Stable isotope probing: Technical considerations when resolving $^{15}N_2$-labeled RNA in gradients

S.L. Addison[1*], I.R. McDonald[2] & G. Lloyd-Jones[1]

[1]Scion, Private Bag 3020, Rotorua 3010, New Zealand. [2]Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton 3240, New Zealand.

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Abstract

RNA based stable isotope probing (SIP) facilitates the detection and identification of active members of microbial populations that are involved in the assimilation of an isotopically labeled compound. ^{15}N-RNA-SIP is a new method that has been discussed in recent literature but has not yet been tested. Herein, we define the limitations to using ^{15}N-labeled substrates for SIP and propose modifications to compensate for some of these shortcomings. We have used ^{15}N-RNA-SIP as a tool for analysing mixed bacterial populations that use nitrogen substrates. After incubating mixed microbial communities with ^{15}N-ammonium chloride or $^{15}N_2$ we assessed the fractionation resolution of ^{15}N-RNA by isopycnic centrifugation in caesium trifluoroacetate (CsTFA) gradients. We found that the more isotopic label incorporated, the further the buoyant density (BD) separation between ^{15}N- and ^{14}N-RNA, however it was not possible to resolve the labeled from unlabeled RNA definitively through gradient fractionation. Terminal restriction fragment length polymorphism (T-RFLP) analysis of the extracted RNA and fluorescent in situ hybridisation (FISH) analysis of the enrichment cultures provided some insight into the organisms involved in nitrogen fixation. This approach is not without its limitations and will require further developments to assess its applicability to other nitrogen-fixing environments.

Introduction

Stable isotope probing (SIP) allows the identification of an actively metabolising population due to incorporation of an isotopically labeled substrate via a particular metabolic pathway (Dumont and Murrell, 2005; Neufeld et al., 2007; Radajewski et al., 2000; Radajewski et al., 2003; Radajewski and Murrell, 2000; Whitby et al., 2005). Active members in a microbial community incorporate the heavy isotope into cellular material, from which labeled nucleic acids (DNA or RNA) can be isolated for analysis after fractionation by isopycnic centrifugation. The migration of specific templates into heavier fractions over time as the pulse of label is consumed can be compared by using an unlabeled control. Stable isotope enrichment is then indicated only if the amount of RNA in specific fractions exceeds the amount that is detected in the unlabeled control (Uhlik et al., 2008).

The structure of labeled and unlabeled communities is resolved by analysing functional marker genes or rRNA genes to determine community composition. SIP provides a very useful tool for exploring microbial communities under *in situ* conditions, however one of the drawbacks associated with SIP and especially DNA-SIP is that cross-feeding can allow non-target organisms to accumulate isotopic label leading to misidentification. It is here that RNA-SIP offers an advantage over DNA-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. 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 labeled, further decreasing cross-feeding effects. Separation is then based on the newly synthesised RNA from community members that have assimilated the isotopically labeled substrate. The resolution of labeled RNA provides access to the gene sequences which are expressed by functional organisms during substrate assimilation. Manefield and co-workers 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.

The feasibility of SIP has been demonstrated using small ^{13}C compounds but has recently evolved to include ^{18}O compounds (Schwartz, 2007) and ^{15}N compounds (Buckley et al., 2007b; Buckley et al., 2007a; Buckley et al., 2008; Cadisch et al., 2005; Cupples et al., 2007; Roh et al., 2009). ^{15}N containing substrates used for DNA-SIP include; $^{15}NH_4Cl$ (Buckley et al., 2007a; Cupples et al., 2007), $^{15}NH_4NO_3$ (Cadisch et al., 2005), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Roh et al., 2009), and $^{15}N_2$ (Buckley et al., 2007b; Buckley et al., 2008). ^{15}N-substrates have yet to be used successfully in a RNA-SIP experiment.

A 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. The average stoichiometry calculated per nucleotide of C relative to N in RNA is 9.5 carbon molecules to 3.75 nitrogen molecules, which allows the incorporation of 2.5 times more heavy isotope from ^{13}C labeling compared to ^{15}N. Fully ^{15}N-labeled DNA in CsCl shows a density gain of ~0.016 g ml^{-1} (Birnie and Rickwood, 1978) and fully ^{13}C-labeled DNA shows a density gain of ~0.04g ml^{-1} (Lueders et al., 2004a), both of

which can be resolved from unlabeled material through a CsCl gradient. Fully ^{13}C-labeled RNA in CsTFA shows a density gain of ~0.04 g ml^{-1} (Manefield et al., 2002a) over unlabeled material (Lueders et al., 2004a).

In this study we have compared ^{15}N-labeled RNA with unlabeled RNA in CsTFA to determine whether the gain in density is sufficient for resolution of ^{15}N-labeled RNA in a CsTFA gradient. The use of ^{15}N-labeled substrates offers 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. We have applied ^{15}N-RNA-SIP methodology to study nitrogen-fixing communities and identify diazotrophs that are actively engaged in nitrogen fixation from environmental samples using full cycle analysis. The model population used in this study was taken from an environment with known high nitrogen fixation rates (Bruce and Clark, 1994; Clark et al., 1997; Gauthier et al., 2000).

2. Materials and methods

2.1. Bacterial pure culture growth with ^{15}N

Novosphingobium nitrogenifigens $Y88^T$ (DSM 19370) previously isolated from pulp and paper wastewater (Addison et al., 2007), was grown 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 unlabeled ammonium chloride (98+ atom % ^{15}N, Aldrich Chem. Co.).

107 *2.2. Bacterial mixed culture growth and labeling with ^{15}N*

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

113 Mixed cultures for $^{15}N_2$ labeling were collected from New Zealand pulp and
114 paper mill effluent (C:N ratio of 140:1). A 10% vol/vol inoculum of the community
115 was grown in NLMM supplemented with 1 g l^{-1} glucose and 1 g l^{-1} sodium acetate, for
116 24 hours at 30°C, 150 rpm. A 10% inoculum was sub-cultured into two 160 ml sealed
117 flasks in which the headspace (100 ml) was first flushed for 2 minutes with argon gas
118 and then 40 ml was replaced with 20 ml O_2 and 20 ml N_2 (unlabeled N_2 in one flask
119 and $^{15}N_2$ (98+ atom % ^{15}N, ISOTEC) in the other). Flasks were incubated for 24 hours
120 at 30°C, 150 rpm, with a further 10 ml O_2 added to both flasks after 10 hours. An
121 acetylene reduction assay was conducted on a sub sample to confirm the presence of
122 the nitrogenase enzyme as an indicator of nitrogen fixation (Sprent and Sprent, 1990).
123

124 *2.3. RNA extraction and quantification*

125 RNA from pure laboratory-grown cultures and mixed cultures were extracted
126 using an RNA/DNA mini kit that uses mechanical bead beating to disrupt cells,
127 alkaline lysis followed by column purification (Qiagen, Hilden, Germany). Cells were
128 washed in phosphate-buffered saline with 0.25 g biomass re-suspended in 0.5 ml of
129 240 mM potassium phosphate buffer (pH 8.0) and 0.5 ml of
130 phenol:chloroform:isoamyl alcohol (25:24:1). Cell suspensions were transferred to
131 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. The aqueous phase was separated by centrifugation and the RNA purified by RNA/DNA mini kit protocol for bacteria (Qiagen, Hilden, Germany). RNA was stored at -80°C and used within one month of extraction to avoid degradation of RNA. 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 the ultrasensitive fluorescent nucleic acid stain RiboGreen (Molecular probes, Invitrogen) according to manufacturer's instructions. Briefly, a series of standards are made from supplied stock RNA and Ribogreen solution is added to all samples and standards and measured on a fluorometer at an excitation of 485 nm and emission of 520 nm, detecting down to 1 ng/mL RNA.

2.4. Isotopic enrichment using isotope ratio mass spectrometry (IRMS)

The ^{15}N and ^{14}N enriched samples were analysed by the 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 mg N carrier was used in the samples with the detection limit of the machine being no less than 20 mg 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.

2.5. Gradient centrifugation and fractionation

Density gradient centrifugation was performed in 6.5 ml polyallomer Cone-Top tubes in a T-1270 Sorvall rotor spun at 40000 r.p.m (146 000 g_{av}) at 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} stock solution (Amersham Pharmacia Biotech) was combined to a final volume of 4.62 ml with 150 µl of deionised formamide (Manefield et al., 2002b), 1000 ng RNA and pure water (Gibco). The centrifuge tubes were overlaid with mineral oil (Sigma).

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. 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 1 volume of isopropanol, washed with 70% ethanol and re-eluted into 10 µl for determination of RNA using the RiboGreen assay.

2.6. Community profiling with terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP analysis of density-resolved rRNA was performed with 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. The T-RF profile was run through Phylogenetic Assignment Tool (PAT) and the T-RF's were assigned groups based on data from restriction enzyme digests (Kent et al., 2003).

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182 *2.7. Fluorescent in situ hybridisation (FISH) and image analysis*

183 Samples from the enriched cultures grown with N_2 were fixed immediately in
184 paraformaldehyde (PFA) as described by (Amann, 1995). All hybridisations were
185 performed as described by Manz et al. (1992), with 8 µl of sample dried on a Teflon
186 coated slide and dehydrated for 2 minutes each in 50%, 80% and 90% ethanol series.
187 The slides were hybridised at 46°C for 2 h in hybridisation buffer containing 0.9 M
188 NaCl and formamide (percentage of formamide as described in cited references Table
189 1), 20 mM Tris-HCl, 0.01% SDS, and the oligonucleotide probe at a concentration of
190 50 ng μl^{-1}. After washing briefly with distilled water and air dried, the slides were
191 mounted in Vectashield® mounting medium (Vector Laboratories, Inc. Burlingame,
192 California, USA). Oligonucleotide probes used in this study are described in Table 1,
193 and were purchased from Thermo Scientific (Germany); these were modified on the
194 5' end, with either indocarbocyanine dye Cy3 or Cy5 and the 3' end labeled with
195 Amino-C6. Unlabeled competitor oligonucleotides for BET42a and GAM42a were
196 used to improve the specificity of the hybridisation as described previously (Manz et
197 al., 1992). Cell counts of images were calculated using *daime* to determine the
198 average number of β- and γ-*proteobacteria* present (Daims et al., 2006).

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3. Results and Discussion

The use of ^{15}N substrates for labeling RNA has been proposed as a method for which technical difficulties exist due to the low amount of labeling that can be produced with nitrogen substrates (Buckley et al., 2007a; Whiteley et al., 2006). Other limitations have been discussed previously with respect to ^{15}N-SIP (Buckley et al., 2007a; Cadisch et al., 2005) including the resolution of $^{14}N/^{15}N$ bands, different GC contents and the effects of the percent of ^{15}N label. To evaluate the methodology, pure culture bacterial RNA labeled with ^{15}N-ammonium chloride and RNA from an environmental sample from a New Zealand pulp and paper mill effluent grown with $^{15}N_2$ as the nitrogen source was used.

3.1. ^{15}N-Ammonium chloride labeled gradient evaluation

RNA isolated from a pure culture was used to establish conditions for separating labeled (^{15}N) and unlabeled (^{14}N) RNA. ^{15}N-labeled RNA was isolated from *Novosphingobium nitrogenifigens* Y88^T (DSM 19370) grown with ^{15}N-ammonium chloride as sole nitrogen source. Isotope ratio mass spectrometry (IRMS) analysis showed the labeled biomass contained 95.0 atom % ^{15}N. RNA extracts from labeled and unlabeled cultures were loaded individually into CsTFA gradient material and isopycnically separated. The average buoyant densities (BD) of CsTFA resolved gradient fractions were determined gravimetrically and the RNA enriched fraction for ^{15}N had a 0.03 $\pm$ 0.004 g ml^{-1} (n = 3) higher BD compared to the ^{14}N control. Fig. 1 shows representative labeled and unlabeled RNA from *N. nitrogenifigens* Y88^T resolved through separate CsTFA gradients (BD shift by ^{15}N-labeling of RNA of 0.032 g ml^{-1}).

A mixed community taken from a bioreactor treating pulp and paper mill effluent was grown supplemented with ^{15}N-ammonium chloride. RNA was extracted to determine whether ^{15}N-labeled and unlabeled mixed community RNA could be separated based on BD. IRMS analysis of mixed community biomass showed enrichment of 80.2 % atom ^{15}N. ^{15}N-labeled RNA from a mixed community resolved at a heavier density than the unlabeled control. 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.

3.2. Environmental sample $^{15}N_2$-labeled gradient separation assessment

The labeled and unlabeled incubations gave 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 in this biomass was 0.37 atom % compared with 99.63 atom % for ^{14}N. Gradients run individually with labeled and unlabeled RNA from the mixed community showed that ^{15}N-labeling of RNA increased BD by 0.013 $\pm$ 0.002 g ml^{-1} (n = 3) compared to the ^{14}N control. A representative gradient (Fig. 2a) demonstrates that individually run unlabeled RNA migrated to a BD of 1.777 g ml^{-1} and labeled RNA migrated to a BD of 1.788 g ml^{-1}, showing a difference of 0.011 g ml^{-1}.

When the mixed community $^{14}N_2$-RNA and $^{15}N_2$-RNA were run together in a single gradient the separation achieved was less than when run on separate gradients (Fig. 2b). The mixed RNA peak was spread over a density range 1.7682–1.7836 g ml^{-}

250 [1] which is smaller than the sum of the two unmixed parent RNAs. Longer spin times
251 of 66 hours did not increase the resolution of the RNA. When run separately the RNA
252 spanned 5-6 fractions but when combined the RNA spanned only 3-4 fractions. This
253 observation suggests that there is co-mingling of ^{14}N- and ^{15}N-RNA. It is unlikely an
254 environmental sample would be fully labeled so the resolution of ^{14}N- and ^{15}N-RNA
255 in a single gradient is important. A mix of ^{14}N- and ^{15}N-labeled RNA was run together
256 in one gradient and compared with the pooled RNA density results from the
257 separately run gradients. This shows that even when run separately the major peak
258 density is similar to when ^{14}N- and ^{15}N-labeled RNA is run together. This
259 demonstrates that it would be difficult to achieve separate peaks when labeled and
260 unlabeled RNA are run together in a gradient.

261 This study used an environment with known high nitrogen fixation rates to
262 achieve a high proportion of labeling (Bowers et al., 2008; Gauthier et al., 2000). It is
263 likely that for separation of labeled and unlabeled RNA from an environment with
264 lower fixation rates it would be difficult to obtain enough heavy labeled biomass for
265 separation. Experiments with ^{15}N-labeled RNA from both pure culture and mixed
266 communities demonstrated that gradient fractionation and resolution between labeled
267 and unlabeled RNA was suggestive, but not conclusive, for showing separation of the
268 nitrogen-fixing portion of the community. This may be because only 32% of the RNA
269 was labeled in the mixed community. Cadisch et al. (2005) were unable to separate
270 ^{15}N-labeled DNA from unlabeled with less than 40% incorporation, with another
271 paper demonstrating similar results (Roh et al., 2009). The issues involved in
272 resolving the mixed community $^{15}N_2$-RNA compared with the pure culture ^{15}N-RNA
273 indicates that a higher percentage of incorporation may lead to better resolution in a
274 gradient. A mixed community will also generate a broader spread of RNA due to the

heterogeneity of community rRNAs compared to those from a pure culture (Amann et al., 1990; Lueders et al., 2004a).

3.3. Correlation between gradient fractionation and T-RFLP profiling

At a BD of 1.788 g ml^{-1} RNA was enriched in the ^{15}N-gradient compared to the corresponding RNA in the ^{14}N-gradient. Successful incorporation of the heavy isotope was based on IRMS analysis. Initial RNA loading concentrations of the gradients were identical. At this BD a terminal-restriction fragment (T-RF) profile could be generated from the ^{15}N-RNA gradient, while none could be obtained for the corresponding fraction in the ^{14}N-RNA (Figure 3). The inability to produce a T-RF profile for the ^{14}N-gradient at densities >1.783 g ml^{-1}, suggests isotopic enrichment with ^{15}N in this region in the ^{15}N-gradient. This is taken to be due to enrichment of ^{15}N in the RNA leading to a higher buoyant density. This serves to highlight that this methodology has potential to highlight populations that are significantly enriched in ^{15}N, within a range of ^{15}N incorporation levels (32–95%). Additional experiments and validation would be needed to determine whether the methodology described here would be applicable to other environments with lower fixation rates.

RNA is spread across any given gradient, with low background levels of unlabeled 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). Our results showed low levels of RNA throughout the gradients but these levels were so low that RT-PCR was unable to amplify products in the unlabeled heavy density 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

300 bands, and that this could be caused by the interactions of different rRNA molecules
301 during gradient centrifugation not being fully prevented. The heterogeneity of rRNA
302 molecules and some reversal to an un-denatured state in density gradients can result in
303 rRNA from different species displaying buoyant densities that range over 0.08 g ml^{-1}
304 (Lueders et al., 2004a). These phenomena explain some of the likely co-occurring of
305 labeled and unlabeled RNA seen within the $^{15}N_2$ mixed community sample. This low
306 resolution in gradient separation might be resolved by employing a second
307 ultracentrifugation step with bisbenzimide as an intercalating agent to alter the
308 buoyant density of RNA from high G+C micro organisms (Buckley et al., 2007a).
309
310 *3.4. Community profiling of mixed community sample*
311 Diversity within the highest RNA peaks from the two gradients ($^{15}N_2$- and
312 $^{14}N_2$-labeled mixed communities) were similar, with the same major and minor T-RFs
313 represented in similar quantities in the profiles (Figure 3). There were some
314 differences in the proportions, but the two major T-RFs (86 and 492) were present at
315 the same proportion of the community for both ^{14}N- and ^{15}N-gradients (68% and 70%
316 respectively).
317 Analysis of the ^{15}N-enriched RNA fraction by T-RFLP (Table 2) revealed
318 major T-RF signatures at 492, 85 and 500 bp, which represented 46.9 %, 23.2% and
319 14.5% of the community profile. The major signature T-RF 492 shows that γ-
320 proteobacterial lineages, possibly as *Klebsiella* and *Pseudomonas*, are dominant in the
321 nitrogen-fixing population. The closest taxonomic group for T-RF 85 was *Azoarcus*
322 (β-*proteobacteria)* and *Flavobacterium* (Bacteroidetes), two genera that are known to
323 contain nitrogen-fixing bacteria. The organisms matching to T-RF 500 are not known

as archetypal wastewater bacteria and may represent novel nitrogen-fixing wastewater bacteria.

To confirm the presence and abundance of the organisms identified in the T-RF profile, FISH was performed with the use of generic bacterial group probes. The T-RF profile identified that the community was dominated by *γ-proteobacteria*, FISH with GAM42a probe confirmed these results with an average of 87.5 $\pm$ 4.0 % of the community highlighted by this probe compared to the EUB mix probe. The BETA42a probe was used to confirm the presence and abundance of β-*proteobacteria* within the enrichment community with these organisms representing 10.5 $\pm$ 2.0 % of the community. This number is lower than that found for *γ-proteobacteria* and confirms the profile from the enriched $^{15}N_2$ fraction.

Nucleic acid fingerprinting methods can be used to determine the isotopic enrichment by particular microbial groups by comparing fingerprints of gradient fractions from isotopically labeled samples relative to the unlabeled controls (Lueders et al., 2004b; Lueders et al., 2004a). To identify the organisms that assimilated the label, it is necessary to match T-RFs from the enriched gradient fraction with sequences obtained from clone libraries: the possibility that two or more organisms may share a particular T-RFs makes it difficult to interpret these data (Kent et al., 2003; Takeshita et al., 2007). The T-RFLP data from the labeled $^{15}N_2$ mixed community fraction showed a mixture of possible organisms for each T-RF with the dominance of γ-*proteobacteria*, including both *Klebsiella* and *Pseudomonas*. FISH analysis confirmed the dominance in the community of γ-*proteobacteria* and the negligible existence of any *Enterobacter*. The average percentages of the groups found in the community with FISH are similar to that predicted with T-RFLP; ~85% (FISH) and ~60% (T-RFLP) of γ-*proteobacteria* and ~10% (FISH) and ~5% (T-

RFLP) β-*proteobacteria*. *Klebsiella* are known to be able to fix nitrogen and have been shown using culture-based approaches to be present in nitrogen-fixing wastewaters (Gauthier et al., 2000). Numerous studies have looked at different wastewater systems and used DNA based approaches showing α-*proteobacteria* and β-*proteobacteria* dominance (Bowers et al., 2008; Reid et al., 2008; Wagner and Loy, 2002), however, these studies did not examining the active portion of the community and did not involve an enrichment to select for nitrogen-fixing bacteria on a defined carbon source. The identification of the active predominance of nitrogen-fixing γ-*proteobacteria* from wastewater opens the path for the identification of undiscovered nitrogen-fixing organisms.

4. Conclusion

^{15}N-RNA-SIP is a new method that has been discussed in recent literature and this is the first study which investigates the validity of SIP methodology for the separation of ^{15}N-labeled RNA. After incubating mixed microbial communities with ^{15}N-ammonium chloride or $^{15}N_2$ we assessed the fractionation resolution of ^{15}N-RNA by isopycnic centrifugation in caesium trifluoroacetate (CsTFA) gradients. We found that the more isotopic label incorporated, the further the buoyant density (BD) separation between ^{15}N- and ^{14}N-RNA, however it was not possible to resolve the labeled from unlabeled RNA definitively through gradient fractionation. Terminal restriction fragment length polymorphism (T-RFLP) analysis of the extracted RNA and fluorescent in situ hybridisation (FISH) analysis of the enrichment cultures provided some insight into the organisms involved in nitrogen fixation. These initial experiments indicate that this method has potential for mixed microbial communities with a higher degree of labeling however this method is not without its limitations and

374 will require further developments to assess its applicability to other nitrogen-fixing
375 environments.
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489 71.

490

491 **LIST OF FIGURES**

492 **Fig. 1.** Representative analysis of CsTFA density gradient of *N. nitrogenifigens* $Y88^T$

493 RNA labeled with ^{14}N- (▲) or $^{15}NH_4Cl$ (□) substrate centrifuged individually.

494

495 **Fig.2. (a).** Representative analysis of CsTFA density gradient of mixed community

496 RNA labeled with $^{14}N_2$ (▲) or $^{15}N_2$ (□) substrate centrifuged individually. **(b).** A mix

497 of ^{14}N and ^{15}N-labeled RNA run together (-■-) and the addition of individually run

498 gradients (-○-).

499

500 **Fig. 3.** Terminal restriction fragment length polymorphism (T-RFLP) analysis across

501 CsTFA density gradients of mixed community RNA labeled with $^{15}N_2$ or $^{14}N_2$

502 substrate and centrifuged individually.

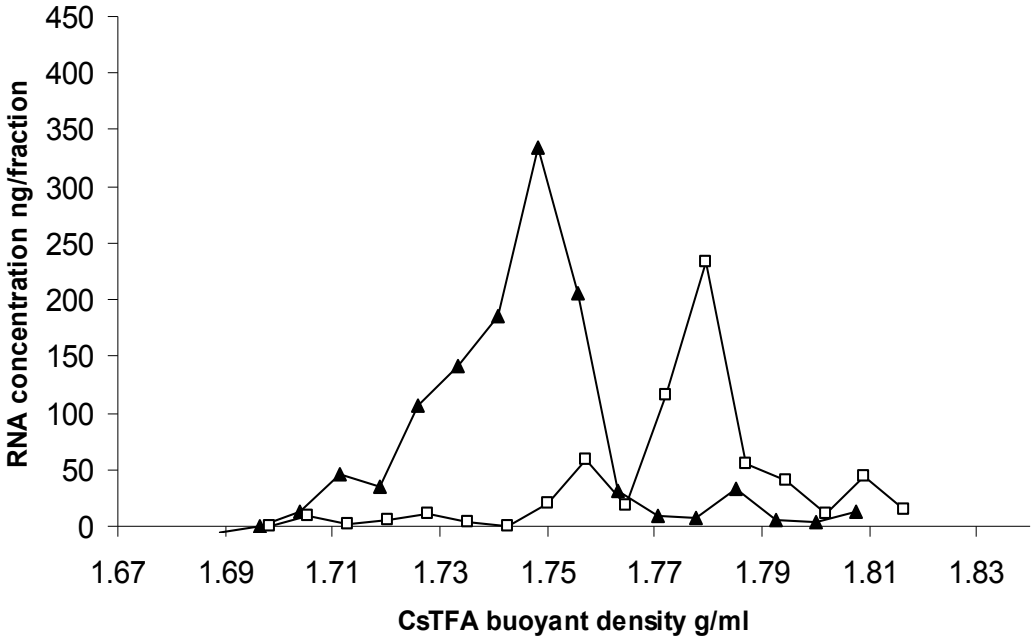
503

504

505

506

507 **Fig.1.**



508

509

510 **Fig. 2.**

511

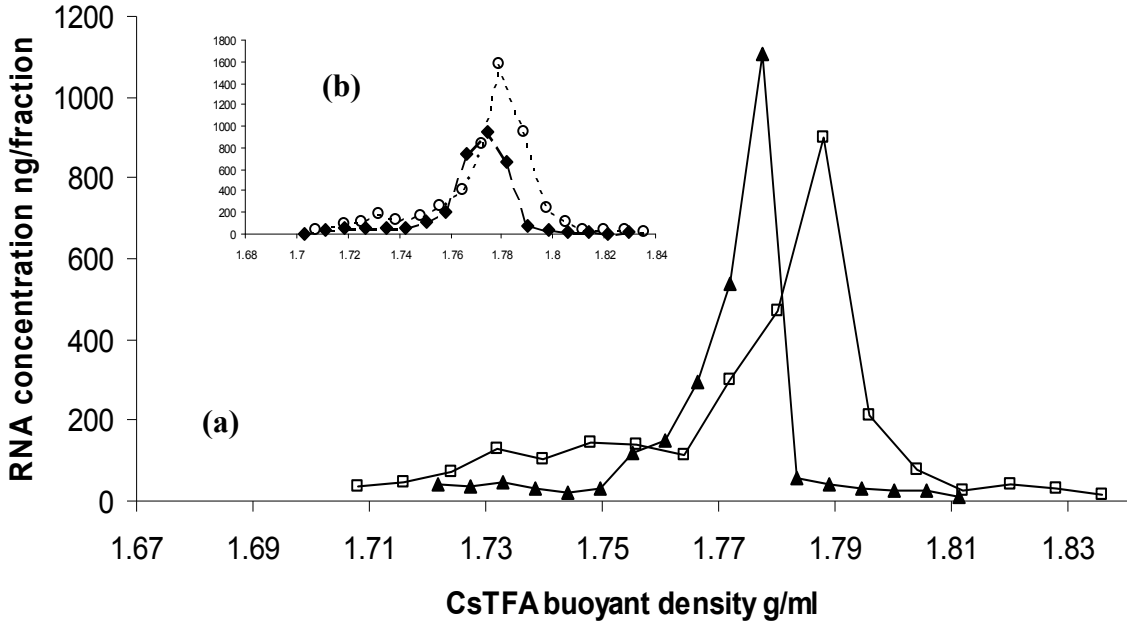

RNA concentration ng/fraction
1200
1000
800
600
400
200
0
1.67
1.69
1.71
1.73
1.75
1.77
1.79
1.81
1.83
CsTFA buoyant density g/ml
(a)
(b)
1800
1600
1400
1200
1000
800
600
400
200
0
1.68
1.7
1.72
1.74
1.76
1.78
1.8
1.82
1.84

Fig. 3.

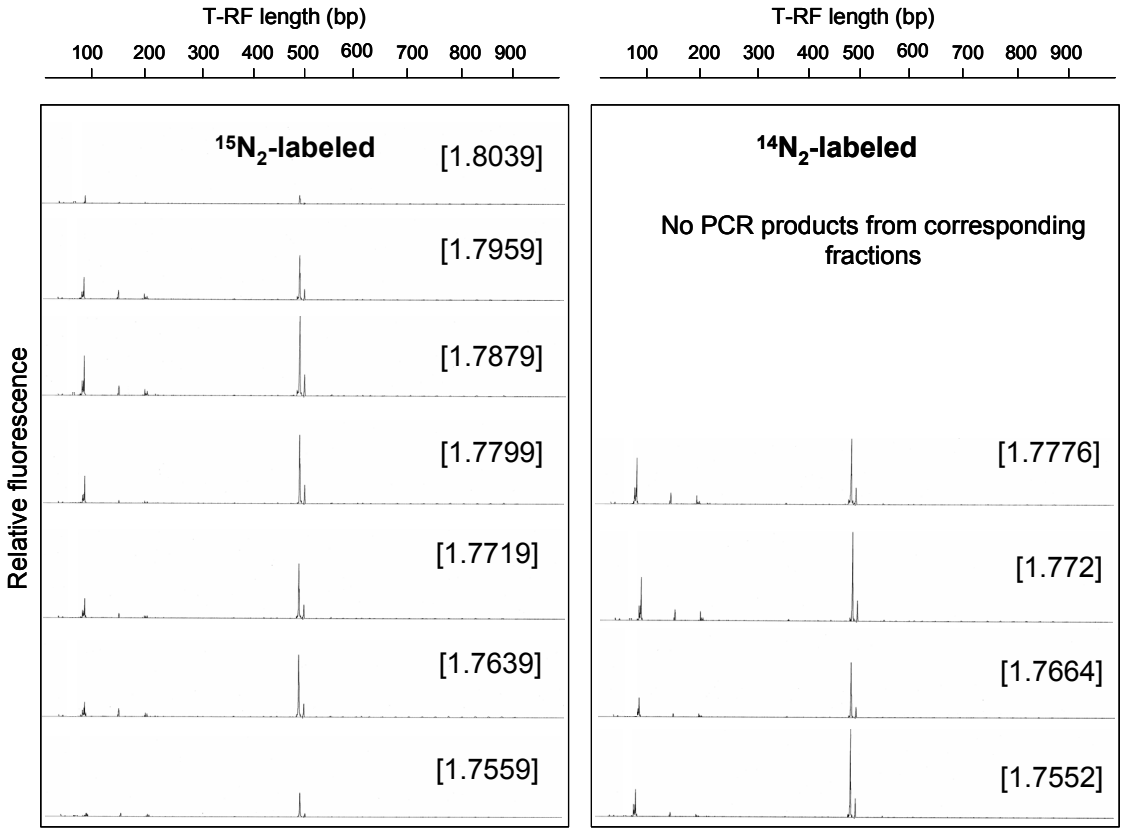

T-RF length (bp)
100 200 300 400 500 600 700 800 900
T-RF length (bp)
100 200 300 400 500 600 700 800 900
Relative fluorescence
15N2-labeled
[1.8039]
[1.7959]
[1.7879]
[1.7799]
[1.7719]
[1.7639]
[1.7559]
14N2-labeled
No PCR products from corresponding fractions
[1.7776]
[1.772]
[1.7664]
[1.7552]

Table. 1. Fluorescent in situ hybridisation probes used in this study

Probe name	Sequence (5'-3')	Formamide %	Target microorganisms	Reference
EUB338	GCTGCCTCCCGTAGGAGT	0-50	Most bacteria	(Amann et al., 1990)
EUB338 II	GCAGCCACCCGTAGGTGT	0-50	*Planctomycetales*	(Daims et al., 1999)
EUB338 III	GCTGCCACCCGTAGGTGT	0-50	*Verrucomicrobiales*	(Daims et al., 1999)
BET42a	GCCTTCCCACTTCGTTT	35	*β-proteobacteria*	(Manz et al., 1992)
GAM42a	GCCTTCCCACATCGTTT	35	*γ-proteobacteria*	(Manz et al., 1992)
ENT183	CTCTTTGGTCTTGCGACG	20	*Enterobacteriaceae*	(Friedrich et al., 2003)

534 **Table. 2.** T-RFLP results from high density fraction (1.7879 g ml^{-1}) in the $^{15}N_2$ labeled gradient with pseudo phylogenetic classification.

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*
200	2.2	*Chryseobacterium*
205	1.4	*Acinetobacter, Burkholderia, Pseudomonas*
488	3.3	*Achromatium, Acidovorax, Pseudomonas*
492	46.9	*Acinetobacter, Klebsiella, Pseudomonas*
495	2.0	Very diverse range of groups
500	14.6	*Actinobacillus*

535

536 *Major T-RFs in bold

537 Only T-RFs with peak height >1 % of total peak height of electropherograms were considered.

538