2.2 Materials and method

2.2.1 Determination of quantities of DNA required for visualisation

Azospirillum lipoferum CRT1 was grown in a mineral salt medium (*Azospirillum*-Medium 221, DSMZ (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germay, http://www.dsmz.de/media/med221.htm). Cultures were grown on an orbital shaker at 100 rpm for 20 h at 30 ^{o}C. DNA was extracted in sub-samples containing app. 1 × 10^{9} cells using the DNeasy® DNA-purification kit (QUIAGEN). Quantification of DNA concentration was done using a NanoDrop ND-1000 spectrophotometer. DNA extracts were mixed with 0.5 × TE buffer to obtain the following amounts of DNA in 4.5 mL Tris-0.5 mM EDTA (TE): 53, 26, 13, 3 and 2 µg; thereafter CsCl was added at 1 g mL^{-1} solution followed by 200 µL ethidium bromide solution (10 mg mL^{-1}). CsCl density gradient centrifugation was performed using a SORVALL centrifuge with rotor TV-689UA at 265000 g for 20 h at 20 ^{o}C. DNA bands were visualised with UV light.

2.2.2 Preparation and separation of DNA from Pseudomonas putida cultured on media containing 0 to 99% ^{15}N enrichment

Pseudomonas putida paw8 was grown in a mineral salts (Roberts et al., 1998) medium to which 100 (99.63) atom % ^{14}N- and 99 atom % ^{15}N- labelled NH_4NO_3 were added to provide 1 mg N mL^{-1} and 10 at% ^{15}N enrichment increments between 0 (0.3663) and 99 at%^{15}N. Cells were washed off overnight P. putida cultures growing on Luria-Bertani agar plates, and inoculated into 5 mL aliquots of each medium. Cultures were grown overnight at 35 ^{o}C. DNA was extracted from the cells following lysis with lysozyme and treatment with proteinase and RNase followed by precipitation by isopropanol. DNA from the P. putida cultures was quantified using a NanoDrop ND-1000 spectrophotometer. Approximately 10 µg of P. putida DNA labelled with between 10 and 99 at% ^{15}N was added to 10 µg 100 (99.63) at% ^{14}N labelled P. putida DNA. The DNA was mixed with a CsCl solution (1 g mL^{-1} TE buffer) and ethidium bromide (10 mg mL^{-1}) in 10 mM TE buffer. Centrifugation was performed using a BeckmanVTi 65.1 rotor at 265000 g for 18 h at 20 ^{o}C (Radajewski et al., 2000), or 140000 g for 69 h (Hutchens et al., 2004). DNA bands were visualised under UV light.

2.2.3 Preparation and collection of ^{15}N labelled DNA from soil samples

^{15}N labelled DNA from soil was extracted from an experiment where highly ^{15}N enriched (>90 at%) ryegrass (*Lolium perenne* L.) was incubated in a topsoil collected from Long Close field on the site at Warwick HRI, Wellesbourne. The soil is a sandy-loam of the Wick Series with 14 % clay, a pH of 6.5 and an organic C content of 0.8 % (Whitfield, 1974). Labelled ryegrass was obtained by propagation in a coarse sand/clay mixture under controlled glasshouse conditions (16 h day length, maximum day temperature 25 ^{o}C, minimum night temperature 15 ^{o}C). The plants were nourished with Hewitt's nutrient solution (Hewitt, 1952), in which the sole N source was 99 at% $^{15}NH_4{}^{15}NO_3$. Three replicates, each consisting of 500 g fresh weight portions of soil mixed with 25 g FW ryegrass were set-up, and incubated at 15 ^{o}C in the dark for 7 days.

DNA was extracted from 1 g FW portions of soil by bead-beating using a Fast Prep DNA spin kit for soil (Bio 101). DNA was centrifuged in CsCl as described above, at 265000 g for 18 h at 20 ^{o}C. After visualisation under UV light, the base of the DNA gradient was identified. A hypodermic needle was inserted below the lower band and another at the top of the tube. Pressure was applied through the upper needle to force the gradient out of the lower needle. Fractions (*ca.* 100µL) were removed from just below the lower band to above the top band in the gradient. Ethidium bromide was removed from the DNA fractions using isoamyl alcohol saturated with H_2O and NaCl. Samples were dialysed on a nitrocellulose membrane for 20 min to remove residual CsCl, and then suspended in TE buffer. The ^{15}N enrichment of DNA fractions was determined by spiking 30 ng DNA with 20 µg unlabelled NH_4SO_4 and analysed using an automated CN analyzer (Robprep) coupled to a 20-20 Europa Mass Spectrometer (PDZ Europa Ltd, Crewe).

2.3 Results and discussion

2.3.1 Visualization of DNA in CsCl

The successful application of the stable isotope probing methodology ideally requires that DNA is visible in the CsCl medium. Staining with ethidium bromide and exposure to UV light shows sufficient quantities of DNA as clearly visible bands. In our experiments we found that >13 µg DNA resulted in clearly identifiable bands in the CsCl tubes (Figure 1). Smaller quantities down to 2 µg DNA were only faintly visible. These results reflect

similar observations by other authors (Lueders et al., 2004) who observed that about 15 µg DNA was necessary for clear visualisation of DNA using ethidium bromide in CsCl gradients. Work with rRNA suggested that lower quantities (e.g. 500 ng) may be sufficient for rRNA in cesium trifluoroacetate gradients (Manefield et al., 2002b). The relatively large quantities of DNA required using ethidium bromide in CsCl gradients limit somewhat the use of the DNA-SIP method if only small quantities of DNA are available. It is thus desirable to aim for larger yields, which can be achieved by multiple DNA extractions and pooling of samples where necessary. It is however possible that, if a control ^{14}N-DNA band is present, extraction of even very small 'invisible' amounts of DNA below the ^{14}N-DNA band will result in sufficient DNA for PCR amplification purposes and subsequent further community/species analysis.

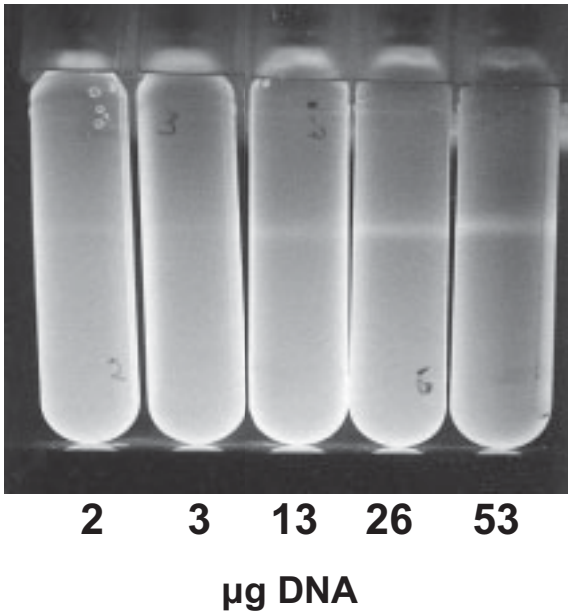

Figure 1. Visualisation of varying quantities of ^{14}N-DNA from *Azosprillum lipoferum* in CsCl gradient (256K g for 20 hours).

2.3.2 Separation of ^{14}/^{15}N labelled DNA

When a mixture of 100 at% ^{15}N-labelled and unlabelled DNA from *P. putida* was subject to isopycnic centrifugation at 265K g for 18 h, two clearly separated DNA bands were visible and the distance between the two bands was approximately 4 mm (Figure 2). These results confirm observations with highly ^{13}C labelled DNA where also a clear separation of DNA bands in CsCl gradient was obtained using unlabelled/highly enriched

DNA (Radajewski et al., 2003). However, whereas a separation between highly labelled/unlabelled ^{13}C-DNA of about 1 cm was achieved (Radajewski et al., 2003) our results suggest that under standard isopycnic centrifugation conditions (265K g for 18 h) only maximum a 4 mm separation of $^{14/15}$N-DNA is achievable. This is because the proportional difference of $^{14/15}$N is lower than of $^{12/13}$C and DNA contains more C than N atoms (average C:N ratio 2.1:1) hence affecting their respective buoyant densities.

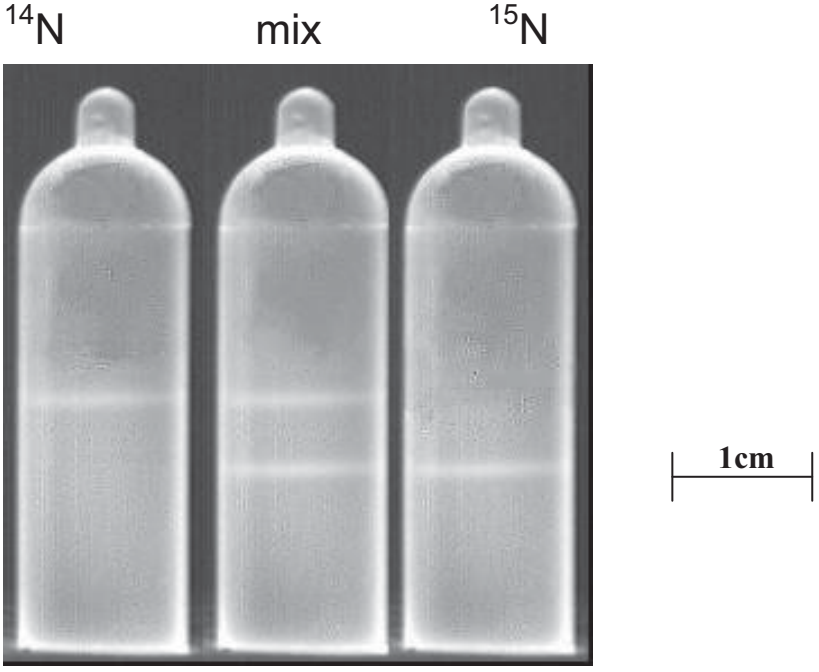

Figure 2. ^{14}N (unlabelled) and ^{15}N (99%) enriched DNA of *Pseudomonas putida* or a mixture of unlabelled and highly enriched DNA in a CsCl gradient (256K g for 18 h).

2.3.3 ^{15}N isotopic DNA enrichment required

In order to determine the minimum ^{15}N DNA enrichment required to obtain a clear separation between labelled and unlabelled DNA we centrifuged unlabelled and a mixture of unlabelled and increasingly ^{15}N labelled DNA (10-99 at%) on a CsCl gradient at 265K g for 18h. A clear separation of bands was achieved with ^{15}N DNA enrichments ≥ 50 at% ^{15}N (Figure 3A). Below that bands of labelled and unlabelled DNA were undistinguishable by eye. These results suggest that the ^{15}N DNA SIP method needs high ^{15}N enrichment for successful application. The 50 at% ^{15}N identified in this experiments is much higher than the minimum enrichment of 20 at% suggested for application of DNA-SIP when using ^{13}C labelling (Radajewski et al., 2000) or as low as 10 at% ^{13}C for RNA-SIP (Manefield et al., 2002b).

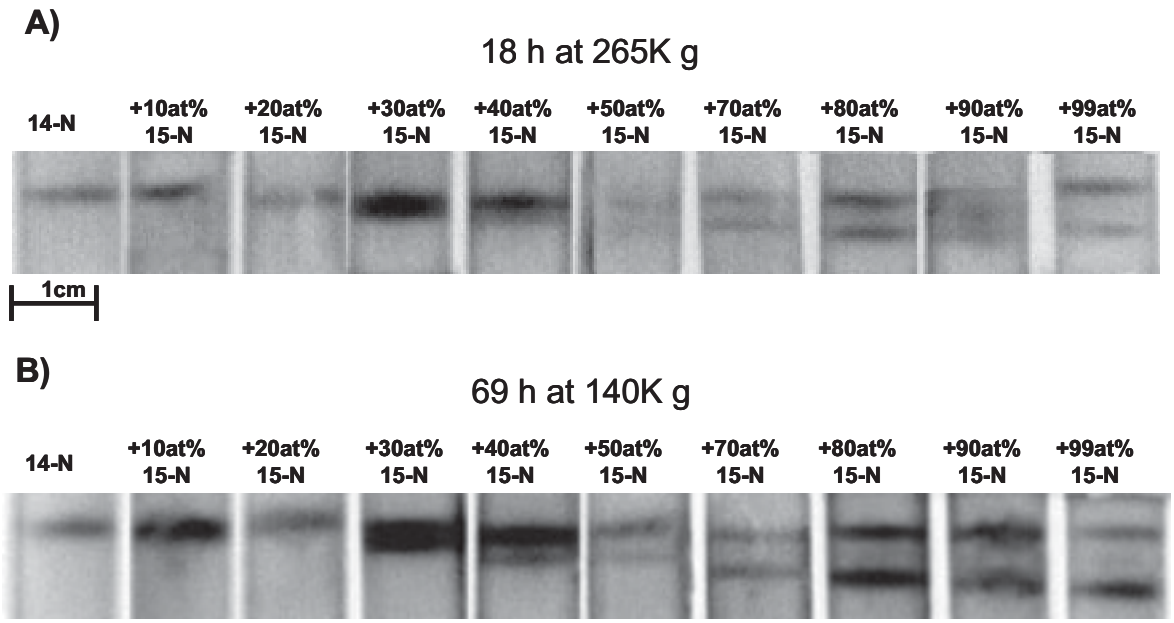

Figure 3. Separation of unlabelled (^{14}N) and increasingly enriched (10-99 at% ^{15}N) DNA of *Pseudomonas putida* in a CsCl gradient obtained at A) 265K g for 18 h or B) 140K g for 69 h.

2.3.4 Evaluation of an alternative approach to enhance separation of ^{14}/^{15}N-DNA bands

The fact that with the conventional centrifugation conditions of 265K g at 18 h a maximum separation of 4 mm between fully labelled and unlabelled bands is achieved severely limits its application in many ecological studies where often only low microbial enrichments are achieved unless the system is artificially highly loaded with the labelled substrate. We tested an alternative centrifugation approach which used much longer spinning time (69 h) at somewhat lower speed (38K rpm or 140K g). The results in Figure 3B demonstrates that with this method the maximum separation of unlabelled and highly ^{15}N labelled DNA can be extended to 5 mm. This improved separation of differently labelled DNA allowed a clear differentiation of enrichments as low as 40 at%^{15}N. This is an improvement compared to the minimum 50 at%^{15}N identified under the higher speed but shorter centrifugation. Similar experiences have been achieved with ^{13}C labelled DNA (Hutchens et al., 2004) although the results were more encouraging by nearly doubling the separation of differently labelled DNA strands.

These separations were achieved with single species DNA composition. However, the nucleic (nitrogenous) composition of DNA can vary depending on the proportion of adenine-thymine (AT) to cytosine-guanine (CG) combinations then the N concentration of DNA could theoretically vary between 13.9-15.8 % depending on species. This also

implies that the molecular weight and buoyant density of DNA changes depending on the CG /AT composition (Birnie, 1978) as well as with its respective labelling with ^{15}N. For example, with a CG content of microbial DNA varying between 35-70% the respective buoyant density varies between 1.69-1.73 g cm^{-3} (Rolfe and Meselson, 1959). Thus also variation in the nucleic content of the DNA has a significant effect on the position of the band in the density medium (see error bars in Figure 4 depicting theoretical maximum/minimum GC content) particularly for ^{15}N-SIP and to a lesser degree also for ^{13}C-SIP (Radajewski et al., 2003). Hence a clear separation at 40 at%^{15}N DNA enrichment, although theoretically still possible, may be difficult to achieve in practice given the width of DNA bands (related to their quantity and variation in %G+C content) in the media. Hence, there is a need for further improvements in techniques that lead to a better separation between DNA strands in density gradients.

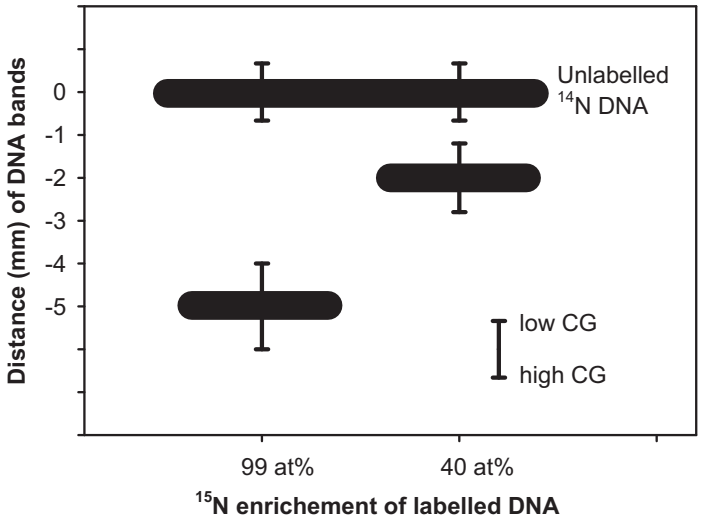

Figure 4. Estimated effect of varying nucleic composition of DNA on separation efficiency of differently ^{15}N enriched DNA. Error bars depict theoretical maximum differences in GC content of DNA based on buoyant density, i.e. high or low GC content.

2.3.5 Verification of isotopic enrichment of heavy bands from soil residue experiment

To test the applicability of the ^{15}N stable isotope probing method in a soil environment, highly enriched *L. perenne* residue was incubated in soil. After 7 days, DNA was extracted and exposed to centrifugation (265K g for 18 h) in a CsCl gradient. Unlike in the pure culture experiments a clear but more widespread band appeared in the CsCl media (Figure 5). This was expected as it was hypothesised that not all organisms would feed solely on the substrate and that the turnover of ^{15}N due to cross feeding of DNA from

originally unlabelled organisms would dilute the DNA from organisms feeding primarily on the labelled substrate. Analysis of the actual enrichments of the isolated DNA fractions showed that the DNA at the bottom of the visible band was heavily enriched with ^{15}N of up to 90 at%. The evidence that this highly ^{15}N enriched DNA was primarily of microbe and not plant origin comes from both microbial biomass and its ^{15}N enrichment measurements and specific primers/DGGE results (data not presented) which suggested that by day 7 little if any plant DNA remained. Enrichment subsequently declined rapidly with increasing distance from the bottom sample. In this experiment we were not able to harvest sufficient amount of unlabelled (100 at% ^{14}N) DNA for isotope ratio mass-spectrometric analysis (which for the current equipment needed to be around 30 µg to obtain reliable results). However, lower levels of enriched DNA might be detectable through the use of GC/C/IRMS or emergent technologies such as HPLC/IRMS. The reason that there was no significant ^{14}N DNA band is that the added material supplied much more microbially available substrate than the native soil organic matter and hence resulted in good ^{15}N enriched microbial DNA yields. These results clearly suggest that sampling strategy of DNA from the CsCl media is crucial and that a careful stepwise separation of DNA at different distances from the reference is advisable.

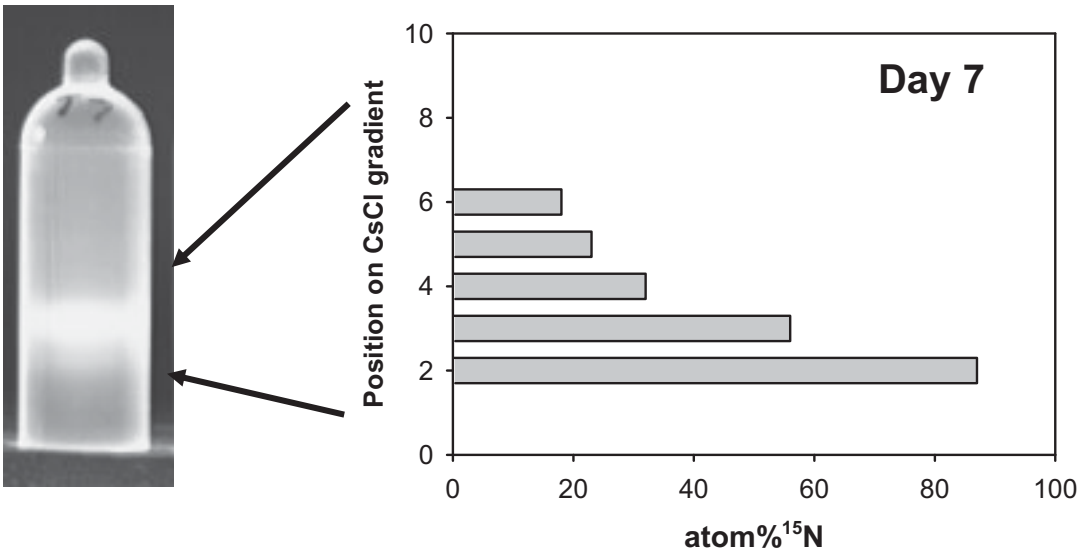

Figure 5. Isotopic ^{15}N enrichment pattern of DNA bands extracted from a CsCl gradient (position 0 starts just below visible band, data only available where sufficient DNA recovered) 7 days after application of highly enriched *Lolium perenne* residue to soil.