials.

Restriction enzyme *EcoRI* was utilised to remove vector ends and a further restriction enzyme with a 4-bp recognition site – *MspI* – was selected for RFLPs. A 4-bp restriction enzyme was chosen at random as it was calculated to cleave at least twice within the a*moA* AOB and AOA sequences – both of which are 491-bp/594-bp in size respectively, a 4-bp restriction enzyme cleaves once in every 256-bp – providing a greater resolution.

In **fig. 3.5** representative clones from previously sequenced *amoA* AOB and AOA (as seen in **Tables: 3.1** and **3.2** and **Figs.: 3.3** and **3.4**) were selected, based on their respective phylogenetic trees to match known sequences with an RFLP. With AOB *amoA* sequences, the resolution of the RFLPs allowed for determination of the genus and even species level (**Fig. 3.5)**, the RFLPs clearly matched the different genera/species that had been determined by sequencing previously (**Table: 3.1** and **Fig.: 3.3**). Representative clones were selected, based on the AOB and AOA phylogenetic tree and estimated fragment sizes are in **Table: 3.4**.

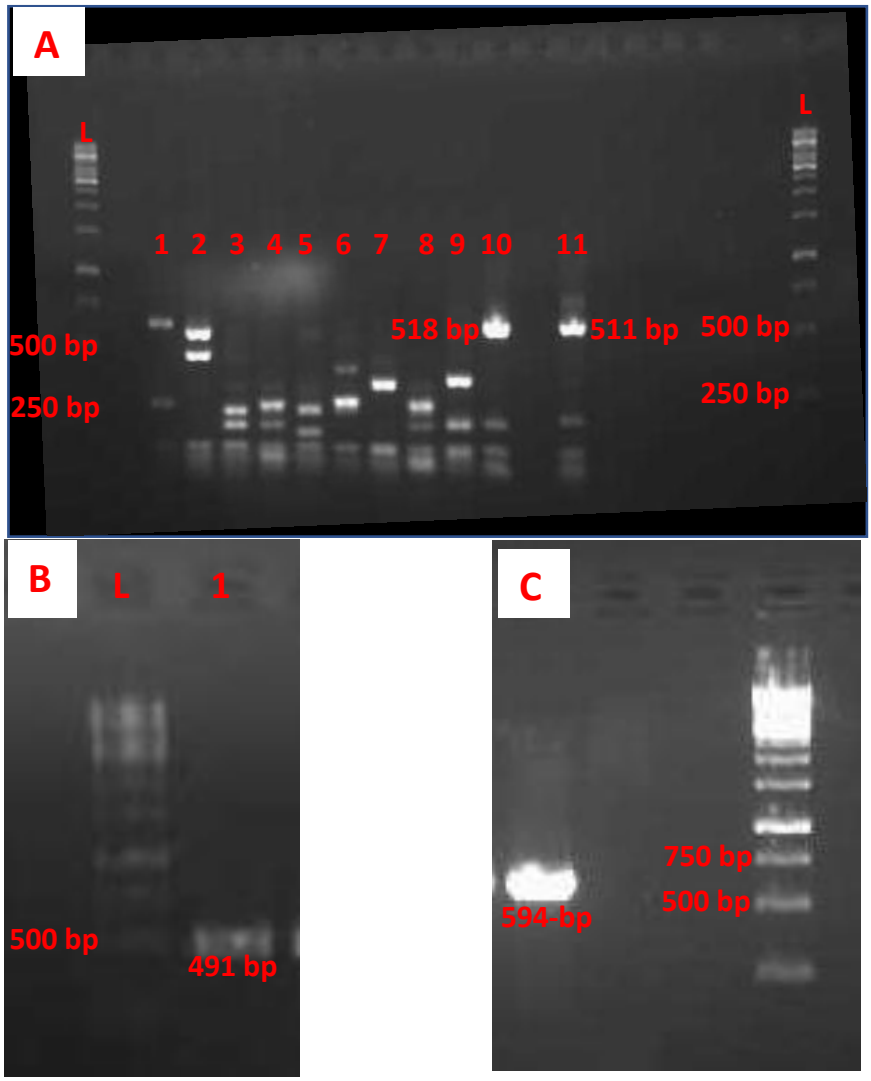

Figure 3.5 *(**A**) RFLPs of amoA AOB and AOA previously sequenced clones from RB6 (22/01/2019) – Lane **1** is +ve control DNA Brocadia fulgida (from anammox RFLPs) – mentioned later in "**3.3.2 Bias in the hzs assay**' section; (**2**) AOB col 1 – Nitrosomonas sp. A7; (**3**) AOB col 15 – Nitrosospira sp. PJA1; (**4**) AOB col 2 – Nitrosospira sp. LT2Fb; (**5**) AOB col 16 – Nitrosospira sp. Nsp1; (**6**) AOB col 3 – Nitrosospira sp. RY3C; (**7**) AOB col 18 – Nitrosomonas mobilis strain nc2; (**8**) AOB col 6 – Nitrosospira sp. Nsp.1; (**9**) AOA col 27 – Nitrososphaera gargensis; (**10**) AOA col 8 – Nitrososphaera viennensis (according to BLASTn (DNA)); (**11**) AOA col 9 – Nitrosocosmicus franklandus. (**B**) Standard PCR band when utilising AOB amoA primers at 491-bp (**1**). (**C**) Standard PCR band when utilising AOA amoA primers at 594-bp (**1**). Ladder (**L**) used in **A**, **B** and **C** is Generuler 1 kb DNA Ladder.*

Table 3.3 *Predicted fragments formed by restriction enzymes (EcoRI and MspI) in RFLP reactions (**Fig. 3.5**) on reprensentative sequences picked from AOB and AOA phylogenetic trees (**Fig. 3.3** and **Fig. 3.4**).*

Sequences / lane #	**Fragment Lengths (bp)**	**Full Fragment size (bp)**
AOB Col 1 / 2	2 - 448	450
AOB Col 15 / 3	13 – 195 - 235	441
AOB Col 2 / 4	11 – 107 – 186 - 207	441
AOB Col 16 / 5	107 - 79 - 21 - 225	432
AOB Col 3 / 6	235 - 208	442
AOB Col 18 / 7	4 - 111 - 324	438
AOB Col 6 / 8	12 - 96 - 79 - 21 - 234	443
AOA Col 27 / 9	148 - 347 - 105 - 108	709
AOA Col 8 / 10	518 - 77 - 110	706
AOA Col 9 / 11	511 - 89 - 108	708

The digest with *EcoRI* and *MspI* could not differentiate between two of the AOA genera *Nitrososphaera viennensis* (**lane 10**) and *Nitrosocosmicus franklandus* (**lane 11**) and digestion may have been incomplete as indicated by the size of the largest fragment being almost the size of a full-size insert (594-bp). As can be seen in **Table 3.4**: AOA col 8 in **lane 10** and AOA col 9 in **lane 11** contained one large fragment and two smaller fragments making it hard to determine a difference in the final product. **Figs 3.6** and **3.7:** are two representative images of new transformants containing *AmoA* AOA DNA extracted from RB6 on the 12/3/19 (described later), this includes DNA extracted from the roots and rhizosphere from the RB6 extraction in January – all unknown as had not yet been sequenced. These indicated either low diversity, or a low resolution of RFLP. From here it was decided to utilise only 16 lane agarose gels (**Fig. 3.6**) as it was harder to determine differences in 20-lane agarose gels (**Fig. 3.7**).

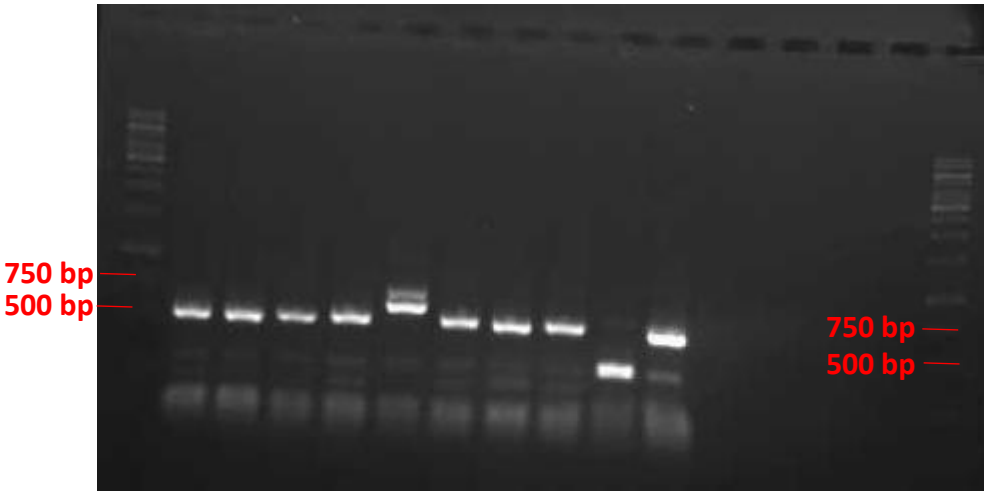

Figure 3.6 *AmoA AOA unknown sequences RFLPs from RB6 12/3/19 extractions and January RB6 root and rhizosphere extraction. Utilised restriction enzymes EcoRI and MspI.*

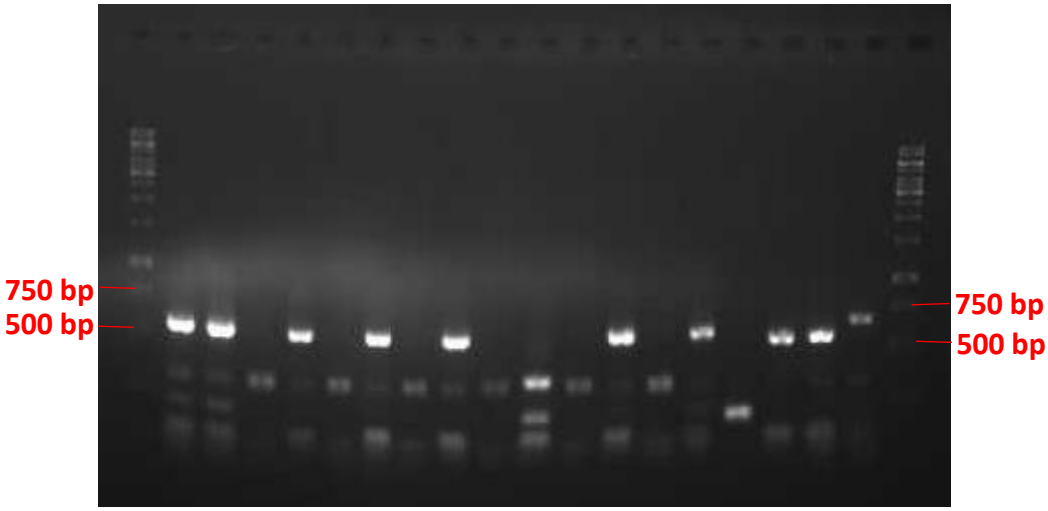

Figure 3.7 *AmoA AOA unknown sequences RFLPs from RB6 12/3/19 extractions and January RB6 root and rhizosphere extraction. Utilised restriction enzymes EcoRI and MspI.*

3.2.2 RFLP refinement of protocol for AOA

With each of the restriction digests from **Fig. 3.8** there was a fragment near to the same size as the original PCR product (594-bp) with fainter bands underneath suggesting incomplete digestion. Therefore, specific parameters of the digestion were modified: volume of restriction enzyme was doubled, a smaller 50-bp ladder (GeneRuler 50-bp DNA Ladder ThermoScientific) was used and a 2% (w/v) 1 x TBE buffer agarose gel was employed, separating

smaller fragments with greater resolution and the restriction digest incubation time increased to 1.5 hr. This did not improve the digestion of the highest molecular weight fragment, which was around the size of the original insert 594-bp and the product that had been cut was faint. It was therefore hard to determine any differences between RFLPs.

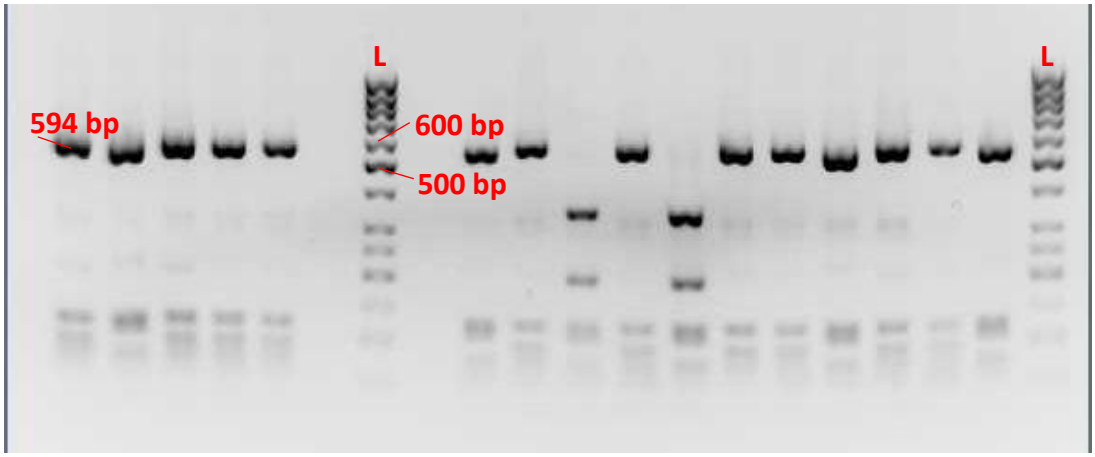

Figure 3.8 *AmoA AOA sequences RFLPs from RB6 12/3/19 extractions and January (22/1/19) RB6 root and rhizosphere extraction. (**L**) – ladder GeneRuler 50-bp DNA ladder ThermoScientific. Restriction enzymes utilised: EcoRI and MspI.*

In **Fig. 3.9** restriction enzyme *EcoRI* along with another 4-bp restriction enzyme *RsaI* that appeared more frequently within the *AmoA* AOA sequence when analysed *in silico* (**Table 3.4** depicts the expected fragments from the *in silico* analysis), were used and after incubation (1 hr) were run on 2% (w/v) 1 x TBE buffer agarose gel along with the smaller 50-bp ladder as in **Fig**. **3.8**. As can be seen in **Fig. 3.9**, there was a much higher resolution, the expected full fragment size, including M13 ends, is 857-bp.

Lanes 2, **5**, **6**, **8** and **9** contain sequences that were within the *Nitrosocosmicus* genus, with slight variations in the RFLP fragment sizes reflecting how clones branch in the phylogenetic tree (**fig. 3.4**). In particular, clones 24 and 25, (**lanes 8** and **9** respectively) that branch together with 98.44% relatedness to *Nitrosocosmicus arcticus* according to BLASTn results, have the same RFLP. In contrast, clones 9, 12 and 22 (**lanes 2**, **5** and **6** respectively) branched together, with clones 12 and 22, being 100% related to *Nitrosocosmicus franklandus* and 9 being of 98.38% relatedness.

Clones 8, 10, 11, and 23 are related to the genus *Nitrososphaera*. **Lanes 3** and **4**, indicate clones 10 and 11, which branch together on the phylogenetic tree (**fig. 3.4**) both highly related to *Nitrososphaera gargensis* (98.38% and 97.91% respectively), the RFLPs reflected this. Whereas clone 8 (**lane 1**) was slightly separated but clearly similar to these species. It was also highly related to *Nitrososphaera gargensis* (94.68%) with the amino acid sequence. However, the DNA sequence indicated a higher relatedness to *Nitrososphaera viennensis* (88.51%) which could account for some differences between these clones in their RFLPs. Clone 23 (**lane 7**) and 27 (**lane 10**) was even more highly related to *Nitrososphaera gargensis* at 98.96% and 98.95% identity respectively.

From these RFLPs, it could be clearly seen which clones were affiliated with the genus *Nitrosocosmicus* or *Nitrososphaera* as this trend was consistent with unknown sequences also (not shown) the protocol was used for further RFLPs.

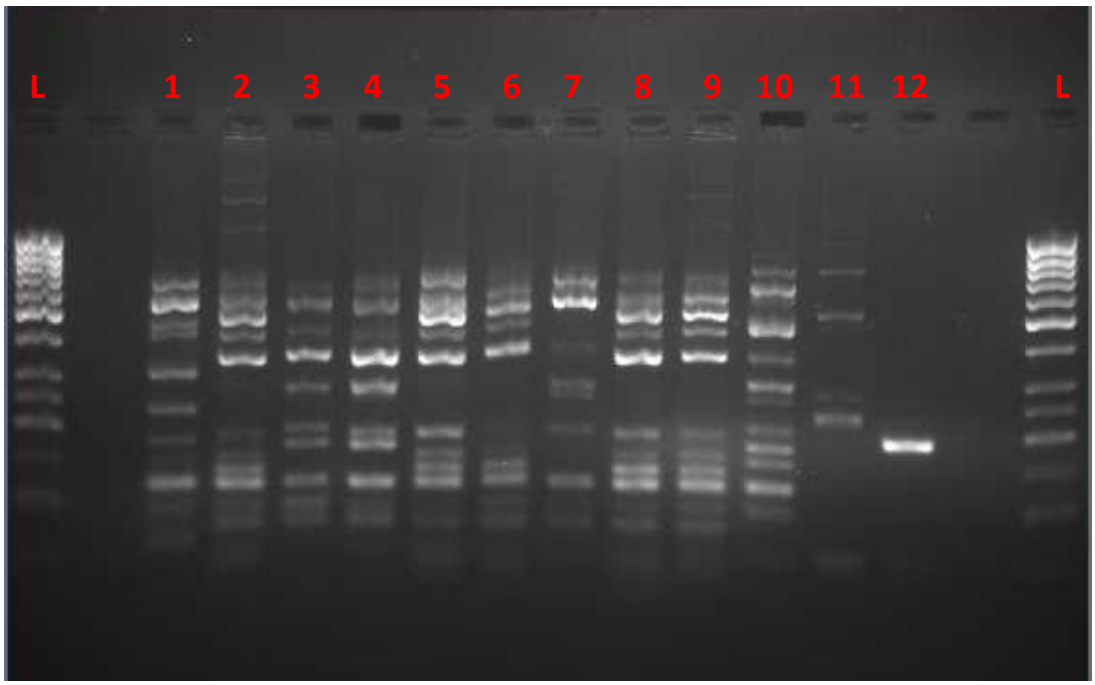

Figure 3.9 *RFLPs on AmoA AOA known sequences from RB6 January extractions, utilizing restriction enzymes: RsaI and EcoRI. (**1**) AOA col 8 – Nitrososphaera viennensis (**2**) AOA col 9 – Nitrosocosmicus franklandus; (**3**) AOA col 10 – Nitrososphaera gargensis; (**4**) AOA col 11 – Nitrososphaera gargensis; (**5**) AOA col 12 – Nitrosocosmicus franklandus; (**6**) AOA col 22 – Nitrosocosmicus franklandus; (**7**) AOA col 23 – Nitrososphaera gargensis; (**8**) AOA col 24 – Nitrosocosmicus arcticus; (**9**) AOA col 25 – Nitrosocosmicus arcticus; (**10**) AOA col 27 – Nitrososphaera gargensis; (**11**-**12**) unsequenced colonies from Bb12 (**L**) GeneRuler 50-bp DNA Ladder ThermoScientific.*

Table 3.4 *Predicted fragments formed by restriction enzymes (EcoRI and RsaI) in RFLP reactions (**Fig. 3.9**) on reprensentative sequences picked from AOA phylogenetic trees (**Fig. 3.4**).*

Sequences / lane #	**Fragment Lengths (bp)**	**Full Fragment size (bp)**
AOA Col 8 / 2	299 - 153 - 85 - 169	706
AOA Col 9 / 2	21 - 141 - 365 - 181	708
AOA Col 10 / 3	157 - 7 - 81 - 284 - 182	711
AOA Col 11 / 4	154 - 9 - 79 - 286 - 184	712
AOA Col 12 / 5	18 - 141 - 365 - 185	709
AOA Col 22 / 6	18 - 141 - 365 - 187	711
AOA Col 23 / 7	242 - 286 - 183	711
AOA Col 24 / 8	17 - 141 - 365 - 187	710
AOA Col 25 / 9	16 - 141 – 365 - 183	705
AOA Col 27 / 10	155 - 86 - 210 - 258	709

3.2.3 Diversity of ammonia oxidising archaea (AOA) and bacteria (AOB) in BioBed 12 (Bb12)

Now with methods in place for RFLPs, diversity of AOA and AOB was next investigated applying the *amoA* primers to the DNA extracted from the RB6 (12/3/19) at the time converting 81% of all influent NH_3 and removing 64% of total nitrogen and unvegetated Bb12 trial converting only 32% of all influent NH_3 and removing 20% of total nitrogen, in addition influent and effluent water samples from multiple other wetland trials were tested too – only by *amoA* primers to test for presence/absence of AOMs. Due to the presence of humic acids and other PCR inhibitors in samples, an alternative DNA extraction method was used as described in Materials and Methods. From PCR alone, it was observed that there was weaker amplification in the top

layers (unsaturated aerobic) of RB6 and Bb12 (12/3/19), contrary to what was indicated in PCR performed on samples extracted from RB6 in January.

Continuing with RFLP, the previously optimised protocol with *RsaI* and *EcoRI* was performed on the PCR amplicons. Again, as **fig. 3.10** indicates, the resolution decreased. There were clear differences, but it was not as clear as in **fig**. **3.9**.

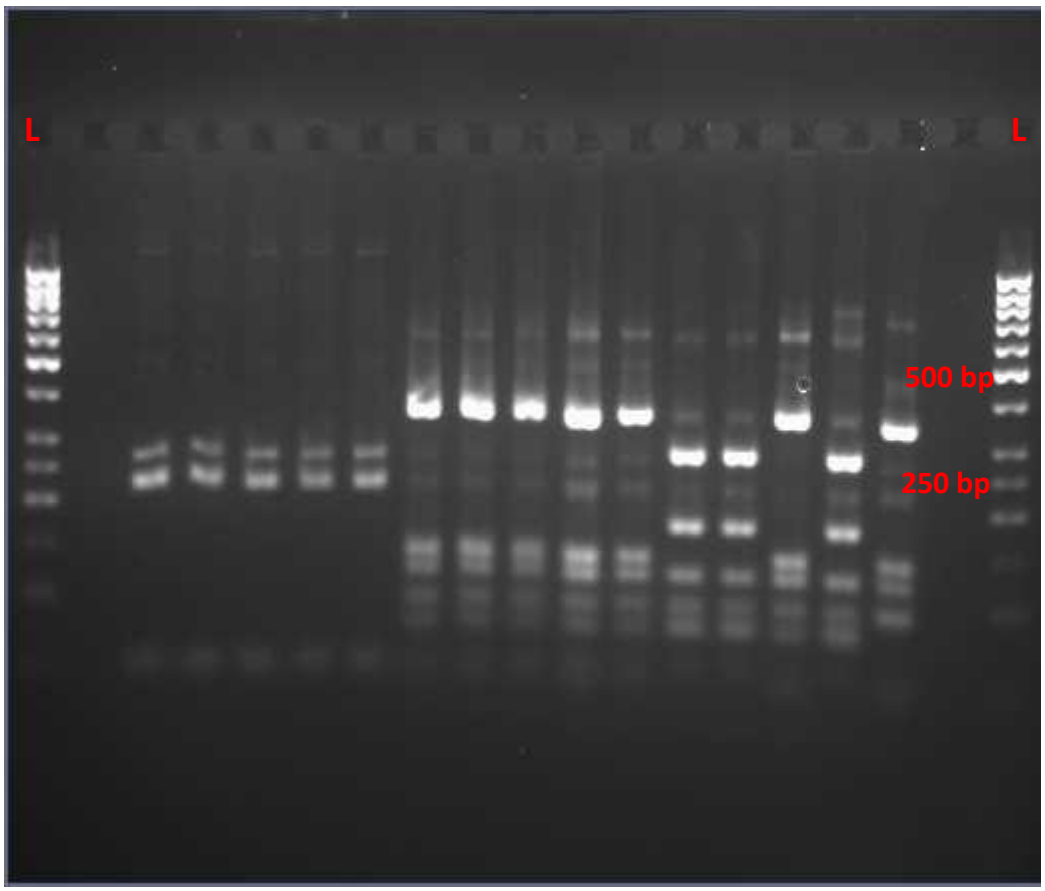

Figure 3.10 *AmoA AOA sequence RFLPs from RB6 and Bb12 sequences from March extractions. Restriction enzymes utilised RsaI and EcoRI. (**L**) GeneRuler 50-bp DNA Ladder ThermoScientific.*

Due to the lack of digestion observed in **Fig. 3.10**, the same RFLP protocol was attempted with a longer incubation time of 1.5 hrs in an effort to digest the highest molecular weight DNA and to observe the difference between the different OTUs. **Fig. 3.11** indicated that restriction digestions performed on PCR-cleaned product produced more distinct bands, although there were still methodological problems with incomplete digestion. Regardless of

methodological limitations, representative sequences could be selected for sequencing of AOA and AOB *amoA* products from RB6 and Bb12.

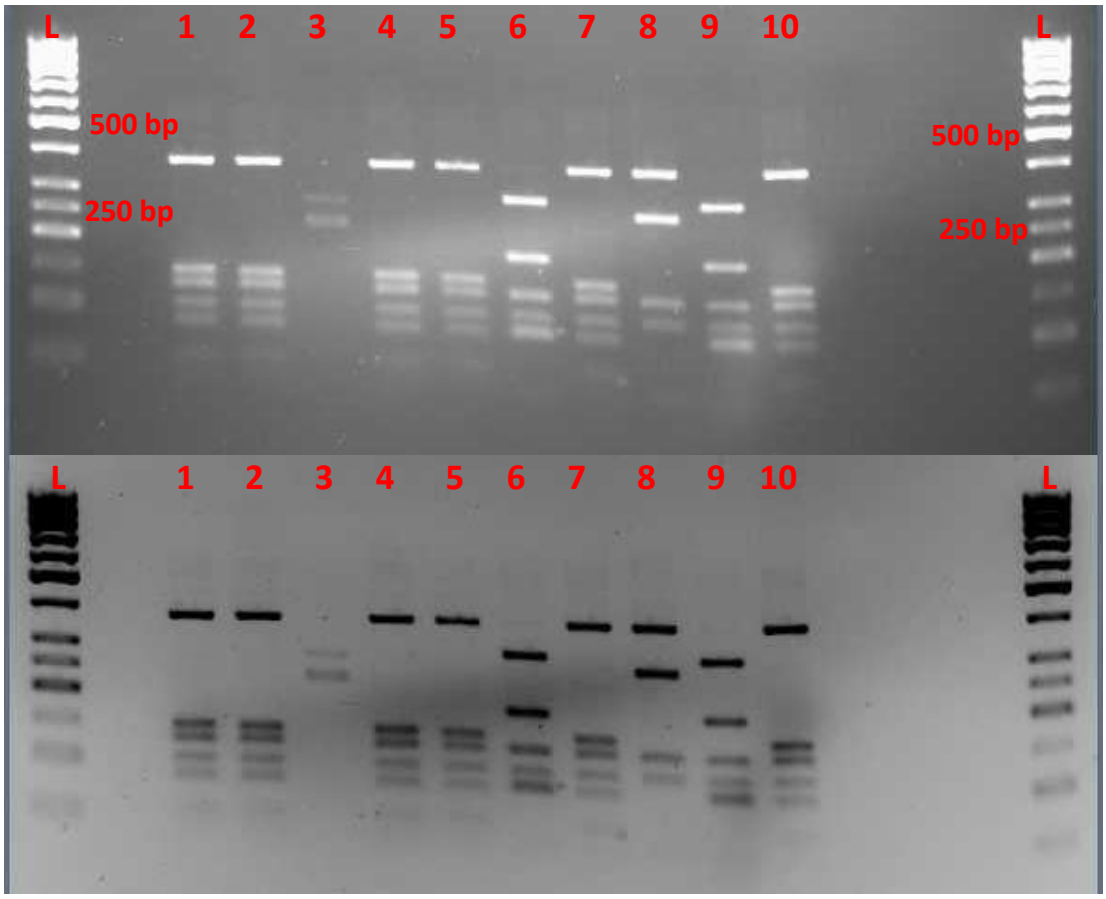

Figure 3.11 *: AmoA AOA sequence RFLPs from RB6 - sequences from January root and rhizosphere extractions and March saturated anaerobic extractions, on PCR-cleaned material. Utilised restriction enzymes RsaI and EcoRI. (**1**) anaerobic saturated col 1; (**2**) root col 3; (**3**) root col 5; (**4**) root col 6; (**5**) root col 7; (**6**) rhizosphere col 3; (**7**) rhizosphere col 1.2; (**8**) rhizosphere col 3.2; (**9**) rhizosphere col 4.2; (**10**) rhizosphere col 5.2 (**L**) GeneRuler 50-bp DNA Ladder ThermoScientific..*

3.3 Anammox

3.3.1 Optimisation of molecular tools to study anammox

In order to develop robust tools for studying anammox within the constructed wetland trials, several sets of previously published PCR primers were tested. Three different published primer sets are detailed in the **2. Materials and Methods**.

The initials tests were run on *amx*694F/960R targeting 16S rRNA Hu *et al.* (2010) and *hzsA*_1597F/_1857R targeting hydrazine synthase Harhangi *et al.* (2012) using control DNA from cultures of *Kuenenia stuttgartiensis* and *Brocadia fulgida* (**fig. 3.12**). There was non-specific amplification with the *amx*694F/960R primer set (**fig. 3.12**) in lanes **1**-**2** there appeared to be a band at the correct size (highlighted at 625-bp) however in all lanes (**1**-**5**) there appeared to be contamination as highlighted below and smears between the band of the correct sizes and this contamination. There were multiple attempts to optimize the *amx*694F/960R primer set, calculating and testing annealing temperatures using a gradient; using the touchdown setting on the PCR machine reducing the annealing temperature every cycle from 68^{o}C (where amplification was first observed in the gradient) to 58^{o}C (where amplification was brightest in the gradient). Also changed the primer concentration and reduced the time for each part of the PCR cycle. (**Fig. 3.13**) indicates the last test done using +ve control DNA *Brocadia fulgida* and *Kuenenia stuttgartiensis* but eventually this primer set was disregarded due to specificity problems and efforts were focused on the functional marker genes, *hzs* and *hzo*.

Primers *hzsA*_1597F/_1857R gave a clean band at 260-bp for *Brocadia fulgida* (lanes **6** and **7**) however *Kunenenia stuttgartiensis* had another band just above the band of the correct size (260-bp) (lanes **8** and **9**). For the *hzsA*_1597F/_1857R primers, further PCR with *K. stuttgartiensis* was performed to test whether non-specific amplification could be overcome.

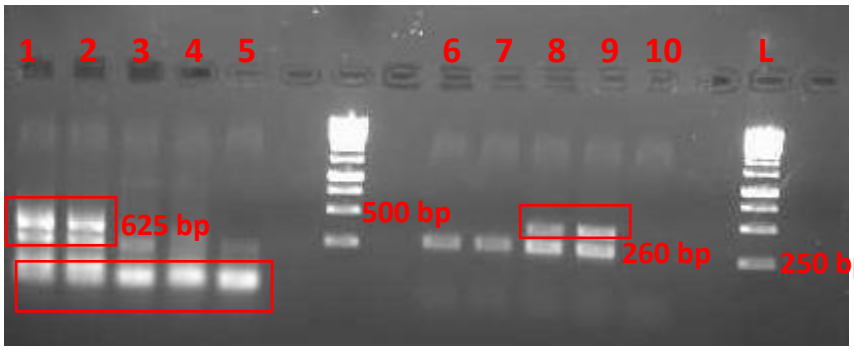

Figure 3.12 *Testing primers amx (lanes **1**-**5**) and hzsA (lanes **6**-**10**) on positive control DNA Brocadia fulgida and Kuenenia stuttgartiensis. (**1**-**2**) Brocadia fulgida extractions 1 and 2 respectively; (**3**-**4**) Kuenenia stuttgartiensis extractions 1 and 2 respectively; (**5**) -ve PCR mastermix with addition of 1 µL of nuclease-free water in place of DNA; (**6**-**7**) Brocadia fulgida extractions 1 and 2 respectively; (**8**-**9**) Kuenenia stuttgartiensis extractions 1 and 2 respectively; (**10**) -ve PCR mastermix with addition of 1 µL of nuclease-free water in place of DNA; (**L**) GeneRuler 1 kb DNA Ladder.*

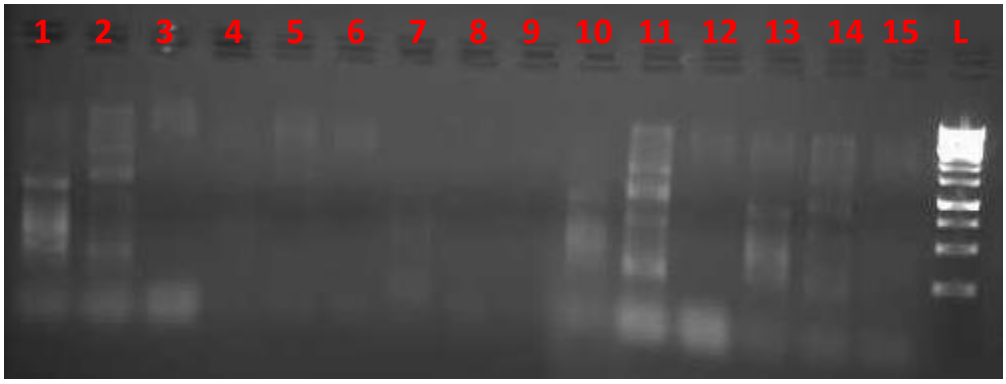

Figure 3.13 *Amx primers testing. (**1**-**3**) 1 µL of primer with annealing temperature of 58°C on Kuenenia stuttgartiensis and Brocadia fulgida and -ve control (PCR mastermix with addition of 1 µL of nuclease-free water in place of DNA) respectively; (**4**-**9**) replications using different stocks of nuclease-free water to see if contamination was the issue at the same temperature and with the same amount of primer; (**10**-**12**) 1 µL of primer with an annealing temperature of 62°C on K. stuttgartiensis and B. fulgida and -ve control; (**13-15**) replication using different stocks of nuclease-free water to see if contamination was the issue at the same temperature and with the same amount of primer; (**L**) GeneRuler 1 kb DNA Ladder.*

A temperature gradient was run to optimise the annealing temperature for this PCR (**fig. 3.14**). Temperature gradient PCR was performed *hzsA*_1597F/_1857R primers on K. *stuttgartiensis* in an attempt to remove the non-specific band above the one at the expected size of 260-bp. The brightest PCR product (lane **6**) was at the temperature 59.2°C, closest to the protocol temperature of 60°C. However, even with the band becoming fainter

above or below this temperature, it was not possible to remove this band at any temperature.

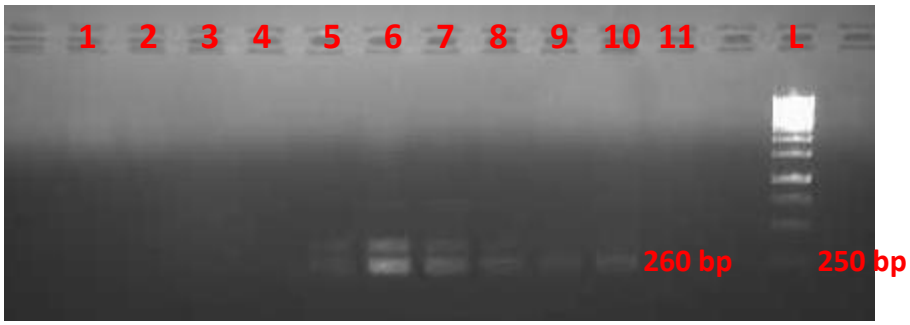

Figure 3.14 *hzsA primers with gradient on +ve DNA K. stuttgartiensis, utilising protocol temperature as a midpoint. (**1**) 66°C, (**2**) 65.1°C, (**3**) 64.2°C, (**4**) 62.9°C, (**5**) 61.2°C, (**6**) 59.2°C, (**7**) 57.4°C, (**8**) 56°C, (**9**) 55°C, (**10**) 54°C, (**11**) -ve control PCR mastermix with addition of 1 µL of nuclease-free water in place of DNA; (**L**) GeneRuler 1 kb DNA Ladder.*

In trying to remove the band that appeared above the one of the expected size of 260-bp and improve amplification brightness, optimisation was again performed on extracted *K. stuttgartiensis* DNA. Implemented shorter times for each step of the PCR reaction, this time: 3 mins, 30 secs, 30 secs, 1 min, for 30 cycles and 5 mins and also utilised double the concentration (2 µL) of primer versus the original volume of (1 µL) but still utilised the rest of the protocol master mix as usual. Amplification was observed (**fig. 3.15**) and the band was brighter in the second lane where double the primer concentration had been utilised.

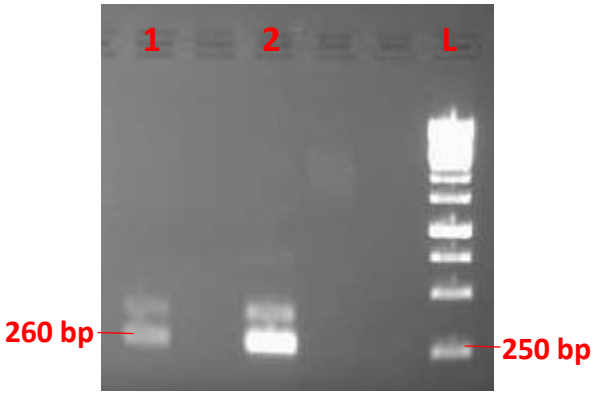

Figure 3.15 *Testing hzsA primers on Kuenenia stuttgartiensis in lane **1**: with 1 µL of primers; lane **2**: with 2 µL of primers; (**L**) GeneRuler 1 kb DNA Ladder.*

This protocol was used on RB6 soil DNA January extractions (**fig. 3.16**). Despite the faintness of the bands, it was clear there was anammox present in all the depth layers of the constructed wetland, with no visible difference in strength of PCR amplification between the layers. The original published protocol (Harhangi *et al.*, 2012) was also tested. It was observed that that the PCR product amplified from *K. stuttgartiensis* was fainter whereas *B. fulgida* and the environmental RB6 samples did not amplify at all. As the amplification was weak, alternative DNA extraction method was attempted using the FastDNA™ Spin Kit for Soil (MP Biomedicals).

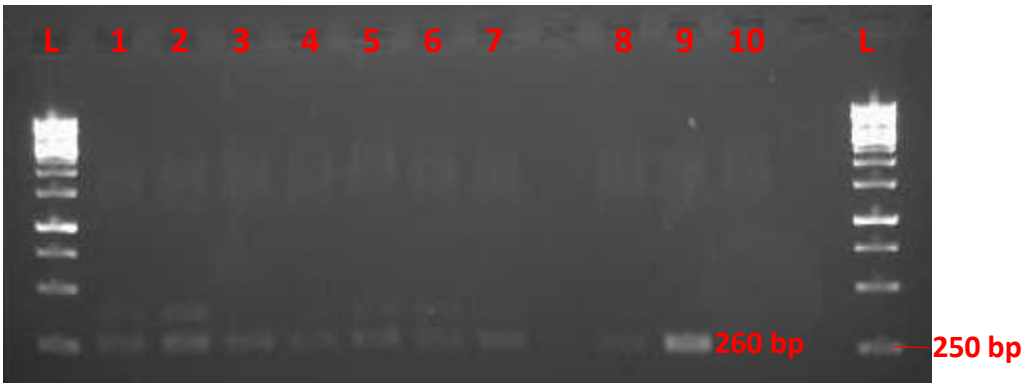

Figure 3.16 *hzsA primers utilised on RB6 DNA extractions from January (22/1/19). Lanes **1**-**2**: unsaturated aerobic layer extractions 1 and 2 respectively; lanes **3**-**4**: interface layer top extractions 2 and 2 respectively; lanes **5**-**6**: interface layer bottom extractions 1 and 2 respectively; lanes **7**-**8**: saturated aerobic layer extractions 1 and 2 respectively; (**9**) +ve Kuenenia stuttgartiensis; (**10**) -ve PCR master mix with 1 µL of nuclease-free water added in place of DNA; (**L**) GeneRuler 1 kb DNA Ladder.*

It was reasoned that PCR inhibitors could also interfere with the amplification, particularly in later extractions (12/3/19) where the top layers (unsaturated aerobic) in both RB6 and Bb12 were fainter with all marker gene primers but quality checks utilising 16S primers (27F/1492R) (Lane, 1991) and running DNA alone on an agarose gel (0.5% (w/v), 1 x TBE buffer) indicated the DNA of these extractions were of good quality. Therefore, serial dilutions were attempted twice using primers *hzsA*_1597F/1857R on the top layer (unsaturated aerobic layer) of RB6 samples (22/1/19) extracted by Griffith's method. Prior to this, DNA samples were usually diluted to a concentration of 10 ng/µL. Serial dilutions were diluted to: 1/5, 1/10 and 1/20 of extracted DNA, with 1/20 dilution being almost equal to the 10 ng/µL. These dilutions were also spiked with a positive control DNA (*K. stuttgartiensis*), in order to see whether amplification of the control DNA

would be diminished as this would indicate the presence of PCR inhibitors. In addition, positive control DNA alone was run alongside, to have a control for usual amplification levels at the same concentrations. Amplification from the control DNA template was reduced in the presence of the extract, confirming that PCR inhibitors were present.

To clean the DNA of the PCR inhibitors, clearly indicated in the spiking experiments, DNA was re-extracted from RB6 (January), utilising the second DNA extraction protocol. The same serial dilution experiments were performed, as **fig. 3.17** indicates the DNA was cleaner using the FastDNA™ Spin Kit for Soil (MP Biomedicals). Due to the fact that amplification was not observed in any of the more concentrated samples of RB6, it was decided to utilise an alternative *hzsA* forward primer, this was *hzsA*_526F (Harhangi *et al.*, 2012) utilised together with the original reverse primer *hzsA*_1857R.

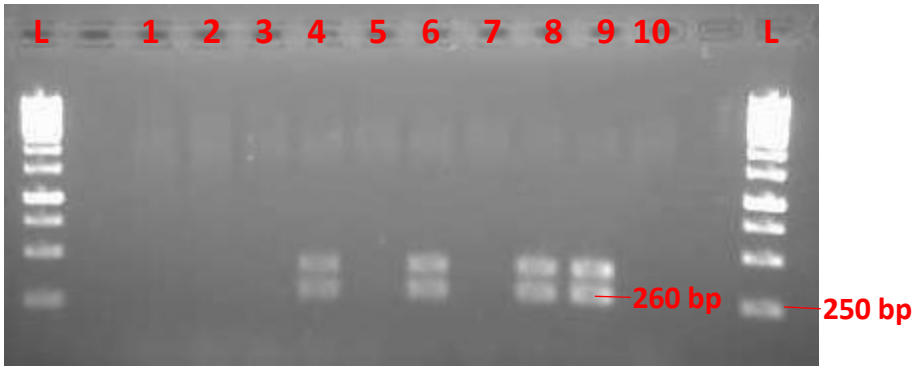

Figure 3.17 *hzsA on RB6 interface level extracted DNA alone and spiked with Kuenenia stuttgartiensis +ve control DNA. Serial dilution concentrations are as follows (**1**) 1/5 (**2**) 1/5 with +ve control, (**3**) 1/10, (**4**) 1/10 with +ve control, (**5**) 1/20, (**6**) 1/20 with +ve control, (**7**) original concentration (154.3 ng/µL) (**8**) original concentration (154.3 ng/µL) with +ve control, (**9**) +ve control, (**10**) -ve control PCR master mix with 1 µL nuclease-free water added instead of DNA; (**L**) GeneRuler 1 kb DNA Ladder.*

3.3.2 The optimised protocol for detection of anammox using hydrazine synthase (*hsz*)

The previously published protocol (Harhangi *et al.,* 2012) using *hzsA*_526F/_1857R was utilised on DNA samples from RB6 (22/1/19) purified by either the FastDNA™ Spin Kit for Soil (MP Biomedicals) (lanes **3**-**10**) and two samples by the original Griffiths' method (lanes **1**-**2**) (**fig. 3.18**).

There was strong amplification observed for all samples and throughout all layers. Further testing with the same serial dilution experiments as earlier showed amplification in all samples. Amplification on RB6 samples indicated some unspecific binding was present and again a temperature gradient of the annealing temperature was performed, as well as shortening the times for each step of the cycle. From this, it was clear that the protocol temperature of 54°C produced the most specific and strongest amplification, and amplification was improved.

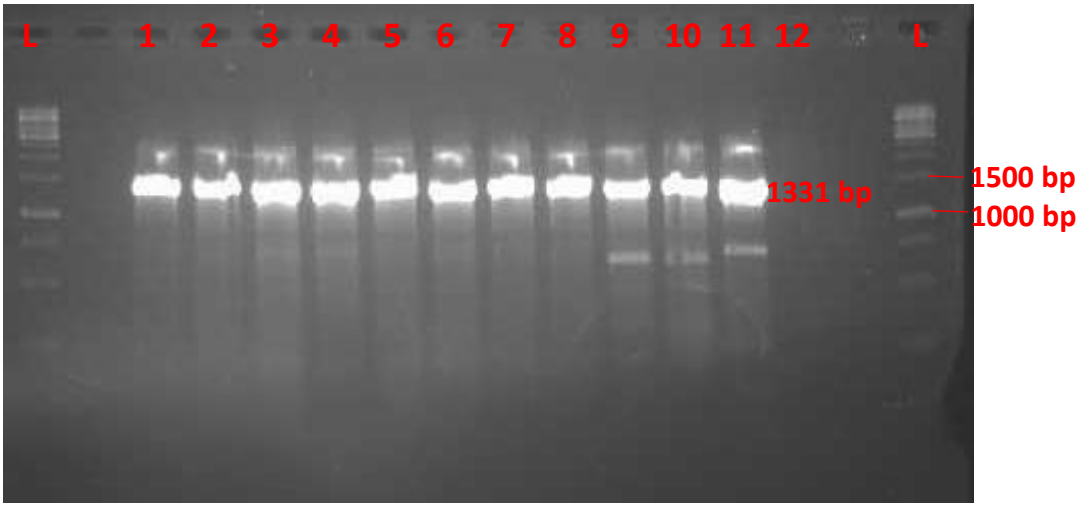

Figure 3.18 *PCR utilising hzsA primers with new forward primer (hzsA_526F). Lanes* ***1****-****2:*** *containing extractions by the Griffiths method (31/01/19) from the unsaturated aerobic (****1****) and saturated aerobic (****2****) layers. The rest were from the column purified samples (22/02/19). Lanes* ***3****-****4****: were unsaturated aerobic layer (1.1 + 1.2); (****5****-****6****) the top interface layer (2.1 + 2.2); (7-****8****) the bottom interface layer (3.1 + 3.2) and (****9****-****10****) the saturated level (4.1 + 4.2); (****11****) +ve Kuenenia stuttgartiensis; (****12****) -ve PCR master mix with 1 µL of nuclease-free water added instead of DNA.*

3.3.3 Bias in the *hzs* assay

During tests with the *hzsA*_526F/_1857R primers, it was observed that *B. fulgida* was not amplifying well and the band produced was much fainter than that observed for *K. stuttgartiensis*. First tested to ensure both *B. fulgida* DNA extractions were of high quality, utilising universal 16S primers (27F/1492R) (Lane, 1991) on both positive control DNA templates. Quality checks indicated the extractions were of a good quality and amplified well. These experiments suggested that the primers may be biased towards *K. stuttgartiensis.* Suspicions were further confirmed through the production of a

clone library from RB6 DNA extractions (22/1/19), utilising the *hzsA*_526F/-_1857R primers. The clones from all layers came back as *K. stuttgartiensis* based on BLASTn (BLASTn) search.

It was found that not all sequences were 100% *K. stuttgartiensis.* Almost half were but most were of an unknown clade, that were of 95% relatedness to *K. stuttgartiensis*, which is a clade that has been previously reported by Humbert and colleagues (2010). One other clone came back as *Jettenia caeni*.

To test for bias in the *hzsA*_526F/_1857R primers, clones were produced from *hzsA*_526F/_1857R amplified known positive control DNA *K. stuttgartiensis* and *B. fulgida* and another set of clones produced from a 1:1 mix of both positive control DNA. Prior to testing these clones, it was observed that amplification from *B. fulgida* alone was fainter than both the bands from *K. stuttgartiensis* alone and the 1:1 mix of both genera.

After picking clones and performing colony PCR to retrieve the clones, restriction digests was performed utilising *EcoRI* and 4-bp restriction enzyme *MspI*, which had been investigated *in silico* first. **Fig. 3.19** shows the control DNA alone had distinctly different RFLPs, *K. stuttgartiensis* in lanes **9** and **10** and *B. fulgida* in lanes **11** and **12**. The rest of the lanes were from the 1:1 mix of *K. stuttgartiensis* DNA and *B. fulgida* DNA, amplified by the *hzsA*_526F/1857R primers. All clones came back as *K. stuttgartiensis*, indicating a clear bias for *K. stuttgartiensis* versus *B. fulgida*. *In silico* analysis indicated a 1-bp mismatch on the reverse primer with *B. sinica* but not *B. fulgida*. It was especially peculiar that *K. stuttgartiensis* appeared so often, without being accompanied by a species from the *Brocadia* genus. It had been mentioned prior (Humbert *et al.*, 2010; Sonthiphand *et al.*, 2010) that the *Brocadia* genus is often found alongside *Kuenenia* in wastewater treatment systems as this genus can also tolerate high amounts of NH_3. Due to this discrepancy, it was decided to use *hzs* primers in tandem with another, separate functional marker gene of anammox (hydrazine

oxidoreductase (*hzo*)), to compare the sequences amplified between the two primer sets.

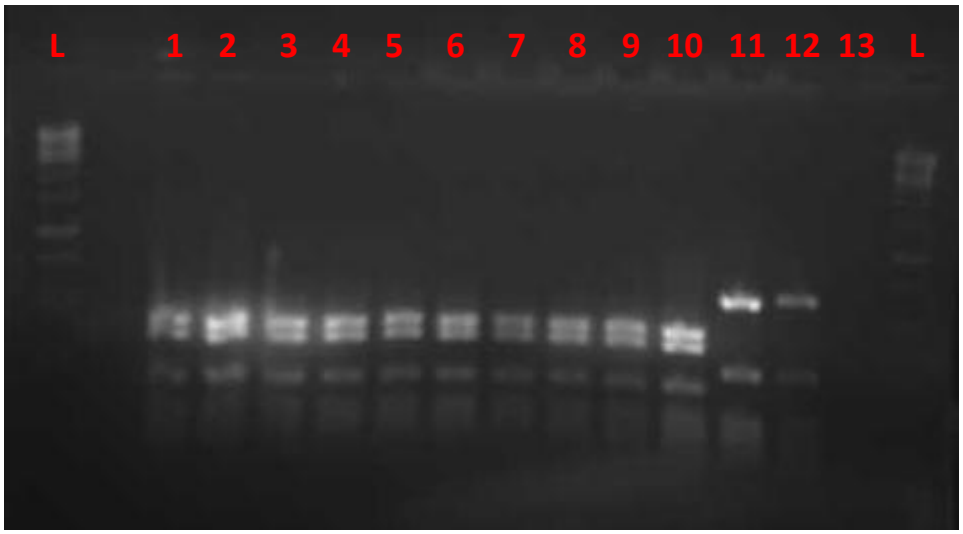

Figure 3.19 *RFLPs on clones from hzsA_526F_1857R amplified control DNA Kuenenia stuttgartiensis, Brocadia fulgida and 50/50 mix of both control DNA. (**1**-**8**) 50/50 mix of control DNA; (**9**-**10**) +ve control DNA Kuenenia stuttgartiensis; (**11**-**12**) +ve control DNA Brocadia fulgida; (**13**) -ve PCR master mix with 1 µL nuclease-free water added in place of DNA.*

3.3.4 Optimisation of anammox detection using primers for hydrazine oxidoreductase (*hzo*)

Hydrazine oxidoreductase (HZO) performs the subsequent step in the anammox reaction after hydrazine synthase. Although widely used for detection and study of anammox organisms, this target gene has the caveat of being related to hydroxylamine oxidoreductases, meaning potentially more non-target amplification. Nevertheless, studying this functional marker gene could provide an important control in the light of the observed bias for the *hzs* primer set. Therefore, the primer set *hzo*1F/1R was tested on anammox control DNA *K. stuttgartiensis* and *B. fulgida*.

The previously published protocol (Long *et al.*, 2012) produced faint PCR amplicons for both genera, with a non-specific product above the correctly-sized PCR product of 243-bp. The second protocol from a different publication (Lansdown *et al.*, 2016) was attempted. This produced faint PCR products for *B. fulgida* and no amplification was observed for *K.*

stuttgartiensis. A temperature gradient was performed for the annealing temperature. The most optimal amplification was seen at 60^{o}C and no unspecific binding was observed.

To improve brightness of amplification, 1 µL of primer pair *hzo-1F/1R* was used (originally 0.8 µL), as this worked better on environmental DNA where there was less DNA template available. In addition, a higher concentration of DNA was used to provide more DNA template for amplification. **Fig. 3.20** indicates original extractions of the unsaturated aerobic layer (1.1) from RB6 (12/3/19), versus the zymo-cleaned extractions, with increasing concentration in a serial dilution. In **lanes 1-4** are the original extractions at original concentration of 39.1 ng/µL, ¾, ½ and 10 ng/µL respectively, and **lanes 7-10** are the zymo-cleaned extractions at the original concentration of 39.1 ng/µL, ¾. ½ and 10 ng/µL respectively. Zymo-cleaned extractions resulted in slightly better amplification in the ¾ diluted sample however this made little difference when compared with the difference seen between original samples and zymo-cleaned samples.

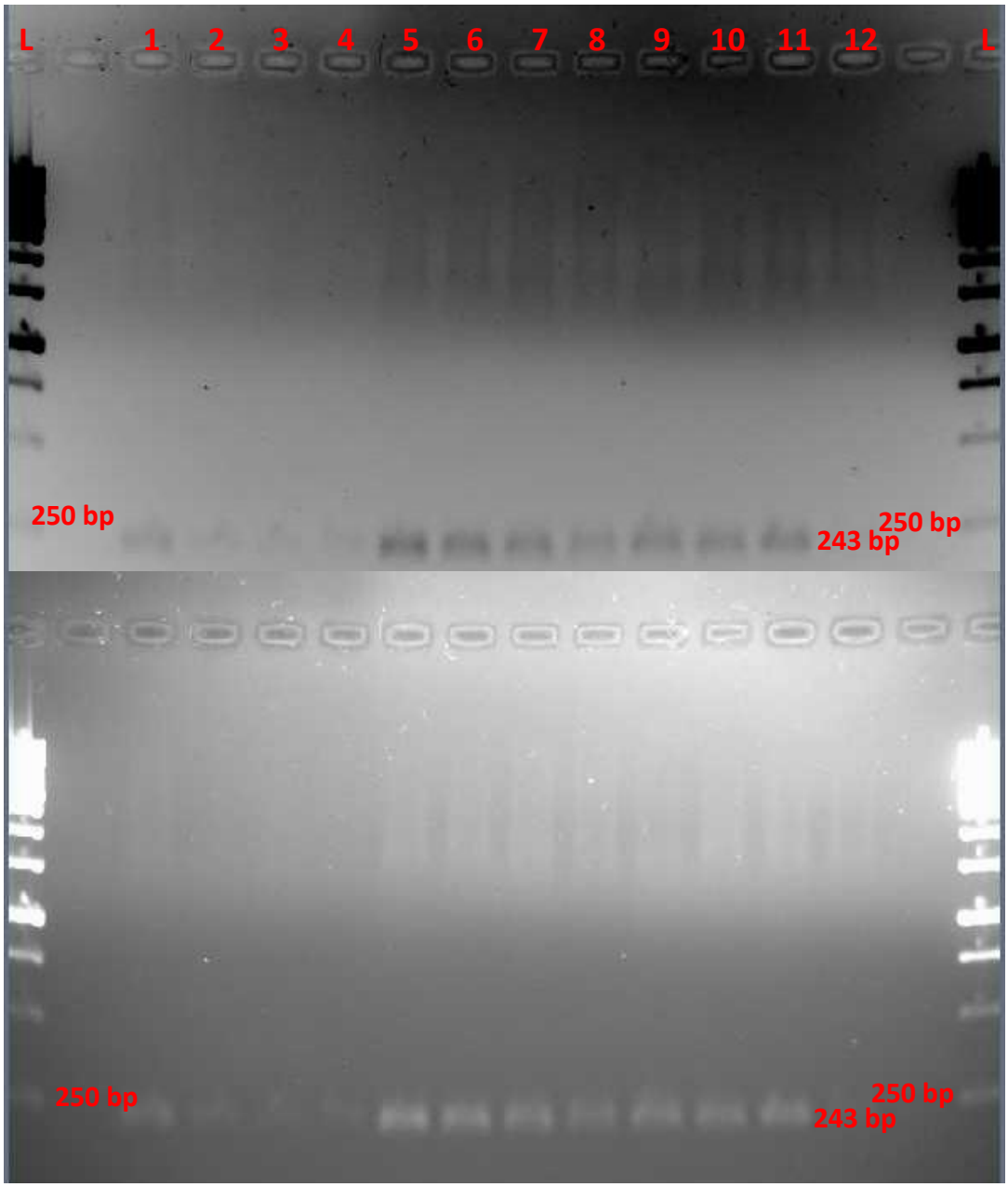

Figure 3.20 *hzo primers on unsaturated layer from RB6 March extractions, original and zymo-cleaned. (**1-4**) Original DNA extractions at: 39.1 ng/µL, ¾ dilution, ½ dilution and 10 ng/µL respectively; (**7-10**) zymo-cleaned DNA extractions at: ¾ dilution, ½ dilution and 10 ng/µL respectively; (**5**+**11**) +ve control DNA Kuenenia stuttgartiensis; (**6**+**12**) -ve PCR master mix with 1µL of nuclease-free water added in place of DNA; (**L**) GeneRuler 1 kb DNA Ladder.*

3.3.5 Examining the diversity of anammox in the constructed wetlands using *hzs* and *hzo* functional marker genes

Clone libraries were produced from PCR amplicons of both *hzs* and *hzo* from DNA extracted from RB6. With *hzsA* primer set (**Table 3.5** and **fig. 3.21**), the initial BLAST results suggested clones are likely affiliated with *Kuenenia stuttgartiensis*. More detailed phylogenetic analysis indicated that sequences fell into two distinct clades, with more than half of the sequences corresponding to a distinct, uncultivated clade. This uncultivated clade has been observed previously in association with roots and wastewater treatment sites (Humbert *et al.*, 2010). Like in this study, this uncultivated clade co-occurred with *K. stuttgartiensis* and the *hzs* genes of this clade share 95% identity with *K. stuttgartiensis*, the sequences of which were highly related clones discovered by the hydrazine synthase primer in an unpublished paper by Li and colleagues (2019).

With *hzo* primer set (**Table 3.6** and **fig. 3.22**), all sequences were highly related to *Kuenenia stuttgartiensis* in RB6. The clones produced using the *hzo* primer set from RB6 support the findings generated using the *hzs* primer set. This also indicates that the results using *hzs* as a marker are unlikely to be due to the amplification bias observed in earlier experiments and are likely to represent the real anammox community in this constructed wetland. Combined results using the two functional markers: *hzs* and *hzo*, thus indicated that the anammox community in RB6 is dominated by organisms of genus *Kuenenia* and with *hzs* potentially another uncultivated clade.

Also, in the unvegetated Bb12, most *hzo* sequences were highly related to *Kuenenia stuttgartiensis.* However, all clones from the interface of Bb12 and one from the saturated aerobic zone in RB6 were most closely related to various species of the genus *Brocadia*. This genus is often found in association with wastewater treatment.

Again, for the most part, not any one genera dominated any part of the constructed wetland, with a mostly equal distribution of *Kuenenia*

stuttgartiensis throughout the constructed wetlands, apart from the sequences all from the interface in Bb12 – found by the *hzo* primer set.

Table 3.5 *Blast results from hzsA sequences amplified from RB6 (22/1/19) and Bb12 (12/3/19).*
** unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic); saturated aerobic = layer below water table (interface) of wet soil next to roots (aerobic); interface = in line with water table (meeting of unsaturated (dry) and saturated (wet) matrix soil); saturated anaerobic = bottommost layer below the water table (interface) of wet soil with no association with the roots (anaerobic).*
*** colony*

Sample: Clone #	**Blast Result: Highest Match Known Species**	**Query Cover**	**Per Identity**
RB6	*Kuenenia stuttgartiensis*		
Unsaturated Aerobic*:			
Col 5-2		100.00%	99.50%
Saturated Aerobic*:			
Col 1-1		100.00%	100.00%
Col 7-1		100.00%	99.50%
Bb12			
Unsaturated Aerobic:			
Col 3		100.00%	99.00%
Col 4		100.00%	99.50%
Col 5		100.00%	100.00%
Interface*:			
Col 5		100.00%	100.00%
Saturated Aerobic:			
Col 1		100.00%	100.00%
Col 4		100.00%	100.00%
Saturated Anaerobic*:			
Col 5		100.00%	100.00%

Table 3.6 *Blast results from hzsA sequences amplified from RB6 (22/1/19) and Bb12 (12/3/19).*
** unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic); saturated aerobic = layer below water table (interface) of wet soil next to roots (aerobic); interface = in line with water table (meeting of unsaturated (dry) and saturated (wet) matrix soil); saturated anaerobic = bottommost layer below the water table (interface) of wet soil with no association with the roots (anaerobic).*
*** colony.*

Sample: Clone #	Blast Result: Highest Match Known Species	Query Cover	Per Identity
RB6	*Kuenenia stuttgartiensis (closest relation but most likely the uncultivated clade mentioned in Humbert et al. 2010)*		
Unsaturated Aerobic:			
Col 1-1			
Col 2-1		100.00%	96.04%
Col 3-1		100.00%	95.05%
Col 7-1		100.00%	94.21%
Col 2-2		100.00%	96.04%
Saturated Aerobic:		100.00%	94.55%
Col 3-1			
Col 6-1		100.00%	95.05%
Col 1-2		100.00%	95.05%
Col 2-2		100.00%	96.04%
Col 3-2		100.00%	95.05%
Col 4-2		100.00%	95.54%
Col 6-2		100.00%	94.06%
Bb12		100.00%	94.97%
Interface:			
Col 1		100.00%	96.04%
Col 3		100.00%	96.04%
Col 4		100.00%	96.04%
Saturated Aerobic:			
Col 2		100.00%	96.04%
Col 3		100.00%	94.35%

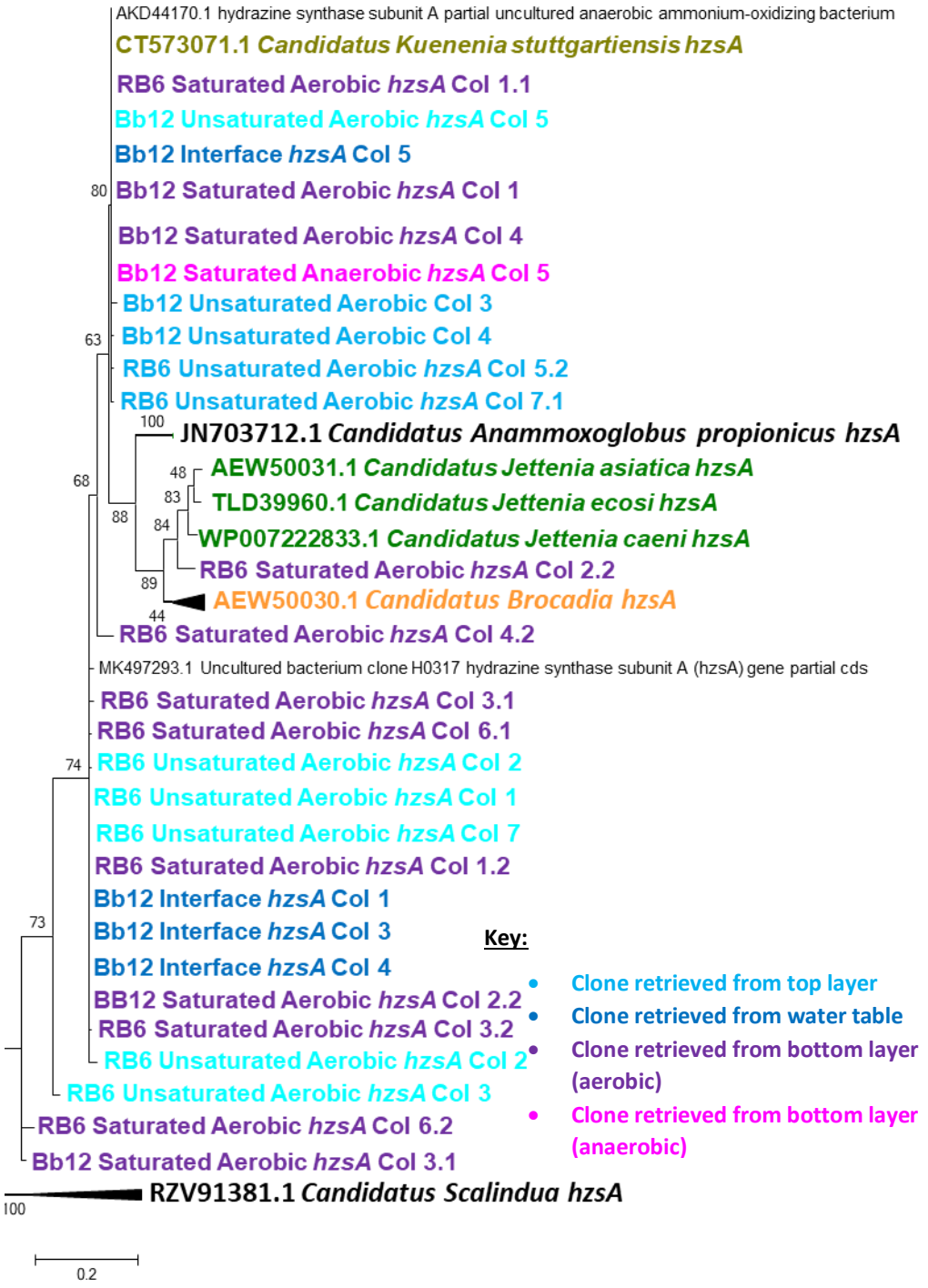

Figure 3.21 *Phylogenetic tree produced from RB6 (22/1/19) and Bb12 (12/3/19) hzsA clones. Clones retrieved from the unsaturated aerobic layer were taken from soil matrix above the water table in dry soil (highlighted in bright blue). Clones retrieved from the interface were taken from the water table (between the dry and wet soil matrix) (highlighted in blue). Clones retrieved from the saturated aerobic layer were taken from soil matrix below the water table next to Phragmites australis roots (highlighted in purple). Clones retrieved from the saturated anaerobic layer were taken from soil matrix below the water table not in association with roots (highlighted in pink). Length of scale bar represents a difference of 20% between different sequences.*

Table 3.7: *Blast results from hzo sequences amplified from RB6 and Bb12 (both 12/3/19) and root/rhizosphere material from RB6 (22/1/19).*
** interface = in line with water table (meeting of unsaturated (dry) and saturated (wet) matrix soil); saturated aerobic = layer below water table (interface) of wet soil next to roots (aerobic); saturated anaerobic = bottommost layer below the water table (interface) of wet soil with no association with the roots (anaerobic); unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic).*
*** colony.*

Sample: Clone #	**Blast Result: Highest Match Known Species**	**Query Cover**	**Per Identity**
RB6	*Kuenenia stuttgartiensis*		
Interface*:			
Col 8-1		100.00%	98.65%
Col 8-2		100.00%	98.65%
Saturated Aerobic*:			
Col 1-2		100.00%	97.30%
Saturated Anaerobic*:			
Col 1-1		100.00%	98.65%
Col 2-1		100.00%	98.65%
Root:			
Col 1-1		100.00%	98.65%
Col 1-2		100.00%	98.65%
Rhizosphere:			
Col 1-1		100.00%	98.65%
Bb12			
Unsaturated Aerobic*:			
Col 1		100.00%	98.65%
Col 2		100.00%	98.65%
Col 3		100.00%	98.65%
Col 4		100.00%	97.30%
Col 5		100.00%	98.65%

Table 3.8: *Blast results from hzo sequences amplified from RB6 and Bb12 (both 12/3/19) and root/rhizosphere material from RB6 (22/1/19).*
** interface = in line with water table (meeting of unsaturated (dry) and saturated (wet) matrix soil); saturated aerobic = layer below water table (interface) of wet soil next to roots (aerobic); saturated anaerobic = bottommost layer below the water table (interface) of wet soil with no association with the roots (anaerobic); unsaturated aerobic = top layer above water table (interface) in dry soil (aerobic).*
*** colony*

Sample: Clone #	Blast Result: Highest Match Known Species	Query Cover	Per Identity
Saturated Aerobic:	*Kuenenia stuttgartiensis*		
Col 1		100.00%	98.65%
Col 2		100.00%	98.65%
Col 3		100.00%	98.65%
Saturated Anaerobic:			
Col 1		100.00%	98.65%
Col 2		100.00%	98.65%
Col 3		100.00%	98.65%
Col 4		100.00%	98.65%
Col 5		100.00%	98.65%
RB6	*Brocadia fulgida*		
Saturated Aerobic:			
Col 1		100.00%	94.59%
Bb12			
Interface:			
Col 1		100.00%	94.59%
Col 2		100.00%	93.24%
Col 3		100.00%	93.24%
Col 4		100.00%	93.24%
Col 5		100.00%	89.19%

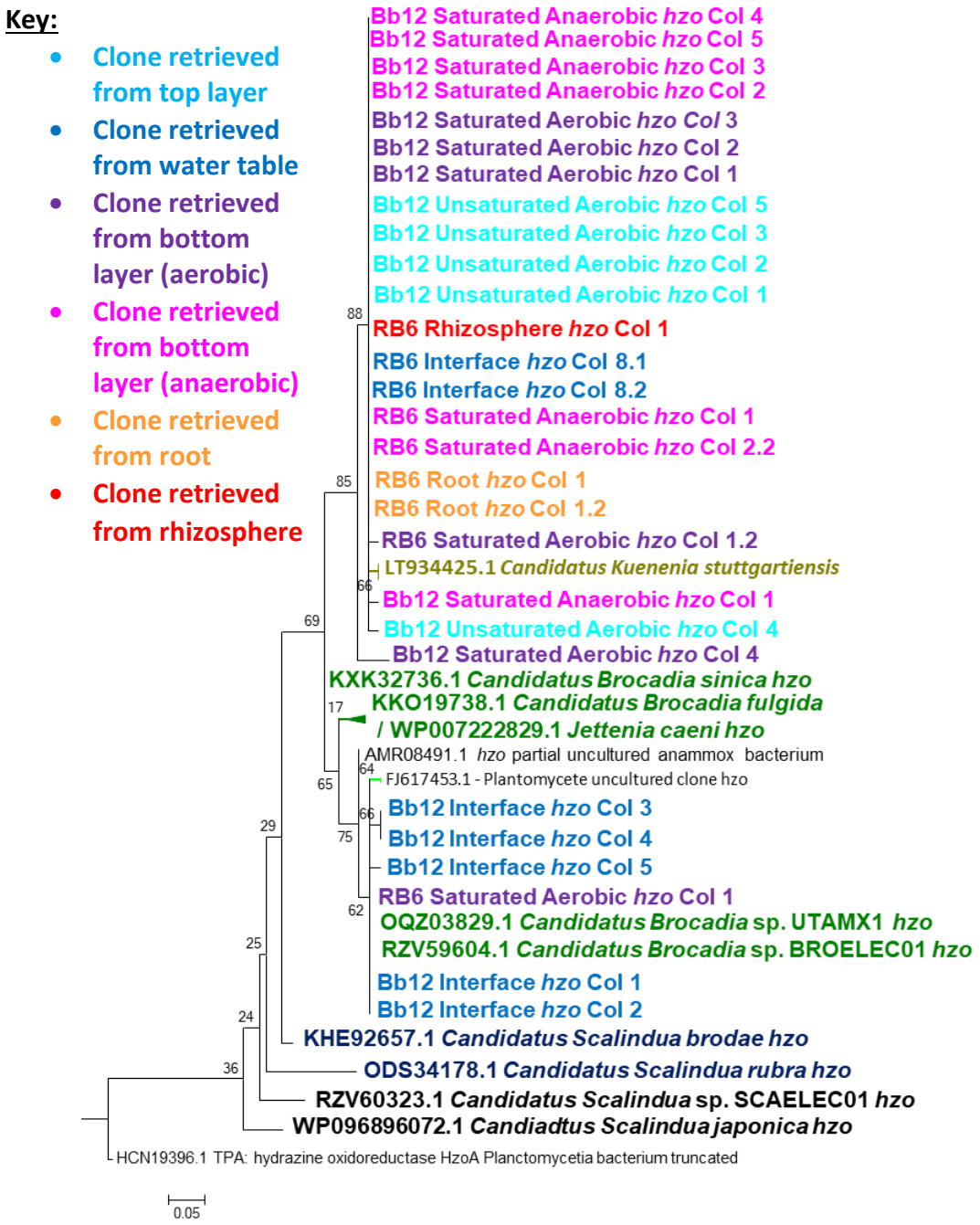

Figure 3.22 *Phylogenetic tree produced from RB6 and Bb12 (12/3/19) hzo clones. Clones retrieved from the unsaturated aerobic layer were taken from soil matrix above the water table in dry soil (highlighted in bright blue). Clones retrieved from the interface were taken from the water table (between the dry and wet soil matrix) (highlighted in blue). Clones retrieved from the saturated aerobic layer were taken from soil matrix below the water table next to Phragmites australis roots (highlighted in purple). Clones retrieved from the saturated anaerobic layer were taken from soil matrix below the water table not in association with roots (highlighted in pink). Clones retrieved from root (highlighted in orange) and rhizosphere (highlighted in red). Length of scale bar represents a difference of 5% between different sequences.*

4. Overall Discussion

4.1 Environmental drivers of the ammonia oxidiser diversity in the constructed wetlands

Age of landfill site, stage of decomposition and operational conditions highly affect the microbial community structure in the landfills, with a shift occurring as easily degradable organic matter disappears, and the community becomes more highly specialised (Remmas *et al.*, 2017). This microbial community shift has been previously described in a landfill treatment system coupled to an anammox bioreactor Sequencing Batch Reactor (SBR) with a high ammonia concentration of 1,333 mg N L^{-1}, which is similar to the ammonia levels found in Mayton Wood Closed Landfill site (1,200 mg L^{-1}). The landfill site was a mature one, with effluent levels comparable to that of Mayton Wood and the ammonia oxidising microbial community was observed to be dominated by AOB sequences.

AMO is a key enzyme in the ammonia oxidation pathways of both AOB and AOA. This membrane-bound enzyme catalyses the first step of the nitrification pathway in the aerobic oxidation of ammonia to hydroxylamine (NH_2OH), which is then further oxidised to nitrite (NO_2-) and is often utilised as a functional marker gene in the study of examining the presence and diversity of AOA and AOB. Since the nitrite produced by this reaction is a pre-requisite for anammox, this study utilised AMO as a first indicator that it is possible for the anammox reaction to occur (Rotthauwe *et al.*, 1997; Tourna *et al.*, 2008; Lehtovirta-Morley *et al.*, 2016). However, there are potential caveats in that nitrite can be produced by denitrification from nitrate (NO_3-) and some anammox bacteria themselves can produce nitrite through consumption of acetate and propionate (Sonthiphand *et al.*, 2014). In this study, AMO sequences fell into two genera for both AOA and AOB. AOA communities were dominated by *Nitrososphaera* and *Nitrosocosmicus* and AOB communities by *Nitrosomonas* and *Nitrosospira*, given the small sample size, amplicon sequencing would be required to see if the same patterns were observed. All detected sequences were affiliated to soil species, with

no marine species found in the small subset of sequences retrieved from Reed Bed 6 (RB6). However, this would be due to the fact the primers utilised in this study to capture AOB diversity (Rotthauwe *et al.*, 1997) cover soil/freshwater species only (β-proteobacteria). Despite this, it seems apparent that the salt concentrations would be too low (see section on '**4.1.3 Salinity**') for marine species to thrive.

4.1.1 Ammonia concentration

Nitrosomonas have been found in sewage disposal plants, eutrophic freshwater and brackish water (Pommerning-Roser and Koops, 2005) indicating that some representatives of this genus can withstand high levels of ammonia, up to >400 mM. This is consistent with the fact that 100% landfill leachate at the study site has measured at most 70.4 mM, which is well within the tolerated range. In contrast, although *Nitrosospira* sequences have been found in soils with high ammonium concentrations, many *Nitrosospira* representatives are inhibited by much lower concentrations of ammonium than *Nitrosomonas*. Other soil *Nitrosospira* strains have been reported to tolerate a range between 7-50 mM of NH_4^+ (Prosser and Nicol, 2012). In general, AOA are much more sensitive to high concentrations of ammonium than AOB and are often found in low nitrogen environments, such as oceans and river sediments, although they are found also in farmlands (Yin *et al.*, 2012). Although many AOA are sensitive to high levels of ammonia, some *Nitrososphaera* strains can tolerate up to 20 mM NH_4^+ and the recently described genus *Nitrosocosmicus* is able to tolerate much higher levels of ammonium >100 mM (Mussman *et al.*, 2011; Sauder *et al.*, 2016). However, at these high substrate levels *Nitrosocosmicus* becomes inhibited and growth rates can be reduced to as much as 60%, which is assumed to be due to the greater substrate affinity many AOA have compared to AOB. In Reed Bed 6 (RB6) the leachate was diluted to 60% of the original strength (42.4 mM NH_4+ maximum) and it would be interesting to explore which genera of AOB and AOA are dominant in BioBed 12 (Bb12) which was fed 100% landfill leachate (70.4 mM NH_4+). Presence of AOA in high ammonia environments is not completely unprecedented as Yin and

colleagues (2012) observed AOA in wastewater with up to 241.6 mg L^{-1} NH_4^+ and found no correlation between low NH_4^+ concentrations and AOA abundance.

4.1.2 Oxygen concentration

AOA studied to date have a higher affinity for oxygen than AOB and AOB have a higher affinity for oxygen over nitrite oxidising bacteria (NOB) (Yin *et al.*, 2018). Therefore, it seems likely that AOA would be competitive in environments with low dissolved oxygen levels. Indeed, Park and colleagues (2010) suggested that dissolved oxygen is more important to preferential growth of AOA over AOB than the substrate concentration. To test O_2 concentration effects sampling of the bottommost anaerobic layer of RB6 would need to be perfected and testing of *amoA* primers on samples from Bb12 which below the water table is most likely anaerobic.

4.1.3 Salinity

No marine species of either AOB or AOA were observed however the primers utilised for AOB (Rotthauwe *et al.*, 1997) cover soil/freshwater species in the β-proteobacteria phylogeny only. Still it can be surmised this is still likely due to the salinity being lower than that typical for marine environments: 100% leachate measured at a salinity of 9,000 ppm maximum compared to the 36,000 ppm typically measured in sea water. In addition, all AOA genera returned by the *CrenamoA*-23F/616R primer pair were all of soil/freshwater species. This is speculation however and it would be best to use primers that have better coverage of all AOB genera. *Nitrosomonas eutropha* can withstand a salinity of up to 400 mM and marine species are found in systems with a salinity greater than 400 mM (Yamamoto *et al.*, 2010). *Nitrosospira* can tolerate salinities of up to 500 mM and landfill leachate at its highest concentration was measured at 154 mM so these microorganisms can easily tolerate the salinity of the system (Yamamoto *et al.*, 2010). During the summer water evaporates and the constructed wetland systems often dry out becoming highly saline. It is unclear how high the

salinity becomes under these conditions and this is pending further investigation (Yamamoto *et al.*, 2010). In wastewater treatment sites, most sequences are related to AOB *Nitrosomonas* or *Nitrosospira*. In a saline nitrification reactor, under low DO and high nitrogen loading, all sequences detected were affiliated to the *Nitrosomonas* genus (Ye and Zhang, 2011). It is interesting to note that, especially at its extremes, moisture plays a role for both salinity and oxygen availability and is a topic for future investigation. Optimal moisture is important for ammonia oxidisers. For instance, AOB and AOA abundances decreased in rice fields with low moisture and in grasslands with increasing soil moisture (Yin *et al.*, 2018; Chen *et al.*, 2008). It is also interesting to note that when constructed wetlands were sampled in spring (03/2019) as opposed to winter (01/2019), *amoA* PCR amplification appeared weaker when estimated qualitatively on an agarose gel. While this observation is pending confirmation by qPCR, it could suggest seasonal variation in the abundance of aerobic ammonia oxidisers due to temperature or moisture and warrants future investigation.

4.1.4 pH of the constructed wetlands

All genera detected in this study are generally found at a pH within a range of 6-8.5 for both AOB and AOA. Many AOA have also been found in acidic soils (4.0-7.0) (Yin *et al.*, 2018), but *Nitrosomonas* generally prefer slightly alkaline soils (Pommerning-Roser and Koops, 2005). As the pH in the landfill leachate is slightly alkaline (generally around 7.5-7.8) (Morling, 2007), this is perfectly within the range that these ammonia oxidisers can adapt to, meaning that AOA do not have a competitive advantage in these conditions (Pommerning and Koops, 2005).

4.1.5 Temperature

AOB are usually found to dominate in mesophilic environments with high ammonium, consistent with the constructed wetlands trials. AOA are found across a wider range of temperatures than AOB, from polar environments with temperatures of 0.2^{o}C to hot springs with temperatures of 74^{o}C. *Ca.*

Nitrososphaera gargensis a slightly thermophilic AOA was found to be the most dominant species in cattle manure composting process (Yamamoto *et al.*, 2010). Seasonal temperature changes are associated with a decrease in AOB abundance in landfill leachate and this trend is particularly pronounced in the raw leachate before it has been fed into the constructed wetlands. This is interesting given that the AOB *Nitrosomonas* can be found in a minimum temperature of 5ºC. This is corroborated by a previous study by Sims and colleagues (2012), who observed that AOB were more sensitive to low temperatures in constructed wetland systems for wastewater treatment than AOA and the AOB community changes were not reflected in the efficiency of ammoniacal nitrogen removal (96% in summer and 93% in winter). NO_2^- levels however increased from 4 mg L^{-1} to 11 mg L^{-1} from winter to summer. A similar trend has been observed in the NCC trials with elevated temperatures, although not explicitly in the summer months. In agreement with this, another study reported a decrease in the AOB abundance in cold temperatures while AOA abundance remained unchanged throughout seasons (Yin *et al.*, 2018). In addition to pH, salinity, ammonia concentration and temperature, it is likely that there are other environmental parameters which affect the microbial community composition in the constructed wetlands, e.g. heavy metals. Archaeal respiration is based on copper-based electron-transport, unlike the iron-heme-dependent AOB (Walker *et al.*, 2010). In the constructed wetland system studied in this work, AOA and AOB were unlikely to be copper or iron-limited. Iron concentrations varied between 2,680 µg L^{-1} and 15,200 µg L^{-1} and copper remained constant at approximately 5 µg L^{-1}. There is insufficient data to speculate whether toxicity by either of these metals or other metals played a role in shaping the ammonia oxidising communities in these constructed wetlands.

4.2 Detection of anammox organisms

This study optimised and used two functional marker gene primer sets, *hzsA* and *hzo*, to determine anammox diversity in the constructed wetlands. Both primer sets confirmed that anammox bacteria were present in all layers of the two constructed wetland trials studied. It is interesting and surprising that

anammox microorganisms were found in all layers of the constructed wetlands, even the unsaturated layer which was assumed to be aerobic, although previous studies suggest that anammox could be found in aerobic environments, e.g. chalk sediment (Lansdown *et al.*, 2016) indicating a requirement for an interface between anoxic sediments and aerobic waters for access to a nitrite supply as clay sediments which are much less porous than chalk did not contain anammox bacteria. These are important findings, in that anammox bacteria are present, however amplicon sequencing would be required to confirm if the trends seen in this study can be replicated and RNA studies in RT-qPCR to gain an understanding of which of the genera are most active in NH_3 removal. This will build upon what has been found in this study to begin optimising the anammox process in this set up.

Anammox diversity was consistent when evaluated with both functional marker genes, suggesting that both primer sets were robust and suitable for studying anammox. Sequencing of the *hzs* clones derived from both the unvegetated Bb12 and the vegetated RB6 (which was planted with *Phragmites australis*), indicated that there were no obvious differences in the anammox community between the two trials. The majority of *hzs* sequences were affiliated to the genus *Kuenenia.* This is in agreement with a previous study (Humbert *et al.*, 2010), where *K. stuttgartiensis* was found in freshwater lakes, rhizosphere soil and often in wastewater with high ammonium concentrations. Intriguingly, most other anammox *hzs* sequences fell into a distinct uncultivated clade related to *Kuenenia*. Sequences of this uncultivated clade were previously observed by Humbert and colleagues (2010) in a salt marsh. These *hzs* sequences were of 95% similarity to *Kuenenia* and have been found to co-occur with *Kuenenia*. One sequence from the saturated aerobic layer in Reed Bed 6 (RB6) was most closely related to *Jettenia caeni*.

Reassuringly, the majority of *hzo* sequences were also related to genus *Kuenenia* in all layers of RB6 and most layers of Bb12. However, in the interface between the unsaturated aerobic layer and the saturation zone in the unplanted Bb12, all sequences were most closely related to genus

Brocadia. This is an important observation as the bias of the *hzs* primer set against *Brocadia* was demonstrated in this thesis. This bias is likely to be the reason why *Brocadia*-related sequences were not obtained from this same sample using the *hzs* primers. This study thus underlines the need for thorough scrutiny and optimisation of primers for molecular ecology surveys. Nevertheless, with the exception of this sample, the results from the two functional gene primer sets were consistent with one another. This finding however could also indicate a difference in the environments between the vegetated and unvegetated constructed wetlands as *Brocadia* was not found with either primer set in RB6 and the uncultrivated clade with a 95% similarity to *K. stuttgartiensis* was not found in Bb12, perhaps due to the lack of vegetation that would usually be found in a salt marsh.

4.3 Environmental drivers of anammox diversity

On a global scale, *Brocadia* is the most dominant of all anammox in natural ecosystems, followed by *Jettenia*. *Brocadia*, *Jettenia* and *Kuenenia* and are the most common anammox genera in terrestrial, freshwater systems and man-made wastewater treatment systems, likely due to their metabolic versatility and their tolerance against certain environmental parameters, particularly high ammonium concentration (Wang *et al.,* 2019). *Brocadia* is usually found in engineered environments and has a lower affinity for ammonia and nitrite and a higher tolerance for O_2 than other characterised anammox bacteria. Especially the oxygen tolerance could explain why sequences affiliated to *Brocadia* were found in the interface of Bb12 where O_2 is likely to be higher than anywhere else in the saturated zone but not appearing in the unsaturated zone itself as the genus still requires a certain level of moisture. *Brocadia* and *Kuenenia* often co-occur in the environment as they appear to in Bb12 with both genera having high ammonia and O_2 tolerance than that of other anammox bacteria genera (Wang *et al.*, 2019). In a previous study, anammox showed a negative correlation with soil depth (Sonthiphand *et al.,* 2014) and where the sediment is not porous enough (Lansdown *et al.*, 2016) likely due to a very low oxygen concentration, which would have restricted the activity of AOB and AOA and therefore the nitrite

supply. This suggests that in order to couple nitrification and anammox, there is a need for abundant oxic-anoxic interfaces (Lansdown *et al.*, 2016). The area of such interfaces is higher in the presence of plant roots in RB6 (in this study all sequences in association with the roots were found to be related to *Kuenenia*), compared to the unvegetated Bb12 and it would be prudent to determine number and activity of anammox in these two constructed wetland systems by qPCR, amplicon sequencing and RT-qPCR.

Terrestrial genera, such as *Kuenenia* and *Brocadia* are more versatile in their metabolism than *Scalindua*. They contain the genes necessary for utilisation of certain inorganic carbon for additional energy/electron source (i.e. acetate, and propionate) (Kartal *et al.,* 2013). However, *Brocadia* lacks the ability to perform dissimilatory nitrate reduction to ammonium, whereas the *Kuenenia* genomes contain a nitrate reductase, which can be used to produce nitrite for the anammox metabolism (Kartal *et al.*, 2013). Representatives of *Brocadia* and *Kuenenia* have also been suggested to perform anaerobic respiration of iron and manganese oxides – which are plentiful in the landfill leachate (Kadlec and Wallace, 2009; Morling, 2007). These attributes may influence the diversity of the anammox community in the constructed wetlands.

Soil moisture level is assumed to be an important driver of the anammox community (Humbert *et al.*, 2010; Wang *et al.*, 2019). In wetland systems however, diversity can decrease as water can diffuse local environmental gradients decreasing the effects above. It may be the case that running water through the system from the top periodically disturbs the gradients thought to be essential to anammox function, perhaps a fill-and-drain mechanism whereby the wetland is filled then left to drain would improve the efficacy of the system (Kadlec and Wallace, 2009). Temperature is thought to be less important (Sonthiphand *et al.,* 2014). Anammox have a moderately high affinity to NH_4+, similarly to AOA, 7 uM for NH_4^+ and 5 µM for NO_2^- for *Brocadia* and 0.2-0.3 µM with NO_2- for *Kuenenia*. The affinity of anammox bacteria for nitrite is higher than that of NOB and anammox would be efficient at out-competing NOB and denitrifiers (Wang *et al.*, 2019).

Marine areas are found to be dominated by *Scalindua* and diversity increases with the heterogeneous nature of soil, when compared with coastal waters. *Kuenenia* and *Brocadia* are negatively correlated with salinity (Wang *et al.*, 2019). In agreement with the results for aerobic ammonia oxidisers, anammox organisms detected in the constructed wetland trials belonged to freshwater and terrestrial genera. It is likely that the salinity of the constructed wetlands is too low to select for marine ammonia oxidisers and anammox bacteria.

As with AOA and AOB, there was a weaker PCR amplification product obtained with anammox primers from the samples acquired from the top unsaturated aerobic layer in spring that were estimated qualitatively on agarose gels. Although this finding still requires confirmation by qPCR, a potentially lower abundance of anammox in the top-most layer of the constructed wetlands could be related to the lack of ponding at the time of sampling. During sampling in the winter, the trial beds were ponding and the top layer of the constructed wetland. which was water-logged. yielded bright PCR bands. In a previously studied aquifer system, most anammox activity occurred in the saturated soils, likely due to low oxygen in these environments and the moisture requirements of these microorganisms (Wang *et al.,* 2019). The same study also reported that anammox bacteria were undetectable in unsaturated soils until upwelling of groundwater soaked these soils and activated anammox bacteria in these layers (Wang *et al.*, 2019).

4.4 Conclusions

In this thesis, it was hypothesised that the microorganisms responsible for the process of ammonia removal (ammonia oxidizing archaea/bacteria in conjunction with anammox bacteria) would be detected in the soil matrix retrieved from the constructed wetland trials. This was indeed the case however it was also hypothesised that the contrasting environments in: moisture both above and below the water table and between a vegetated and unvegetated constructed wetland would indicate two very distinct

microbiology communities however this was not observed. To prove the hypotheses two functional marker gene PCR primers sets targeting anammox were successfully optimised (Aim 1). This enabled investigations into anammox organisms in the NCC constructed wetland trials at Mayton Wood. The presence of anammox bacteria in two of the wetland trials was confirmed using the newly optimised PCR primer sets (Aim 2).

All primer sets (*amoA, hzsA* and *hzo*) indicated that there was no difference in the microbial diversity between the different layers of the constructed wetland (Aim 3). Furthermore, no difference was observed between the vegetated and unvegetated wetland trials studied in this thesis except for the presence of the genus *Brocadia* in the interface of Bb12 and the lack of the uncultivated clade of 95% similarity to *Kuenenia* in Bb12 that was seen in RB6. Is was quite surprising given the differences between moisture content in the different layers of the wetland however that differences were not observed between the saturated and unsaturated layers. Previously published work also reported changes to aerobic ammonia oxidising communities in rice paddy soils in response to vegetation (Chen *et al.,* 2008). In addition, Yang and colleagues (2014) observed a shift in the ammonia oxidising communities between vegetated and unvegetated marshes.

This could be an indication that the constructed wetland does not influence diversity however a greater number of sequences would need to be tested by amplicon sequencing to gain a greater understanding of the microbial community. The results in this study alone indicates the constructed wetland is most likely seeded by landfill leachate which already contains these microorganisms or the microorganisms could have been introduced in the matrix when the wetland was constructed. Without testing the landfill leachate, and original compost and sand, it is difficult to speculate how the abundances of key microbes have changed. Regardless of how the microorganisms were introduced, it is interesting to see no obvious selection of the community by the factors studied in this work. One caveat of this work is that only a small subset of clones were sequenced. PCR amplicon sequencing with the functional marker genes and general 16S primers would

provide a much greater depth of sampling and a better resolution of the microbial communities and should be performed in future. In addition, this study only demonstrated the presence of the organisms but not their activity. Although sequence diversity may remain constant across the wetland, some microbial clades may be more active in certain parts of the wetland. Therefore, RT-qPCR should be carried out in the future to determine the transcriptional activity of these microorganisms.

4.5 Limitations of the work

Due to the small subset of sequences sampled in this study it is hard to determine any meaningful trends from the data and therefore the results are reviewed speculatively in this thesis. In the section '**4.6 Future work**' it is determined how to expand upon the results and confirm any speculations made. It would be prudent to perform amplicon sequencing, utilising the protocols put in place from the optimisations performed on *hzsA* and *hzo* primers to cover anammox bacterial diversity. General 16S primers designed to cover both bacterial and archaeal 16S rRNA sequences would be useful to determine the general microbiological community within the constructed wetland trials, giving an overall representation of abundances of AOB, AOA and anammox bacteria in relation to one another and the wider microbiological community. Primer set 16S 515F/806R (v4) (Walters *et al.*, 2015) tested *in silico* in the SILVA 16S database (https://www.arb-silva.de/) indicated even coverage of bacterial AOB and the *Thaumarchaeota* and *Planctomycetes* phyla covering AOAs and anammox bacteria respectively. Lastly, using marker gene primers *amoA* for AOB and AOA to determine the abundances of nitrite suppliers and how this affects anammox abundances. In the case of AOB *amoA* primers, it would be necessary to find those that cover terrestrial, fresh water and marine species to confirm whether marine species are indeed missing from the constructed wetlands trials. Although it was speculated in the '**4.1.3 Salinity**' section, that perhaps as no marine AOA species were found, that it was likely that there would be no marine AOB present either.

Further testing by RT-qPCR would be utilised after confirming the microbiological community within these constructed wetland trials to determine the most active species involved in nitrogen cycling. Lastly, it would be sensible to perform process measurements on the soil matrix in the constructed wetland trials themselves – as these measurements were only performed on the landfill leachate (100%). Firstly, to determine if there is a difference in environmental factors such as pH, salinity, temperature etc. between the unsaturated and saturated levels of each wetland and between the vegetated and unvegetated constructed wetlands, which would be expected. Secondly, it would be useful to determine process measurements at the time of sampling for a snapshot of the conditions and microbiological community associated with this. Both the information garnered from RT-qPCR studies and process measurements can lead to a further understanding of the conditions required to enhance anammox and nitrogen cycling performance and therefore NH_3 and total nitrogen removal.

4.6 Future work

With the molecular tools in place and with the knowledge that the anammox bacteria are present in the wetland trials, there are several future steps which should be taken to further understand the microbial ammonia conversions in the trials at Mayton Wood. This will help optimise and enhance the performance of the constructed wetlands and feed information into scaling up the process. The following should be carried out as a priority:

1. Determination of microbial (AOB, AOA, anammox) abundances by qPCR to validate trends and observations from this work.
2. Amplicon sequencing to sample microbial diversity at greater depth.
3. Process measurements using ^{15}N isotopic tracers.

It will be crucial to link the molecular tools to process measurements as this will provide very robust evidence on both the identity of anammox organisms and their contribution to the ammonium removal. There are a plethora of other future experiments which could help fine-tune the performance of the

constructed wetlands. Measurement of dissolved oxygen levels in the constructed wetlands would be vital in finding the balance to achieve partial nitrification-anammox. It would be interesting to further examine the effect of plants by studying the microbial communities in the rhizosphere. Previous studies have found a selection of specific ammonia oxidisers in the rhizosphere (Chen *et al.*, 2008). Although in this study no difference was observed in the anammox diversity between vegetated and unvegetated constructed wetlands, aerobic ammonia oxidisers were not examined in the unvegetated trial but were found in the bottommost layers of the constructed wetland. Plant roots could potentially provide an important niche of coupling aerobic ammonia oxidation and anammox by creating a microaerophilic environment. Furthermore, it would be useful to investigate the causes of ponding and explore potential other matrices for constructing wetlands. One potential way to enhance the performance of the wetlands is seeding by activated sludge as previous described (Zhu *et al.*, 2011). While there are many adjustments that can be made in the future, the work in this thesis has laid a solid foundation for the future work and demonstrated both the suitability of the molecular tools and the presence of anammox bacteria in the trials.

5. List of Abbreviations

AMO	Ammonia monooxygenase
AmoA	Ammonia monooygenase alpha subunit
Anammox	Anaerobic ammonia oxidisation
AOA	Aerobic oxidising archaea
AOB	Aerobic oxidising bacteria
AOM	Aerobic oxidising microorganism
Bb	Biobed
BNR	Biological nitrogen removal
BOD	Biological oxygen demand
Bp	Base pair
Cl-	Chloride
COD	Chemical oxygen demand
CW	Constructed wetland
DNA	Deoxyribonucleotide acid
FWS	Free water surface
HDH	Hydrazine dehydrogenase
HSSF	Horizontal subsurface flow
HZO	Hydrazine oxidoreductase
HZS	Hydrazine synthase
hzsA	Hydrazine synthase alpha subunit
N_2	Dinitrogen gas
N_2O	Nitrous oxide
NaCl	Sodium chloride (salt)
NaOCl	Sodium hypochlorite (bleach)
NCC	Norfolk County Council
NH_2OH	Hydroxylamine
NH_3	Ammonia
NH_4+	Ammonium
NH_4+-N	Ammoniacal nitrogen
NIR	Nitrite reductase
NO	Nitric oxide
NO_2-	Nitrite
NO_3-	Nitrate
NOB	Nitrite oxidising bacteria
O_2	Oxygen
OTU	Operational taxonomic unit
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
RB	Reed bed
RFLP	Restriction fragment length polymorphism
SBR	Sequencing batch reactor
SOB	Super Optimal Broth
TBE	Tris/borate/EDTA buffer
TOC	Total organic carbon
TON	Total organic nitrogen
VF	Vertical flow

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7. Appendices

Table 7.1 *Chemistry of raw leachate in January (22/1/19)*
** These are measured monthly – closest measurement 5/2/19*
*** Last measurement by National Laboratory Service on 4/9/18*

Chemical	**Concentration**
Ammoniacal Nitrogen (NH_4+-N)	937 mg L^{-1} N
Biological Oxygen Demand	93 mg L^{-1} O *
Chemical Oxygen Demand	1390 mg L^{-1} O *
Chloride	1450 mg L^{-1} Cl
Conductivity	12960 µS cm^{-1} *
Nitrite	0.5 mg L^{-1}
pH	7.69 *
Total Organic Carbon	344 mg L^{-1} C **
Total Oxidised Nitrogen	0.3 mg L^{-1} N

Table 7.2 *Chemistry of raw leachate in March (12/3/19)*
** These are measured monthly – closest measurement 5/3/19*
*** Last Measurement by National Laboratory Service on 4/9/18*

Chemical	**Concentration**
Ammoniacal Nitrogen (NH_4+-N)	1120 mg L^{-1} N
Biological Oxygen Demand	119 mg L^{-1} O *
Chemical Oxygen Demand	1310 mg L^{-1} O *
Chloride	1530 mg L^{-1} Cl
Conductivity	13750 µS cm^{-1} *
Nitrite	<0.1 mg L^{-1}
pH	7.66 *
Total Organic Carbon	344 mg L^{-1} C **
Total Organic Nitrogen	0.3 mg L^{-1} N