

1 **Stable isotope probing: Technical considerations when resolving $^{15}\text{N}_2$ -**
2 **labeled RNA in gradients**

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8

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10

11 **Abstract**

12

13 RNA based stable isotope probing (SIP) facilitates the detection and
14 identification of active members of microbial populations that are involved in the
15 assimilation of an isotopically labeled compound. ^{15}N -RNA-SIP is a new method that
16 has been discussed in recent literature but has not yet been tested. Herein, we define
17 the limitations to using ^{15}N -labeled substrates for SIP and propose modifications to
18 compensate for some of these shortcomings. We have used ^{15}N -RNA-SIP as a tool for
19 analysing mixed bacterial populations that use nitrogen substrates. After incubating
20 mixed microbial communities with ^{15}N -ammonium chloride or $^{15}\text{N}_2$ we assessed the
21 fractionation resolution of ^{15}N -RNA by isopycnic centrifugation in caesium
22 trifluoroacetate (CsTFA) gradients. We found that the more isotopic label
23 incorporated, the further the buoyant density (BD) separation between ^{15}N - and ^{14}N -
24 RNA, however it was not possible to resolve the labeled from unlabeled RNA
25 definitively through gradient fractionation. Terminal restriction fragment length
26 polymorphism (T-RFLP) analysis of the extracted RNA and fluorescent in situ
27 hybridisation (FISH) analysis of the enrichment cultures provided some insight into
28 the organisms involved in nitrogen fixation. This approach is not without its
29 limitations and will require further developments to assess its applicability to other
30 nitrogen-fixing environments.

31

32 **Introduction**

33

34 Stable isotope probing (SIP) allows the identification of an actively metabolising
35 population due to incorporation of an isotopically labeled substrate via a particular
36 metabolic pathway (Dumont and Murrell, 2005; Neufeld et al., 2007; Radajewski et
37 al., 2000; Radajewski et al., 2003; Radajewski and Murrell, 2000; Whitby et al.,
38 2005). Active members in a microbial community incorporate the heavy isotope into
39 cellular material, from which labeled nucleic acids (DNA or RNA) can be isolated for
40 analysis after fractionation by isopycnic centrifugation. The migration of specific
41 templates into heavier fractions over time as the pulse of label is consumed can be
42 compared by using an unlabeled control. Stable isotope enrichment is then indicated
43 only if the amount of RNA in specific fractions exceeds the amount that is detected in
44 the unlabeled control (Uhlik et al., 2008).

45 The structure of labeled and unlabeled communities is resolved by analysing
46 functional marker genes or rRNA genes to determine community composition. SIP
47 provides a very useful tool for exploring microbial communities under *in situ*
48 conditions, however one of the drawbacks associated with SIP and especially DNA-
49 SIP is that cross-feeding can allow non-target organisms to accumulate isotopic label
50 leading to misidentification. It is here that RNA-SIP offers an advantage over DNA-
51 SIP.

52 RNA-SIP was first used to identify phenol-degrading microbes from an
53 aerobic industrial bioreactor (Manefield et al., 2002a; Manefield et al., 2002b). These
54 studies showed that RNA-SIP holds significant potential for exploring active
55 populations from a variety of environments. RNA-SIP can reduce the unwanted
56 influence of cross feeding since RNA synthesis rates, which are higher than those of

57 DNA, allow for greatly decreased incubation times reducing the opportunity for
58 significant cross-feeding (Manefield et al., 2002b). Not only is RNA rapidly
59 synthesised, but it is the most active population that becomes labeled, further
60 decreasing cross-feeding effects. Separation is then based on the newly synthesised
61 RNA from community members that have assimilated the isotopically labeled
62 substrate. The resolution of labeled RNA provides access to the gene sequences which
63 are expressed by functional organisms during substrate assimilation. Manefield and
64 co-workers showed that the isotope incorporation into the biomass and the rate of the
65 incorporation into RNA exceeded that of DNA by more than 8-fold over the same
66 time period.

67 The feasibility of SIP has been demonstrated using small ^{13}C compounds but
68 has recently evolved to include ^{18}O compounds (Schwartz, 2007) and ^{15}N compounds
69 (Buckley et al., 2007b; Buckley et al., 2007a; Buckley et al., 2008; Cadisch et al.,
70 2005; Cupples et al., 2007; Roh et al., 2009). ^{15}N containing substrates used for DNA-
71 SIP include; $^{15}\text{NH}_4\text{Cl}$ (Buckley et al., 2007a; Cupples et al., 2007), $^{15}\text{NH}_4\text{NO}_3$
72 (Cadisch et al., 2005), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Roh et al.,
73 2009), and $^{15}\text{N}_2$ (Buckley et al., 2007b; Buckley et al., 2008). ^{15}N -substrates have yet
74 to be used successfully in a RNA-SIP experiment.

75 A potential drawback of ^{15}N -RNA-SIP is the lower density gain that is
76 possible from the incorporation of ^{15}N compared to ^{13}C isotopes. The average
77 stoichiometry calculated per nucleotide of C relative to N in RNA is 9.5 carbon
78 molecules to 3.75 nitrogen molecules, which allows the incorporation of 2.5 times
79 more heavy isotope from ^{13}C labeling compared to ^{15}N . Fully ^{15}N -labeled DNA in
80 CsCl shows a density gain of $\sim 0.016 \text{ g ml}^{-1}$ (Birnie and Rickwood, 1978) and fully
81 ^{13}C -labeled DNA shows a density gain of $\sim 0.04 \text{ g ml}^{-1}$ (Lueders et al., 2004a), both of

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

85 In this study we have compared ¹⁵N-labeled RNA with unlabeled RNA in
86 CsTFA to determine whether the gain in density is sufficient for resolution of ¹⁵N-
87 labeled RNA in a CsTFA gradient. The use of ¹⁵N-labeled substrates offers the
88 potential to use dinitrogen ¹⁵N₂ to identify nitrogen-fixing bacteria, as well as the
89 identification of organisms capable of utilising ammonium, nitrate, nitrite, and
90 nitrogen-containing organic compounds as sole nitrogen sources. We have applied
91 ¹⁵N-RNA-SIP methodology to study nitrogen-fixing communities and identify
92 diazotrophs that are actively engaged in nitrogen fixation from environmental samples
93 using full cycle analysis. The model population used in this study was taken from an
94 environment with known high nitrogen fixation rates (Bruce and Clark, 1994; Clark et
95 al., 1997; Gauthier et al., 2000).

96

97 **2. Materials and methods**

98

99 *2.1. Bacterial pure culture growth with ¹⁵N*

100 *Novosphingobium nitrogenifigens* Y88^T (DSM 19370) previously isolated
101 from pulp and paper wastewater (Addison et al., 2007), was grown overnight at 30°C,
102 150 rpm in nitrogen-limited minimal medium (NLMM) (containing, l⁻¹, 0.4 g
103 KH₂PO₄, 0.1 g K₂HPO₄, 0.2 g MgSO₄, 0.1 g NaCl, 10 mg FeCl₃, 2 mg Na₂MoO₄, 5 g
104 glucose, 50 mg yeast extract and pH 7.2 ± 0.1) supplemented with 0.5 g ¹⁵N- or
105 unlabeled ammonium chloride (98+ atom % ¹⁵N, Aldrich Chem. Co.).

106

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

108 Mixed cultures for ¹⁵NH₄Cl 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 ¹⁴N- or ¹⁵N-ammonium chloride (98+ atom % ¹⁵N, Aldrich Chem. Co.).

113 Mixed cultures for ¹⁵N₂ 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 O₂ and 20 ml N₂ (unlabeled N₂ in one flask
119 and ¹⁵N₂ (98+ atom % ¹⁵N, ISOTEC) in the other). Flasks were incubated for 24 hours
120 at 30°C, 150 rpm, with a further 10 ml O₂ 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

132 and lysed by agitation in a FastPrep bead beating system for 30 s at 5.5 m/s. The
133 aqueous phase was separated by centrifugation and the RNA purified by RNA/DNA
134 mini kit protocol for bacteria (Qiagen, Hilden, Germany). RNA was stored at -80°C
135 and used within one month of extraction to avoid degradation of RNA. Aliquots of
136 RNA extracts were visualised by standard agarose gel electrophoresis to verify the
137 quality of extracted RNA preparations. RNA was quantified in extracts using the
138 ultrasensitive fluorescent nucleic acid stain RiboGreen (Molecular probes, Invitrogen)
139 according to manufacturer's instructions. Briefly, a series of standards are made from
140 supplied stock RNA and Ribogreen solution is added to all samples and standards and
141 measured on a fluorometer at an excitation of 485 nm and emission of 520 nm,
142 detecting down to 1 ng/mL RNA.

143

144 *2.4. Isotopic enrichment using isotope ratio mass spectrometry (IRMS)*

145 The ¹⁵N and ¹⁴N enriched samples were analysed by the Waikato Stable
146 Isotope Unit (University of Waikato, New Zealand). After freeze-drying, an internal
147 standard of urea was added to an accurately weighed amount (~3 mg) of freeze dried
148 biomass. A 30 mg N carrier was used in the samples with the detection limit of the
149 machine being no less than 20 mg N. The urea was standardized against a certified
150 standard and calibrated relative to atmospheric nitrogen. Samples were analysed using
151 a Dumas elemental analyser (Europa Scientific ANCA-SL) interfaced to an isotope
152 mass spectrometer (Europa Scientific 20-20 Stable Isotope Analyser) to give the atom
153 % ¹⁵N.

154

155 *2.5. Gradient centrifugation and fractionation*

156 Density gradient centrifugation was performed in 6.5 ml polyallomer Cone-
157 Top tubes in a T-1270 Sorvall rotor spun at 40000 r.p.m ($146\ 000\ g_{av}$) at a
158 temperature of 16°C for 42 hours. Caesium trifluoroacetate (CsTFA) was used as the
159 gradient forming material; 3.72 ml of a $1.99\ g\ ml^{-1}$ stock solution (Amersham
160 Pharmacia Biotech) was combined to a final volume of 4.62 ml with 150 μ l of
161 deionised formamide (Manefield et al., 2002b), 1000 ng RNA and pure water (Gibco).
162 The centrifuge tubes were overlaid with mineral oil (Sigma).

163 Centrifuged gradients were fractionated from below by water displacement
164 using an 1100 HPLC pump (Hewlett Packard) operating at a flow rate of 1 μ l/s. The
165 buoyant density of gradient fractions was determined by weighing measured 200 μ l
166 volumes on a four-figure milligram balance. RNA was isolated from gradient
167 fractions by precipitation with 1 volume of isopropanol, washed with 70% ethanol and
168 re-eluted into 10 μ l for determination of RNA using the RiboGreen assay.

169

170 *2.6. Community profiling with terminal restriction fragment length polymorphism (T-* 171 *RFLP)*

172 T-RFLP analysis of density-resolved rRNA was performed with primers 27F-
173 FAM/1492R (Lane, 1991), by RT-PCR using a one-step RT-PCR system (Superscript
174 III One-Step RT-PCR system, Invitrogen). 15 μ l of the resulting PCR product was
175 digested with a mix of *MspI* and *HhaI* (Roche) in 30 μ l reaction volumes as per the
176 manufacturer's instructions. The digested PCR products were resolved on an
177 Amersham Biosciences MegaBACE DNA Analysis System alongside a 600 bp
178 ladder. The T-RF profile was run through Phylogenetic Assignment Tool (PAT) and
179 the T-RF's were assigned groups based on data from restriction enzyme digests (Kent
180 et al., 2003).

181

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

183 Samples from the enriched cultures grown with N₂ 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).

199

200 3. Results and Discussion

201

202 The use of ^{15}N substrates for labeling RNA has been proposed as a method for
203 which technical difficulties exist due to the low amount of labeling that can be
204 produced with nitrogen substrates (Buckley et al., 2007a; Whiteley et al., 2006). Other
205 limitations have been discussed previously with respect to ^{15}N -SIP (Buckley et al.,
206 2007a; Cadisch et al., 2005) including the resolution of $^{14}\text{N}/^{15}\text{N}$ bands, different GC
207 contents and the effects of the percent of ^{15}N label. To evaluate the methodology, pure
208 culture bacterial RNA labeled with ^{15}N -ammonium chloride and RNA from an
209 environmental sample from a New Zealand pulp and paper mill effluent grown with
210 $^{15}\text{N}_2$ as the nitrogen source was used.

211

212 3.1. ^{15}N -Ammonium chloride labeled gradient evaluation

213 RNA isolated from a pure culture was used to establish conditions for
214 separating labeled (^{15}N) and unlabeled (^{14}N) RNA. ^{15}N -labeled RNA was isolated
215 from *Novosphingobium nitrogenifigens* Y88^T (DSM 19370) grown with ^{15}N -
216 ammonium chloride as sole nitrogen source. Isotope ratio mass spectrometry (IRMS)
217 analysis showed the labeled biomass contained 95.0 atom % ^{15}N . RNA extracts from
218 labeled and unlabeled cultures were loaded individually into CsTFA gradient material
219 and isopycnicly separated. The average buoyant densities (BD) of CsTFA resolved
220 gradient fractions were determined gravimetrically and the RNA enriched fraction for
221 ^{15}N had a $0.03 \pm 0.004 \text{ g ml}^{-1}$ ($n = 3$) higher BD compared to the ^{14}N control. Fig. 1
222 shows representative labeled and unlabeled RNA from *N. nitrogenifigens* Y88^T
223 resolved through separate CsTFA gradients (BD shift by ^{15}N -labeling of RNA of
224 0.032 g ml^{-1}).

225 A mixed community taken from a bioreactor treating pulp and paper mill
226 effluent was grown supplemented with ^{15}N -ammonium chloride. RNA was extracted
227 to determine whether ^{15}N -labeled and unlabeled mixed community RNA could be
228 separated based on BD. IRMS analysis of mixed community biomass showed
229 enrichment of 80.2 % atom ^{15}N . ^{15}N -labeled RNA from a mixed community resolved
230 at a heavier density than the unlabeled control. Gradients for mixed community RNA
231 showed a gain in BD for ^{15}N -RNA of $0.02 \pm 0.004 \text{ g ml}^{-1}$ ($n = 3$) compared to the ^{14}N
232 control.

233

234 *3.2. Environmental sample $^{15}\text{N}_2$ -labeled gradient separation assessment*

235 The labeled and unlabeled incubations gave strong positive results for the
236 acetylene reduction assay, which is indicative of active nitrogen fixation. The RNA
237 extracted from mixed communities, incubated with either $^{15}\text{N}_2$ or $^{14}\text{N}_2$, was resolved
238 using CsTFA density gradient fractionation. IRMS analysis of total cell biomass
239 confirmed nitrogen fixation with incorporation of 32.6 % atom ^{15}N from $^{15}\text{N}_2$. The
240 natural abundance of ^{15}N in this biomass was 0.37 atom % compared with 99.63 atom
241 % for ^{14}N . Gradients run individually with labeled and unlabeled RNA from the
242 mixed community showed that ^{15}N -labeling of RNA increased BD by $0.013 \pm 0.002 \text{ g}$
243 ml^{-1} ($n = 3$) compared to the ^{14}N control. A representative gradient (Fig. 2a)
244 demonstrates that individually run unlabeled RNA migrated to a BD of 1.777 g ml^{-1}
245 and labeled RNA migrated to a BD of 1.788 g ml^{-1} , showing a difference of 0.011 g
246 ml^{-1} .

247 When the mixed community $^{14}\text{N}_2$ -RNA and $^{15}\text{N}_2$ -RNA were run together in a
248 single gradient the separation achieved was less than when run on separate gradients
249 (Fig. 2b). The mixed RNA peak was spread over a density range $1.7682\text{--}1.7836 \text{ g ml}^{-1}$

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 ¹⁴N- and ¹⁵N-RNA. It is unlikely an
254 environmental sample would be fully labeled so the resolution of ¹⁴N- and ¹⁵N-RNA
255 in a single gradient is important. A mix of ¹⁴N- and ¹⁵N-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 ¹⁴N- and ¹⁵N-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 ¹⁵N-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 ¹⁵N-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 ¹⁵N₂-RNA compared with the pure culture ¹⁵N-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

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

277

278 *3.3. Correlation between gradient fractionation and T-RFLP profiling*

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

292 RNA is spread across any given gradient, with low background levels of
293 unlabeled RNA expected throughout all the gradient fractions (on average 0.7% of
294 maximum quantities) and is most apparent when using PCR to detect templates
295 (Lueders et al., 2004a; Uhlik et al., 2008). Our results showed low levels of RNA
296 throughout the gradients but these levels were so low that RT-PCR was unable to
297 amplify products in the unlabeled heavy density fractions. Manefield et al. (2002b)
298 reported similar results and concluded that density gradients typically used to isolate
299 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 ¹⁵N₂ mixed community sample. This low
306 resolution in gradient separation might be resolved by employing a second
307 ultracentrifugation step with bisbenzimidazole 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 (¹⁵N₂- and
312 ¹⁴N₂-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 ¹⁴N- and ¹⁵N-gradients (68% and 70%
316 respectively).

317 Analysis of the ¹⁵N-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

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

326 To confirm the presence and abundance of the organisms identified in the T-
327 RF profile, FISH was performed with the use of generic bacterial group probes. The
328 T-RF profile identified that the community was dominated by *γ-proteobacteria*, FISH
329 with GAM42a probe confirmed these results with an average of 87.5 ± 4.0 % of the
330 community highlighted by this probe compared to the EUB mix probe. The BETA42a
331 probe was used to confirm the presence and abundance of *β-proteobacteria* within the
332 enrichment community with these organisms representing 10.5 ± 2.0 % of the
333 community. This number is lower than that found for *γ-proteobacteria* and confirms
334 the profile from the enriched $^{15}\text{N}_2$ fraction.

335 Nucleic acid fingerprinting methods can be used to determine the isotopic
336 enrichment by particular microbial groups by comparing fingerprints of gradient
337 fractions from isotopically labeled samples relative to the unlabeled controls (Lueders
338 et al., 2004b; Lueders et al., 2004a). To identify the organisms that assimilated the
339 label, it is necessary to match T-RFs from the enriched gradient fraction with
340 sequences obtained from clone libraries: the possibility that two or more organisms
341 may share a particular T-RFs makes it difficult to interpret these data (Kent et al.,
342 2003; Takeshita et al., 2007). The T-RFLP data from the labeled $^{15}\text{N}_2$ mixed
343 community fraction showed a mixture of possible organisms for each T-RF with the
344 dominance of *γ-proteobacteria*, including both *Klebsiella* and *Pseudomonas*. FISH
345 analysis confirmed the dominance in the community of *γ-proteobacteria* and the
346 negligible existence of any *Enterobacter*. The average percentages of the groups
347 found in the community with FISH are similar to that predicted with T-RFLP; ~85%
348 (FISH) and ~60% (T-RFLP) of *γ-proteobacteria* and ~10% (FISH) and ~5% (T-

349 RFLP) *β-proteobacteria*. *Klebsiella* are known to be able to fix nitrogen and have
350 been shown using culture-based approaches to be present in nitrogen-fixing
351 wastewaters (Gauthier et al., 2000). Numerous studies have looked at different
352 wastewater systems and used DNA based approaches showing *α-proteobacteria* and
353 *β-proteobacteria* dominance (Bowers et al., 2008; Reid et al., 2008; Wagner and Loy,
354 2002), however, these studies did not examining the active portion of the community
355 and did not involve an enrichment to select for nitrogen-fixing bacteria on a defined
356 carbon source. The identification of the active predominance of nitrogen-fixing *γ*-
357 *proteobacteria* from wastewater opens the path for the identification of undiscovered
358 nitrogen-fixing organisms.

359

360 **4. Conclusion**

361 ¹⁵N-RNA-SIP is a new method that has been discussed in recent literature and
362 this is the first study which investigates the validity of SIP methodology for the
363 separation of ¹⁵N-labeled RNA. After incubating mixed microbial communities with
364 ¹⁵N-ammonium chloride or ¹⁵N₂ we assessed the fractionation resolution of ¹⁵N-RNA
365 by isopycnic centrifugation in caesium trifluoroacetate (CsTFA) gradients. We found
366 that the more isotopic label incorporated, the further the buoyant density (BD)
367 separation between ¹⁵N- and ¹⁴N-RNA, however it was not possible to resolve the
368 labeled from unlabeled RNA definitively through gradient fractionation. Terminal
369 restriction fragment length polymorphism (T-RFLP) analysis of the extracted RNA
370 and fluorescent in situ hybridisation (FISH) analysis of the enrichment cultures
371 provided some insight into the organisms involved in nitrogen fixation. These initial
372 experiments indicate that this method has potential for mixed microbial communities
373 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.

376

377

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382

383 **References**

384 Addison, S.L., Foote, S.M., Reid, N.M., Lloyd-Jones, G., 2007. *Novosphingobium*
385 *nitrogenifigens* sp. nov., a polyhydroxyalkanoate-accumulating diazotroph isolated
386 from a New Zealand pulp and paper wastewater. *Int. J Syst. Evol. Microbiol.* 57:
387 2467-2471.

388 Amann, R., 1995. In situ identification of micro-organisms by whole cell
389 hybridization with rRNA-targeted nucleic acid probes. In *Molecular Microbial*
390 *Ecology Manual*. Akkermans,A.D.L., van Elsas,J.D., and de Bruijn,F.J. (eds).
391 London: Kluwer Academic Publishers, 1-15.

392 Amann, R., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A.,
393 1990. Combination of 16S ribosomal-RNA-targeted oligonucleotide probes with
394 flow-cytometry for analyzing mixed microbial-populations. *Appl. Environ. Microbiol.*
395 56: 1919-1925.

396 Birnie, G.D., Rickwood, D., 1978. Centrifugal separations in molecular and cell
397 biology. Boston: Butterworths.

398 Bowers, T.H., Reid, N.M., Lloyd-Jones, G., 2008. Composition of *nifH* in a
399 wastewater treatment system reliant on N₂ fixation. *Appl. Microbiol. Biotechnol.* 79:
400 811-818.

401 Bruce, M.E., Clark, T.A., 1994. *Klebsiella* and nitrogen fixation in pulp and paper
402 mill effluents and treatment systems. *Appita* 47: 231-237.

403 Buckley, D.H., Huangyutitham, V., Hsu, S.F., Nelson, T.A., 2007a. Stable isotope
404 probing with ^{15}N achieved by disentangling the effects of genome G + C content and
405 isotope enrichment on DNA density. *Appl. Environ. Microbiol.* 73: 3189-3195.

406 Buckley, D.H., Huangyutitham, V., Hsu, S.F., Nelson, T.A., 2007b. Stable isotope
407 probing with $^{15}\text{N}_2$ reveals novel non-cultivated diazotrophs in soil. *Appl. Environ.*
408 *Microbiol.* 73: 3196-3204.

409 Buckley, D.H., Huangyutitham, V., Hsu, S.F., Nelson, T.A., 2008. $^{15}\text{N}_2$ -DNA-stable
410 isotope probing of diazotrophic methanotrophs in soil. *Soil Biol. Biochem.* 40: 1272-
411 1283.

412 Cadisch, G., Espana, M., Causey, R., Richter, M., Shaw, E., Morgan, J.A.W. et al.,
413 2005. Technical considerations for the use of ^{15}N -DNA stable-isotope probing for
414 functional microbial activity in soils. *Rapid Commun. Mass Spectrom.* 19: 1424-
415 1428.

416 Clark, T.A., Dare, P.H., Bruce, M.E., 1997. Nitrogen fixation in an aerated
417 stabilization basin treating bleached kraft mill wastewater. *Water Environ. Res.* 69:
418 1039-1046.

419 Cupples, A.M., Shaffer, E.A., Chee-Sanford, J.C., Sims, G.K., 2007. DNA buoyant
420 density shifts during ^{15}N -DNA stable isotope probing. *Microbiol. Res.* 162: 328-334.

421 Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-
422 specific probe EUB338 is insufficient for the detection of all Bacteria: Development
423 and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* 22: 434-
424 444.

425 Daims, H., Lucker, S., Wagner, M., 2006. *daime*, a novel image analysis program for
426 microbial ecology and biofilm research. *Environ. Microbiol.* 8: 200-213.

427 Dumont, M.G. and Murrell, J.C. (2005) Stable isotope probing - Linking microbial
428 identity to function. *Nat Rev Micro* 3: 499-504.

429 Friedrich, U., Van Langenhove, H., Altendorf, K., Lipski, A., 2003. Microbial
430 community and physicochemical analysis of an industrial waste gas biofilter and
431 design of 16S rRNA-targeting oligonucleotide probes. *Environ. Microbiol.* 5: 439.

432 Gauthier, F., Neufeld, J.D., Driscoll, B.T., Archibald, F.S., 2000. Coliform bacteria
433 and nitrogen fixation in pulp and paper mill effluent treatment systems. *Appl.*
434 *Environ. Microbiol.* 66: 5155-5160.

435 Kent, A.D., Smith, D.J., Benson, B.J., Triplett, E.W., 2003. Web-based phylogenetic
436 assignment tool for analysis of terminal restriction fragment length polymorphism
437 profiles of microbial communities. *Appl. Environ. Microbiol.* 69: 6768-6776.

438 Lane, D.J., 1991. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial*
439 *Systematics*. Stackebrandt, S. and Goodfellow, M. (eds). Chichester: Wiley, 115-175.

440 Lueders, T., Manefield, M., Friedrich, M.W., 2004a. Enhanced sensitivity of DNA-
441 and rRNA-based stable isotope probing by fractionation and quantitative analysis of
442 isopycnic centrifugation gradients. *Environ. Microbiol.* 6: 73-78.

443 Lueders, T., Pommerenke, B., Friedrich, M.W., 2004b. Stable-Isotope probing of
444 microorganisms thriving at thermodynamic limits: Syntrophic propionate oxidation in
445 flooded soil. *Appl. Environ. Microbiol.* 70: 5778-5786.

446 Manefield, M., Whiteley, A.S., Ostle, N., Ineson, P., Bailey, M.J., 2002a. Technical
447 considerations for RNA-based stable isotope probing: an approach to associating
448 microbial diversity with microbial community function. *Rapid Commun. Mass*
449 *Spectrom.* 16: 2179-2183.

450 Manefield, M., Whiteley, A.S., Griffiths, R.I., Bailey, M.J., 2002b. RNA stable
451 isotope probing, a novel means of linking microbial community function to
452 phylogeny. *Appl. Environ. Microbiol.* 68: 5367-5373.

453 Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.H., 1992. Phylogenetic
454 oligodeoxynucleotide probes for the major subclasses of Protoeobacteria - Problems
455 and solutions. *Syst. Appl. Microbiol.* 15: 593-600.

456 Neufeld, J.D., Dumont, M.G., Vohra, J., Murrell, J.C., 2007. Methodological
457 considerations for the use of stable isotope probing in microbial ecology. *Microb.*
458 *Ecol.* 53: 435-442.

459 Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C., 2000. Stable-isotope probing
460 as a tool in microbial ecology. *Nature* 403: 646-649.

461 Radajewski, S., McDonald, I.R., Murrell, J.C., 2003. Stable-isotope probing of
462 nucleic acids: a window to the function of uncultured microorganisms. *Curr. Opin.*
463 *Biotechnol.* 14: 296-302.

464 Radajewski, S., Murrell, J.C., 2000. Stable isotope probing for detection of
465 Methanotrophs after enrichment with $^{13}\text{CH}_4$. *Meth. Mol. Biol.* 179:149-157.

466 Reid, N.M., Bowers, T.H., Lloyd-Jones, G., 2008. Bacterial community composition
467 of a wastewater treatment system reliant on N₂ fixation. *Appl. Microbiol. Biotechnol.*
468 79: 285-292.

469 Roh, H., Yu, C.P., Fuller, M.E., Chu, K.H., 2009. Identification of hexahydro-1,3,5-
470 trinitro-1,3,5-triazine-degrading microorganisms via ¹⁵N-stable isotope probing.
471 *Environ. Sci. Technol.* 43: 2505-2511.

472 Schwartz, E., 2007. Characterization of growing microorganisms in soil through
473 stable isotope probing with H₂¹⁸O. *Appl. Environ. Microbiol.* 73: 2541-2546.

474 Sprent, J.I., Sprent, P., 1990. *Nitrogen fixing organisms: pure and applied aspects.*
475 London & New York: Chapman Hall.

476 Takeshita, T., Nakano, Y., Yamashita, Y., 2007. Improved accuracy in terminal
477 restriction fragment length polymorphism phylogenetic analysis using a novel internal
478 size standard definition. *Oral Microbiol. Immun.* 22: 419-428.

479 Uhlik, O., Jecna, K., Mackova, M., Leigh, M.B., Demnerova, K., Macek, T., 2008.
480 Stable isotope probing as a tool for the detection of active microorganisms in
481 xenobiotics degradation. *Chem. Listy* 102: 474-479.

482 Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage
483 treatment systems. *Curr. Opin. Biotechnol.* 13: 218-227.

484 Whitby, C.B., Bailey, M.J., Whiteley, A.S., Murrell, J.C., Killham, K., Prosser, J.I.,
485 Lappin-Scott, H., 2005. Stable isotope probing links taxonomy with function in
486 microbial communities. *ASM News* 71: 169-173.

487 Whiteley, A.S., Manefield, M., Lueders, T., 2006. Unlocking the 'microbial black box'
488 using RNA-based stable isotope probing technologies. *Curr. Opin. Biotechnol.* 17: 67-
489 71.

490

LIST OF FIGURES

491

492 **Fig. 1.** Representative analysis of CsTFA density gradient of *N. nitrogenifigens* Y88^T
493 RNA labeled with ¹⁴N- (▲) or ¹⁵NH₄Cl (□) substrate centrifuged individually.

494

495 **Fig.2. (a).** Representative analysis of CsTFA density gradient of mixed community
496 RNA labeled with ¹⁴N₂ (▲) or ¹⁵N₂ (□) substrate centrifuged individually. **(b).** A mix
497 of ¹⁴N and ¹⁵N-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 ¹⁵N₂ or ¹⁴N₂
502 substrate and centrifuged individually.

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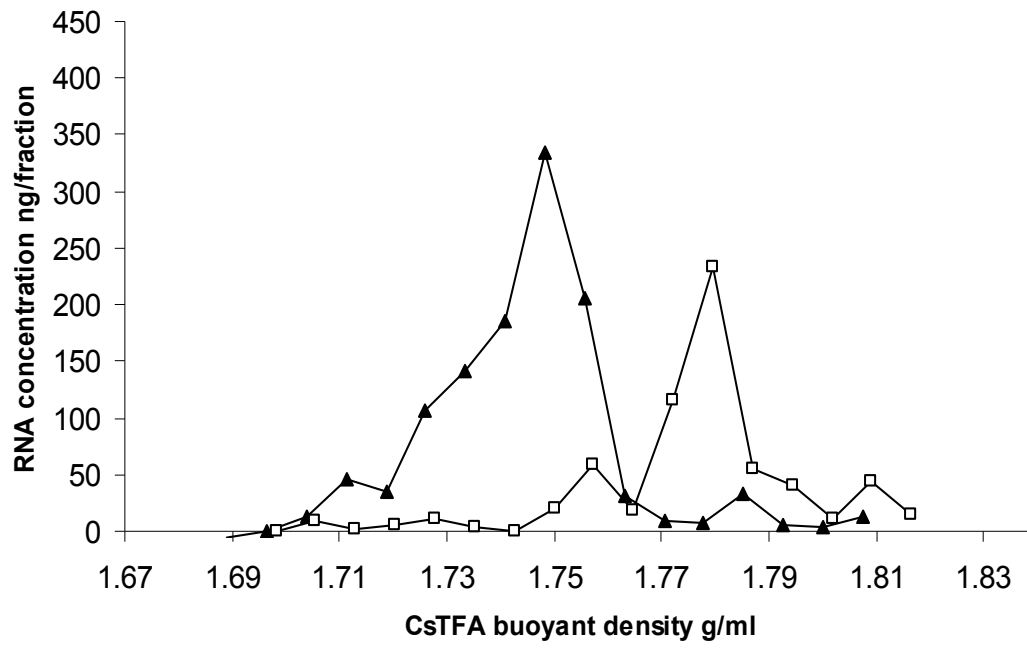
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Fig.1.



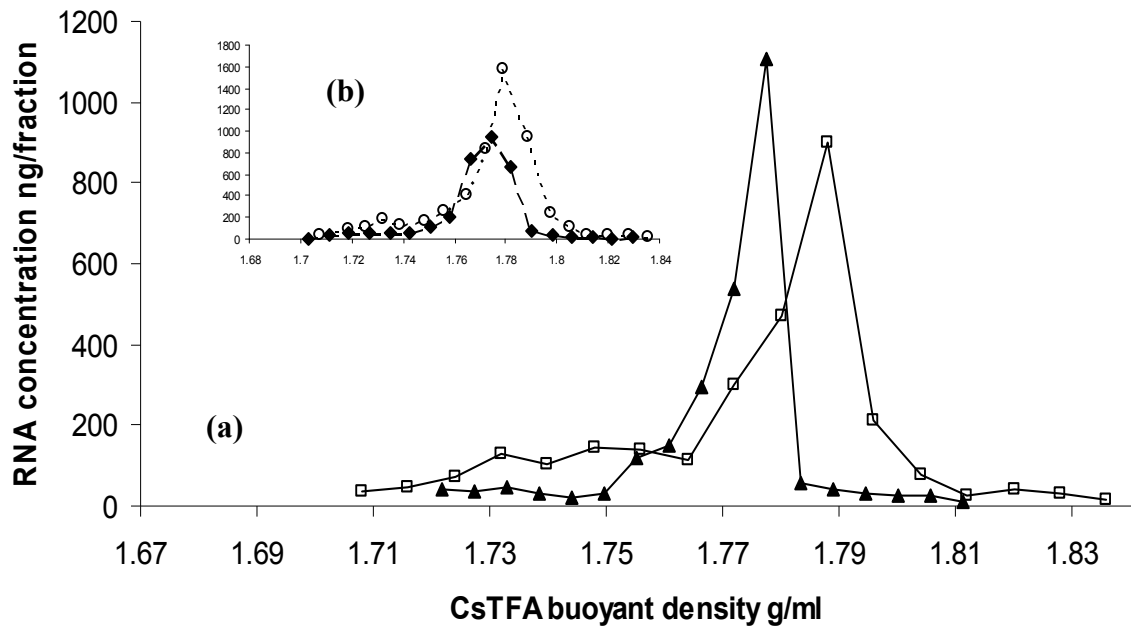
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Fig. 2.

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Fig. 3.

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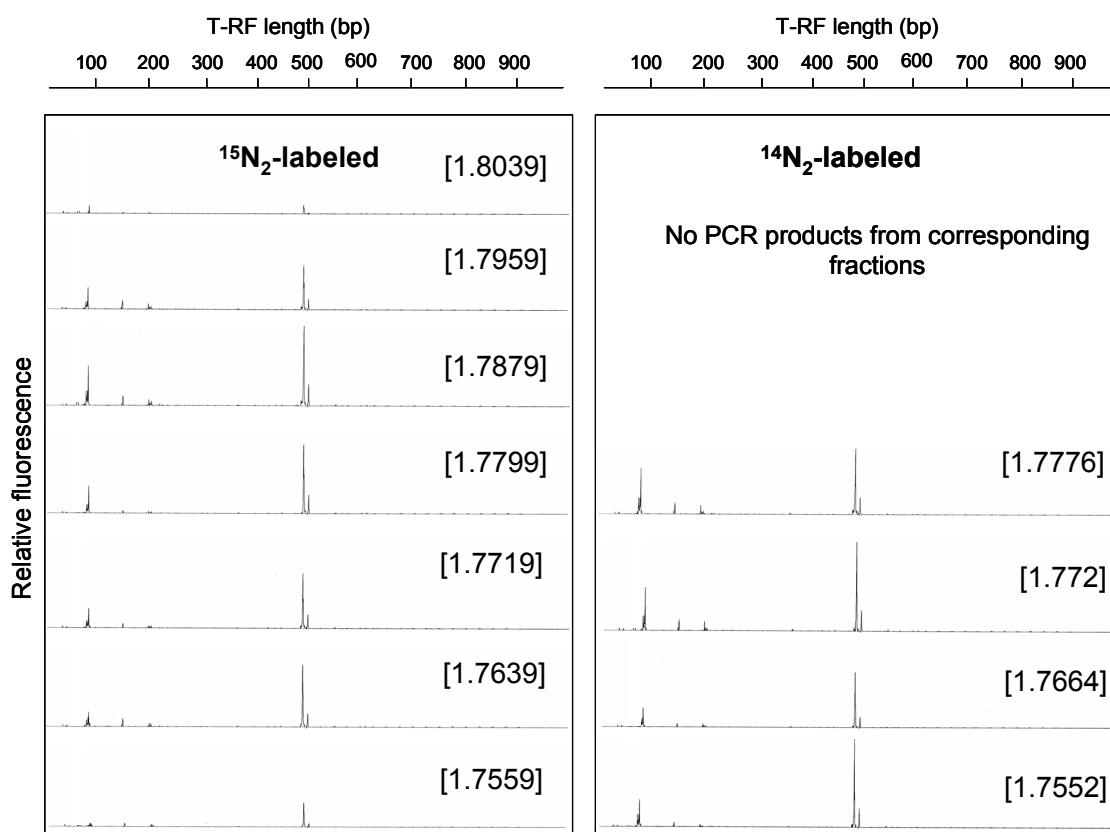
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526 **Table 1.** Fluorescent in situ hybridisation probes used in this study

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

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534 **Table 2.** T-RFLP results from high density fraction (1.7879 g ml⁻¹) in the ¹⁵N₂ labeled gradient with pseudo phylogenetic classification.

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

535

536 *Major T-RFs in bold

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

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