^{15}N STABLE ISOTOPE PROBING OF PULP AND PAPER WASTEWATERS

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ABSTRACT

Stable isotope probing (SIP) is an established technique that can be applied to identify the metabolically active micro-organisms within a microbial population. The SIP method utilises an isotopically-labelled substrate and PCR techniques to discern the members of a microbial community that incorporate the isotope into their DNA or RNA. The current literature gap around using ^{15}N isotopes with RNA-SIP offers real potential and advantages for targeting and identifying active members from mixed communities involved in global biogeochemical nitrogen cycling.

This study specifically investigated whether nitrogen based compounds can be used as substrates in RNA-SIP methodologies and whether they can in turn be used to probe mixed community environments known to be actively fixing nitrogen. The nitrogen-limited systems targeted represented an ideal opportunity to assess the suitability of ^{15}N-RNA-SIP approaches due to their known high nitrogen fixation rates. Identifying these nitrogen-fixing bacteria could provide a better representation analysis of the community, leading to an improved prediction on how to manage and optimise the treatment performance of target waste systems and to exploit the unique bioconversion properties of these types of organisms.

Initially, the project undertook methodological proof of concept by using a soluble nitrogen source, $^{15}NH_4Cl$, to label the RNA of *Novosphingobium nitrogenifigens* and a mixed microbial community. Successful separation of the ^{14}N- (control) and ^{15}N-RNA was achieved for both pure and mixed communities using isopycnic caesium trifluoroacetate (CsTFA) gradients in an ultracentrifuge. The usefulness of this technique to identify active diazotrophs in real environmental samples was tested using a nitrogen-fixing community from a pulp and paper wastewater treatment system. After growing the mixed culture with $^{15}N_2$ as the sole nitrogen source, the labelled RNA was extracted and fractionated using isopycnic centrifugation in CsTFA gradients.

The community composition of the active nitrogen-fixing community in the $^{15}N_2$ enriched fraction was analysed by establishing a 16S rRNA gene clone library containing over 200 members. These were analysed by comparison with published sequences and by phylogenetic analysis.

It was found that the more isotopic label substrate incorporated, the further the buoyant density (BD) separation between ^{15}N- and ^{14}N-RNA. *Novosphingobium nitrogenifigens* gave an average BD shift of 0.03 $\pm$ 0.004 g ml^{-1} (95.0 atom % ^{15}N) with $^{15}NH_4Cl$. For mixed communities the average BD shift was 0.02 $\pm$ 0.004 g ml^{-1} (80.0 atom % ^{15}N) with $^{15}NH_4Cl$ and 0.013 $\pm$ 0.002 g ml^{-1} (32.6 atom % ^{15}N) when using $^{15}N_2$. Clone library analysis of 16S rRNA genes present in the enriched ^{15}N-RNA fraction of the mixed community was shown to consist of a diverse population of bacteria as indicated by a Shannon Weaver index value of >2.8. Three dominant genera (*Aeromonas, Pseudomonas* and *Bacillus*) were identified by comparison with published sequences and phylogenetic analysis. Many other groups not known as archetypal nitrogen-fixing bacteria were also identified, demonstrating that $^{15}N_2$-RNA-SIP provides a useful tool for the identification of important and previously unknown contributors to nitrogen fixation in a range of environments.

Overall, this project has established that nitrogen based RNA-SIP is a powerful tool that can be used successfully and reproducibly with both pure and complex mixed microbial communities to study active diazotrophs in environmental samples.

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Borat likes very much!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ASB	Aerated stabilisation basin
BD	Buoyant density
BOD	Biological oxygen demand
bp	Base pair
BNF	Biological nitrogen fixation
C/N	Carbon to nitrogen ratio
CsCl	Caesium chloride
CsTFA	Caesium trifluoroacetate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNA-SIP	Deoxyribonucleic acid stable isotope probing
FISH	Fluorescent *in situ* hybridisation
FISH-MAR	Microautoradiography
IRMS	Isotope ratio mass spectrometry
$\mathbf{N_2}$	Dinitrogen
$\mathbf{^{14}N_2}$	Unlabelled dinitrogen
$\mathbf{^{15}N_2}$	Heavy labelled dinitrogen
NLMM	Nutrient limited minimal media
Nr	Total reactive nitrogen
OD	Optical density
OTU	Operational taxonomic unit
PAT	Phylogenetic assessment tool
PCR	Polymerase chain reaction
PLFA-SIP	Phospholipid fatty acid stable isotope probing
PPW	Pulp and paper wastewater
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNA-SIP	Ribonucleic acid stable isotope probing
RT-PCR	Reverse transcriptase polymerase chain reaction
SIP	Stable isotope probing
SSU rRNA	Single stranded ribosomal ribonucleic acid
T-RFLP	Terminal restriction fragment length polymorphism
T-RF	Terminal restriction fragment

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 BIOLOGICAL WASTEWATER TREATMENT SYSTEMS

Wastewater from many pulp and paper mills, municipal sewage plants and other carbon rich effluents are commonly treated using biological treatment. Biological treatment involves the use of microbial populations to convert the material within the waste streams, therefore decreasing the toxicity and reducing the biological oxygen demand (BOD) before discharging into receiving waters (Smith *et al*., 2003; Yu and Mohn, 2001). Bacteria are primarily responsible for undertaking this conversion, although also present are algae, protozoa, viruses and fungi. Biological treatment can be achieved through a large number of configurations, including aerated stabilisation basins, anaerobic treatment systems, activated sludge and sequencing batch reactors (Dionisi *et al*., 2006; Gauthier *et al*., 2000; Slade *et al*., 2004a).

The maintenance of bacteria in treatment systems depends on the concentrations of essential nutrients for growth including carbon, nitrogen and phosphorus. Nitrogen is necessary for providing bacteria with building blocks for the synthesis of cell material. Pulp and paper wastewaters are high in carbon but low in available fixed nitrogen due to the naturally occurring high C/N ratio typical for wood (Gauthier *et al*., 2000).

Within the pulp and paper industry the most commonly used wastewater treatment system is in the form of an aerated lagoon, also known as an aerated stabilisation basin (ASB). These types of systems contain lower concentrations of nitrogen and phosphorus than conventionally recommended for aerobic treatment systems (Clark *et al*., 1997). Aerated stabilisation basins are operated without extra nutrient addition under high carbon, low nitrogen conditions and rely on either settled biomass feeding back soluble nutrients or the fixation of atmospheric nitrogen (Slade *et al*., 2004a).

In nitrogen limiting environments, especially pulp and paper wastewaters, the ability of nitrogen-fixing micro-organisms to provide their own nitrogen enables them to have a competitive advantage over those micro-organisms that cannot. The survival and effectiveness of the mixed microbial communities in the biological treatment of high C/N ratio wastewaters therefore becomes dependent on the community's overall ability to fix nitrogen.

Clark *et al*. (1997) suggested that under certain circumstances, atmospheric nitrogen fixation by bacteria allow ASB systems to operate effectively without any additional nitrogen supplementation. The N_2 fixed in these lagoons was shown to be more than 600 kg of nitrogen day^{-1}, capable of supplying the entire nitrogen requirements of the system.

Therefore, in order to keep operating costs down, the natural nitrogen-fixing micro-organisms within such a treatment system could be actively utilised and manipulated to help improve and manage the process. In typical ASB wastewater biological treatment systems the treatment performance, and thus the microbial community, could be controlled by the regulation of conditions of dissolved oxygen, overall nutrient concentrations and pH.

1.2 ROLE OF BIOLOGICAL NITROGEN FIXATION IN PULP AND PAPER WASTEWATER TREATMENT SYSTEMS

N-ViroTech® is a novel treatment process developed at Scion, Rotorua (previously Forest Research), which is designed to utilise communities of nitrogen-fixing bacteria to treat nitrogen limited wastewaters. It relies on the manipulation of growth conditions within the biological system to maintain a nitrogen-fixing population while still removing large amounts of BOD. Nitrogen fixation is an important ecological process because it makes atmospheric dinitrogen available for cycling to relieve nitrogen limitation in the ecosystem (Zehr *et al*., 2003). Biological nitrogen fixation requires readily available and utilisable carbon substrates for energy sources, low fixed nitrogen concentrations and low dissolved oxygen concentrations.

High nitrogen can repress nitrogen fixation while high dissolved oxygen concentrations impose considerable constraints on the nitrogenase enzyme which is highly oxygen sensitive (Dixon and Kahn, 2004; Slade *et al*., 2004b).

Advantages of the N-ViroTech® process include the elimination of nitrogen supplementation, reducing costs within the system; self-regulation of nutrient requirements by the bacteria; and improved environmental performance because of reduced nutrient loadings in the final effluent being discharged (Slade *et al*., 2004a; Slade *et al*., 2004b). The N-ViroTech® process ensures high carbon, low nitrogen and low dissolved oxygen conditions, making an amicable environment for nitrogen-fixing microbial communities to prosper.

The operation and optimisation of nitrogen-fixing biological treatment systems requires an understanding of the key micro-organisms involved in the nitrogen fixation process. A thorough knowledge of the ecology of the micro-organisms is required to reveal factors that influence the efficiency and stability of wastewater treatment plants for developing improved treatment process performance strategies. Identification and quantification of these key organisms requires the use of a variety of molecular microbiology techniques.

Previous attempts at Scion to identify and characterise the diversity of nitrogen fixers in nitrogen-fixing biological treatment systems have relied on culturing and/or DNA based molecular biology. Gene amplification, 16S rRNA (bacterial identification signature gene) and *nifH* (codes for the nitrogenase nitrogen fixation protein) gene clone library construction, and genetic diversity studies have been used. 16S rRNA gene clone libraries have shown that a large diversity of bacteria present in nitrogen-fixing biological treatment systems (Reid *et al*., 2008). However a *nifH* clone library to investigate the diversity of *nifH* in a wastewater treatment system showed that the community that possessed *nifH* had a high density of *nifH* sequences which were of low diversity (Bowers *et al*., 2008). Construction of clone libraries only inferred that nitrogen fixation was able to be carried out by certain bacteria through the presence of *nifH*, but it was not able to identify the active nitrogen-fixing bacteria from a mixed community.

1.3 STABLE ISOTOPE PROBING

Culture-dependent methods may identify <3% of the total bacterial population (Amann *et al*., 1995). Therefore, culture-independent methods need to be used to give a more accurate representation of the bacterial species in a mixed population linked to a specific characteristic, in this case, nitrogen fixation. Bacterial population diversity can be assessed by using methods such as cloning, Terminal-Restriction Fragment Polymorphism (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescent *in situ* Hybridization (FISH). The use of such DNA based techniques provides an estimation of the genetic potential of the microbial community but does not identify those bacteria actively undertaking the targeted process of nitrogen fixation, the conversion of N_2 to NH_3.

The technique of Stable Isotope Probing (SIP) has been highlighted as a means of identifying those bacteria responsible for nitrogen fixation using DNA or RNA based SIP (Buckley *et al*., 2007b). SIP provides a culture-independent method of discovering populations that perform a specific metabolic process in certain environments (Cebron *et al*., 2007). A substrate enriched in a rare stable isotope is added to an environmental sample and the micro-organisms that metabolise the substrate will incorporate the isotope into their biomass. This enriched biomass can then be separated out and identified based on the analysis of labelled biomarkers (Dumont *et al*., 2006). SIP involves extracting and separating the labelled 'heavy' constituents from the unlabelled counterparts on the basis of buoyant density (Buckley *et al*., 2008). This gives an alternative method to link taxonomic identity with function, which is independent of culturing methods.

SIP can be used to label both DNA and RNA and subsequent analyses are dependent on the aims of the experiment. DNA-SIP was first introduced by Radajewski *et al*. (2000), and the protocol has only undergone slight modifications during the past five years. Both DNA-SIP and RNA-SIP have utilised C-isotopes, however N-isotopes have only been used in a few recent DNA-SIP experiments (Buckley *et al*., 2007a; Buckley *et al*., 2007b; Buckley *et al*., 2008; Cadisch *et al*., 2005; Cupples *et al*., 2007). Examples that use N-isotopes for RNA-SIP are not currently present in the literature.

Limited numbers of studies have attempted to explicitly identify the active nitrogen-fixing micro-organisms from communities present in wastewater treatment systems. This proposal outlines an innovative approach to separate and identify the nitrogen-fixing micro-organisms amongst mixed communities using the RNA-SIP method. The high nitrogen fixation rates observed in pulp and paper wastewater treatment processes made these systems ideal models for demonstrating the potential of a nitrogen-based RNA-SIP methodology.

1.4 RESEARCH OBJECTIVE

To develop and test a RNA-Stable Isotope Probing (RNA-SIP) method using nitrogen based substrates to detect and identify active nitrogen-fixing bacteria from pulp and paper wastewaters. This was undertaken using the following research approach:

1. Optimise methodology to label and separate ^{14}N- and ^{15}N-RNA from a pure culture and a mixed community sample using a caesium trifluoroacetate (CsTFA) gradient.

2. Identify active nitrogen-fixing bacteria from a $^{15}N_2$-labelled pulp and paper wastewater sample using gradient centrifugation, fractionation, cloning and sequencing.

Details of how each of these steps were undertaken are outlined in Figure 1.

^{15}N- RNA-STABLE ISOTOPE PROBING PROTOCOL DEVELOPMENT

- RNA extraction from pure cultures grown on ^{14}N
- Test CsTFA gradient with RNA from pure cultures
- Optimise CsTFA gradient to achieve best resolution of RNA fractions
- Label pure culture RNA with $^{15}NH_4Cl$
- Separate ^{14}N- and ^{15}N-RNA from pure culture through CsTFA gradients
- Investigate degree of separation between ^{14}N- and ^{15}N-RNA from a pure culture

MIXED COMMUNITY ^{15}N-RNA-SIP PROTOCOL DEVELOPMENT

- Label mixed community RNA with $^{15}NH_4Cl$
- RNA extraction from mixed cultures
- Separate ^{14}N- and ^{15}N-RNA from a mixed community through CsTFA gradients
- Investigate degree of separation between ^{14}N- and ^{15}N-RNA from a mixed culture

IDENTIFICATION OF NITROGEN FIXERS FROM $^{15}N_2$-LABELLED MIXED COMMUNITY

- Identify and collect mixed community sample from nitrogen-fixing wastewater
- Label mixed community RNA with $^{15}N_2$
- Isotope incorporation analysis of cells
- Separate ^{14}N- and ^{15}N-RNA from a mixed community through CsTFA gradients
- T-RFLP comparison between fractions from ^{14}N- and ^{15}N-RNA
- Sequencing and identification of nitrogen-fixing clones from pulp and paper wastewater

Figure 1.1 Overview of research approach.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

This introduction section reviews the current literature around nitrogen fixation, stable isotope probing (SIP), findings from previous SIP studies and the various applications of this methodology. The implications of this approach for studying nitrogen-fixing organisms are also presented.

2.2 IMPORTANCE OF BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation is a vitally important global process with the conversion of nitrogen carried out in a variety of different conditions by bacteria from most phylogenetic groups (Dixon and Kahn, 2004). The ability to fix nitrogen has been found in most bacterial phylogenetic groups including firmicutes, actinobacteria, cyanobacteria and all subdivisions of the proteobacteria (Dixon and Kahn, 2004). In the world's oceans, nitrogen fixation is commonly associated with cyanobacteria (Capone and Knapp, 2007). These results are not portrayed in recent phylogenetic analyses of nitrogen-fixing communities (Bostrom *et al*., 2007), leading to the assumption that other heterotrophic bacteria are dominating global nitrogen-fixation. Fixation is necessary to convert atmospheric dinitrogen (N_2) into forms usable by other organisms to relieve ecosystem nitrogen limitation (Zehr *et al*., 2003). Dinitrogen (N_2) fixation links with other biogeochemical cycles, such as denitrification, mineralisation and nitrification to control overall nitrogen levels in the ecosystem. Organisms capable of fixing nitrogen use N_2 and reduce it to ammonia which is then converted to the organic form of nitrogen (amino acids). Total reactive nitrogen (Nr) is composed of contributions from biological nitrogen fixation (BNF) (46-67%), atmospheric sources (lightning, 11-16%) and anthropogenic sources (22-30%) (Capone, 2008).

With the decline of fossil fuels and, therefore, petrochemically-derived fertilisers, it has become imperative that nitrogen is converted by other sources, namely BNF, to maintain the balance of global nitrogen cycling.

Original nitrogen fixation studies used acetylene reduction assays (C_2H_2) and mass balance calculations to extrapolate the values for environmental rates of nitrogen fixation. The nitrogenase enzyme in nitrogen fixers reduces acetylene to ethylene and the amount of ethylene produced is proportional to the amount of enzyme present. These results are only indicative of nitrogen fixation rates and not truly representative because only a small proportion of the community may be fixing nitrogen at any given time, making it hard to measure these very low concentrations. This method also can only be used as a measure of nitrogen fixation activity and not for the identification of the organisms from a mixed community that are responsible for nitrogen fixation. A more accurate method of establishing nitrogen fixation rates in a sample has been achieved by coupling $^{15}N_2$ enrichment and acetylene reduction assay (Morris *et al*., 1985).

Previously, noncultivated diazotrophs could only be identified through *nifH* detection. This gene codes for the key nitrogenase reductase protein involved in nitrogen fixation. Unfortunately, detection of *nifH* does not confirm phylogeny of the organism, providing very limited information that can be used in the identification and characterisation of diazotrophs (Raymond *et al*., 2004; Zehr *et al*., 2003). In the absence of alternative nitrogen sources, diazotrophs use N_2, which if substituted for $^{15}N_2$ results in the incorporation of 'heavy' labelled N for growth. $^{15}N_2$-DNA-SIP has been used to link heavy labelled 16S rRNA genes to the process of nitrogen fixation while providing a valuable technique for exploring non-cultivated diazotrophs from a range of environments separate from cultivation (Buckley *et al*., 2007b).

2.3 MICROBIAL ECOLOGY

The field of microbial ecology focuses on examining micro-organisms in their natural environments to identify which micro-organisms are carrying out specific metabolic processes (Boschker and Middelburg, 2002; Dumont and Murrell, 2005; Lu *et al*., 2005).

Culturing of organisms allows for their identification and for studies into their various functions, but often results in enrichment of pure cultures which do not accurately represent the habitat from which they were isolated. Cultured organisms only represent <3% of total bacteria and are not representative of an entire environmental sample (Amann *et al*., 1995), making it vitally important to use culture-independent methods to effectively elucidate the composition of microbial populations.

2.4 STABLE ISOTOPE PROBING (SIP)

SIP provides a culture-independent method of discovering populations that perform a specific metabolic process in certain environments (Cebron *et al*., 2007; Dumont and Murrell, 2005; Neufeld *et al*., 2007; Radajewski *et al*., 2000; Radajewski *et al*., 2003; Radajewski and Murrell, 2000; Whitby *et al*., 2005). A substrate highly enriched in a rare stable isotope is added to an environmental sample and the micro-organisms that metabolise the substrate will incorporate the isotope into their biomass and can then be separated out and identified based on the analysis of labelled biomarkers (Dumont *et al*., 2006; McDonald *et al*., 2005). The structure of labelled and unlabelled communities is resolved by analysing functional marker genes or phylogenetic genes (for example rRNA) to determine phylogeny with PCR or reverse transcriptase PCR (RT-PCR). Amplified products can be separated by denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or cloned directly for phylogenetic characterisation (Kreuzer-Martin, 2007).

SIP provides a useful tool for exploring microbial communities under *in situ* conditions. It does not require the cultivation of micro-organisms and is able to exploit the wide range of gene probes available for the characterisation of the functionally active population of micro-organisms (Radajewski and Murrell, 2000). However, it is restricted to those organisms with the highest growth rates.

SIP gives an alternative method for unambiguously linking taxonomic identity with function independent of culturing methods which only identify <3% of total bacteria. An important question for SIP to answer currently is ‘who eats what?’ but Neufeld *et al*. (2007) hypothesise that it will soon shift to ‘who eats what, where and when?’

This demonstrates the huge potential of SIP for studying not only primary organisms but uses the perceived negative aspect of cross feeding to studying compound cycling among mixed communities or through food webs.

SIP has progressed a long way over the last 10 years with the number of publications increasing significantly each year. In 2004 there were 14 papers and in 2007 this number had increased to 34 publications (ISI Web of Science). Throughout these 10 years, over 100 articles have been published with 15 of these being review articles. This highlights the significance of the information that can be discovered using stable isotope probing.

SIP involves the following experimental steps: (i) assimilation of a labelled substrate and labelling of the nucleic acids, (ii) extraction of nucleic acids, (iii) separation of the labelled nucleic acids by density gradient ultracentrifugation, (iv) phylogenetic fingerprinting, cloning/sequencing and phylogenetic analysis (Figure 2.1). Any stable heavy isotope can be used if it is integrated into the nucleic acids.

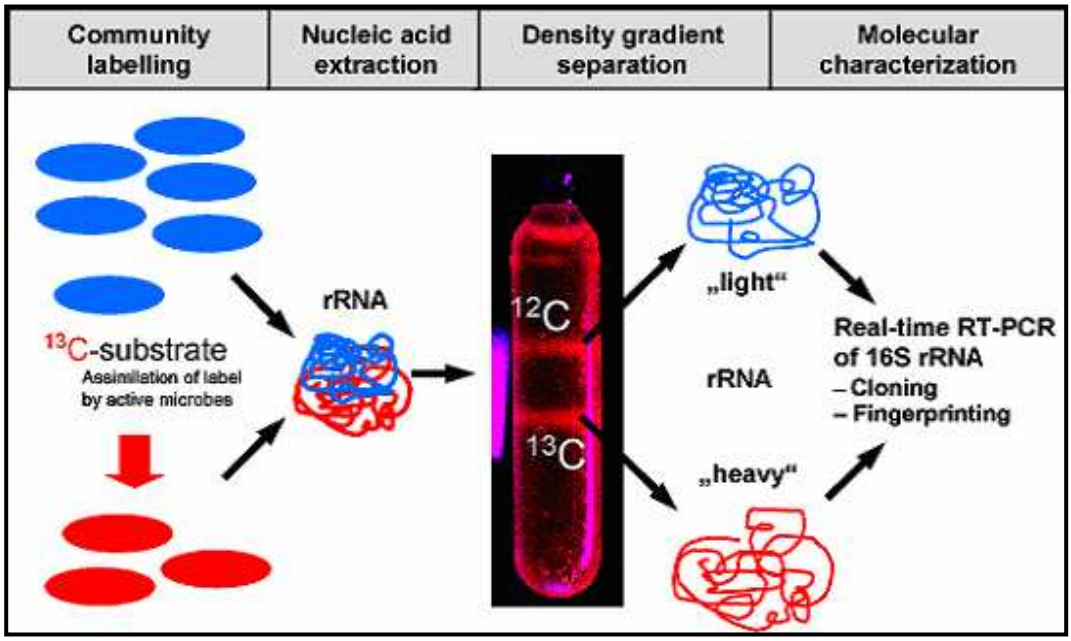

Figure 2.1. C-labelled rRNA stable isotope probing procedure.

The overall efficiency of SIP is dependent on the effectiveness and biases of each step in the process, from label assimilation to nucleic acid extraction and PCR (Kreuzer-Martin, 2007).

There are three stable isotope approaches routinely used: phospholipid fatty acid-SIP (PLFA-SIP), DNA-SIP and RNA-SIP. SIP was first introduced using PLFA to link populations to specific processes (Boschker *et al*., 1998). DNA-SIP was first introduced by Radajewski *et al*. (2000), using SIP to identify methylotrophs after enrichment with $^{13}CH_3OH$. SIP methodology has changed very little since this time with only slight modifications occurring to include RNA based SIP (Manefield *et al*., 2002b).

Stable isotopes do not undergo radioactive decay, making their nuclei stable with constant masses (Ginige, 2008). Some isotopes of low abundance occur naturally including ^{2}H, ^{13}C and ^{15}N. These isotopes are used in the same way by organisms as their higher abundant isotopes (^{1}H, ^{12}C and ^{14}N) and can therefore be substituted to link specific metabolic processes to specific organisms. When planning to use a SIP based approach for studying mixed communities it is important to carefully assess a range of factors. It is highly dependent on the environment, the substrate being utilised and the duration of the incubation (Neufeld *et al*., 2007). For example, with a short incubation, no labelling will occur whilst a long incubation could possibly lead to cross-feeding of the label being passed down the food chain beyond the primary population using the labelled substrate (Madsen, 2006).

Nucleic acids from different organisms have different buoyant densities based partially on the full % GC content of the organism and also likely due to other unknown reasons. Lueders *et al*. (2004a) presented a table of different organisms and their average buoyant densities of both DNA and SSU rRNA and the relative % GC. Buoyant densities of different organisms, especially for SSU rRNA varied greatly from 1.755 g ml^{-1} for *Pseudomonas putida* (53% GC) to 1.78 g ml^{-1} for *Methylobacterium extorquens* (55% GC). These small shifts in individual densities can increase the density spread of labelled and unlabelled DNA across a gradient, and mean that unlabelled DNA could co-occur with labelled DNA in some environmental samples.

2.4.1 DNA-SIP

The principal of DNA-SIP was first introduced by Meselson and Stahl (Meselson and Stahl, 1958), where they labelled *E. coli* on a labelled NH_4Cl media coupled with CsCl density gradient centrifugation. This paper led the way for DNA-SIP which was developed by Radajewski *et al*. (2000). DNA-SIP has been used subsequently for numerous stable isotope studies and has lead to a wide breadth of knowledge on a variety of different environments, including identifying organisms unknown for their involvement in some metabolic processes (Friedrich, 2006).

CsCl gradients can typically hold 10 μg ml^{-1} of DNA, allowing visualisation of the ^{12}C and ^{13}C bands with ethidium bromide fluorescence (Radajewski *et al*., 2000). Gallagher *et al*. (2005) found that the use of carrier DNA in their SIP experiments reduced incubation times while allowing for a quick labelling of the organisms utilising a labelled substrate present in an environmental sample.

DNA-SIP requires long incubations with the isotope of choice. Most SIP studies use conditions emulating the natural environment while avoiding long incubation periods. Often there is a compromise between the length of time necessary to achieve sufficient labelling and the possibility of cross-feeding occurring (Cebron *et al*., 2007). A prerequisite for DNA-SIP is the need for DNA synthesis and cell division, so that sufficient label is incorporated in the DNA for gradient separation (Neufeld *et al*., 2007). It has been suggested that at least two cell generations need to occur to sufficiently incorporate the label into DNA, increasing this number increases the successful fractionation of labelled DNA but can increase the enrichment bias of the experiment (Lueders *et al*., 2004c).

2.4.2 RNA-SIP

RNA-SIP was first used to identify phenol-degrading microbes from an aerobic industrial bioreactor (Manefield *et al*., 2002a; Manefield *et al*., 2002b). These studies showed that RNA-SIP holds significant potential for exploring active populations from a variety of environments. Separation is then based on the newly synthesised RNA from community members that have assimilated the isotopic labelled substrate.

RNA-SIP can reduce the unwanted influence of cross feeding since RNA synthesis rates, which are higher than those of DNA, allow for greatly decreased incubation times, reducing the opportunity for significant cross-feeding (Manefield *et al*., 2002b). Not only is RNA rapidly synthesised, but it is the most active population that becomes labelled, further decreasing cross-feeding effects. However, final analyses can be biased by selecting for the highest growth/incorporation rate group of organisms.

The resolution of labelled RNA provides access to the gene sequences which are expressed by functional organisms during substrate assimilation. One key advantage of RNA-SIP is the natural amplification of 16S rRNA, the phylogenetic signature model in active cells (Radajewski *et al*., 2003). Manefield *et al*. (2002b) showed that the isotope incorporation into the biomass and the rate of the incorporation into RNA exceeded that of DNA by more than 8-fold over the same time period.

Another advantage for use of this method is the isolation of entire transcriptomes along with the 16S rRNA of community members utilising the isotopic substrate. After separation on a caesium trifluoride (CsTFA) gradient through centrifugation of the ^{13}C-rRNA and ^{12}C-rRNA molecules and analysis by RT-PCR of the 16S rRNA molecule, phenol degradation was implicated by a species of *Thauera* (Manefield *et al*., 2002b).

RNA-SIP cannot be performed using caesium chloride (CsCl) gradients because it precipitates during centrifugation at the buoyant density (BD) of ~2.0 g ml^{-1}, necessary for rRNA banding. CsTFA gradients allow an appropriate BD for the banding of rRNA, but limits loading capacity (Manefield *et al*., 2002b). It is also very important to perform complete fractionation of gradients for RNA-SIP. A very clear and comprehensive RNA-SIP method has been previously described (Whiteley *et al*., 2007) and includes methods for using quantitative RT-PCR for the analysis of the gradient fractions.

G + C content variation can become a real issue for DNA based SIP with labelled and unlabelled DNA co-occurring in a gradient. This is not such a problem for rRNA based SIP because the range of G + C contents is much lower than found with DNA (G + C between 50-60%, Buckley *et al*., 2007a).

RNA has extensive regions of secondary and tertiary structure making variability in denaturing conditions of rRNA molecules in density gradients important because they can affect the rRNA secondary structure. This can result in different species having buoyant densities ranging over 0.08 g ml^{-1} (Lueders *et al*., 2004c). Therefore, under some circumstances labelled and unlabelled RNA is likely to co-occur but is not as likely due to differences in G + C contents as it is with DNA.

2.4.3 Advantages of using RNA vs DNA

RNA is labelled faster than DNA due to RNA synthesis rates being higher than DNA. This has been demonstrated to be true by monitoring the label in RNA and DNA of the same organisms (Manefield *et al*., 2007). This is a function of the copy number and continual turnover of RNA, showing that cellular activity is independent of replication (Manefield *et al*., 2002b). RNA is growth regulated in a number of bacteria leading to faster labelling of RNA than compared with the genome and is more indicative of active populations (Kemp, 1995; Kerkhof and Ward, 1993; Madsen, 2006; Mahmood *et al*., 2005). The number of ribosomes in a cell is known to correlate with growth rate (Wellington *et al*., 2003). RNA may be sensitive enough to allow the identification of populations that become activated but have not divided or are growing (Lueders *et al*., 2004c). RNA synthesis is often very rapid and can be a more responsive biomarker and is therefore, indicative of actual active populations within the community (Madsen, 2006; McDonald *et al*., 2005). All of these reasons make RNA-SIP a faster method than DNA-SIP, greatly decreasing any cross-feeding effects.

2.4.4 Isotopes

The feasibility of SIP has been demonstrated using mostly small ^{13}C compounds but has recently evolved to include ^{18}O compounds (Schwartz, 2007) and ^{15}N compounds (Buckley *et al*., 2007a; Buckley *et al*., 2007b). The selection of the labelled compound is very important to SIP and it should be relevant to the microbial community of interest, impact least on the natural state of the community and its environment and be able to label enough atoms to allow separation and detection (Madsen, 2006).

2.4.4.1 Isotopic Labelling

For successful nucleic acid SIP experiments it is crucial to have a high degree of isotopic enrichment for labelling of nucleic acids. There are certain factors that can influence the amount of labelling including; substrate being assimilated, duration of label addition, relative abundance of naturally occurring unlabelled substrate and, rate of DNA synthesis (if DNA-SIP is being used) (Lueders *et al*., 2004c).

Obviously, the labelled substrate needs to be metabolised by the cell so it is fully incorporated into cell biomass. SIP is based on the assumption that sufficient amounts of the heavy isotope will be incorporated into the nucleic acids to permit successful separation by isopycnic centrifugation. If the substrate is used for other cell processes and not incorporated sufficiently into nucleic acids then the label becomes too dilute for detection in cell biomass. Insufficient substrate concentration and incubation times can result in incomplete ^{13}C labelling of nucleic acids. Using a control with unlabelled substrate as a comparison to the ^{13}C labelled can override the issue of incomplete labelling (Hori *et al*., 2007).

2.4.4.2 ^{15}N Isotopes

The natural abundance of nitrogen isotopes is 99.63% for ^{14}N and 0.37% for ^{15}N. The low natural abundance for ^{15}N makes it an ideal isotope for tracking its addition to organisms in an environment. ^{15}N has been used as a stable isotope for the last 40 years as an unambiguous indicator of nitrogen fixation, especially in diazotrophs associated with plants and legumes concomitant with transfer of fixed nitrogen to the plant host (Meselson and Stahl, 1958).

Cadisch *et al*. (2005) were the first to successfully demonstrate the feasibility of using ^{15}N-DNA-SIP with density centrifugation and found that enrichment was ideally >50 atom % but could be lowered to approximately 40 atom % ^{15}N. The use of ^{15}N substrates has now been used with DNA-SIP in five studies; $^{15}NH_4Cl$ (Buckley *et al*., 2007a) (Cupples *et al*., 2007), $^{15}NH_4NO_3$ (Cadisch *et al*., 2005), and $^{15}N_2$ (Buckley *et al*., 2007b; Buckley *et al*., 2008). It has yet to be attempted for RNA-SIP, although this has been highlighted as an area for expansion of the SIP application tool-box (Whiteley *et al*., 2006).

^{15}N-labelled substrates have been used less commonly among SIP researchers than ^{13}C. There are a number of limitations when using ^{15}N-labelled substrates. For example, the change in DNA buoyant density for fully ^{15}N-labelled DNA is 0.016 g ml^{-1}, which is less than half of the change that occurs naturally through G + C genome variation (Buckley *et al*., 2007a). Labelled and unlabelled DNA can possibly co-occur in this situation and this needs careful assessment in the development of the methodology. Using RNA instead of DNA may reduce the change that occurs naturally because RNA has less variation in G + C content than DNA.

A further potential drawback of ^{15}N-RNA-SIP is the lower density gain that is possible from the incorporation of ^{15}N compared to ^{13}C isotopes since these are more abundant in nucleic acids. The average stoichiometry of C relative to N in RNA is 9.5 carbon molecules to 3.75 nitrogen molecules, a C/N ratio of 2.5:1. Fully ^{15}N-labelled DNA in CsCl showed a density gain of ~0.016 g ml^{-1} (Birnie and Rickwood, 1978) and fully ^{13}C-labelled DNA showed a density gain of ~0.04 g ml^{-1} (Lueders *et al*., 2004a), both of which could be resolved from unlabelled material through a CsCl gradient. Fully ^{13}C-labelled RNA in CsTFA shows a density gain of ~0.04 g ml^{-1} (Manefield *et al*., 2002a) over unlabelled material.

The use of ^{15}N-labelled substrates offers the specific opportunity to use dinitrogen $^{15}N_2$ to identify nitrogen-fixing bacteria, as well as the identification of organisms capable of utilising ammonium, nitrate, nitrite, and nitrogen-containing organic compounds as sole nitrogen sources or any other part of the nitrogen cycle (Friedrich, 2006).

$^{15}N_2$-SIP offers several advantages over other SIP applications. The first is that incubations with the labelled substrate can be performed at realistic concentrations as atmospheric N_2 can be completely replaced with simulated air containing $^{15}N_2$ as the only nitrogen source. The second is that nitrogen fixation is inhibited in the presence of the mineral forms of nitrogen so $^{15}N_2$ can be used exclusively for growth, reducing significantly the chance for isotopic dilution. Lastly, the majority of nitrogen fixed by free-living diazotrophs is immobilised in microbial biomass (Buckley *et al*., 2008).

2.4.5 Gradient Materials

Two types of gradient material are routinely used to separate RNA and DNA for stable isotope probing centrifugation; caesium chloride (CsCl) and caesium trifluoroacetate (CsTFA). One problem with using CsCl for RNA separation is that the buoyant density of RNA exceeds the density of a saturated CsCl solution and is pelleted out (Zhang *et al*., 2003). CsTFA solutions have a higher aqueous solubility and can accomplish a higher density than CsCl, making it possible to keep RNA soluble in the gradient rather than precipitating out as in CsCl solutions. The trifluoroacetate anion leads to a higher density of nucleic acid preparation because the maximum CsTFA molarity at saturation is 10 M compared with 7.36 M for CsCl. This results in a maximum density difference between the two of 2.6 g ml^{-1} for CsTFA and 1.9 g ml^{-1} for CsCl (Zhang *et al*., 2003).

In contrast to CsCl gradients, CsTFA only allows loading quantities below 250 ng ml^{-1}, as above this value rRNA can aggregate in the gradient (Lueders *et al*., 2004c).

RNA-SIP therefore requires a more rigorous gradient evaluation procedure including smaller fraction sizes and accurate measurements for density and RNA concentrations than DNA-SIP. Only high-quality rRNA extracts should be loaded onto RNA gradients to obtain meaningful results because gradients need to be fractionated and the fractions analysed for rRNA content (Whiteley *et al*., 2006). CsTFA gradients contain formamide which helps denature the rRNA secondary structure while the RNA is in the gradient material. This helps reduce and possibly eliminate any differences in secondary structure causing co-occurrence of labelled and unlabelled RNA in the gradients. The use of CsTFA gradients for rRNA molecules has also been found to produce the most linear, shallow and reproducible density gradients with no detectable impact on overall RNA integrity (Manefield *et al*., 2002b). The main advantage of the use of CsTFA over CsCl gradients is that RNA is able to be recovered from a single soluble fraction of the CsTFA with very minimum manipulations. CsTFA is also soluble in ethanol so when precipitation of the RNA with alcohol is occurring, salt precipitation from the RNA is not, thereby reducing the need for further purification steps after precipitation (Zhang *et al*., 2003).

2.4.6 Gradient Comparisons

Creating and effecting control against false positives can be achieved through a comparison of T-RFLP profiles over a range of BD for both labelled and unlabelled samples. This is extremely important when analysing samples where small BD shifts are expected due to low incorporation levels (Cupples and Sims, 2007).

Gradient evaluation has shown that extended ultracentrifugation (30-60 hours) is necessary to separate 'light' from 'heavy' rRNA molecules otherwise 'heavy' fractions can still be contaminated with unlabelled RNA (Lueders *et al*., 2004a; Lueders *et al*., 2004c; Manefield *et al*., 2002a; Manefield *et al*., 2002b)

Lueders *et al*. (2004a) found that ^{12}C- and ^{13}C-rRNA form more distinct and separate bands when they are centrifuged separately than when they are combined in the same gradient. Combining ^{12}C-rRNA from a pure archaea culture and ^{13}C-rRNA from a pure bacterium culture, Lueders *et al*. (2004a) found that when the RNA extracts were combined and centrifuged in a CsTFA gradient, the rRNA bands were found to be closer together and showed increased overlap than when they were centrifuged separately. This is due to the limited capacity of CsTFA gradients to focus mixed rRNA species into precisely defined bands and these often spread over 3 – 4 fractions. This problem emphasises the use of quantitative analysis of gradient fractions to provide better resolution than visualising with ethidium bromide or gradient fractionation coupled with fingerprinting techniques (Lueders *et al*., 2004a) (Manefield *et al*., 2002b).

Despite these factors contributing to variability, the use of RNA-SIP can be a labour intensive technique and therefore, replicate centrifugation gradients or data from replicate incubations are not normally obtained (Lueders *et al*., 2004a).

2.5 Analysing Mixed Communities

Stable isotope probing is often coupled with other methods for analysis of the organisms involved in specific metabolic processes. The most common complementary method is to identify the organisms involved by sequencing a clone library of the 16S rRNA gene (bacteria and archaea).

This gene is a molecular marker that is now a well established method for determining the structural phylogenetic relationships for bacteria and archaea, avoiding the need for culturing and isolation of microbial species for identification purposes. This gene is highly conserved and gives an identity of the organism, with further information coming from sequencing specific functional genes. For example in this project the *nifH* gene could also be sequenced to yield more information around nitrogen fixation.

There are numerous fingerprinting methods including, FISH (fluorescent in situ hybridisation), FISH-MAR (microautoradiography), DGGE (denaturing gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism) and quantitative RT-PCR. On their own these methods either cannot (or only in a few cases can only indirectly) answer the question of what function and traits these organisms have in an environment (Keller and Zengler, 2004). However, these methods can act as accessories to stable isotope probing enable insights into the functions that specific micro-organisms have in an environment.

These methods are an efficient and accurate means for providing a complete picture of the community diversity within the biases of enrichment that occur during the SIP incubation. Some of these methods, especially quantitative RT-PCR, offer a quantitative way of measuring the actual changes to groups in the community using RNA-SIP methods. Quantitative PCR (Q-PCR) also allows the monitoring of SSU rRNA distribution across an entire gradient (Lueders *et al*., 2004a). FISH-MAR together with SIP has been suggested as an effective approach for determining *in vivo* physiology of mixed community micro-organisms from environments (Ginige *et al*., 2004). SIP can act as a precursor to 16S rRNA gene sequencing as it has the ability to unequivocally identify the organisms participating in specific metabolic processes. It is this benefit that can be exploited by the current project for identifying active nitrogen-fixing bacteria from an environmental sample.

Most of the methods upstream of SIP require 16S rRNA gene cloning for identification of the organisms present. This is because the 16S rRNA gene based approach is the most widely used technique for identification and community analysis, thereby offering phylogenetic information based on a large database of sequence information (Toshifumi *et al*., 2006).

Clone library sequencing is advantageous for downstream applications as the identification of close relatives can provide clues towards the development of appropriate culture conditions required for the growth of currently uncultured organisms. There are a number of factors that need to be considered when preparing clone libraries. If clones are screened with restriction digest before sequencing and only the clones with unique restriction patterns are chosen, this could lead to higher numbers of chimeras than if random clones are sequenced (Singleton *et al*., 2005).

A recent paper (Ashelford *et al*., 2006) highlights the importance of screening clone libraries for chimeras, demonstrating that recent large 16S rRNA gene clone libraries contain significant number of chimeras. Mallard is a program that uses the Pintail algorithm to distinguish chimeras with the screening of whole 16S rRNA gene libraries simultaneously and quickly (Ashelford *et al*., 2006).

The gene sequences obtained from 16S rRNA gene cloning can also be used for the construction of specific PCR primers for other applications, such as to target and quantify particular species in a population by Real-Time PCR.

2.6 Summary

The driving force for culture-independent methods is the realisation that only <3% of microbes can be cultivated. The development of stable isotope probing methods for the unambiguous identification of organisms performing specific metabolic activities offers real opportunities for exploring the other 97% of microbes.

Dinitrogen RNA-SIP is a method that can identify bacteria actively fixing nitrogen without the need for traditional microbiology approaches, such as culturing. SIP can then be coupled with further molecular approaches such as DGGE, T-RFLP, FISH, FISH-MAR, cloning, microarrays and metagenomics to help unravel the organisms actively involved in specific processes.

RNA-SIP offers the advantage of having short incubation times with high cell turnover numbers independent of cell division. RNA-SIP with ^{15}N compounds is currently unexplored and offers an opportunity to develop the method for nitrogen compounds including $^{15}N_2$ for the identification of nitrogen-fixing bacteria.

Previous literature around SIP has lead to clues and methods for exploring ^{15}N-RNA-SIP. As a target example for method development, the high carbon and low nitrogen concentrations of pulp and paper wastewater are already known to select for suitable nitrogen-fixing bacteria. It has also been shown to have high nitrogen fixation rates enhancing the chances for successful $^{15}N_2$-RNA-SIP experiments, offering the highest amount of labelling, and in turn, producing the greatest separation possible between labelled and unlabelled RNA.

Developing such a tool for the identification of the active nitrogen-fixing bacteria from a mixed community will enable future studies to follow global nitrogen cycling from a variety of different environments. An understanding of the nitrogen-fixing bacteria such communities will enable the correlation of the effects of changing environmental conditions with community composition and consequently, treatment system performance.

CHAPTER 3: ^{15}N-AMMONIUM CHLORIDE RNA STABLE ISOTOPE PROBING PROTOCOL DEVELOPMENT

3.1 INTRODUCTION

Stable isotope probing (SIP) is a method than can elucidate the active portion of a mixed community by measuring the amount of labelled substrate incorporated into nucleic acids. ^{15}N-substrates have been used in the past to label DNA, but have not been used to label RNA. In this Chapter, it was sought to demonstrate if $^{15}NH_4Cl$, which is used by all bacteria for growth, can be incorporated into pure culture RNA, and if separation by buoyant density centrifugation is possible for ^{14}N- and ^{15}N-RNA. To determine if this method could be used for environmental samples, labelling of mixed community cultures with $^{15}NH_4Cl$ was also undertaken and separation attempted of labelled and unlabelled RNA.

For this project to be successful, the extraction of good quality RNA from pure culture isolates and mixed community samples was essential. Secondly, optimisation of CsTFA gradients was required to achieve best resolution of RNA fractions. Once RNA had been shown to be resolved in CsTFA gradients then the RNA was labelled with $^{15}NH_4Cl$, and ^{14}N- and ^{15}N-RNA from a pure culture was separated through CsTFA gradients. Finally the degree of separation between ^{14}N- and ^{15}N-RNA from a pure culture was investigated to show that by using ^{15}N-labelled substrates it was possible to separate RNA based on buoyant density and to then apply the same protocol to mixed community samples.

3.2 METHODS

3.2.1 Bacterial Samples used for Method Development

3.2.1.1 Pure culture

The model organism for these studies was *Novosphingobium nitrogenifigens* $Y88^T$ (DSM 19370) (hereafter referred to as Y88), which was isolated from pulp and paper wastewater (Addison *et al*., 2007). This was grown in pure culture overnight at 30°C, 150 rpm in nitrogen-limited minimal medium (NLMM) (containing, l^{-1}, 0.4 g KH_2PO_4, 0.1 g K_2HPO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 10 mg $FeCl_3$, 2 mg Na_2MoO_4, 5 g glucose, 50 mg yeast extract and pH 7.2 $\pm$ 0.1) supplemented with 0.5 g ^{15}N- or unlabelled ammonium chloride (98+ atom % ^{15}N, Aldrich Chem. Co.).

3.2.1.2 Mixed culture

Mixed cultures for $^{15}NH_4Cl$ labelling were collected from New Zealand pulp and paper mill effluents (C/N ratio of 140:1) undergoing biological treatment in a bioreactor operated under nitrogen-limited conditions. A sample of pulp and paper mill effluent (10% inoculum) was grown overnight at 30°C, 150 rpm in NLMM supplemented with 0.5 g ^{14}N- or ^{15}N- ammonium chloride (98+ atom % ^{15}N, Aldrich Chem. Co.).

3.2.2 RNA Extraction

RNA from pure laboratory-grown cultures and mixed cultures was extracted using an RNA/DNA mini kit (Qiagen, Hilden, Germany). Cells were washed in phosphate-buffered saline with 0.25 g biomass re-suspended in 0.5 ml of 240 mM potassium phosphate buffer (pH 8.0) to which was added 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1). Cell suspensions were transferred to bead beater vials containing 0.5 g each of 0.1 mm and 0.3 mm silica-zirconium beads and lysed by agitation in a FastPrep bead beating system for 30 s at 5.5 m s^{-1}.

The aqueous phase was recovered by centrifugation and the RNA purified using RNA/DNA mini kit protocols (Qiagen, Hilden, Germany). Aliquots of RNA extracts were visualised by standard agarose gel electrophoresis to verify the quality of extracted RNA preparations. RNA was quantified in extracts using RiboGreen quantification dyes (Molecular probes, Invitrogen) according to manufacturer's instructions.

3.2.3 Isotope Ratio Mass Spectrometry (IRMS) Analysis

The ^{15}N and ^{14}N enriched samples were analysed commercially by Waikato Stable Isotope Unit (University of Waikato, New Zealand). After freeze-drying, an internal standard of urea was added to an accurately weighed amount (~3 mg) of freeze dried biomass. A 30 µg N carrier was used in the samples with the detection limit of the machine being no less than 20 µg N. The urea was standardized against a certified standard and calibrated relative to atmospheric nitrogen. Samples were analysed using a Dumas elemental analyser (Europa Scientific ANCA-SL) interfaced to an Isotope Mass Spectrometer (Europa Scientific 20-20 Stable Isotope Analyser) to give the atom % ^{15}N.

3.2.4 Gradient Centrifugation, Fractionation and Quantification

Density gradient centrifugation was performed in 6.5 ml polyallomer Cone-Top tubes in a T-1270 Sorvall rotor spun at 40 000 r.p.m (110 000 g_{av}) and a temperature of 16°C for 42 hours. Caesium trifluoroacetate (CsTFA) was used as the gradient forming material; 3.72 ml of a 1.99 g ml^{-1} CsTFA solution (Amersham Pharmacia Biotech) was combined with 750 µl of H_2O, 150 µl of deionised formamide (Manefield *et al*., 2002b), and 1000 ng RNA to give a final volume of 4.62 ml. To fill the remainder of the tube, the CsTFA solution was overlaid with mineral oil (Sigma). The starting gradient material buoyant density was 1.78 g ml^{-1}, measured gravimetrically.

Centrifuged gradients were fractionated from below by water displacement using an 1100 HPLC pump (Hewlett Packard) operating at a flow rate of 1 µl s^{-1}.

The buoyant density of gradient fractions was determined by weighing measured 200 µl volumes on a four-figure milligram balance. RNA was isolated from gradient fractions by precipitation with one volume of isopropanol, washed with 70% ethanol and re-eluted into 10 µl for determination of RNA using the RiboGreen assay.

3.3 RESULTS

3.3.1 Y88 Labelling and Extraction

IRMS analysis compared RNA from the cultures of ^{14}N- and ^{15}N-labelled Y88. These results showed that the $\delta\ ^{15}N$ for unlabelled sample was 23.18 and labelled sample was 125543.90. The atom % ^{15}N changed from 0.375 for unlabelled to 95.0 for labelled. These results confirm that ^{15}N was incorporated into Y88 RNA and that there was close to complete labelling of nitrogen atoms in the RNA, thus increasing the chances of separation between labelled and unlabelled RNA.

RNA was extracted successfully from labelled and unlabelled Y88 cultures (Figure 3.1) and quantified fluorometrically to reveal ^{14}N 2700 ng μl^{-1} and ^{15}N 4000 ng μl^{-1} RNA extracted. These RNA samples were used in separations of all pure culture gradients.

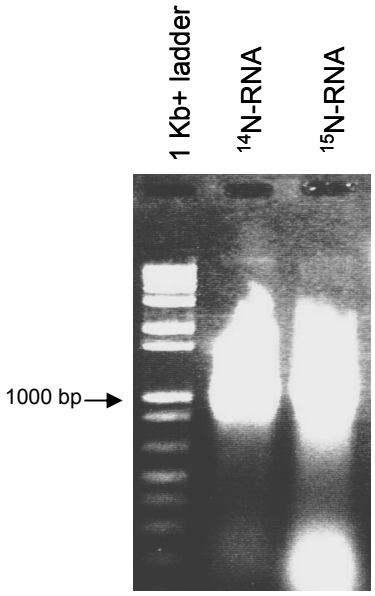

Figure 3.1. RNA extractions from Y88 cultures.

3.3.2 Y88 Gradient Separation and Fractionation

High RNA extraction concentrations allowed the centrifugation and separation of Y88 RNA for establishing RNA banding patterns in CsTFA gradients. Gradients P1 – P3 were used to establish separation and fractionation of unlabelled RNA in CsTFA gradients. Results from the optimisation of pure culture gradients are summarised in Appendix II.

Gradients were each fractionated into 200 µl fractions and then weighed to find density measurements. Through small pipetting and weighing errors the buoyant densities of the fractions were not always exact. To overcome this, the buoyant density results were used to generate a line of best fit for comparing results, producing the most accurate way for comparison between gradients.

Gradients P4 – P6 all used ^{14}N-RNA to determine the BD where Y88 RNA naturally banded and gradients P7 – P9 used ^{15}N-RNA to determine the banding BD of labelled Y88 RNA. Table 3.1 shows the density of the major RNA peaks for gradients P4 – P9.

Table 3.1. RNA density results for ^{14}N- and ^{15}N-RNA Y88 gradients.

Isotopic Label	Gradient	Buoyant Density of Highest RNA Concentration (g ml^{-1})	RNA >50% Concentrated among Fractions
^{14}N	**P4**	1.7659	3 – (65%)
^{14}N	**P5**	Un-measurable	–
^{14}N	**P6**	1.7483	3 – (62%)
^{15}N	**P7**	1.7796	3 – (64%)
^{15}N	**P8**	1.7826	4 – (69%)
^{15}N	**P9**	1.749	4 – (50%)

P = Pure culture

These results show the importance of doing replicate gradients for each RNA sample. Inconsistency between each gradient is due to the initial position of the needle in the gradients, some slight variations in the stock gradient material and possible variations in gravimetric analysis of the fractions.

Overall gradient results showed that the average buoyant densities (BD) of CsTFA resolved gradient fractions for ^{15}N had a 0.03 $\pm$ 0.004 g ml^{-1} (n = 3) higher BD compared to the ^{14}N control. Figure 3.2 shows representative labelled (P8) and unlabelled (P6) RNA from Y88 resolved through separate CsTFA gradients with a BD shift by ^{15}N-labelling of RNA of 0.032 g ml^{-1}.

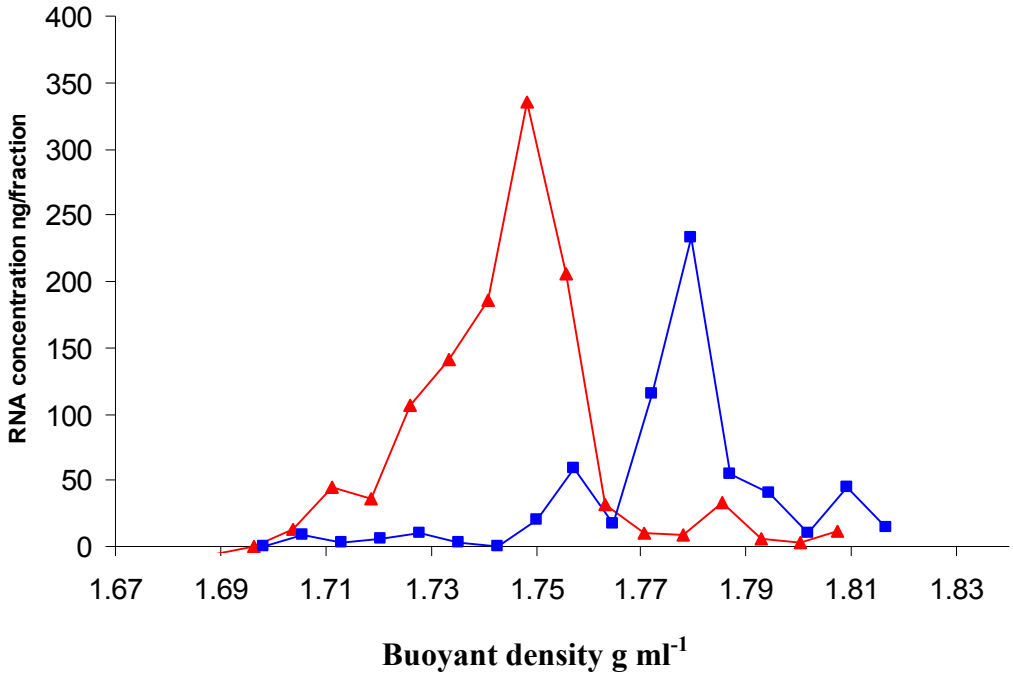

Figure 3.2. Representative analysis of CsTFA density gradient of Y88 RNA labelled with ^{14}N- or $^{15}NH_4Cl$ substrates. ^{14}N- (▲) or ^{15}N-RNA (■) was centrifuged individually and detected fluorometrically.

An equal mix of labelled and unlabelled RNA was resolved less well on a CsTFA gradient compared to unmixed samples (Figure 3.3). A single peak spreading across the density fractions was wider than the peaks represented for individually centrifuged gradients. The gradients also showed reduced total concentrations of RNA compared to the amount actually loaded, and had higher levels of background RNA.

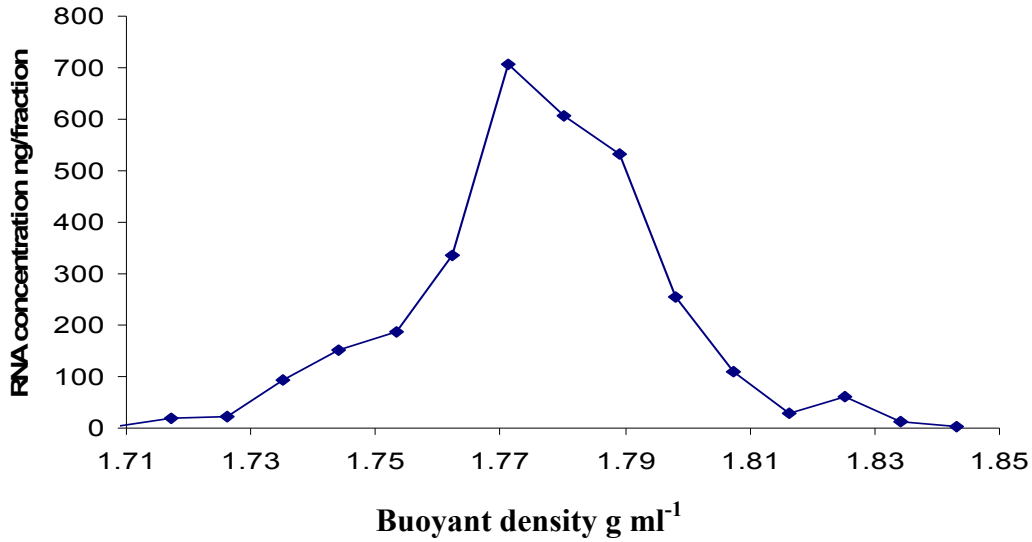

Figure 3.3. Fractionation of combined ^{14}N- and ^{15}N-RNA pure culture gradient.

Results show that the RNA was spread across five fractions (Figure 3.3), representing 78% of the total RNA in the gradient. In the separately RNA run gradients the ^{14}N labelled RNA concentrated at a buoyant density between 1.74 – 1.76 g ml^{-1} and the ^{15}N labelled RNA concentrated higher at a buoyant density between 1.77 – 1.79 g ml^{-1}. If this data is compared with Figure 3.2 then the highest peak at 1.77 g ml^{-1} possibly represented a mix of ^{14}N- and ^{15}N-labelled RNA. Most of the peaks below this point should have comprised only ^{14}N-RNA (with some possible small ^{15}N background) and the peaks at and above 1.78 g ml^{-1} would represent ^{15}N-labelled RNA. All three of the ^{14}N and ^{15}N mixed RNA gradients showed similar profiles, demonstrating that when the RNA was mixed together before gradient separation this resulted in a spread across the gradient decreasing the resolution between labelled and unlabelled RNA.

These gradient evaluations showed the successful separation of labelled and unlabelled RNA for a pure culture when run separately, supporting further method development and applications to more complex mixed community samples.

3.3.3 Labelling and RNA Extraction of Mixed Community

IRMS analysis compared RNA from the mixed community cultures when ^{14}N- and ^{15}N-labelled. These results showed that δ ^{15}N for the unlabelled sample was 635.42 and for the labelled sample δ ^{15}N was 80812.63.

The atom % ^{15}N changed from 0.398 for unlabelled to 80.0 for labelled. These results confirmed that ^{15}N was incorporated into mixed community RNA and that there was high labelling of nitrogen atoms in the RNA. This increased the potential to obtain suitable separations between labelled and unlabelled RNA.

High concentrations of RNA were extracted for the mixed community culture; ^{14}N-unlabelled 3100 ng μl^{-1} and ^{15}N-labelled 2840 ng μl^{-1} (Figure 3.4).

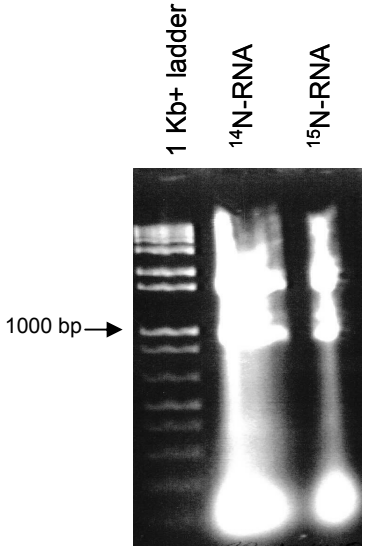

Figure 3.4. RNA extractions from mixed community labelled and unlabelled cultures.

3.3.4 Gradient Fractionation for Mixed Community RNA

Good quality and quantities of RNA were extracted from both ^{14}N- and ^{15}N-labelled mixed cultures. This was used for gradient fractionation to compare the resolution of labelled and unlabelled RNA from mixed community cultures. Six gradients in total were run with three unlabelled (^{14}N) and three labelled (^{15}N) RNA. The gradients were fractionated and results are shown in Table 3.2.

Table 3.2. RNA density results for ^{14}N- and ^{15}N-ammonium chloride RNA mixed community gradients.

Isotopic Label	Gradient	Buoyant Density of Highest RNA Concentration (g ml^{-1})	RNA >50 % Concentrated among Fractions
^{14}N	**M1**	1.7538	4 – (70%)
^{14}N	**M2**	1.7455	4 – (66%)
^{14}N	**M3**	Un-measurable	–
^{15}N	**M4**	Un-measurable	–
^{15}N	**M5**	1.760	4 – (53%)
^{15}N	**M6**	1.7737	4 – (55%)

M = Mixed community

The results (Table 3.2) show that the RNA was more highly concentrated across fractions for unlabelled gradients (>65%) than for labelled gradients (<55%). This is likely due to the labelling only being 80% ^{15}N. These results also show that the buoyant density for ^{15}N-labelled RNA gradients successfully resolved at a heavier density than the ^{14}N-unlabelled gradient counterparts.

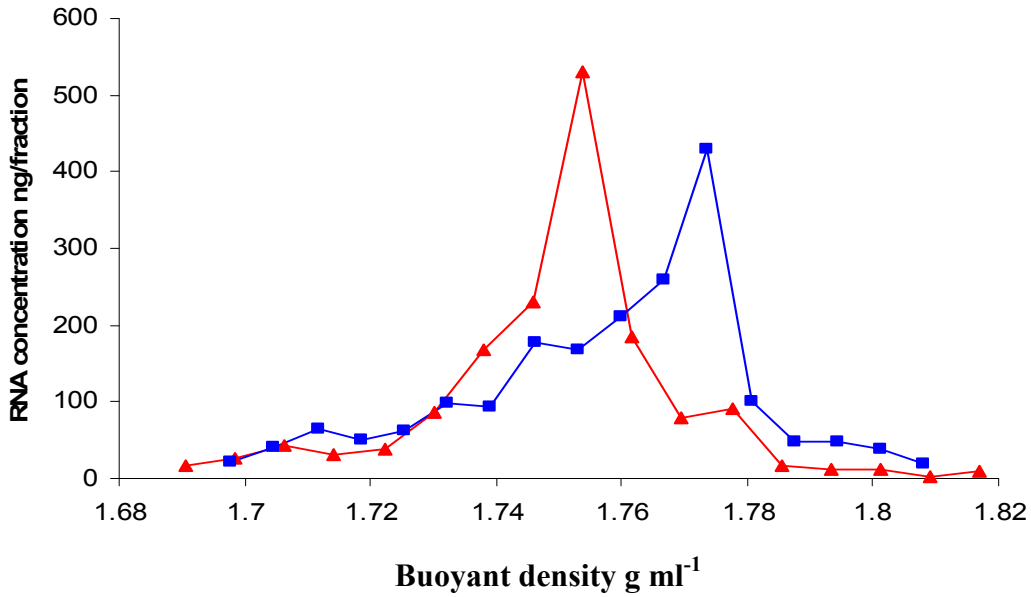

Figure 3.5. Representative analysis of CsTFA density gradient of mixed community RNA labelled with ^{14}N- (▲) or $^{15}NH_4Cl$ (■) substrates. ^{14}N (M1) and ^{15}N (M6) RNA was centrifuged individually and detected fluorometrically.

A representative graph for mixed community gradient separation shows that there was some overlap between unlabelled and labelled RNA between M1 and M6 (see Figure 3.5). The overlap occurred in the density range where unlabelled RNA is resolved; 1.75 – 1.76 g ml^{-1}. An average of 80% ^{15}N-labelling in the heavy labelled gradient was shown with IRMS analysis. This does not mean that 20% of RNA was unlabelled. All organisms might have been labelled, but only with an average of 80% atom labelling. This could create some overlap with the density change in labelling shifting only slightly but is more likely due to a mixed community generating a broader spread of RNA due to the heterogeneity of community rRNAs compared to those from a pure culture.

The main RNA peaks still showed good resolution and separation between the labelled and unlabelled cultures. A representative gradient showed that unlabelled RNA migrated to a peak BD of 1.7538 g ml^{-1} and labelled RNA migrated to a BD of 1.7737 g ml^{-1}, showing a difference in BD by ^{15}N-labelling of RNA of 0.0199 g ml^{-1}.

Overall, ^{15}N-labelled RNA from the mixed community resolved at a heavier density than the unlabelled controls. Gradients for mixed community RNA showed a gain in BD for ^{15}N-RNA of 0.02 $\pm$ 0.004 g ml^{-1} (n = 3) compared to the ^{14}N-control.

3.4 DISCUSSION

The gradients that utilised a mix of ^{14}N-unlabelled and ^{15}N-labelled RNA did not show conclusive evidence of separation and were all represented by very small RNA concentrations which was spread throughout the gradients. All the gradients showed the presence of trace background levels of RNA throughout the resolved density gradients at concentrations detectable by fluorometer measurements. This occurred despite loading only small amounts of rRNA and the presence of formamide to resolve secondary structures.

Greater than 65% of RNA for all gradients resolved in the main RNA peak (5 – 6 fractions). Manefield *et al*. (2002b) reported similar results and concluded that density gradients typically used to isolate RNA based on buoyant density have limited ability to focus RNA into tightly defined bands, and this could be caused by the interactions of different rRNA molecules during gradient centrifugation not being fully prevented.

One factor that could be manipulated to improve this resolution is the g-force of the centrifugation, so that either diffuse bands that are widely separated or tight bands that are closer together can be obtained (Schwartz, 2007). The results from the current study show an intermediate level of resolution, with bands that are tight enough to easily identify and far enough apart for separation to be obtained.

Densities differed slightly between the actual fraction numbers for each different gradient due to the initial position of the needle. However, the pure culture gradients showed that a shift from light to heavy density RNA occurred over 3 – 4 gradient fractions. This correlated to the data obtained by Manefield *et al*. (2002b) and Lueders *et al*. (2004c).

Due to inter-run variability between gradients, this study showed that comparative ^{14}N and ^{15}N gradients must be run concurrently to allow resolution of the ^{15}N-labelled RNA fractions. Lueders *et al*. (2004a) reported that ^{12}C- and ^{13}C-rRNA formed more distinct and tighter bands when centrifuged separately than when they were combined in the same gradient. This was also true for Y88 gradients when ^{14}N- and ^{15}N-RNA were run together on one gradient. The rRNA bands moved closer together and they showed increased overlap than when they were centrifuged individually. This shows that there were possibly interactions between the rRNA of different buoyant densities, but why this occurred is still unknown.

The initial growth experiments for mixed community samples performed before labelling provided information for further experiments using $^{15}N_2$ as the label (Chapter 4). This included the conditions needed for the lowest possible nitrogen addition and minimum inoculum levels that still provided enough growth in sealed vessels given use of a gaseous nitrogen source. The best growth was obtained from 1% inoculum in 1 g l^{-1} ammonium chloride for mixed community ammonium chloride labelling.

The mixed gradients showed less separation between the ^{14}N- and ^{15}N-RNA than shown for pure culture gradients. This shift is likely due to the decrease in ^{15}N-labelling for mixed cultures compared with pure culture RNA. It is also expected that a mixed community will generate a broader spread of RNA due to the heterogeneity of community rRNAs compared to those from a pure culture.

3.5 SUMMARY

These initial RNA-SIP experiments determined the reproducibility and efficiency in separating $^{14}NH_4Cl$ and $^{15}NH_4Cl$ labelled RNA from a pure culture into defined bands of different buoyant densities. They showed that RNA could be successfully extracted from Y88 and optimisation of the CsTFA gradient from these samples gave good fractionation and banding patterns. IRMS analysis confirmed that the label had been incorporated into the RNA in the ^{15}N-labelled RNA when compared with the ^{14}N-control RNA.

The robustness of the protocol was confirmed by the large buoyant density difference and small variability found between the banding of resolved RNA for ^{14}N-unlabelled and ^{15}N-labelled RNA. The average buoyant densities (BD) of CsTFA resolved gradient fractions for the RNA enriched fraction for ^{15}N had a 0.03 ± 0.004 g ml^{-1} ($n = 3$) higher BD compared to the ^{14}N control.

The successful separation of labelled and unlabelled RNA from a pure culture with maximum labelling enabled the method to be developed further to encompass a more complex sample using mixed microbial communities. Labelled and unlabelled RNA isolated from a mixed community bioreactor was used to establish conditions for separating labelled (^{15}N) and unlabelled (^{14}N) RNA. The methods for pure culture gradient separation were applied to mixed community samples.

It was found that there was successful separation of ^{15}N-ammonium chloride labelled RNA from ^{14}N-unlabelled RNA. The results from mixed community gradients labelled with ^{14}N- or ^{15}N-ammonium chloride showed that ^{15}N-labelled RNA resolved at a heavier density than ^{14}N-unlabelled RNA. Gradients for mixed community RNA showed a gain in buoyant density for ^{15}N-RNA of 0.02 ± 0.004 g ml^{-1} ($n = 3$) compared to the ^{14}N control.

Overall, the initial method development studies showed that ^{15}N-RNA-SIP was a viable technique for both pure and mixed culture community analyses. Based upon successful labelling with a soluble nitrogen source, ammonium chloride, the method was further developed for use with gaseous dinitrogen for direct measurement of nitrogen fixation activity.

CHAPTER 4: IDENTIFYING DIAZOTROPHS FROM PULP AND PAPER WASTEWATER WITH $^{15}N_2$-RNA-SIP

4.1 INTRODUCTION

The use of ^{15}N-labelled substrates for SIP offered the potential to use dinitrogen ($^{15}N_2$) to identify nitrogen-fixing bacteria as well as the identification of organisms capable of utilising ammonium, nitrate, nitrite, and nitrogen-containing organic compounds as sole nitrogen sources. This chapter aimed to determine if $^{15}N_2$-RNA-SIP methodology could be applied to an environmental sample for the identification of diazotrophs that are actively engaged in nitrogen fixation. The model population used in this study was taken from pulp and paper wastewaters, an environment with known high nitrogen fixation rates (Clark *et al*., 1997).

This application of $^{15}N_2$-RNA-SIP to an environmental sample relied on the presence of sufficient nitrogen-fixing rates of the community involved to incorporate the highest possible label in the short incubation times while still providing enough growth for RNA extraction. This was imperative for the further separation of the RNA in CsTFA gradient material. When successful fractionation of $^{14}N_2$- and $^{15}N_2$-RNA has been completed, the enriched RNA fraction from the $^{15}N_2$ gradient could be used for cloning to identify the 16S rRNA gene sequences from the micro-organisms actively involved in nitrogen fixation from pulp and paper wastewater. Identifying these bacteria would provide a better cross-sectional analysis of the community, leading to an improved prediction on how to manage and advance the treatment performance of target waste systems and to exploit the unique bioconversion properties of these types of organisms.

4.2 METHODS

4.2.1 Environmental Sample

Mixed cultures for $^{15}N_2$ labelling were collected from a New Zealand pulp and paper mill wastewater treatment system using ASBs and with an initial effluent (C/N ratio of 140:1). The sampling point was the Kinleith Pond 19 inlet.

4.2.2 Growth Conditions for SIP

Optimum growth conditions were achieved as described below. Other growth conditions and results are shown in Appendix II.

A 10% v/v inoculum (10 ml in 100 ml media volume) of the community was grown for 24 hours in NLMM supplemented with 1 g l^{-1} glucose and 1 g l^{-1} sodium acetate at 30°C and 150 rpm. A 10% (6 ml) inoculum was sub-cultured into two 160 ml sealed flasks in which the headspace (100 ml) had first been flushed for two minutes with argon gas and then 40 ml of headspace was replaced with 20 ml O_2 and 20 ml N_2 (unlabelled N_2 in one flask and $^{15}N_2$ (98+ atom % ^{15}N, ISOTEC) in the other). After incubation for 10 hours, a further 10 ml O_2 was added to both flasks. An acetylene reduction assay was conducted on a sub-sample to confirm the presence of the nitrogenase enzyme as an indicator of nitrogen fixation (Sprent and Sprent, 1990).

All assays were tested with a blank sample, positive control of ethylene and two samples for each culture (see full method in Appendix I).

4.2.3 RNA Extraction of Environmental Sample

RNA from the cultures was extracted using an RNA/DNA mini kit (Qiagen, Hilden, Germany). Cells were first washed in phosphate-buffered saline with 0.25 g biomass re-suspended in 0.5 ml of 240 mM potassium phosphate buffer (pH 8.0) to which was added 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1). Cell suspensions were transferred to bead beater vials containing 0.5 g each of 0.1 mm and 0.3 mm silica-zirconium beads and lysed by agitation in a FastPrep bead beating system for 30 s at 5.5 m s^{-1}.

The aqueous phase was separated by centrifugation and the RNA purified by RNA/DNA mini kit protocols (Qiagen, Hilden, Germany).

Aliquots of each RNA extracts were visualised by standard agarose gel electrophoresis to verify the quality of extracted RNA preparations. RNA was quantified in extracts using RiboGreen quantification dyes (Molecular probes, Invitrogen) according to manufacturer's instructions.

4.2.4 IRMS Analysis

The ^{15}N- and ^{14}N-enriched samples were analysed commercially by Waikato Stable Isotope Unit (University of Waikato, New Zealand). After freeze-drying, an internal standard of urea was added to an accurately weighed amount (~3 mg) of freeze dried biomass. A 30 µg N carrier was used in the samples with the detection limit of the machine being no less than 20 µg N. The urea was standardized against a certified standard and calibrated relative to atmospheric nitrogen. Samples were analysed using a Dumas elemental analyser (Europa Scientific ANCA-SL) interfaced to an Isotope Mass Spectrometer (Europa Scientific 20-20 Stable Isotope Analyser) to give the atom % ^{15}N.

4.2.5 Gradient Centrifugation, Fractionation and Quantification of Environmental Samples

Density gradient centrifugation was performed in 6.5 ml polyallomer Cone-Top tubes in a T-1270 Sorvall rotor spun at 40 000 rpm (110 000 g_{av}) and a temperature of 16°C for 42 hours. Caesium trifluoroacetate (CsTFA) was used as the gradient forming material; 3.72 ml of a 1.99 g ml^{-1} CsTFA solution (Amersham Pharmacia Biotech) was combined with 750 µl of H_2O, 150 µl of deionised formamide (Manefield *et al*., 2002b), and 1000 ng RNA to give a final volume of 4.62 ml. To fill the remainder of the tube, the CsTFA solution was overlaid with mineral oil (Sigma). The starting gradient material buoyant density was 1.78 g ml^{-1}, measured gravimetrically.

Centrifuged gradients were fractionated from below by water displacement using an 1100 HPLC pump (Hewlett Packard) operating at a flow rate of 1 µl s^{-1}.

The buoyant density of gradient fractions was determined by weighing 200 µl volumes on a four-figure milligram balance. RNA was isolated from gradient fractions by precipitation with 1 volume of isopropanol, washed with 70% ethanol and re-eluted into 10 µl for determination of RNA using the RiboGreen assay.

The following gradients (Table 4.1) were all prepared, centrifuged, fractionated and quantified as per the procedure for gradient preparation outlined in Appendix I.

Table 4.1. Gradient set-ups for mixed community N_2 labelling.

Gradient	Sample	RNA concentration loaded	Fraction sizes µl
R1 – R3	$^{14}N_2$	1000 ng	200
R4 – R6	$^{15}N_2$	1000 ng	200
R7 – R9	$^{14}N_2 + {}^{15}N_2$	500 ng each	200

R = Real environment.

4.2.6 Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analyses of density-resolved rRNA were performed with the primers 27F-FAM/1492R (Lane, 1991) by RT-PCR using a one-step RT-PCR system (Superscript III One-Step RT-PCR system, Invitrogen). 15 µl of the resulting PCR product was digested with a mix of MspI and HhaI (Roche) in 30 µl reaction volumes as per the manufacturer's instructions. The digested PCR products were resolved on an Amersham Biosciences MegaBACE DNA Analysis System alongside a 600 bp ladder (Waikato DNA Sequencing Facility, University of Waikato). The T-RF profile was run through Phylogenetic Assignment Tool (PAT) and the T-RFs were assigned groups based on data from restriction enzyme digests (Kent *et al*., 2003).

4.2.7 Enriched $^{15}N_2$ Fraction Clone Library Preparation and Sequencing

The enriched fraction from a $^{15}N_2$-labelled gradient was selected for cloning with 27F-FAM/1492R (Lane, 1991) by RT-PCR using a one-step system (Superscript III One-Step RT-PCR system, Invitrogen). This product was cloned using TOPO-TA Kit (Invitrogen) and 300 clones were picked for further analysis. These clones were grown in LB broth overnight and the plasmids were extracted (Saunders and Burke, 1990). Only white clones were selected for sequencing. A 16S rRNA gene amplification was performed for each of the clones and the product was sequenced on a 3010 HiDye Sequencer (Applied Biosystems, Scion).

4.2.8 Nitrogen Fixation Capability nifH Gene Amplification

Nitrogen fixation capability from the enriched fraction of a $^{15}N_2$-labelled gradient was demonstrated by the detection of the *nifH* gene (encoding the iron protein of nitrogenase, a key enzyme in nitrogen fixation) through the amplification of a 360 bp *nifH* fragment using primers PolF and PolR as described by Poly *et al*. (2001).

4.2.9 Sequence Analysis of Clones

Sequences obtained were first annotated and cleaned up using Sequence Scanner Version 1.0 (Applied Biosystems). This study utilised existing techniques for identifying chimeric sequences. The Mallard approach uses the pintail algorithm to match sequences in an entire library against one another to show which sequences are chimeric (Ashelford *et al*., 2006). Chimera_Check blasts the sequence against known sequences in the Ribosomal Database Program (RDP) and splits the sequence into two halves (http://rdp8.cme.msu.edu/cgis/chimera.cgi). The sequences (excluding any chimeric ones) were blasted against the ncbi nr/nt collection 16S rRNA sequence database using blastn (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). These searches gave matches for the closest organism to classify the clones into groups.

Once sequences were screened and annotated the resulting sequences were aligned using a 16S rRNA web based alignment program Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-NAST). The generated file was then imported into ARB and the alignment was checked and edited manually (Ludwig *et al*., 2004) before generating neighbour joining phylogenetic trees with 1000 replicates and bootstrap values.

DOTUR is a program for Defining Operational Taxonomic Units and estimating species richness (Schloss and Handelsman, 2005). Sequences were grouped into OTUs with sequences >97% identity typically assigned to same species and those with >95% identity are typically assigned to the same genus. A PHYLIP generated distance matrix was used as an input file into DOTUR, which assigned sequences to OTUs for every possible distance. DOTUR was able to then calculate values which construct randomised rarefaction curves of observed OTUs, diversity indices, and richness estimators.

4.3 RESULTS

4.3.1 Environmental Sample Growth

A series of experiments were performed to optimise the growth conditions for nitrogen-fixing pulp and paper wastewater samples. The full results can be found in Appendix II. A fresh sample from Kinleith Pond 19 Inlet was collected and a 100 ml starter culture was grown overnight before being sub-cultured.

Results from the cultures showed that the $^{14}N_2$ culture grew to an OD_{600nm} of 0.604 after 24 hours, and the $^{15}N_2$ culture grew to an OD_{600nm} of 0.513. Based on the original starting OD of 0.027 this is an increase of around 4.5 generations in the cultures. Both cultures showed that some flocs were present even after vigorous shaking and the use of baffled bottles, so growth was likely to be underestimated. Acetylene reduction assay results from these cultures showed no peak in the ethylene area in the blank and the positive control showed a very strong ethylene peak (Figure 4.1). Both $^{14}N_2$ and $^{15}N_2$ samples show very strong ethylene peaks where the acetylene has been converted to ethylene through activity of the nitrogenase enzyme. The results indicate that the cultures were efficiently fixing nitrogen when the cells were harvested for RNA extraction.

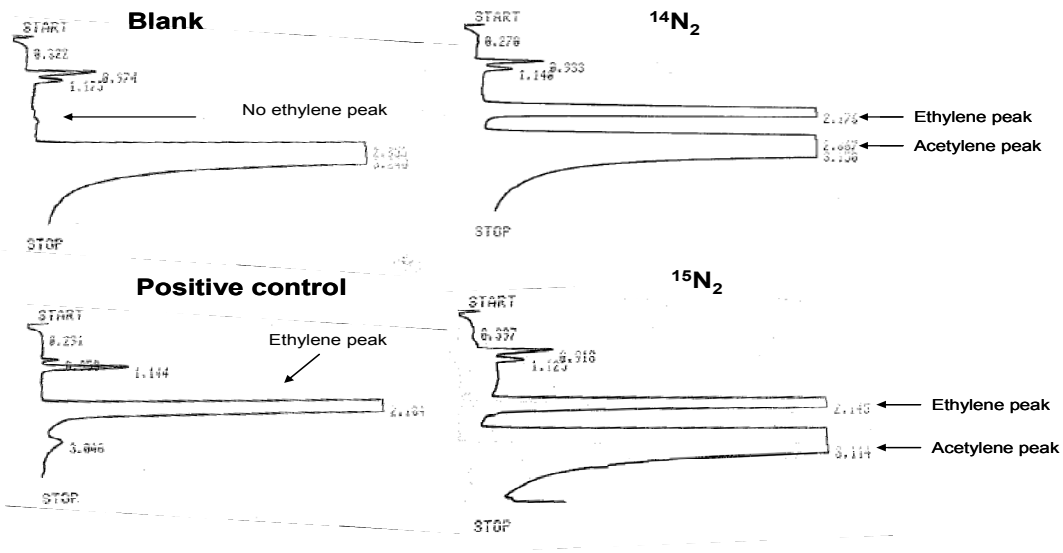

Figure 4.1. Acetylene reduction assay results after 24 hours growth for $^{14}N_2$- and $^{15}N_2$-labelled cultures from Pond 19 Inlet real wastewater sample.

4.3.2 RNA Extraction

The labelled and unlabelled cultures were centrifuged and part of the pellet was freeze dried and sent for IRMS analysis. The remainder of the pellet was used for simultaneous RNA and DNA extractions (Figure 4.2).

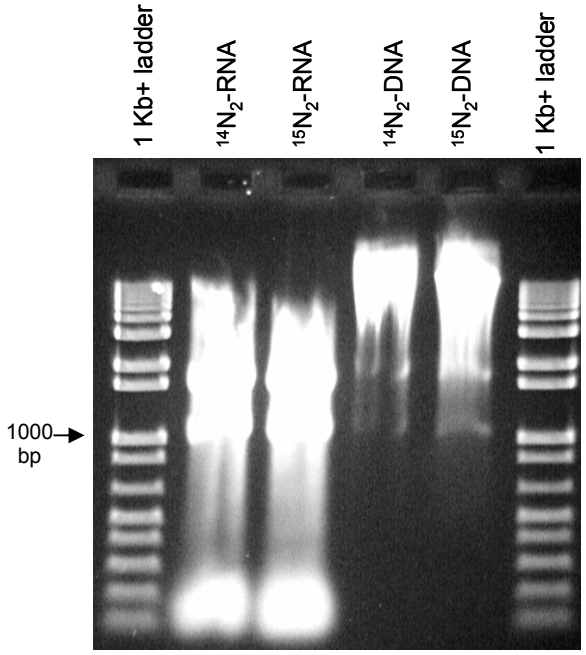

Figure 4.2. RNA and DNA extractions from mixed community labelled ($^{15}N_2$) and unlabelled ($^{14}N_2$) cultures.

High concentrations of RNA were extracted from both labelled and unlabelled cultures (Figure 4.2). DNA was extracted simultaneously and stored in the freezer for possible future use if required. The extraction from the $^{14}N_2$ culture produced 1069 ng μl^{-1} of RNA. That from $^{15}N_2$ produced 1014 ng μl^{-1} of RNA.

4.3.3 IRMS Analysis

IRMS analysis compared freeze dried biomass from the mixed community cultures with $^{14}N_2$ and $^{15}N_2$. These results showed that δ ^{15}N for $^{14}N_2$ biomass sample was 1.69 and $^{15}N_2$ biomass sample was 130717. The atom % ^{15}N was 32.6 for $^{15}N_2$ (labelled) biomass and $^{14}N_2$ (unlabelled) was 0.3 atom % ^{15}N. These results confirm that ^{15}N was incorporated into mixed community RNA and that there was a sufficient degree of labelling of nitrogen atoms in the RNA.

4.3.4 $^{14}N_2$ and $^{15}N_2$ Gradient Separation and Fractionation

$^{14}N_2$ and $^{15}N_2$ gradients were run separately to assess the resolution of labelled and unlabelled RNA from mixed community cultures. Separation was possible for both pure cultures and mixed cultures with labelled NH_4Cl, however, the use of $^{15}N_2$ may not label all the community because nitrogen fixation is an energy expensive process resulting in lower growth yields and lower labelling percentages. The separation was therefore expected to be smaller between labelled and unlabelled RNA in CsTFA gradients (Table 4.2).

Gradient R3 was un-measurable due to leakage of the gradient. Gradient R5 fractionated well but on analysis showed a large spread of RNA across 7 out of the total 17 fractions ranging from BD 1.79 – 1.75 g ml^{-1}. Both of these gradients were excluded from the final analysis due to this large RNA distribution across the gradients.

Gradients R1, R2, R4 and R6 showed that ^{15}N-labelling of RNA increased buoyant density by 0.013 $\pm$ 0.007 g ml^{-1} (n = 4) compared to the ^{14}N control. Removing R2, which showed a large peak at density 1.79 g ml^{-1}, from the same analysis showed the same average 0.013 $\pm$ 0.002 g ml^{-1} (n = 3) but with a smaller range.

Table 4.2. RNA density results for $^{14}N_2$ and $^{15}N_2$ RNA mixed community gradients.

Isotopic Label	Gradient	Buoyant Density of Highest RNA Concentration (g ml^{-1})	RNA >50 % Concentrated among Fractions
^{14}N	R1	1.7767	3 – (74%)
^{14}N	R2	1.7672	3 – (52.7%)
^{14}N	R3	Un-measurable	–
^{15}N	R4	1.7879	3 – (58.5%)
^{15}N	R5	1.7946	4 – (50%)
^{15}N	R6	1.7821	3 – (60.4%)

A representative gradient of R1 and R4 (Figure 4.3) demonstrates that unlabelled RNA migrated to a buoyant density of 1.777 g ml^{-1} and labelled RNA migrated to a buoyant density of 1.788 g ml^{-1}, showing a difference of 0.011 g ml^{-1}.

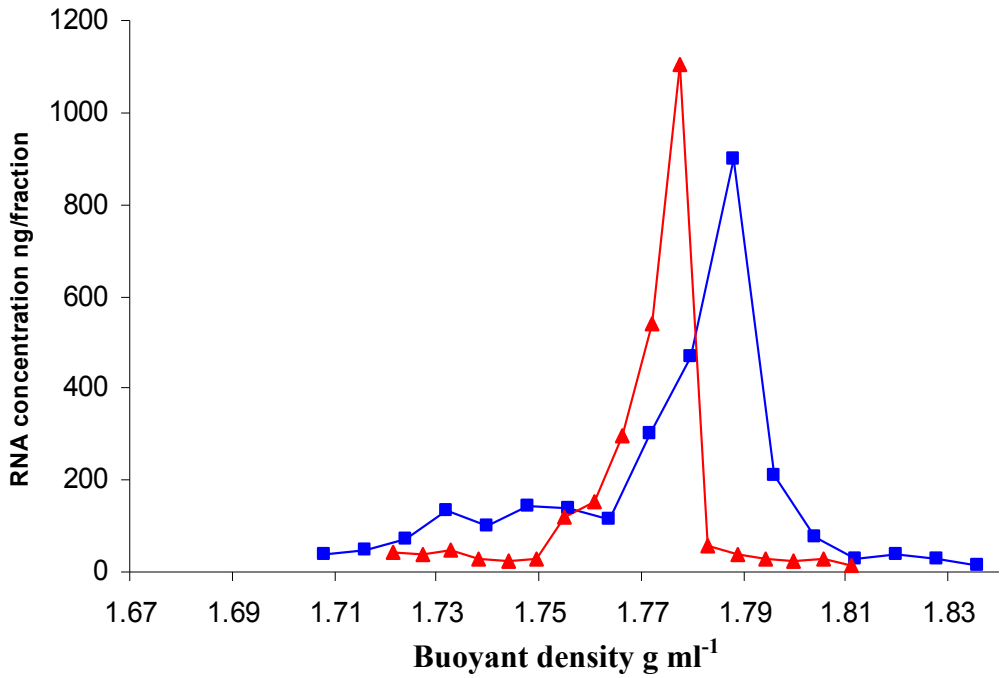

Figure 4.3. Representative analysis of CsTFA density gradient of mixed community RNA labelled with $^{14}N_2$ (▲) and $^{15}N_2$ (■) substrates. ^{14}N (R1) and ^{15}N (R4) RNA was centrifuged individually and detected fluorometrically.

A mix of ^{14}N- and ^{15}N-labelled RNA (500 ng each) were run under the same gradient conditions as previously. The average spread across the peaks was 1.7684 – 1.7836 g ml^{-1} (n = 3), showing a difference of 0.0152 $\pm$ 0.002 g ml^{-1}. The peak was spread mostly across three fractions and represented the same densities as seen by the labelled and unlabelled individually run gradients. The highest peak for these mixed RNA gradients is in the middle of this spread and likely represents a mix of ^{14}N- and ^{15}N-RNA. The mixed sample resulted in a single RNA peak encompassing the densities observed in the unmixed gradients (Figure 4.4).

The mixed RNA peak was spread over a density range of 1.7682 – 1.7836 g ml^{-1} which is wider than the sum of the two unmixed parent RNAs. This spread was further apart than the spread between the resolved peaks for ^{14}N- and ^{15}N-individually run RNA samples. The $^{15}N_2$-labelled gradient (Figure 4.4) was at a slightly heavier density than the mixed sample indicating that when labelled and unlabelled RNA were run together they co-occurred and tended to spread rather than create defined separate peaks. This phenomenon of co-occurring RNA is presently not well understood (Pers. comm. Mike Manefield).

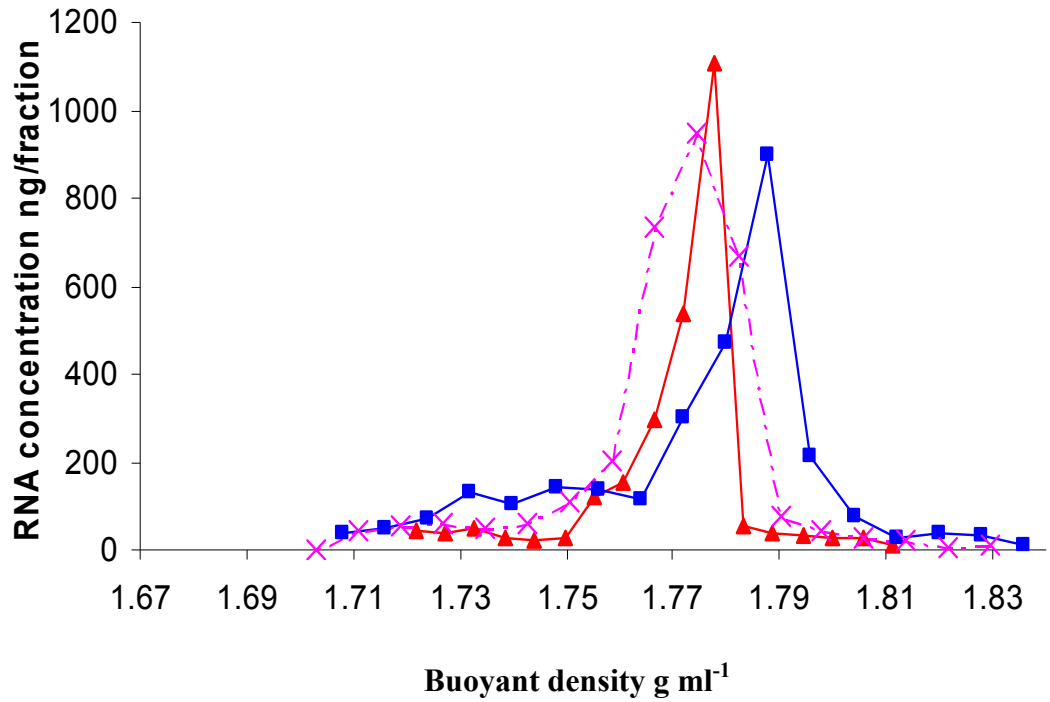

Figure 4.4. Density gradient centrifugation of $^{14}N_2$ (▲) and $^{15}N_2$ (■) gradients run individually compared with a mix of $^{14}N_2$- and $^{15}N_2$-RNA in one gradient (X).

To determine whether a mix of ^{14}N- and ^{15}N-RNA could be resolved and separated beyond the broad peak in Figure 4.3, longer spin times of 66 hours were employed (compared with 42 hours in previous experiments). It has been reported by Uhlik *et al*. (2008) that lower centrifuge speeds for longer spin times lead to smaller buoyant density spans within gradients, producing better separation of partially labelled RNA due to the higher resolution within a selected buoyant density range. Small differences were seen between the two sets of gradients with the longer spin times resulting in the heavier density moving from 1.85 g ml^{-1} through to 1.80 g ml^{-1}, producing more spread of samples within this heavier density. Unfortunately, ^{15}N-labelled RNA was unable to be resolved at this heavy density so the longer spin time created no further improvements in resolution between $^{14}N_2$- and $^{15}N_2$-RNA.

4.3.5 Nitrogen Fixation Capability - nifH Amplification

Due to its degradable nature, RNA from the enriched fractions was not able to be used in a *nifH* RT-PCR. Therefore, 10 µl of the cDNA (one-step RT-PCR) product, which was generated as a preliminary step to 16S rRNA gene product PCR, was used to amplify the *nifH* cDNA. This PCR method was able to produce a positive amplification of the *nifH* gene around 360 bp long as shown in Figure 4.5. The positive amplification demonstrated that the enriched fractions have a strong nitrogen-fixing capability and indicated that nitrogen-fixing genes were present.

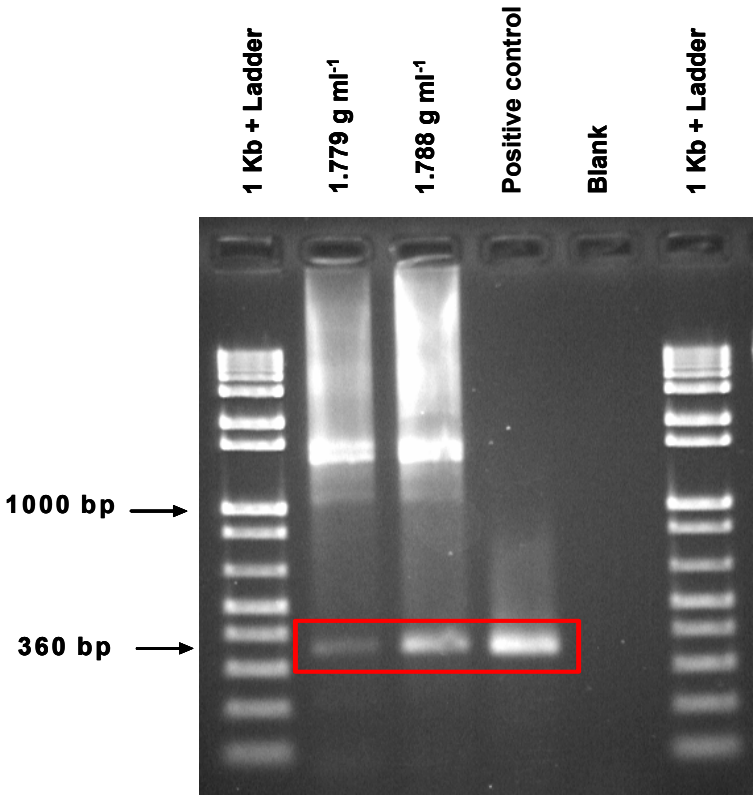

Figure 4.5. PCR of the *nifH* gene from 10 µl of the cDNA generated as a preliminary step to 16S gene rRNA product PCR from the enriched fractions in a $^{15}N_2$-labelled mixed community gradient.

4.3.6 Phylogenetic Analysis of $^{14}N_2$ and $^{15}N_2$ Gradients by T-RFLP Analysis

T-RFLP analyses were conducted across $^{14}N_2$ (R1) and $^{15}N_2$ (R4) gradients with 20 ng diluted RNA. Any fractions with less than 20 ng used 1 µl of RNA from the fractions in the RT-PCR. The T-RFLP analysis worked well with profiles of T-RFs generated for ^{14}N- and ^{15}N-labelled RNA, as shown in Figure 4.6.

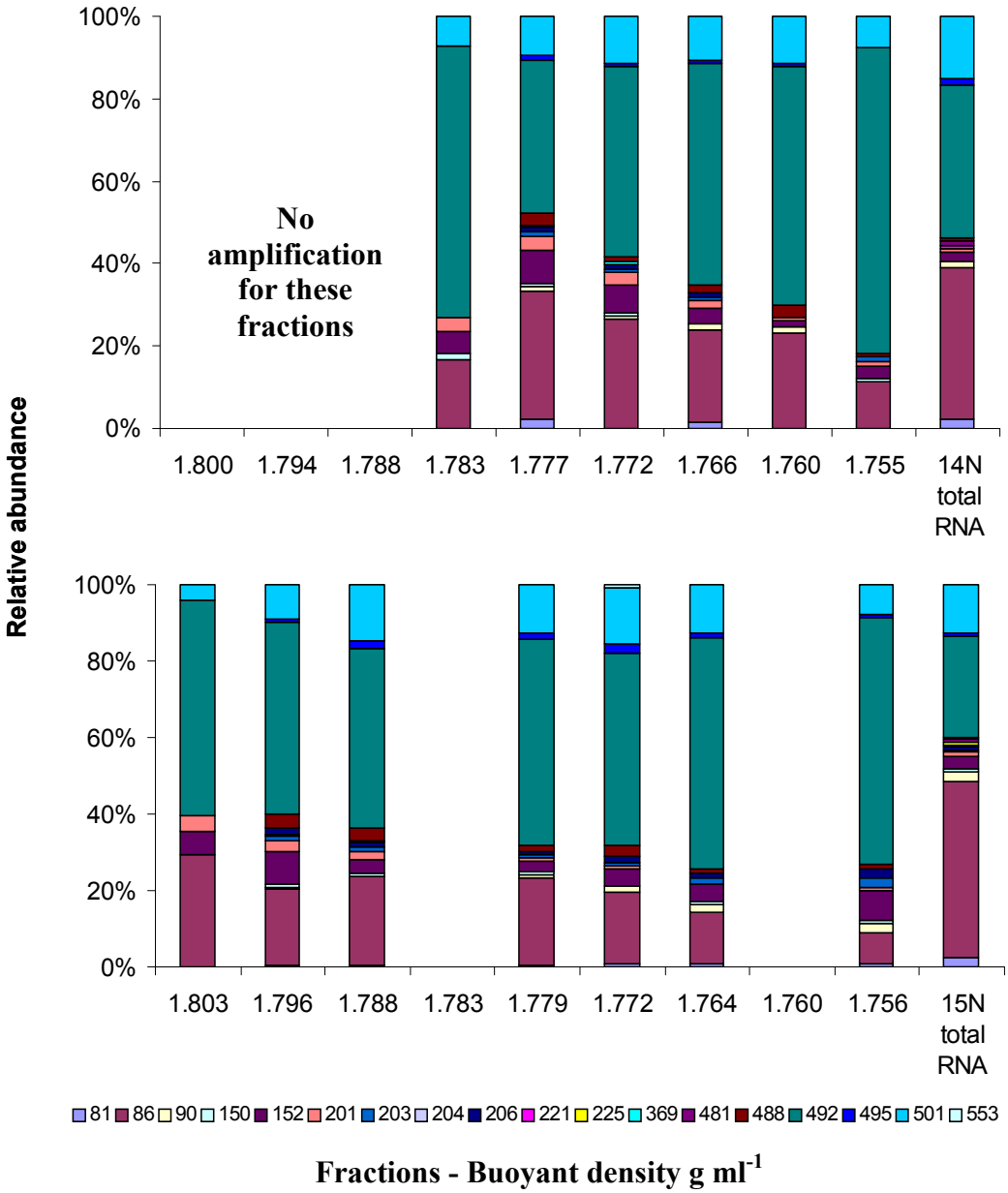

Figure 4.6. T-RFLP results across $^{14}N_2$- and $^{15}N_2$-labelled gradients. RNA was diluted to 20 ng per RT-PCR reaction. Each colour represents the size (bp) of the fragment.

Fractions at the same density were compared between and along the densities of the two gradients. The results show that no profile was obtained for ^{14}N-labelled gradient fraction densities heavier than 1.783 g ml^{-1} because no product was able to be amplified from these fractions. At densities heaver than this, the ^{15}N-labelled RNA was resolved in the $^{15}N_2$-labelled gradients.

^{14}N-gradient fraction 1.777 g ml^{-1} represented the highest ^{14}N-RNA concentration peak whereas 1.788 g ml^{-1} represented the highest RNA concentration peak in the ^{15}N gradient.

A direct comparison between the two gradient's highest RNA peaks showed close similarities with the same major and minor T-RFs represented at similar quantities in the profiles (Figure 4.7). These profiles should represent the same organisms because they were from the same fractions, but shifted to a heavier density within the ^{15}N gradient due to the incorporation of the heavier isotope. There were some differences in the proportions, but the two major T-RFs (86 and 492) combined to the same amount in the community for both the ^{14}N- and the ^{15}N-RNA peaks (68% and 70% respectively). The minor T-RFs were the same between the peak samples and overall only two T-RFs have proportional differences >5% but <9%.

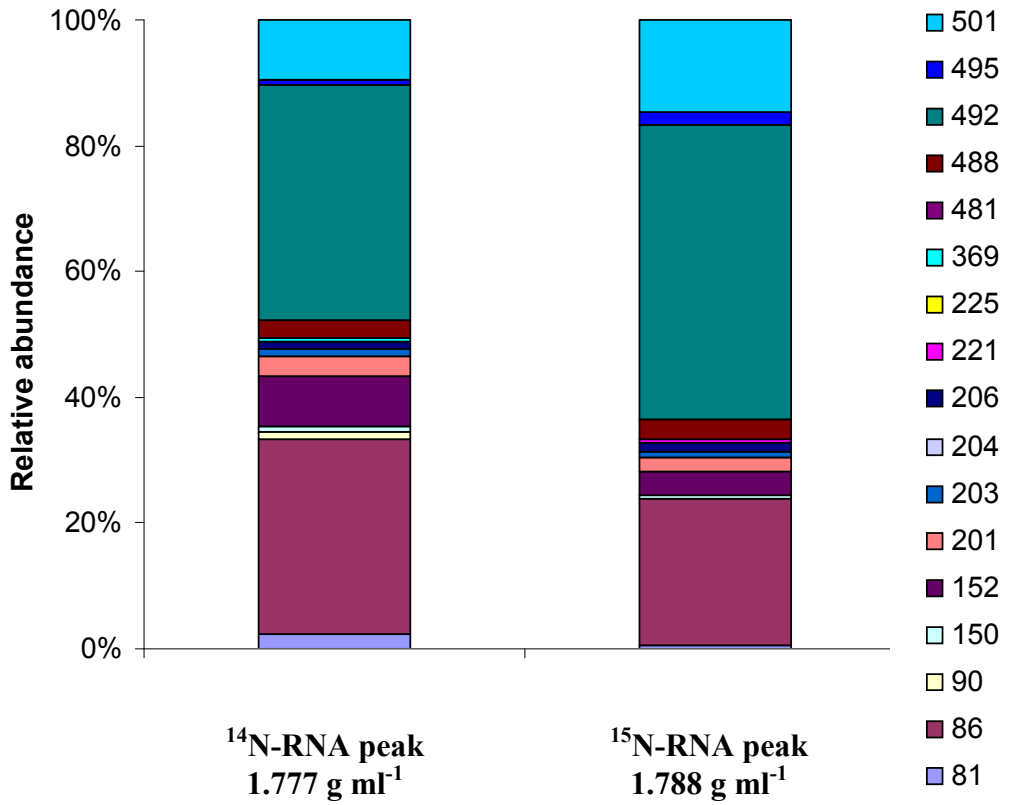

Figure 4.7. Comparison of the T-RFLP profiles from labelled and unlabelled gradient major RNA peaks. Each colour represents the size (bp) of the fragment.

The RNA used for these analyses were normalised by adding only 20 ng per RT-PCR reaction. For fractions with lower concentrations, 1 µl per reaction was used, resulting in no amplification for some fractions and therefore no T-RFLP profile. The normalisation of sample concentration and the use of RNA biased the selection to only active, fast growing, nitrogen-fixing aided growth with N_2 enrichment, leading to similar profiles of organisms present across gradients and between labelled and unlabelled gradients.

At the BD of 1.788 g ml^{-1}, ^{15}N-RNA was significantly enriched (28 times more concentrated) compared to the corresponding ^{14}N-RNA gradient indicating successful incorporation of the heavy isotope. Initial gradient RNA loading concentrations were identical. At this BD, a terminal-restriction fragment (T-RF) profile could be generated from the ^{15}N-RNA sample, while none could be obtained for the ^{14}N-RNA corresponding fraction. Analysis of the ^{15}N-enriched RNA fraction by T-RFLP (Table 4.3) revealed major T-RF signatures at 492, 85 and 500 bp, which represented 46.9%, 23.2% and 14.5% of the community profile respectively.

The major signature at T-RF 492 showed that γ-Proteobacterial lineages, possibly as *Klebsiella* and *Pseudomonas*, were dominant in the nitrogen-fixing population. *Klebsiella* are known to be able to fix nitrogen and have been identified using culture-based approaches in nutrient deficient wastewaters (Gauthier *et al*., 2000). Only a few cultivated *Pseudomonas* species are known to fix nitrogen (Chan *et al*., 1994; Xie *et al.,* 2006), however, this genus is broadly distributed. The closest taxonomic group for T-RF 85 were *Azoarcus* (β-*Proteobacteria*) and *Flavobacterium* (Bacteroidetes), two genera that are known to contain nitrogen-fixing bacteria. The groups of organisms matching to T-RF 500 were not known as archetypal wastewater bacteria and may represent novel nitrogen-fixing wastewater bacteria.

Table 4.3. T-RFLP results from fraction 1.788 g ml^{-1} in $^{15}N_2$-labelled gradient. Closest taxonomic group were assigned using Phylogenetic Assessment Tools (PAT).

Characteristic T-RF lengths (bp)	Relative abundance of T-RFs (% of community)	Closest taxonomic groups
85	**23.2**	***Azoarcus, Flavobacterium***
152	3.9	*Bacillus, Bradyrhizobium*, clones
200	2.2	*Chryseobacterium* clones
205	1.4	*Acinetobacter, Burkholderia, Pseudomonas*, clones
488	3.3	*Achromatium, Acidovorax, Pseudomonas,* clones
492	**46.9**	***Acinetobacter, Klebsiella, Pseudomonas,* clones**
495	2.0	Very diverse range of groups
500	**14.6**	***Actinobacillus, Moritella, Vibrio***

4.3.7 $^{15}N_2$ Enriched Fraction Clone Analyses and Identification

4.3.7.1 Chimeric Sequence Checking

All of the 227 sequences cloned from the enriched $^{15}N_2$-labelled fraction (density 1.788 g ml^{-1}) that were sequenced were run through Mallard which aligns the sequences with *E. coli* K12 16S rRNA gene sequences and then aligns every sequence to one another to identify the deviation from expected and the mean % difference between the sequences. The output graph displayed by Mallard is shown in Figure 4.8. Every point represents one sequence matched to another, with points above the dotted line showing very different and possibly chimeric sequences. Mallard identified a possible 15 sequences as being chimeric and a further 11 as unclear.

To confirm the results obtained from Mallard, the sequences in question were run through the Chimera_Check program. This confirmed that 11 out of the 26 sequences were definitely chimeric and to could be excluded from further clonal analysis.

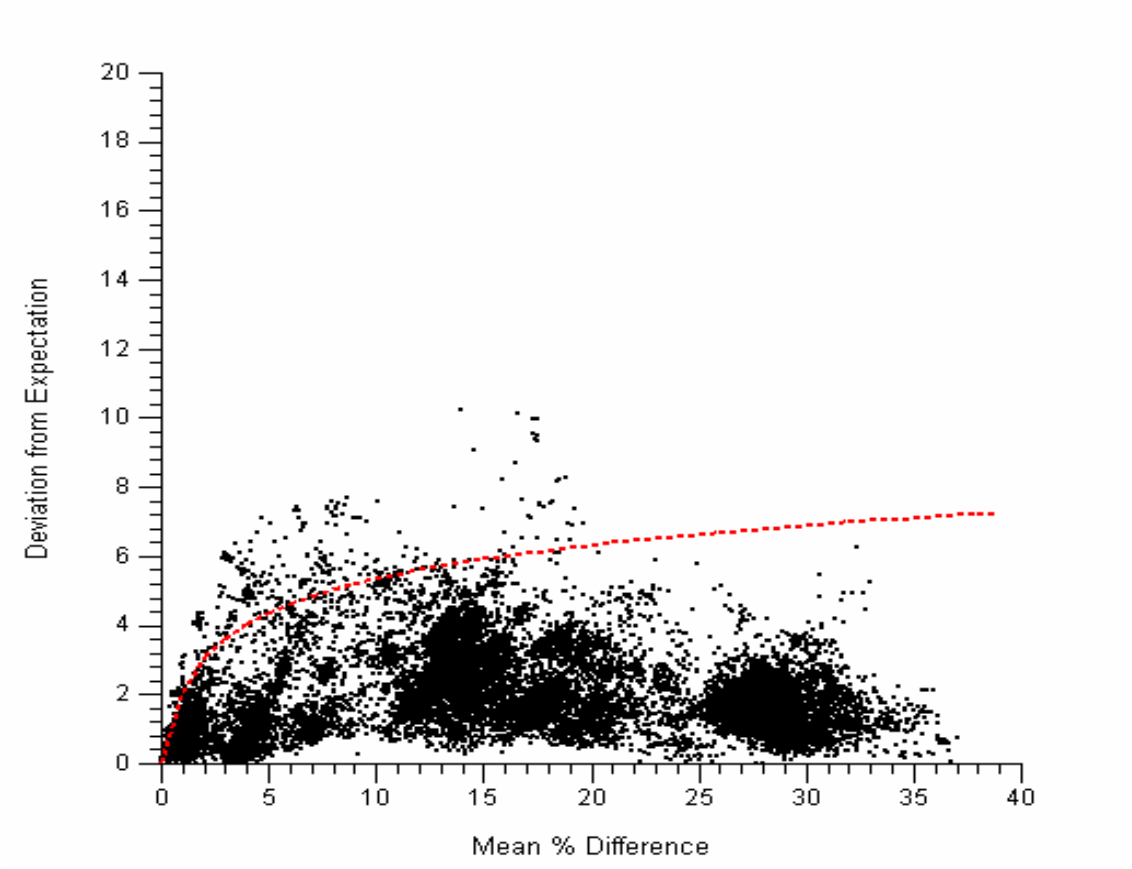

Figure 4.8. Mallard identification of chimeric 16S rRNA sequences. The red dotted line represents the log linear curve with a 99.9% cut off value.

4.3.7.2 Clonal Community Coverage

DOTUR was used as a measure of the community coverage and to determine the amount of coverage performed in the clone library. It also predicted the sample diversity needed to complete a true representation of the community. The chimeric sequences were excluded from this analysis. DOTUR results show that if the sequences were 0% different, more sequences would be needed to get a representative number of clones for the sample (Figure 4.8). Most researchers use either a 1% or 3% difference between sequences. The results demonstrate a plateau in the numbers of OTUs for both 1% and 3% (Figure 4.9).

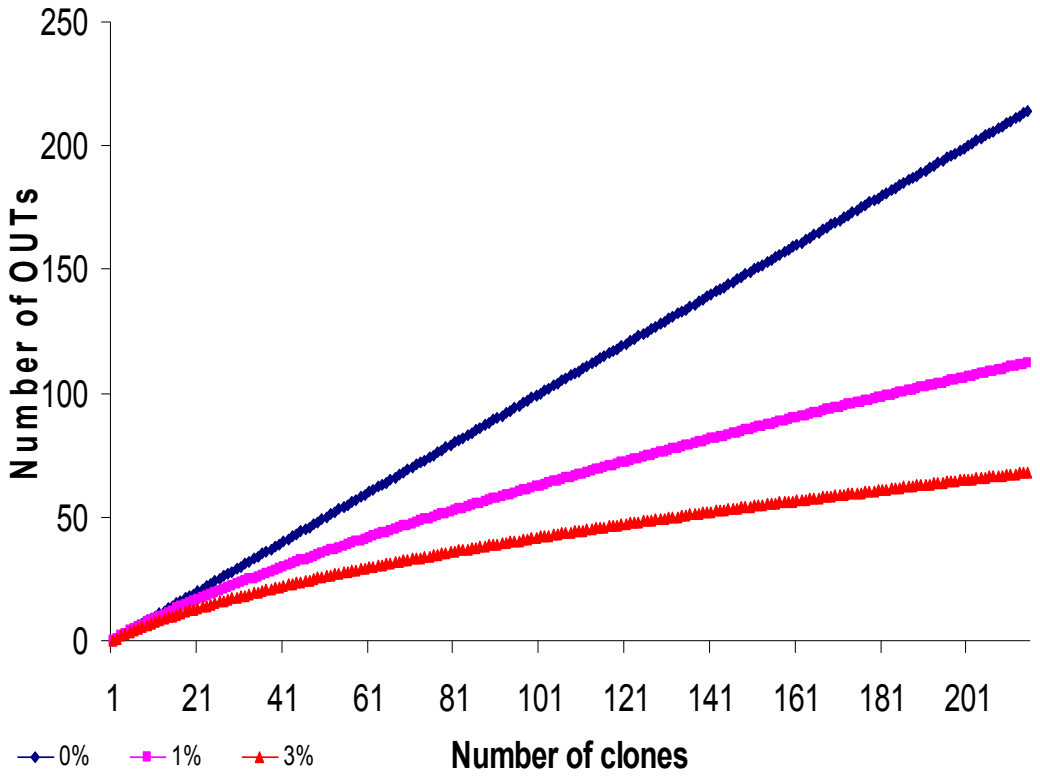

Figure 4.9. Rarefaction plot of clones from enriched $^{15}N_2$ mixed community sample. This was used to assess if the number of clones was representative of the whole community sample.

The analysis of clone libraries was dependent on the number of clones or sequences that were obtained. The higher the number of sequences the better the coverage of the library. Sequence analysis such as DOTUR can measure the species richness and the predicted number of clones needed to reach good coverage for the library to be as complete as possible. Analyses within DOTUR (Table 4.4) showed that out of 216 clones, if the sequences only had 1% difference, there would be 68 different OTUs present. If the sequences were 3% different then only 45 OTUs would be present.

This study also used the Shannon Weaver index to represent the amount of diversity; 0 indicates low diversity and 4 indicates high diversity. Based on the clones in this study, the community showed good diversity with Shannon Weaver indices of 3.34 (1% difference) and 2.85 (3% difference).

Table 4.4. DOTUR analysis of the community coverage from the enriched $^{15}N_2$-gradient fraction.

	1% Sequence difference	3% Sequence difference
Number of OTUs	68	45
Shannon Weaver Index	3.34	2.85
Chao1	180 (115, 335)	91 (61, 176)
ACE	162 (114, 260)	79 (59, 129)

OTU = Operational Taxonomic Units. Numbers in brackets represent 95% lower and upper values.

The Chao1 and ACE indices predict the number of OTUs needed to represent the whole of the community. There were slight differences in these numbers and this was based on the different ways that they are calculated. Both showed that the community has not been covered completely, however, as they are only predictions it is hard to estimate the number of sequences needed to be fully representative. A recent paper by Huber *et al*. (2007) analysed more than 900 000 microbial rRNA amplicons and they found that despite collecting an unparalleled number of sequences the statistical analyses indicated that there was additional bacterial diversity at every taxonomic level.

4.3.7.3 Taxonomic Classification of Clones

The clones identified from the enriched $^{15}N_2$ fraction were sequenced and analysed by BLAST to find the closest organisms match and taxonomic group. A full table of results for each clone can be found in Appendix III. A summary of clones broken up into taxonomic groups and number of clones in each group is shown in Table 4.5.

Table 4.5. Break down of clones into taxonomic groups and number of clones in each group.

Divisional group of clones	Organism with closest match	Number of clones
α-*Proteobacteria*	*Sphingomonas*	1
	Rhodobacter	1
	Zoogloea	1
β-*Proteobacteria*	*Hydrogenophaga*	2
	Leptothrix/Sphaerotillus	2
	Acidovorax	1
	Comamonas	2
γ-*Proteobacteria*	*Aeromonas*	70
	Pseudomonas	99
	Acinetobacter	6
	Azotobacter	3
	Klebsiella	2
	Enterobacter	1
Flavobacterium	*Flavobacterium*	1
Bacteroidetes	*Bacteroidetes*	2
Firmicutes	*Bacillus*	16
	Exiguobacterium	1
Sphingobacteriales	*Flectobacillus*	1
	Sphingobacteriales	1
Unmatched	Clones/unmatched	3

The clones fell into 3 main groups; *Pseudomonas* (99 clones), *Aeromonas* (70 clones) and *Bacillus* (16 clones), with the rest of the clones spread across a wide number of groups. On further investigation, the majority of the clones (83.4%) grouped in the γ-*Proteobacteria* (see Figure 4.10) with a smaller group of clones, 7.8% matching with Firmicutes.

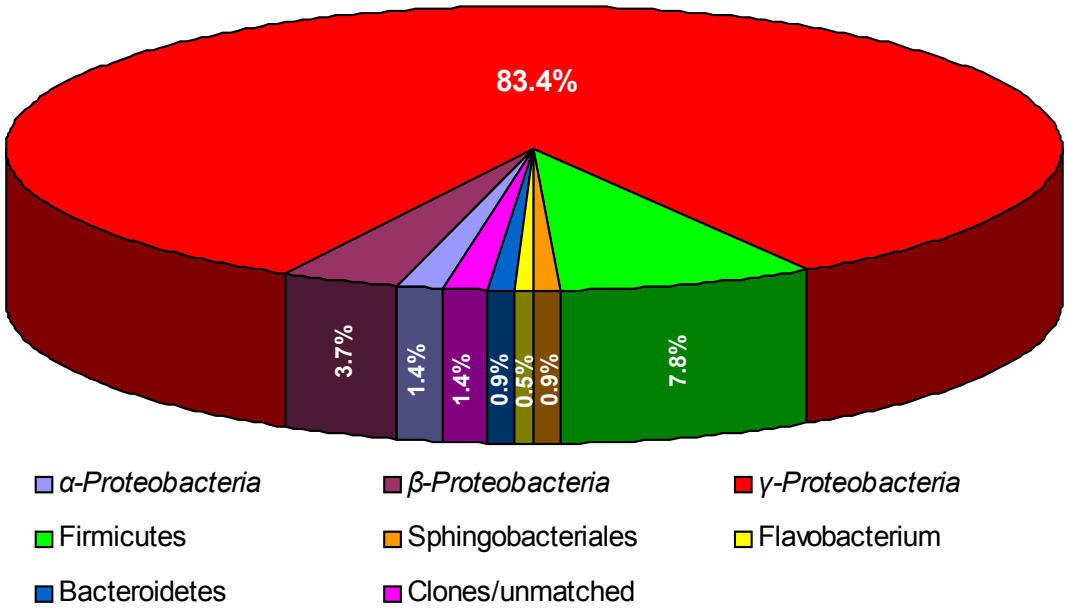

Figure 4.10. Separation of clones into bacterial divisions.

10% (22 out of 216) of the clones represented Gram-positive bacteria and 90% (191 out of 216) represented Gram-negative bacteria. The sequence information from these clones was used to generate a neighbour-joining phylogenetic tree. The large number of clones precluded every sequence being included in the tree, so a representative tree was constructed (see Figure 4.11).

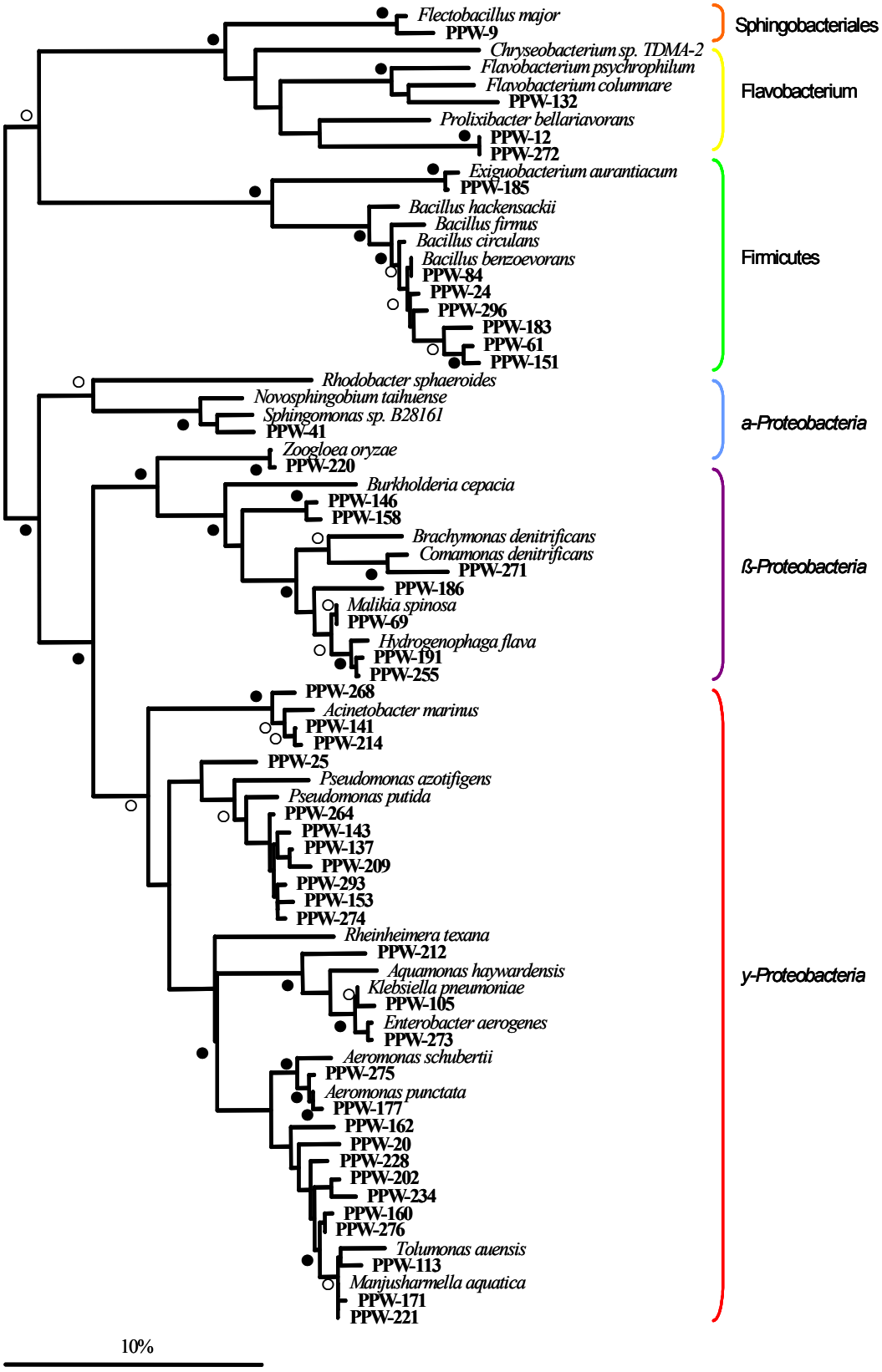

Figure 4.11. Neighbour-joining phylogenetic tree of representative 16S rRNA gene sequences from stable isotope probing pulp and paper wastewater (PPW) clones. Values on tree represent bootstrap values, ● >90% and ○ 70-90% similarity.

The largest group of clones matched to the *Pseudomonas* group. The capacity to fix nitrogen is distributed widely amongst Bacteria and Archaea phyla but had long considered to be absent from the Pseudomonas group (Yan *et al*., 2008). Only a few cultivated *Pseudomonas* species are known to fix nitrogen, including *P. azotofigens*, *P. stutzeri* (Chan *et al*., 1994; Hatayama *et al*., 2005; Yan *et al*., 2008), *P. mendocina* and *P. pseudoalcaligenes* (Xie *et al*., 2006). Interestingly there are only seven *nifH* genes in GenBank that are from *Pseudomonas* sp. and the *Pseudomonas* group currently contains around 150 species. It therefore appears that more members of this group are capable of fixing nitrogen, but few have been investigated and may be difficult to culture, and this ability within the genus is currently poorly understood.

Aeromonas are not traditionally known or recognised as nitrogen-fixing bacteria, however, GenBank shows one *nif* like sequence present (EU035278). The large number of clones represented in this group (70 clones) show variability between the 16S rRNA gene sequences. Xie *et al.* (2006) found that *A. hydrophila* DNA matched with *nif* probes, showing it has nitrogen-fixing capabilities. This group represents a unique opportunity to find a novel group of bacteria within *Aeromonas* capable of fixing nitrogen.

Clones matching to the *Bacillus* group were numerous and represented the majority of Gram-positive bacteria obtained from the clone library. *Bacillus* are found ubiquitously in nature and are not known as archetypal nitrogen fixers, however, many reports have alluded to nitrogen-fixing capabilities. A few studies have investigated this more closely. Xie *et al*. (2006) tested a number of *nif* probes against *Bacillus* sp. DNA and found that a number of *Bacillus* sp. were positive for nitrogen fixation. A total of 11 *nifH* sequences from *Bacillus* sp. are available in GenBank demonstrating the capability for fixing nitrogen by some strains of *Bacillus*.

Clone PPW-185 matched 100% to *Exiguobacterium* sp., which are known as facultative anaerobic alkaliphiles, and form part of the Firmicutes, however, this bacterium is not known for nitrogen-fixing.

A few of the less well represented clones matched to groups with known nitrogen-fixing members. Clone PPW-220 matched closely to *Zoogloea oryzae*, which is a nitrogen-fixing bacterium with positive acetylene reduction assay results and sequencing of *nifH* (Xie and Yokota, 2006b).

There was one clone, PPW-41, which aligned with the *Sphingomonas* group. This group has recently been shown to contain the nitrogen-fixing species *S. azotifigens* (Xie and Yokota, 2006a). *Malikia spinosa* matched closely to PPW-69 and has been grown under nitrogen-fixing conditions (Spring *et al*., 2005). Under nitrogen limitation the strain was able to grow and it was assumed nitrogen fixation was probable with very low amounts of bound nitrogen in the medium (0.1 g l^{-1} yeast extract). Upon further investigation the strain was not able to show positive nitrogen fixation capabilities and was not pursued further. The current study suggests that nitrogen fixation is possible from this bacterial strain.

Clones PPW-12 and PPW-272 matched (91% and 88% respectively) to uncultured bacteria. These clones fitted in between the Flavobacterium and Bacteroidetes groups with the closest match to *Prolixibacter* which is a filamentous, anaerobic organism not know for nitrogen-fixing.

Flectobacillus major is an interesting organism and matches closely (99% similarity) to clone PPW-9. *Flectobacilli* are known as filamentous bacteria isolated from either eutrophic or fresh waters and have been specifically isolated from paper mill slimes (Oppong *et al*., 2003). These organisms have not been shown to be nitrogen-fixing bacteria and may represent yet another novel nitrogen-fixing species previously unidentified for this capability.

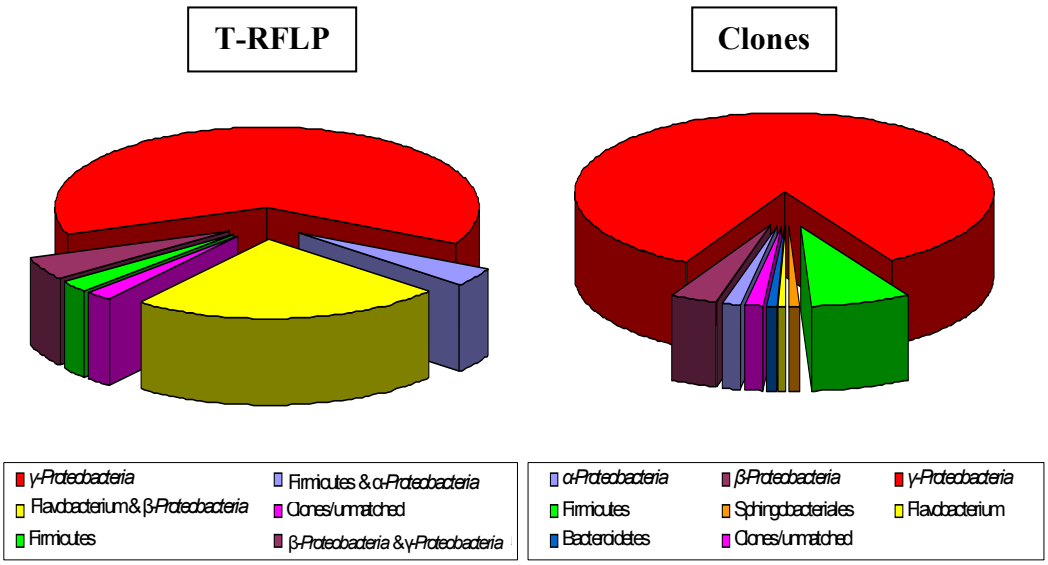

Figure 4.12. Separation of T-RF profile from enriched fraction into bacterial divisions based on Pseudo T-RF digestions compared with actual division from clone sequences.

Comparison of groups of organisms using pseudo T-RF profiles and the clone library sequences both showed the major group was represented by the γ-*Proteobacteria* (Figure 4.12). The T-RFLP profile showed some mixing of the groups and the proportion of other groups was larger than seen for the actual clone library. However, the spread for the pseudo T-RF profile was similar to that seen for the actual clone sequences. T-RFLP only gives an indication of the organisms that could be present based on the database of restriction digests and does not definitively tell what specific organisms are present. When coupled with clone libraries it is possible to display more information and, through analysis, show that the organisms identified as possibly being present by the phylogenetic assessment tool (PAT) are in fact present in similar proportions to the clone library.

4.4 DISCUSSION

4.4.1 Mixed Community Labelling and Gradient Separation

$^{15}N_2$-RNA-SIP methodology was applied to identify diazotrophs that are actively engaged in nitrogen fixation from pulp and paper wastewaters.

Environmental samples collected from a New Zealand pulp and paper mill wastewater treatment system were grown with either $^{15}N_2$ or $^{14}N_2$ as the nitrogen source. The labelled and unlabelled cultures were shown to have strong positive results for the acetylene reduction assay which is indicative of active nitrogen fixation.

The RNA extracted from mixed communities incubated with either $^{15}N_2$ or $^{14}N_2$, was resolved using CsTFA density gradient fractionation. IRMS analysis of total cell biomass confirmed nitrogen fixation with incorporation of 32.6 atom % ^{15}N from $^{15}N_2$. The natural abundance of ^{15}N is 0.37 atom % compared with 99.63 atom % for ^{14}N.

Gradients run individually with labelled and unlabelled RNA from the mixed community showed that ^{15}N-labelling of RNA increased BD by 0.013 ± 0.002 g ml^{-1} ($n = 3$) compared to the ^{14}N control. All the gradients showed the presence of trace background levels of RNA throughout the resolved density gradients at concentrations detectable by fluorometer measurements, despite loading only small amounts of rRNA and the presence of formamide to resolve secondary structures.

However, greater than 65% of the RNA from all individually run gradients resolved into the main RNA peak (5 – 6 fractions). Manefield *et al*. (2002b) reported similar results and concluded that density gradients typically used to isolate RNA based on buoyant density have limited ability to focus RNA into tightly defined bands. This could be caused by the interactions of different rRNA molecules during gradient centrifugation not being fully prevented. RNA has a certain spread on any given gradient with low background levels of unlabelled RNA expected throughout all the gradient fractions (on average 0.7% of maximum quantities) and is most apparent when using PCR to detect templates (Lueders *et al*., 2004a; Uhlik *et al*., 2008). All gradients produced in this study showed low background levels of RNA. These did not interfere with downstream PCR/T-RFLP cloning amplifications because low RNA concentration fractions were unsuccessful for amplification.

Differences between unlabelled ammonium chloride and N_2 mixed communities could be caused by changes in GC content between the different community samples.

The wastewater samples used in this study came from the same sampling site but were collected at different time points and therefore may have had a different distribution of bacteria, meaning a different RNA profile may have altered their natural buoyant density in gradient material.

The CsTFA gradients used to resolve mixed community RNA from both ^{15}N-ammonium chloride and $^{15}N_2$ sources showed less of a shift in BD between labelled and unlabelled peaks when compared to pure culture gradients of *N. nitrogenifigens* Y88. This shift is likely due to the decrease in ^{15}N-labelling for the mixed cultures compared with pure culture RNA. It is also expected that a mixed community would generate a broader spread of RNA due to the heterogeneity of community rRNAs compared to those from a pure culture.

Variation may also have been introduced through minor experimental error in the gravimetric estimation of gradient fraction densities. For example, a micropipette with a 1% measurement error and a balance with +/-1 mg accuracy could plausibly produce estimates of density that vary by 0.02 g ml^{-1}.

Overall, the most probable explanation for the difference between labelled and unlabelled community RNA using the different substrates is the amount of labelled isotope in the RNA. ^{15}N-ammonium chloride is an easily utilised substrate and would become incorporated quickly into all bacterial RNA. On the other hand, $^{15}N_2$ requires longer times to be assimilated and to prepare the necessary pathways to use this substrate. This increases the time dramatically for the bacteria to become fully labelled.

One important part of gradient preparation is whether to run a mix of $^{14}N_2$- and $^{15}N_2$-labelled RNA or to run each in separate gradients. In an environmental sample, generally a mix of labelled and unlabelled will occur making it important to run them together. An equal mix of labelled and unlabelled RNA from a mixed community was resolved on CsTFA gradients and compared to separately run labelled and unlabelled RNA samples. The mixed sample resulted in a single RNA peak encompassing the densities observed in the unmixed gradients.

The peak for these mixed RNA gradients occurs in the middle of this distribution and likely represents a mix of ^{14}N- and ^{15}N-RNA. Using gaseous nitrogen as the only major nitrogen source causes a heavy enrichment in the media for nitrogen-fixing bacteria.

The short incubation times result in not all the ^{14}N in the RNA being replaced with ^{15}N, however, it does mean that the organisms able to fix nitrogen will incorporate the label but not all the nitrogen atoms in the RNA will become labelled.

4.4.2 Enriched Fraction Analyses

The successful separation of $^{15}N_2$-labelled RNA and unlabelled RNA in CsTFA gradients lead to positive 16S rRNA RT-PCR amplifications of the enriched gradient fraction whilst none was obtained from the corresponding fraction in the unlabelled gradient as there was little or no RNA in this fraction (see Figure 4.3). The migration of specific templates into heavier fractions over time as the pulse of label is consumed can be compared by using an unlabelled control pulse. Stable isotope enrichment is then indicated only if the amount of RNA in specific fractions exceeds the amount that is detected in the unlabelled control (Uhlik *et al*., 2008).

The inability to produce a T-RFLP profile for the ^{14}N-gradient at densities >1.783 g ml^{-1} offered a comparison between the control and labelled gradients. The templates moved to a heavier density for the ^{15}N-gradient (1.788 g ml^{-1}) and T-RFLP analysis across the two gradients were expected to produce a spread of the same T-RFs but at slightly different proportions because the same growth conditions were applied, with the only difference being the labelled substrate versus unlabelled substrate.

A clone library was not made from the peak fraction from the ^{14}N incubation as the T-RFLP analysis for this fraction showed very similar bacteria present to other fractions except for changes in observed proportions. This was due to the highly selective nature of the environmental sample and the fact that RNA selected for the highly active, fast growing bacteria. In addition, there was no RNA in the corresponding fraction from the ^{14}N gradient which also suggested that it is highly unlikely that there was any ^{14}N contaminating the ^{15}N fraction used for the library. The positive amplification of the $^{15}N_2$-enriched gradient fraction resulted in a suitable T-RFLP profile and a clone library. The clone library produced both known and also unknown nitrogen-fixing bacterial groups.

Previous DNA-based studies of whole populations using 16S rRNA genes have shown a low abundance of free-living nitrogen-fixing γ-*Proteobacteria* associated with New Zealand pulp mill wastewaters (Reid *et al*., 2008). This study showed a dominance of α-*Proteobacteria* and β-*Proteobacteria* from the whole population. Bowers *et al*. (2008) found a similar spread of α-*Proteobacteria* and β-*Proteobacteria* through *nifH* gene amplicon sequencing from New Zealand pulp and paper wastewaters. Both of these previous studies analysed the whole population including active and non-active organisms.

A summary of 16S rRNA-based diversity studies of wastewater treatment plants and reactors (Wagner and Loy, 2002) revealed that the average spread of bacteria fell predominantly into β-*Proteobacteria* with an even spread between α-*Proteobacteria,* γ-*Proteobacteria*, and Bacteroidetes. Some of the clone libraries analysed used small numbers of clones, reducing the numbers within the libraries and therefore decreasing the community coverage. The different wastewaters resulted in different proportions of the groups of bacteria identified. However, one common feature among the studies was that the same general groups were found in all cases.

There were only small quantities of nitrogen (~5 mg l^{-1}) from the yeast extract used in the growth medium and, since only very low concentration of nitrogen were present in the original wastewater, extremely minimal transfer of any nitrogen to the media should have occurred. This made the media heavily nitrogen limited, forcing the bacteria to use N_2 to grow. RNA-SIP therefore would have lead the active portion of the community to become labelled as $^{15}N_2$ was the only major nitrogen source in the media.

Clone library analysis from the active portion of this nitrogen-fixing pulp and paper wastewater community resulted in a dominance of γ-*Proteobacteria* and smaller numbers of Bacilli and β-*Proteobacteria* clones, showing a different proportion of clones to those identified by Reid *et al*. (2008).

Klebsiella has been shown to be a major component of pulp and paper mill effluents in a number of studies which have utilised culturing techniques (Gauthier *et al*., 2000; Kapley *et al*., 2007). In the study by Kapley *et al*. (2007), it was shown that 24/169 isolates were *Klebsiella*, but in their clone library found 0/194 *Klebsiella* sequences were present.

RNA-SIP in this study utilised culture-independent techniques and it was shown that only two clones matched to *Klebsiella* species out of 220 clones. This supported outcomes of Reid *et al.* (2008) where they did not detect any *Klebsiella* species from a clone library consisting of 50 different clones from the analysed pulp mill effluents.

It is possible that cloning biases may have preferentially amplified non-*Klebsiella* species but it is far more likely that high proportions of *Klebsiella* bacteria in other studies was due to their ease of culturability compared to most other bacterial species present. This accentuates the need for culture-independent methods to accurately analyse populations and their proportions within a community.

4.5 SUMMARY

Gradients run individually with labelled and unlabelled RNA from the mixed community showed that $^{15}N_2$-labelling of RNA increased BD by 0.013 ± 0.002 g ml^{-1} ($n = 3$) compared to the ^{14}N-control. This shift was less than the shift observed for both pure culture and mixed cultures labelled with $^{15}NH_4Cl$ and correlated to a lower percentage of labelled RNA through use of gaseous nitrogen as a nutrient source.

At a BD of 1.788 g ml^{-1} ^{15}N-RNA was significantly enriched (28 times more concentrated) compared to the corresponding ^{14}N-RNA gradient, indicating successful incorporation of the heavy isotope. Stable isotope enrichment was confirmed because the amount of RNA in specific fractions exceeded the amount that was detected in the unlabelled control even when initial gradient RNA loading concentrations were identical. At this BD a terminal-restriction fragment (T-RF) profile and clone library were generated from the ^{15}N-RNA sample, while none could be obtained for the ^{14}N-RNA corresponding fraction.

16S rRNA gene clone sequencing led to the identification of a wide range of bacteria from the enriched ^{15}N gradient RNA fraction. The majority of clones belonged to the *γ-Proteobacteria*, with a smaller number of clones represented in the Firmicutes and *β-Proteobacteria*. Low matches of some clones indicated possible new species, with many identified clones fitting into groups that were not well known for their nitrogen-fixing capabilities.

However, throughout the clone library clones were present that matched to known nitrogen-fixing bacteria including *Zoogloea*, *Klebsiella*, *Acinetobacter* and *Pseudomonas putida*. DOTUR analysis showed good clone coverage and good community diversity with a high Shannon Weaver Index >2.8. Comparison of pseudo T-RFLP results with clone data showed a similar trend, with the *γ-Proteobacteria* representing the largest group in both approaches and similar proportions of the other groups.

The identification of clones from the $^{15}N_2$-enriched fraction has led to useful insights into the bacteria involved in nitrogen fixation from pulp and paper wastewaters. A mix of Gram-positive and Gram-negative bacteria were identified, with Gram-negative *γ-Proteobacteria* dominating the population. The majority of clones were identified as being obligate and facultative aerobes with very few obligate anaerobes.

Overall, the application of $^{15}N_2$-RNA-SIP methodology to pulp and paper wastewaters has demonstrated the ability to successfully resolve labelled and unlabelled RNA in CsTFA gradients in order to reveal the active nitrogen-fixing population.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

5.1.1 ^{15}N-RNA-SIP Protocol Development

In this study SIP experiments have confirmed that ^{15}N-labelled RNA can be resolved through CsTFA gradients to allow the successful use of ^{15}N-RNA-SIP for analysing mixed bacterial populations that use nitrogen substrates. Furthermore this study has demonstrated that ^{15}N-RNA-SIP can be used to isotopically label nitrogen fixing bacteria using $^{15}N_2$ as a nitrogen source. This was possible despite the lower level of incorporation of ^{15}N from $^{15}N_2$ (32.6 atom % ^{15}N) compared to that from a more easily assimilated nitrogen source such as ammonium (80.2 atom % ^{15}N).

After incubating mixed microbial communities with ^{15}N-ammonium chloride or $^{15}N_2$, ^{15}N-RNA was isolated and fractionated by isopycnic centrifugation in caesium trifluoroacetate (CsTFA) gradients. It was found that the more isotopic label incorporated, the further the buoyant density (BD) separation between ^{15}N- and ^{14}N-RNA. Pure cultures of *Novosphingobium nitrogenifigens* gave an average BD shift of 0.03 $\pm$ 0.004 g ml^{-1} (95.0 atom % ^{15}N) with $^{15}NH_4Cl$. For mixed communities an average BD shift of 0.02 $\pm$ 0.004 g ml^{-1} (80 atom % ^{15}N) was found with $^{15}NH_4Cl$ and 0.013 $\pm$ 0.002 g ml^{-1} (32.6 atom % ^{15}N) with $^{15}N_2$. Across all gradients run individually the RNA was resolved into single broad peaks, with the peak centre being significantly different between ^{14}N- and ^{15}N-RNA (t (10) = 2.44, $p < 0.05$).

Gradients that contained a mixed of ^{15}N- and ^{14}N-RNA were unable to be fractionated into two discrete peaks at different buoyant densities. This puts some limitations on the use of this method for future experiments.

5.1.2 Application of ^{15}N-RNA-SIP to Environmental Sample

To demonstrate that ^{15}N-RNA-SIP could be used to identify important contributors to nitrogen cycling, 16S rRNA gene cloning of the $^{15}N_2$-enriched RNA was used to reveal a nitrogen-fixing population dominated by *γ-Proteobacteria* in pulp and paper wastewater.

Clone library analyses of 16S rRNA genes present in the enriched ^{15}N-RNA fraction of the mixed communities found them to consist of a diverse population of bacteria as indicated by a Shannon Weaver index value of >2.8. Three main genera (*Aeromonas, Pseudomonas* and *Bacillus*) were identified by comparison with published sequences and phylogenetic analysis. Many other groups not known as archetypal nitrogen-fixing bacteria were also identified such as *Aeromonas*, *Flectobacillus*, *Exiguobacterium* and *Pseudomonas*, demonstrating that $^{15}N_2$-RNA-SIP provided a useful tool for the identification of important and previously unknown contributors to nitrogen fixation in a range of environments.

Here we have demonstrated the discriminatory power of RNA-SIP for targeting specific active sub-populations, in this case the active nitrogen-fixing community, and in doing so have revealed a distinct nitrogen-fixing population to be active in pulp and paper wastewater.

Overall, this project has established that nitrogen based RNA-SIP is a powerful tool that can be used successfully and reproducibly with both pure and complex mixed microbial communities to study active diazotrophs in environmental samples. Outcomes of this study are now being transferred to industrial end users to aid in optimisation of existing treatment system performance and to enhance opportunities for novel process configurations.

Applications include microbial production of biopolyesters from carbon-rich waste streams, lignin transformations in nitrogen-fixing gut bacteria from native termites, and blue-green algal nutrient cycles in eutrophic lakes.

5.2 RECOMMENDATIONS FOR FUTURE WORK

The ^{15}N-RNA-SIP methodology has significant potential for application to a wide range of nitrogen substrates and environments:

- Further investigation of ^{15}N-RNA-SIP methodology could be done to improve the separation between labelled and unlabelled RNA. This could include altering centrifugation speeds and spin times, using different methods for density measurements, smaller fraction sizes and altering the amount of labelling to show the buoyant density between different degrees of labels.
- Cloning and sequencing of *nifH* from an enriched $^{15}N_2$ fraction could show the distribution of actively expressed *nifH* genes present. This would allow a comparison of distributions of active 16S rRNA gene with active *nifH* gene distribution. It may be possible that limited numbers of different *nifH* genes are present and could show dominance of some genes, indicating horizontal gene transfer. Possible benefits may include identifying dominant nif regions that could be transferred to plasmids and seeded into cultures to promote nitrogen fixation.
- Cells from cultures grown for the $^{15}N_2$- and $^{14}N_2$-labelling studies were collected and stored in glycerol stocks in the -80 ºC freezer. These could be re-grown in NLMM cultures for cultivation of isolates. This might be done to isolate some of the organisms identified in the clone library as novel and not known for their nitrogen-fixing abilities.
- This method shows significant potential in discovering nitrogen-fixing bacteria from a wide range of environments by using $^{15}N_2$ as the only nitrogen source. Other nitrogenous substrates with specific biochemical roles could also be used in this manner. For example, it could target organisms capable of using ^{15}N-labelled carbazole as the carbon source. This compound contains nitrogen in the ring structure and for the organisms to grow they need to break down the carbazole to release the nitrogen. It could lead to the identification of novel organisms capable of breaking down difficult chemicals that persist in the environment.

- The scope for identifying nitrogen-fixers is not limited to bacteria and could expand to include nitrogen-fixing archaea. Archaea have not been studied in nutrient deficient wastewaters and it is not known how large their role is in the breakdown of waste streams.
- Real-time PCR is another technique which could be used in conjunction with RNA-SIP to quantify the gene expression from nitrogen-fixing samples, containing bacteria, archaea, fungi or specific types within these groups.
- Nitrogen cycling is a hot topic especially in impacted environments, such as the Rotorua lakes where excess nitrogen is being leached into the lakes. The methodology described in this project could be used to identify the type of nitrogen leaching into the lakes and then identify organisms proximal to the lake which are capable of using this nitrogen. The Rotorua lakes are suffering badly from years of leaching and the surrounding community is looking for solutions that are both cost effective and will not harm the welfare of various inhabitants in and around the lakes. This offers an opportunity to utilise the natural microbial population present in the lakes. Those organisms that can assimilate this nitrogen and thereby block the nitrogen from going into the lake nutrient cycle could be used for mitigation technologies, hopefully stopping the nitrogen leaching into lakes and reducing toxic algal blooms.
- In today's society there are and have been a large number of pesticides and herbicides that have been applied to land. Large numbers of these compounds contain nitrogen atoms and are not easily broken down and degraded. These could be used as labelled compounds to enrich organisms capable of breaking them down. The identification of these organisms could hold huge potential to reduce pesticide and herbicide contamination in the environment, and could be developed into commercial applications where these compounds are in high concentrations.

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APPENDIX I

Culture Media Formulations

Nutrient agar

Prepared as per manufacturer's instructions.

Nitrogen-limited media

Yeast Extract (Final 50 mg l^{-1} concentration)

5 g in 250 ml milliQ water

Autoclave

Glucose (Final 5 g l^{-1} concentration) – Carbon source varied throughout experiments

25 g in 250 ml milliQ water

Filter sterilise

KH_2PO_4	0.4 g
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$FeCl_3$	10 mg
$Na_2MoO_4.2H_2O$	2 mg
14- and 15-NH_4Cl	Varies
Yeast extract	2.5 ml

Add to 950 ml of milliQ water and autoclave at 121°C for 20 minutes.

Once cool add:

Filtered carbon source	50 ml

Adjust medium to final pH of 7.2 $\pm$ 0.1

Simultaneous RNA/DNA Extraction Methods

1. Wash cells in 1x PBS solution.
2. Re-suspend 0.25 g of biomass in 0.5 ml 240 mM potassium phosphate buffer (pH 8.0) and 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1). Transfer cell suspensions to bead beater vials (same as DNA extraction method).
3. Mix vials in the FastPrep system for 30 seconds at 5.5 m s^{-1}.
4. Centrifuge for 10 min at 14000 rpm and transfer the supernatant to a fresh tube.
5. Add 0.15 ml Buffer QRL1 to the sample (Ensure the B-ME has been added to buffer QRL1 – 10 µL B-ME into 1 ml QRL1).
6. Add 1.35 ml Buffer QRV2. Mix thoroughly by vortexing or shaking.
7. Centrifuge at 14000 rpm for 5 min.
8. During centrifugation prepare the Qiagen tip. Pipette 1 ml of Buffer QRE into the Qiagen-tip to equilibrate. Allow the buffer to enter the column by gravity flow. Allow the buffer to drain completely and discard the flow through. (Do not force out the remaining buffer at the bottom of the tip).
9. Apply supernatant from step 7 to the Qiagen-tip, and allow the resin to enter by gravity flow. Collect the flow-through for simultaneous DNA isolation and leave at room temperature.
10. Pipette 2 ml (2 x 1 ml) of Buffer QRW onto the Qiagen-tip. Allow to enter the resin by gravity flow. (This washes away contaminating proteins, polysaccharides, carbohydrates and cellular metabolites). Do not force out the residual wash buffer. (A working solution of buffer QRU must be prepared by dissolving 29 g of urea in 60ml of buffer QRU, alternatively prepare only the volume needed for your reactions by adding 0.48% (w/v) Urea to Buffer QRU). Buffer QRU/Urea mix is stable for 2 weeks art room temperature. If stored for longer periods of time, the pH of Buffer QRU should be adjusted with HCL to pH 7.0 immediately prior to use.
11. Pipette 1 ml of preheated (45^{o}C) buffer QRU onto the Qiagen-tip, and elute the RNA by gravity flow into a 2ml tube. *
12. Add 1x Vol ice-cold isopropanol. Mix thoroughly by vortexing and place on ice for 10 min.

*** Keep Qiagen-Tip for DNA extraction**

13. Take the flow-through from step 9 and pipette onto the same Qiagen-tip and allow it to enter the resin by gravity flow. Keep the flow through.
14. Pipette the flow-through onto the same Qiagen-tip and allow it to enter the resin by gravity flow.
15. Pipette 3 ml QC buffer on the Qiagen-tip and allow it to enter the resin by gravity flow. Do not force out the residual QC buffer.
16. Elute the genomic DNA by adding 1 ml of preheated (45°C) Buffer QF onto the Qiagen-tip. Collect the flow through in a 2 ml collection tube.
17. Add 0.7 volumes of room-temperature isopropanol. Mix thoroughly and incubate for 10 minutes at room temperature.
18. Centrifuge RNA from step 12 and DNA from step 17 at 14000 rpm for 30 min. Carefully remove the supernatants. (pellet may not be visible, mark on the tube the expected location of the pellet)
19. Add up to 0.5 ml of ice-cold 70% ethanol to the RNA pellet. Vortex and centrifuge at 14000 rpm for 20 minutes. Carefully remove the supernatant.
20. Air-dry the pellets for approximately 10 minutes at room temperature with the tubes resting upside down on a paper towel (over-drying the pellet with make it difficult to dissolve the RNA).
21. Dissolve the RNA in 50 µl of RNase-free water by heating the tube for 3 min at 60^{o}C followed by vortexing for 5 sec and sharply flicking the tube. Repeat this at least two more times. Store the RNA frozen at -20^{o}C.

    Dissolve the DNA in 50 µl of water by heating the tube for 3 minutes at 60°C followed by vortexing for 5 sec and sharply flicking the tube. Repeat at least twice. Store the DNA frozen at -20°C.
22. Run a small amount of the extracts to verify the presence of RNA and DNA.

RNA Quantification

TE is supplied RNase-free as a 20X stock, dilute 1/20 with RNase-free water to achieve the final 1X concentration required.

RNA standard dilution preparation

RNA standard stock (100 µg ml^{-1})	RNase-free 1X TE	Final concentration
6 µl	294 µl	2 µg ml^{-1}

Ribogreen Dilution preparation – prepare within 2 hours of use in RNase-free plastic ware. Multiply this mixture by the number of samples and standards you have.

Ribogreen stock	RNase-free 1X TE	Dilution
0.5 µl	99.5 µl	1/200 dilution

RNA Standard curve preparation

Final RNA concentration (ng ml^{-1})	RNA std dilution (2 µg ml^{-1})	1X TE	Ribogreen dilution (1/200)
1000	100 µl	0 µl	100 µl
750	75 µl	25 µl	100 µl
500	50 µl	50 µl	100 µl
250	25 µl	75 µl	100 µl
100	10 µl	90 µl	100 µl
20	2 µl	98 µl	100 µl
0	0 µl (blank)	100 µl	100 µl

Preparation of unknown concentration total RNA samples

Pipette 2.5 µl of each sample into 97.5 µl of 1x TE and add 100 µl of the Ribogreen dilution to each sample.

Pipette all standards and samples into a black RNAse-free BMG lab Tech fluorometer plate in allocated wells and keep in dark until ready to analyse.

Quantitation using Fluorometer

- Enter program, go into layout and put in layout and standard concentrations, save.

- Select test setup and choose BMG labtechnologies 96 in the microplate line. Alter number of flashes to 020, and check that cycle time is on 120. Set excitation filter at 485 and emission filter at 520 and save.
- Click on traffic light and then gain adjustment, select the most concentrated standard and choose gain adjust. Start test run.
- Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence vs RNA concentration.
- Calculate sample concentrations by multiplying with dilution factor to find ng/ fraction (X4).

Cloning

Kit used: TOPO TA Cloning Kit (with pCR 2.1-TOPO). Catalog number K4500-01, Invitrogen.

LB Medium and Plates

10 g tryptone

5 g yeast extract

10 g NaCl

950 ml MilliQ water

For agar: add 15 g l^{-1} purified agar before autoclaving.

Adjust to pH 7.0 with NaOH and bring volume up to 1 L. Autoclave and cool to 50°C before adding antibiotics.

Antibiotics

Make up solutions of 50 mg ml^{-1} solutions of Ampicillin and Kanamycin in sterile water. Filter sterilise before storing in freezer.

Add 1 ml^{-1} of these solutions to make final 50 µg ml^{-1} concentration in media.

Make up 40 mg ml^{-1} X-gal solution in dimethylformamide (DMF). Store in freezer.

Step 1: PCR product set up

Use as fresh as possible PCR products, A-overhang can drop off over time, reducing ligation and transformation. Best results are obtained from adding A tails the morning of cloning.

Step 2: Setting up Ligation

Set up the TOPO cloning reaction on ice around 1pm in afternoon:

Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
Water	Add to a total volume of 5 µl
TOPO vector	1 µl
Final Volume	**6 µl**

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C). Increasing the incubation time to 20 to 30 minutes, allows more molecules to ligate, increasing the transformation efficiency.
2. Place the reaction on ice. (TOPO cloning reaction can be stored at -20°C overnight).

Step 3: Preparation for Transformation

3. Equilibrate water bath to 42°C.
4. Warm vial of S.O.C media to room temperature.
5. Warm plates at 37°C for 30 minutes.
6. Spread 40 µl of 40 mg ml^{-1} X-gal on each LB plate and incubate at 37°C until ready for use.
7. Thaw **on ice** 1 vial of One Shot cells for each transformation.
8. Add 2 µl of the TOPO cloning reaction into a vial of One Shot Chemically competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
9. Incubate on ice for 5 to 30 minutes.
10. Heat-shock the cells for 30 seconds at 42°C without shaking.
11. Immediately transfer the tubes to ice for 2 minutes.
12. Aseptically add 250 µl of room temperature SOC medium.

13. Cap the tube tightly and shake the tube horizontally at 200 rpm at 37°C for 1 hour.
14. Spread 10 µl (+ 40 µl of SOC) and 50 µl from each transformation onto a pre-warmed plates and incubate overnight at 37°C. Plate two different volumes to ensure that at least one plate will have well-spaced colonies.
15. Store the remaining transformation mix in the fridge. Additional cells may be plated out the next day, if desired.

Step 4: Analysing Transformants

16. An efficient reaction should produce several hundred colonies.
17. Take 2-6 white or light blue colonies and culture them overnight in LB medium containing 50 µg ml^{-1} ampicillin.
18. Isolate plasmid DNA
19. Sequence the construct with the M13 forward and M13 reverse primers. Both are supplied at 0.1 µg $µl^{-1}$ in TE Buffer.

Primer	**Sequence**
M13 Forward	5'-GTAAAACGACGGCCAG-3'
M13 Reverse	5'-CAGGAAACAGCTATGAC-3'

Step 5: Performing the Control Reactions

1. For each transformation prepare two LB plates containing 50 µg ml^{-1} kanamycin. For pUC19 control prepare two plates containing 100 µg ml^{-1} ampicillin.
2. Produce a 750 bp control PCR product using following reaction.

Temp	Time	No. of cycles	ddH_2O	41.5
94	2:00	1	PCR buffer 10 x	5
94	1:00	25	dNTP Mix	0.5
55	1:00	25	Control primers	1
72	1:00	25	Control DNA template	1
72	7:00	1	Taq DNA pol 1 U $µl^{-1}$	1
4		1	**Total**	**50**

3. Analyse the product by gel electrophoresis. If a discrete 750 bp band is present proceed to the control cloning reactions.
4. Set up control TOPO cloning reactions:

Reagent	**"Vector Only"**	**"Vector + PCR Insert"**
Control PCR Product	-	1 µl
Water	4 µ l	3 µl
Salt Solution	1 µ l	1 µl
TOPO vector	1 µl	1 µl

5. Incubate at room temperature for 5 minutes and place on ice.
6. Transform 2 µl of each reaction into separate vials of One Shot competent cells (same as with samples). At same time transform 10 pg per 50 µl of pUC19 of cells using same protocols (transformation efficiency control).
7. Spread 10-50 µl of each transformation mix onto LB plates **containing 50 µg ml^{-1} kanamycin** and X-Gal. For pUC19 cells use LB plates containing 100 µg ml^{-1} ampicillin.
8. Incubate overnight at 37°C.

Hundreds of colonies from the vector + PCR insert should be produced. 95% of these will be white and 90% of these should contain the insert when analysed by EcoR I digestion and gel electrophoresis.

Only a few colonies will be produced in the vector-only reaction, most will be dark blue.

Plasmid Preparation

Method from: S. E. Saunders and J. F. Burke. Nucleic Acids Research **18** (16) 4948

Solution I

To 100 ml water add:

0.9 g glucose

0.37 g EDTA

0.3 g Tris base

Solution II

To 100 ml water add:

0.8 g NaOH

1 g SDS

Solution III

To 100 ml water add:

29.4 g Potassium acetate

11.44 ml Glacial acetic acid

Autoclave all Solutions before use

1. Re-suspend pellet from 2 ml overnight culture in 130 µl solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA).
2. Add 260 µl solution II (0.2 M NaOH, 1% SDS). Gently invert and swirl Eppendorf tube to ensure complete lysis.
3. Add 200 µl solution III (3 M KOAC, 2 M HOAC) and shake vigorously (2-3 sec) and immediately centrifuge 2 min 10 000 g (Same as 10,000 rpm with our rotor).
4. Carefully remove supernatant (500 µl) and precipitate nucleic acids with 1 ml isopropanol, mix, centrifuge immediately 5 min 10 000 g.
5. Wash pellet with 70% ethanol to remove any salts, centrifuge briefly and remove residual ethanol. Air dry pellet for 2 min at room temperature.
6. Re-suspend pellet in 20 µl sterile water.
7. If worried about RNA being present, add 0.5 µl of RNase to sample and incubate at room temperature for 1-2 hours.

PCR Reactions

16S rDNA PCR

Primer Sequences

27F 5'-AGAGTTTGATCCTGGCTCAG -3'

1492R 5' –GGTTACCTTGTTACGACTT -3'

Reaction Mix

Sterile water	17.25 µl
PCR buffer 10X	2.5 µl
dNTPs 2 mM	2.5 µl
27 F 5 µM	0.5 µl
1492R 5µM	0.5 µl
DNA	0.5 µl
Taq DNA Pol 5 U μl^{-1}	0.25 µl

PCR Program

Temperature °C	Time (mins)	Number of cycles
94.0	3:00	1
94.0	1:00	30
55.0	1:00	30
72.0	1:00	30
72.0	5:00	1
4.0	∞	

Electrophoresis

Run out 4 µl of PCR product with 4 µl loading buffer on a 1.5% agarose mini gel with 1 Kb+ ladder for 60 mins at 120 V. Stain for 20-30 mins in ethidium bromide solution, de-stain in running water and visualise under UV illumination.

16S cDNA RT-PCR

Primer Sequences

27F 5'-AGAGTTTGATCCTGGCTCAG -3'

1492R 5' –GGTTACCTTGTTACGACTT -3'

Reaction Mix

2 X Reaction Mix	12.5 µl
Template RNA (0.1 pg-1 µg)	0.5 µl
27 F (10 µM)	0.5 µl
1492R (10 µM)	0.5 µl
SuperScript III RT/Platinum Taq Mix	1 µl
Autoclaved Distilled Water	10 µl

PCR Program

Temperature °C	Time (mins)	Number of cycles
50.0	30:00	1
94.0	2:00	1
94.0	1:00	25
55.0	1:00	25
68.0	1:30	25
68.0	5:00	1
4.0	∞	

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Restriction Digest

Per reaction:

PCR product (FAM labeled primer)	13.5 µl
10X buffer	3.0 µl
Sterile water	10.5 µl
Enzyme (10 U μl^{-1}) MspI	1.5 µl
Enzyme (10 U μl^{-1}) HhaI	1.5 µl

Samples sent to Waikato DNA Sequencing Facility for Genotyping analysis with 60-900 bp ladder.

nifH PCR

PCR primers and protocol developed by Poly *et al*. (2001).

Primer Sequences

nifH (polF) 5' –TGCGATCCSAARGCBGACTC -3'

nifH (polR) 5' –ATSGCCATCATYTCRCCGGA -3'

Reaction Mix

Sterile water	14.25 µl
PCR buffer 10X	2.5 µl
dNTPs 2 mM	2.5 µl
nifH(polF) 5 µM	2.5 µl
nifH (polR) 5µM	2.5 µl
DNA	0.5 µl
Taq DNA Pol 5 U μl^{-1}	0.25 µl

PCR Program

Temperature °C	Time (mins)	Number of cycles
95.0	2:00	1
94.0	1:00	30
55.0	1:00	30
72.0	1:00	30
72.0	5:00	1
4.0	∞	

Acetylene Reduction Assay

1. Turn off the ionization amplifier while you light the flame. Light the flame by having the oxygen pressure slightly lower than the mark (~9 psi) on the gauge and the hydrogen pressure higher (~28 psi) than the mark (~9 psi). Check the flame is lit by holding a glass or shiny metallic object up to the small vent at the back of the flame head (7). Once the flame is lit, set the gauges to the appropriate marks. Decrease the hydrogen pressure slowly so that you don't kill the flame.
2. The nitrogen should still be on already (~10 psi). Helium is not used for this analysis.

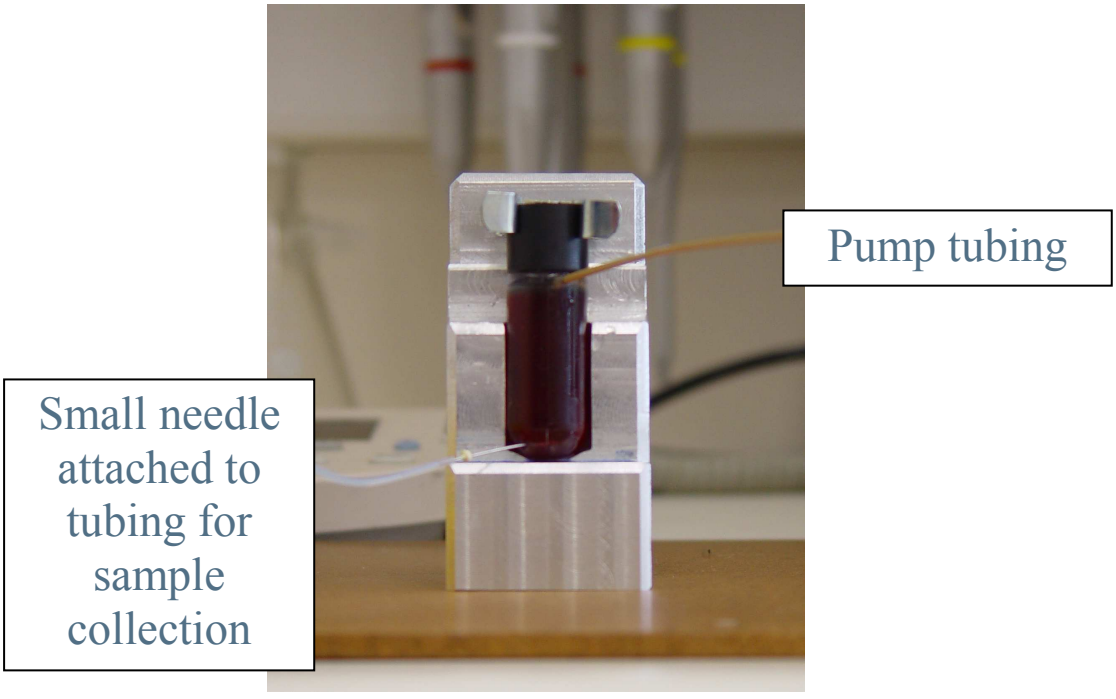
3. Prepare the 50 ml serum bottles by putting a septum and foil cap over the top. Put two syringe needles in the septum. One to put argon into the bottle and the other one to allow the air to escape. Flush the bottle with argon for about 60 seconds.
4. In the fume hood, prepare acetylene by first squeezing as much air as possible out of the gas bladder. Fill a 1 L conical flask with ~ 200 ml of tap water. Add 1 teaspoon of Calcium Carbide and quickly fit with the rubber bung that has tubing attached. Vent the first 30 sec of acetylene then fill the bladder by attaching a syringe needle to the other end of the tubing and pushing the needle through the bladder seal. Leave the full bladder. Remove the bung and let the remaining acetylene vent. Leave the fume hood on.
5. Take 0.1 ml of the prepared acetylene and inject it into the serum bottle.
6. Inject 1 ml of oxygen into the serum bottle.
7. Take 2 x 5 ml of mixed liquor and inject each 5 ml into a serum bottle. Prepare two blanks by using 5 ml of MQ water instead of mixed liquor
8. Put the sealed bottles in a 30ºC water bath for 30 min.
9. Remove ~ 6 ml of headspace gas with a syringe. Stick the syringe needle into a rubber bung to keep the sample sealed before injection.
10. Inject a blank sample into the GC first, then follow with the reactor samples. Inject a sample by turning the injection valve to "L". Remove the needle from the syringe and connect the syringe barrel to the "load loop" port. Feed the gas sample into the injection loop by depressing the syringe plunger. Leave the syringe attached. Simultaneously turn the injection valve to "I" and push start on the integrator.
11. The ethylene peak area for reactor samples that show nitrogenase activity, should be at least 10 times greater than the blank.
12. Inject a pure ethylene standard to check peak distance.

Gradient preparation

1. Prepare a stock solution (20 ml) of CsTFA/Formamide gradient.
    a. 18.6 ml of a 1.99 g ml^{-1} CsTFA solution, 3.75 ml of H_2O, and 750 µl of deionised formamide.

b. Store in the fridge once made and mix well before use.

2. To a polyallomer cone-top centrifuge tube (16 by 45mm) add 4.62 ml of the CsTFA/Formamide solution. Load total 1000 ng RNA and add mineral oil to the top of the centrifuge tube, so that no air is present in the tube. Attach the lid and screw down tightly.
3. Spin in a T-1270 rotor in the ultracentrifuge at 40,000 rpm at 16°C for 42 hours.
4. When stopping the centrifuge, ensure that the break is not on. This will cause the gradient to be disrupted and the fractions will start to mix.
5. Once gradient finished removed from centrifuge, fractionate by pumping water into the top of tube (into the mineral oil fraction). Gradient fractionation is shown in following picture.



6. Collect fractions from the gradient into clean sterile RNAse free tubes, which contain ~200 µl in each one. 17 in total.
7. Set-up a further 17 tubes and accurately weight (to the nearest mg). Transfer 200 µl from the previous tubes into these tubes and weigh accurately. Subtract the tube weight off to give an exact density weight.

8. Precipitate these fractions using 1X volume of ice cold isopropanol, leave on ice for 10 minutes and spin for 30 mins at 14 000 rpm. Remove the supernatant and wash the pellet with 70% ethanol. Spin for a further 15 minutes at 14 000 rpm and remove supernatant. Re-suspend pellet in 10 µl RNase free water.
9. Determine RNA concentrations in each of the fractions with RiboGreen quant. Use 2.5 µl of sample for quant. Samples can be stored in the freezer at this point.

APPENDIX II

^{15}N-Ammonium Chloride Labelled Pure and Mixed Community Cultures

1000 and 5000 ng ^{14}N-RNA were loaded into separate gradients and were fractionated into 400 µl fractions. The following gradients were all prepared, centrifuged, fractionated and quantified as per Appendix I method (Table A.1).

Table A.1. Gradient set-ups for NH_4Cl labelled and unlabelled cultures. P = Pure cultures and M = Mixed community cultures.

Gradient	Sample	RNA concentration loaded	Fraction sizes µl
P1	^{14}N-Y88	5000 ng	400
P2 – P3	^{14}N-Y88	1000 ng	400
P4 – P6	$^{14}NH_4Cl$-Y88	1000 ng	200
P7 – P9	$^{15}NH_4Cl$-Y88	1000 ng	200
P10 – P12	$^{14}NH_4Cl$-Y88 & $^{15}NH_4Cl$-Y88	500 ng each	200
M1 – M3	$^{14}NH_4Cl$ - R6	1000 ng	200
M4 – M6	$^{15}NH_4Cl$ – R6	1000 ng	200

Optimisation of Pure Culture Gradients

Gradients P1 and P2 assessed the differences observed in the amount of RNA loaded into the gradients.

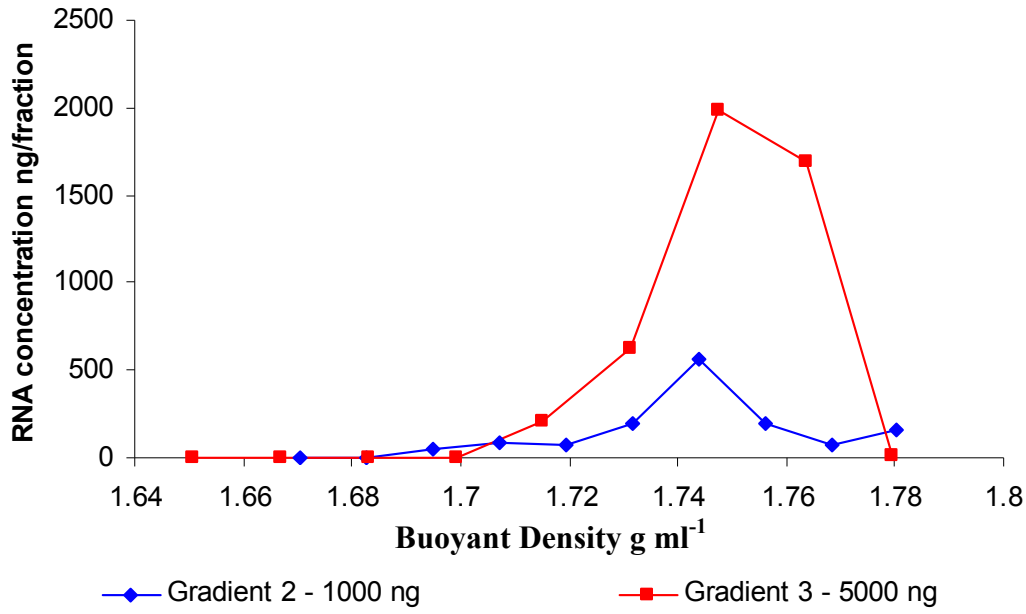


Figure A.1. Fractionation of 1000 ng and 5000 ng RNA from unlabelled Y88 gradients.

Both concentrations of RNA loaded into the gradients showed the RNA banding at the same density for both gradients (Figure A.1). 5000 ng appears to cause overloading of the CsTFA material. 1000 ng total RNA loaded for further gradients. The gradients were fractionated and the fractions precipitated and run out on a gel (Figure A.2).

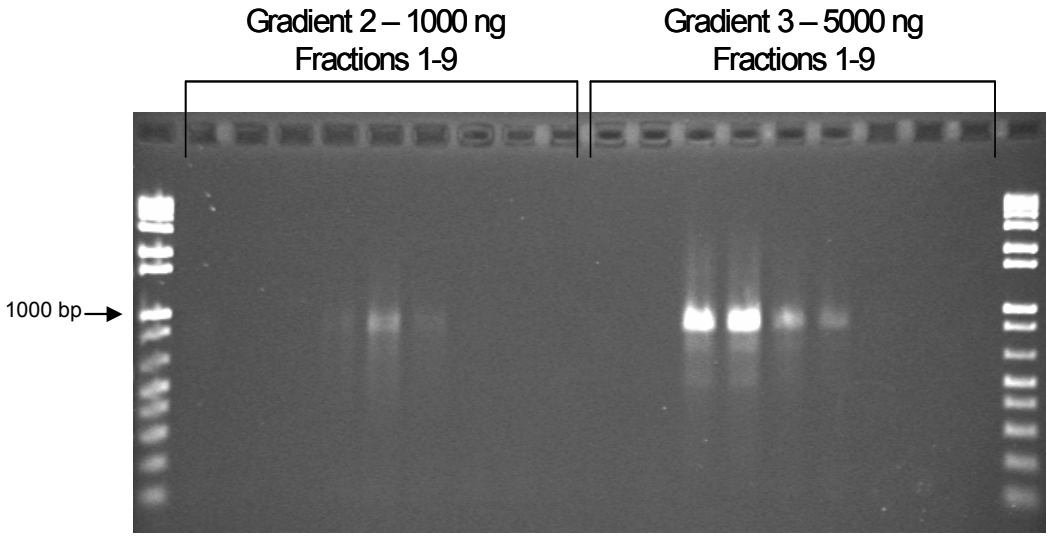


Figure A.2. Gel electrophoresis of RNA gradients fractions from Y88 unlabelled RNA.

^{15}N-Ammonium Chloride Mixed Community Growth Assessment

The growth of the mixed community sample was assessed to enhance the highest amount of growth possible without limiting diversity of the culture. Results for these growth experiments are shown in Table A.2.

Table A.2. Growth results after 24 hours with a variety of variables.

	NH_4Cl concentration	**Inoculum**	**24 Hours OD_{600nm}**
Flasks	None	1%	0.464
	None	0.1%	0.287
	0.1 g l^{-1}	1%	1.431
	0.1 g l^{-1}	0.1%	0.671
	1 g l^{-1}	**1%**	**0.995**
	1 g l^{-1}	0.1%	0.808
Bottles	None	1%	0.383
	None	0.1%	0.342
	0.1 g l^{-1}	1%	0.722
	0.1 g l^{-1}	0.1%	0.525
	1 g l^{-1}	1%	0.830
	1 g l^{-1}	0.1%	0.883

Growth was good for most of the cultures with the best being shown for the ones containing more nitrogen in both flasks and bottles.

After 24 hours the flask 1 g l^{-1} NH_4Cl, 1% inoculum culture was sub-cultured (0.1%) into two flasks; one containing $^{14}NH_4Cl$ and the other containing $^{15}NH_4Cl$. After 24 hours the flasks produced an OD_{600nm} of 2.366 and 2.61 respectively.

Growth Optimisation of Environmental Sample

Table A.3. Growth conditions for real waste sample for determining best growth conditions. Each experiment tested a range of conditions and was completed in order.

Expt	Carbon source	Growth conditions	Nitrogen source
1	0.5 g l^{-1} glucose + 0.5 g l^{-1} sodium acetate	Flasks 30°C, 150 rpm	NH_4Cl -concentration varies
2	0.5 g l^{-1} glucose + 0.5 g l^{-1} sodium acetate	Bottles 10% inoculum Various O_2 volumes 30°C, 150 rpm	$^{14}N_2$ – concentration varies
3	0.5 g l^{-1} glucose + 0.5 g l^{-1} sodium acetate	Baffled vs non-baffled bottles 10% inoculum 30°C, 150 rpm	20 ml $^{14}N_2$
4	1 g l^{-1} glucose + 1 g l^{-1} sodium acetate	Baffled bottles 10% inoculum 20% v/v O_2 + 10% O_2 at T=10 hours 30°C, 150 rpm	$^{14}N_2$ and $^{15}N_2$ 20% v/v headspace

Growth Experiment 1 tested the amount of growth that is possible from a waste stream sample inoculated into synthetic NLMM media containing low nitrogen concentrations and the presence of nitrogen fixation with an acetylene reduction assay. Table A.4 shows the results from this experiment after 24 hours.

Table A.4. Results from growth Experiment 1. Various nitrogen and inoculum concentrations with NLMM, using a mix of 0.5 g l^{-1} glucose and 0.5 g l^{-1} sodium acetate.

NH_4Cl concentration	Inoculum	OD_{600nm} 24 hours	Acetylene reduction assay
None	1%	0.204	+
None	**10%**	**0.422**	+
0.01 g l^{-1}	1%	0.350	+ (weak)
0.01 g l^{-1}	10%	0.628	+
0.05 g l^{-1}	1%	0.734	+ (weak)
0.05 g l^{-1}	10%	0.658	+ (weak)
0.1 g l^{-1}	1%	1.046	-
0.1 g l^{-1}	10%	0.760	-

This first set of experiments showed that the more nitrogen added, the higher the growth and the more inoculum added to low nitrogen containing media, doubled the growth. In higher nitrogen cultures there was little difference in the amount of inoculum added to the final growth. The cultures with more nitrogen showed lower or non existent nitrogen fixation which was expected because cultures use available nitrogen before fixing nitrogen due to the energy expense of fixing.

Experiment 2 tested various nitrogen and oxygen concentrations with 10% inoculum from Pond 19 inlet (sample kept in fridge, 1 month old) in bottles first flushed with argon and then replaced with the nitrogen and oxygen (100 ml headspace and 60 ml media) (See Table A.5).

Table A.5. Results from growth Experiment 2. Various nitrogen and oxygen concentrations with NLMM, using a mix of 0.5 g l^{-1} glucose and 0.5 g l^{-1} sodium acetate.

Bottle	N_2 used (ml)	O_2 used (ml)	OD_{600nm} 24 hours	Acetylene reduction assay
1	20	20	0.228	+
2	20	10	0.212	+
3	10	20	0.222	+
4	10	10	0.210	+
5	-	-	0.143	-
6	0	20	0.207	+
7	Air	Air	0.244	+

The floc production from mixed cultures can make accurate growth measurements difficult, so to reduce floc production with improved aeration in the cultures compared baffled versus non baffled flasks with 20 ml N_2 and 20 ml O_2 in the headspace. After 10 hours, 10 ml extra oxygen was added to the cultures.

Table A.6. Results from growth Experiment 3. Baffled versus non-baffled flasks in starter culture and end culture with NLMM, using a mix of 0.5 g l^{-1} glucose and 0.5 g l^{-1} sodium acetate and 10% inoculum.

Bottle	Flask 1	Flask 2	OD_{600nm} 24 hours	Acetylene reduction assay
1	Baffled	Baffled	0.419	+
2	Baffled	Non-baffled	0.424	+
3	Non-baffled	Baffled	0.389	+
4	Non-baffled	Non-baffled	0.400	+

Measurement of growth in this experiment showed very small differences between the baffled and non-baffled flasks (Table A.6), however, the flocs seen in the baffled flasks were much smaller than in the non-baffled flasks making it easier to get a more accurate measurement of growth. Strong ethylene peaks were shown for all cultures, indicating high nitrogen fixation rates.

APPENDIX III

Table A.7. Overall pulp and paper wastewater (PPW) clone BLAST matches.

Clones	Sequence length	Blastn match %	Closest match	GenBank No
PPW-3	478	465/478 (97%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604321
PPW-5	686	674/677 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604322
PPW-6	693	685/688 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1|	EU604323
PPW-7	747	716/748 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604324
PPW-8	679	655/679 (96%)	Pseudomonas sp. A AY762360.1	EU604325
PPW-9	727	721/727 (99%)	Flectobacillus sp. AJ011917.1	EU604326
PPW-10	652	646/648 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604327
PPW-11	627	627/627 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604328
PPW-12	714	622/677 (91%)	Uncultured bacterium clone SZB84 AM176863.1	EU604329
PPW-13	739	738/739 (99%)	Pseudomonas sp. AJ278108.1	EU604330
PPW-14	706	664/704 (94%)	Pseudomonas luteola strain Marseille AY574976.1	EU604331
PPW-15	687	685/685 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604332
PPW-16	625	617/619 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604333
PPW-17	472	450/472 (95%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604334
PPW-18	545	539/545 (98%)	Uncultured gamma proteobacterium clone DQ446058.2	EU604335
PPW-20	572	548/571 (95%)	Uncultured bacterium clone FB46-15 AY527771.1	EU604336
PPW-21	680	649/679 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604337
PPW-22	663	657/658 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604338
PPW-23	763	736/765 (96%)	Pseudomonas luteola strain Marseille AY574976.1	EU604339
PPW-24	780	776/780 (99%)	Bacillus sp. BT97 DQ358737.1	EU604340
PPW-25	577	531/577 (92%)	Uncultured bacterium clone EF672261.1	EU604341
PPW-27	520	500/521 (95%)	Pseudomonas sp. LFJS3-9 EF660333.1	EU604342
PPW-28	588	565/588 (96%)	Pseudomonas sp. FR1439 AY770691.1	EU604343
PPW-30	353	352/353 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604344
PPW-31	584	579/584 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604345
PPW-33	598	569/598 (95%)	Uncultured bacterium clone DQ817523.1	EU604346
PPW-34	569	548/569 (96%)	Pseudomonas sp. LFJS3-9 EF660333.1	EU604347
PPW-36	518	492/518 (94%)	Bacterium H4 AY345546.1	EU604348
PPW-37	608	577/605 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604349
PPW-38	544	518/542 (95%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604350
PPW-39	617	594/617 (96%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604351
PPW-41	663	647/662 (97%)	Uncultured beta proteobacterium clone DQ316835.1	EU604352
PPW-42	728	724/730 (99%)	Bacillus sp. BT97 DQ358737.1	EU604353
PPW-44	723	719/723 (99%)	Bacillus sp. BT97 16S ribosomal RNA DQ358737.1	EU604354
PPW-45	680	680/682 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604355
PPW-46	612	582/613 (94%)	Pseudomonas luteola strain Marseille AY574976.1	EU604356
PPW-48	761	757/762 (99%)	Pseudomonas sp. AJ278108.1	EU604357
PPW-49	413	383/414 (92%)	Pseudomonas tuomuerense DQ868767.1	EU604358
PPW-50	691	690/691 (99%)	Pseudomonas sp. AJ278108.1	EU604359

PPW-51	636	634/634 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604360
PPW-52	691	686/692 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604361
PPW-53	678	673/676 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604362
PPW-54	723	686/710 (96%)	Pseudomonas sp. AY770691.1	EU604363
PPW-55	728	723/729 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604364
PPW-57	517	488/504 (96%)	Pseudomonas sp. LFJS3-9 EF660333.1	EU604365
PPW-58	544	509/545 (93%)	Uncultured proteobacterium clone DQ234129.2	EU604366
PPW-59	587	575/587 (97%)	Uncultured bacterium AB286482.1	EU604367
PPW-60	744	372/372 (100%)	Aeromonas sharmana strain GPTSA-6 16S ribosomal RNA gene, partial DQ013306.1|	EU604368
PPW-61	589	587/589 (99%)	Bacillus circulans DQ374636.1	EU604369
PPW-62	469	469/469 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604370
PPW-63	492	473/492 (96%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604371
PPW-64	401	401/402 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604372
PPW-65	517	515/516 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604373
PPW-66	499	499/499 (100%)	Aquatic bacterium R1-G8 AB195775.1	EU604374
PPW-68	415	415/415 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604375
PPW-69	538	513/517 (99%)	Aquatic bacterium R1-G8 AB195775.1	EU604376
PPW-70	270	258/270 (95%)	Gamma Proteobacterium AY972868.1	EU604377
PPW-71	349	341/349 (97%)	Uncultured gamma proteobacterium clone DQ409957.1	EU604378
PPW-73	515	515/515 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604379
PPW-76	515	515/515 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604380
PPW-77	314	313/314 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604381
PPW-78	629	627/630 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604382
PPW-79	510	494/510 (96%)	Pseudomonas monteilii strain EF600841.1	EU604383
PPW-80	587	586/587 (99%)	Uncultured Bacillus sp. AM489497.1	EU604384
PPW-81	394	394/394 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604385
PPW-82	141	141/141 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604386
PPW-83	494	475/494 (96%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604387
PPW-84	607	603/606 (99%)	Bacillus circulans DQ374636.1	EU604388
PPW-85	424	405/424 (95%)	Pseudomonas tuomuerense DQ868767.1	EU604389
PPW-86	345	332/345 (96%)	Gamma Proteobacterium BAL281 AY972868.1	EU604390
PPW-87	570	544/568 (95%)	Pseudomonas sp. FR1439 AY770691.1	EU604391
PPW-88	577	574/576 (99%)	Pseudomonas sp.AJ278108.1	EU604392
PPW-90	467	463/465 (99%)	Comamonas sp. DQ851179.1	EU604393
PPW-92	433	419/432 (96%)	Pseudomonas monteilii strain EF600844.1	EU604394
PPW-93	336	323/335 (96%)	Uncultured gamma proteobacterium clone DQ230942.1	EU604395
PPW-94	353	350/352 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604396
PPW-96	580	557/581 (95%)	Pseudomonas sp. FR1439 AY770691.1	EU604397
PPW-98	715	684/712 (96%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604398
PPW-100	696	694/696 (99%)	Bacillus sp. BT97 DQ358737.1	EU604399
PPW-101	462	459/461 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604400
PPW-102	434	433/434 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604401
PPW-104	701	664/690 (96%)	Pseudomonas sp. FR1439 AY770691.1	EU604402
PPW-105	612	608/612 (99%)	Klebsiella pneumoniae strain DQ470487.1	EU604403
PPW-106	668	655/666 (98%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604404
PPW-107	801	799/801 (99%)	Bacillus sp. BT97 DQ358737.1	EU604405

PPW-108	686	684/686 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604406
PPW-109	769	764/770 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604407
PPW-110	611	581/611 (95%)	Azotobacter beijerinckii EF620440.1	EU604408
PPW-112	654	623/654 (95%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604409
PPW-113	690	688/690 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604410
PPW-116	751	747/751 (99%)	Bacillus sp. BT97 DQ358737.1	EU604411
PPW-117	675	644/675 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604412
PPW-118	400	396/400 (99%)	Uncultured bacterium clone DQ447311.1	EU604413
PPW-119	765	743/765 (97%)	Pseudomonas sp. EF660333.1	EU604414
PPW-120	707	674/707 (95%)	Pseudomonas putida AF307869.1	EU604415
PPW-121	700	698/698 (100%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604416
PPW-122	736	704/736 (95%)	Uncultured soil bacterium clone AY699598.1	EU604417
PPW-124	488	467/489 (95%)	Uncultured soil bacterium clone AY699600.1	EU604418
PPW-129	708	678/708 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604419
PPW-130	736	713/736 (96%)	Pseudomonas sp. LFJS3-9 EF660333.1	EU604420
PPW-132	739	745/748 (99%)	Uncultured bacterium clone DQ988321.1	EU604421
PPW-133	391	377/391 (96%)	Gamma Proteobacterium AY972868.1	EU604422
PPW-135	760	731/763 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604423
PPW-136	731	701/731 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604424
PPW-137	736	735/737 (99%)	Pseudomonas sp. AJ278108.1	EU604425
PPW-139	374	360/374 (96%)	Gamma Proteobacterium AY972868.1	EU604426
PPW-141	799	791/792 (99%)	Aquatic bacterium AB195775.1	EU604427
PPW-143	794	760/790 (96%)	Pseudomonas luteola strain Marseille AY574976.1	EU604428
PPW-146	750	723/751 (96%)	Uncultured beta proteobacterium clone AY947999.1	EU604429
PPW-147	587	568/587 (96%)	Uncultured soil bacterium clone AY699600.1	EU604430
PPW-148	802	768/803 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604431
PPW-149	759	757/760 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604432
PPW-150	774	744/775 (96%)	Uncultured soil bacterium clone AY699600.1	EU604433
PPW-151	751	746/751 (99%)	Bacillus sp. BT97 DQ358737.1	EU604434
PPW-152	484	480/484 (99%)	Aquatic bacterium AB195775.1	EU604435
PPW-153	778	737/773 (95%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604436
PPW-155	588	584/585 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604437
PPW-156	630	608/630 (96%)	Pseudomonas sp. EF660333.1	EU604438
PPW-157	736	714/737 (96%)	Pseudomonas sp. EF660333.1	EU604439
PPW-158	823	772/826 (93%)	Uncultured beta proteobacterium clone AY947999.1	EU604440
PPW-159	606	577/606 (95%)	Pseudomonas sp. IBUN MAR1 DQ813307.1	EU604441
PPW-160	776	765/772 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604442
PPW-161	767	739/768 (96%)	Azotobacter beijerinckii EF620440.1	EU604443
PPW-162	682	659/684 (96%)	Uncultured bacterium clone DQ817109.1	EU604444
PPW-163	625	595/625 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604445
PPW-164	734	703/734 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604446
PPW-165	758	726/760 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604447
PPW-167	794	758/796 (95%)	Pseudomonas putida AF307869.1	EU604448
PPW-169	740	735/739 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604449
PPW-170	802	768/804 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604450
PPW-171	778	776/779 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604451
PPW-172	814	811/815 (99%)	Bacillus sp. DQ358737.1	EU604452
PPW-173	774	742/776 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604453
PPW-174	397	389/397 (97%)	Uncultured Bacillus sp. AM489497.1	EU604454

PPW-175	621	619/622 (99%)	Uncultured Bacillus sp. AM489497.1	EU604455
PPW-176	780	774/779 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604456
PPW-177	637	630/637 (98%)	Aeromonas sp. RK 217215 AY987764.1	EU604457
PPW-178	745	741/743 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604458
PPW-179	738	707/740 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604459
PPW-180	408	397/408 (97%)	Uncultured Pseudomonas sp.EU073818.1	EU604460
PPW-181	609	586/609 (96%)	Pseudomonas sp. EF660333.1	EU604461
PPW-182	724	720/723 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604462
PPW-183	721	716/721 (99%)	Bacillus circulans DQ374636.1	EU604463
PPW-185	501	499/499 (100%)	Exiguobacterium sp. DQ407720.1	EU604464
PPW-186	771	771/774 (99%)	Uncultured bacterium clone EF572513.1	EU604465
PPW-187	806	781/806 (96%)	Pseudomonas sp. EF660333.1	EU604466
PPW-188	817	817/821 (99%)	Aeromonas punctata EU082831.1	EU604467
PPW-190	451	445/448 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604468
PPW-191	508	496/509 (97%)	Antarctic bacterium AJ440995.1	EU604469
PPW-192	716	713/714 (99%)	Aquatic bacterium AB195775.1	EU604470
PPW-193	660	657/660 (99%)	Aeromonas sp. AY987764.1	EU604471
PPW-194	773	769/771 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604472
PPW-195	777	746/778 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604473
PPW-199	438	434/435 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604474
PPW-200	691	691/691 (100%)	Bacillus circulans DQ374636.1	EU604475
PPW-201	625	600/625 (96%)	Pseudomonas sp. EF660333.1	EU604476
PPW-202	803	797/801 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604477
PPW-204	800	765/801 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604478
PPW-205	646	642/643 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604479
PPW-206	791	760/791 (96%)	Pseudomonas luteola strain Marseille AY574976.1	EU604480
PPW-207	409	397/408 (97%)	Uncultured Pseudomonas sp. clone EU073818.1	EU604481
PPW-209	634	611/634 (96%)	Pseudomonas sp. EF660333.1	EU604482
PPW-210	359	349/359 (97%)	Pseudomonas sp. AY914075.1	EU604483
PPW-212	826	794/833 (95%)	Enterobacter sp. EF489450.1|	EU604484
PPW-213	698	697/698 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604485
PPW-214	800	791/793 (99%)	Aquatic bacterium R1-G8 AB195775.1	EU604486
PPW-216	404	398/404 (98%)	Uncultured gamma proteobacterium DQ409957.1	EU604487
PPW-217	407	397/403 (98%)	Uncultured gamma proteobacterium DQ409957.1	EU604488
PPW-218	631	601/631 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604489
PPW-220	805	771/792 (97%)	Zoogloea oryzae AB201044.1	EU604490
PPW-221	785	769/779 (98%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604491
PPW-222	763	751/761 (98%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604492
PPW-223	804	769/804 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604493
PPW-228	773	762/771 (98%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604494
PPW-229	804	769/804 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604495
PPW-232	813	755/790 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604496
PPW-234	839	832/836 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604497
PPW-236	411	401/408 (98%)	Uncultured Pseudomonas sp. DQ167048.1	EU604498
PPW-237	407	394/407 (96%)	Uncultured Pseudomonas sp. EU073818.1	EU604499
PPW-239	407	396/403 (98%)	Uncultured gamma proteobacterium DQ409957.1	EU604500
PPW-241	411	396/407 (97%)	Uncultured Pseudomonas sp. EU073818.1	EU604501
PPW-242	759	750/752 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604502
PPW-243	415	397/411 (96%)	Uncultured Pseudomonas sp. EU073818.1	EU604503

PPW-246	695	685/686 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604504
PPW-247	654	624/654 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604505
PPW-249	801	733/812 (90%)	Uncultured bacterium AM176863.1	EU604506
PPW-250	794	759/790 (96%)	Pseudomonas luteola strain Marseille AY574976.1	EU604507
PPW-252	816	802/818 (98%)	Uncultured bacterium AB105442.1	EU604508
PPW-253	742	710/742 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604509
PPW-255	837	834/838 (99%)	Uncultured bacterium clone AY625151.1	EU604510
PPW-257	798	764/798 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604511
PPW-259	808	770/804 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604512
PPW-260	795	760/790 (96%)	Pseudomonas luteola strain Marseille AY574976.1	EU604513
PPW-262	408	394/411 (95%)	Uncultured Pseudomonas sp. EU073818.1	EU604514
PPW-263	406	394/408 (96%)	Pseudomonas sp. EU177802.1	EU604515
PPW-264	888	846/884 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604516
PPW-268	822	818/820 (99%)	Acinetobacter sp. AM235168.1	EU604517
PPW-269	664	634/667 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604518
PPW-270	844	810/839 (96%)	Pseudomonas sp. EU177802.1	EU604519
PPW-271	920	913/919 (99%)	Comamonas sp. DQ851179.1	EU604520
PPW-272	606	550/618 (88%)	Uncultured bacterium AM176863.1	EU604521
PPW-273	884	882/887 (99%)	Klebsiella sp. DQ100465.1	EU604522
PPW-274	874	831/879 (94%)	Pseudomonas sp. EU177802.1	EU604523
PPW-275	910	902/908 (99%)	Aeromonas punctata EU082831.1	EU604524
PPW-276	672	669/670 (99%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604525
PPW-280	768	754/764 (98%)	Aeromonas sharmana strain GPTSA-6 DQ013306.1	EU604526
PPW-281	756	726/759 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604527
PPW-282	870	842/871 (96%)	Aeromonas enteropelogenes EF465529.1	EU604528
PPW-283	867	836/862 (96%)	Uncultured bacterium clone T12-4 AF332355.1	EU604529
PPW-287	868	828/867 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604530
PPW-289	422	406/418 (97%)	Pseudomonas sp. AB007999.1	EU604531
PPW-290	887	847/885 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604532
PPW-293	676	640/673 (95%)	Pseudomonas luteola strain Marseille AY574976.1	EU604533
PPW-294	740	706/738 (95%)	Pseudomonas sp. DQ813307.1	EU604534
PPW-295	407	393/409 (96%)	Uncultured Pseudomonas sp. EU073818.1	EU604535
PPW-296	530	521/524 (99%)	Uncultured Bacillus AM489497.1	EU604536

This thesis was brought to you by the letters A and S and the number 12.