Taxon-specific responses of soil bacteria to the addition of trace or low amounts of glucose

Sasha Jenkins[A], Clare Lanyon[B], Andrew Whiteley[C], Ian Waite[A], Philip Brookes[D], Richard Evershed[E] Anthony O'Donnell[A]

[A]Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley WA 6009, Australia Email sasha.jenkins@uwa.edu.au
[B]Institute for Research on Environment and Sustainability, Newcastle University, Newcastle upon Tyne, NE1 7RU, England, UK
[C]CEH Oxford, Oxford OX1 3SR, UK
[D]Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK
[E]School of Chemistry, University of Bristol, Bristol BS8 1TS, UK

Abstract
When small or trace amounts of labile C substrates enter the soils either a priming effect or a triggering effect is induced but the identities of the microorganisms involved remains largely unknown. Here, we modified the Stable Isotope Probing (SIP) protocol to accommodate low level carbon pulses, such that their fate within the microbial community could be determined. Soils were amended with different concentrations of [^{14}C] glucose (150, 50 and 15 and 0 µg C /g soil) prior to rRNA extraction and equilibrium density centrifugation. Subsequent denaturing gradient gel electrophoresis (DGGE) analysis, sequencing of bands and multivariate statistics was then used to identify the taxon specific responses at different glucose concentrations. [^{14}C] RNA enrichment was restricted to *Bacillus, Pseudomonas, Burkholderia* and β-proteobacteria and the concentration of glucose determined the magnitude of the community response. *Pseudomonas* was only enriched at 150 µg C /g soil whilst *Bacillus* and *Burkholderia* although present at 150 µg C /g soil, were more abundant at trace amounts (15-50 µg C /g soil). Glucose amendment also impacted indirectly on the community changing the [^{12}C] RNA signal of *Bradyrhizobium,* Actinobacteria *Nitrosomonas* and Acidobacteria which probably reflects changes in substrate availability and microbial interactions.

Keywords
RNA radioactive isotope probing (RNA-RIP), [^{14}C] glucose, Bacterial Community Structure, Priming Effect, Triggering Effect, Soil organic matter (SOM) turnover.

Introduction

A significant portion of degradable carbon entering agricultural soils from plants is in the form of glucose which is readily degraded by microorganisms. Soil organic matter (SOM) represents a major carbon sink in the biosphere (Fontaine *et al.* 2003) but its turnover is often limited by the availability of labile C substrates, such as glucose, that are required for growth, maintenance and the production of extracellular enzymes (Schneckenberger *et al.* 2008) in most soils. Although, additions of these substrates to soil has been shown to increase microbial activity, accelerate SOM turnover and induce a priming effect, the high application rates (1000-5000 µg C /g) used in earlier studies (e.g. Wu *et al.* 1993) does not reflect the natural C concentrations in soil since inputs can be small and infrequent (Schneckenberger *et al.* 2008). Therefore, recent studies have sought to amend soil with substrates at concentrations more akin to those found in soils (De Nobili *et al.* 2001; Schneckenberger *et al.* 2008) but there is still much debate over whether this induces a priming effect or triggering effect (De Nobili *et al.* 2001) where CO_2 evolution is derived from endocellular reserves. Furthermore, the mechanisms and the identities of the microorganisms involved remain largely unresolved and better methodologies are required.
Stable Isotope Probing (SIP) approaches offer the possibility of linking microbial identity with metabolic function by coupling specific biogeochemical tracers to highly resolved phylogenetic markers, such as the 16S rRNA gene (Whiteley *et al.* 2006). However, the relatively high level of substrate used to label nucleic acids prior to density separation (Whiteley *et al.* 2006) has rendered it unsuitable for soil priming studies so far. The aim of the work reported here was to modify the RNA-SIP procedure to accommodate low level carbon pulses within the range 15–150 µg C /g that are more in line with available C concentrations in soil. To this end, soils were amended with [^{14}C] glucose prior to nucleic acid extraction and equilibrium density centrifugation ([^{14}C] RIP, radioisotope probing). Confirmation that RNA-SIP could discriminate between 'heavy' [^{14}C] RNA and 'light' [^{12}C] RNA at these low C levels was verified by autoradiography, visualised and quantified. Subsequent DGGE analysis of the fractions and subsequent multivariate statistics was used to investigate the relationship between bacterial community structure and different glucose concentrations.

Methods

Microcosm set-up and RNA-radioisotope labelling (RIP) procedure

Soils were amended with different concentrations of [^{14}C] glucose (150, 50, 15 μg C /g soil and control [no amendment, 0 μg C /g]) and incubated at 22 ° C for 4 days to ensure adequate enrichment. Following [^{14}C] glucose amendment, nucleic acids were extracted using the phenol-chloroform-CTAB method (Griffiths *et al.* 2000). The 'heavy' [^{14}C] labelled RNA was separated from the 'light' [^{12}C] unlabelled RNA was separated using equilibrium density centrifugation. The existing [^{13}C] RNA-SIP protocol (Whiteley *et al.* 2007) was adapted to accommodate the use of [^{14}C] labelled glucose and then optimised for low C additions. The extracted RNA was added to Caesium trifluoracetate (CsTFA) and spun in an Optima TLX ultracentrifuge at 60,000 rev/min and 20 °C for 37 h to form a density gradient. Two blank reactions were included in every run to provide a reference gradient so that a density profile could be calculated after fractionation. Gradients were fractionated from below by displacement with water by using a Beckman Fraction Recovery System and a syringe pump at a flow rate of 3.3 μl/s. Fractions were collected every 30 s, giving 30 fractions per gradient and the RNA was isolated from each fraction by precipitation with ice-cold isopropanol. The [^{14}C] RNA radioisotope probing (RIP) approach was evaluated and verified by autoradiography to confirm the location of the [^{14}C] fractions.

Molecular community profiling

The RNA from each fraction was subjected to reverse transcription (RT)-PCR and the PCR-products were analysed by denaturing gradient gel electrophoresis (DGGE). Unique bands were excised from the DGGE gels, re-amplified and sequenced. Replicate DGGE gels (x3) were digitized and analysed using Bionumerics gel analysis software to generate a data matrix of relative taxon abundance (band intensity) for each community (lanes). A corresponding data matrix of explanatory variables (glucose concentration, buoyant density) was prepared and canonical correspondence analysis (CCA) was used to explore the relationship between community structure and glucose concentrations.

Results and discussion

The [^{14}C] RNA-RIP approach clearly resolves the 'heavy' fractions ([^{14}C] labelled RNA) from the 'light' fractions ([^{12}C] unlabelled RNA) which was verified using autoradiography. Interestingly, the RNA-RIP has highlighted the existence of [^{14}C] intermediate fractions. A range of buoyant densities could have resulted from variations in the amount of [^{14}C] enrichment between glucose assimilating taxa or differences in G+C content between bacterial taxa. This 'effect' will be accentuated here by the [^{14}C] RNA (compared to [^{13}C] RNA) since the separation distance is greater along the gradient.

As expected, DGGE analysis showed a distinct shift in community structure following the addition of [^{14}C] glucose at all concentrations and was characterised by an increase or decrease in the relative abundance of particular bands (Figure 1). The canonical correspondence analysis (CCA) biplots in Figure 2 showed that the community response in both the 'heavy' and 'light' fractions differed according to the concentration of glucose amendment. For both fractions, the differences in community structure were most pronounced in soils amended with 150 μg C /g soil. In the 'heavy' RNA fractions there was a clear separation between soils amended with 150 μg C /g soil and those receiving 50 and 15 μg C /g soil; the community structures in the latter were more similar to each other. This suggests that at low or trace C inputs (so-called trigger molecule concentrations) the microbial community response is quite distinct from that seen at higher concentrations. A possible explanation is that there is insufficient energy to activate the majority of the community at this level of amendment (Wu 1993; De Nobili *et al.* 2001) and the energy is used for internal microbial metabolism instead possibly inducing a 'triggering effect' (De Nobili *et al.* 2001).

To investigate these differences further, bands that changed in relative intensity following amendment were excised from the DGGE gels, reamplified and sequenced. [^{14}C] RNA enrichment was restricted to *Bacillus, Pseudomonas, Burkholderia* and β-proteobacteria. This is not unexpected since competition for a limited resource typically results in the dominance of one or a few populations with the highest growth rates (Fontaine *et al*. 2003). Also, these bacterial groups are particularly adept at responding to a variety of labile C compounds entering soils across a range of different ecosystems and have been described as r-strategists or (Bernard *et al.* 2007). *Pseudomonas* was only enriched at 150 μg C /g soil which is consistent with their putative roles as r-strategists whilst *Bacillus* and *Burkholderia* although present at 150 μg C /g soil, were most prevalent at the lower glucose concentrations (15-50 μg C /g soil) which could mean they have evolved survival strategies to remain 'metabolically alert' during periods of starvation (De Nobili *et al.* 2001). Glucose amendment also impacted indirectly on the community by changing the abundance of taxa in the 'light' fractions and this probably reflects changes in substrate availability and microbial interactions. The

increase in the abundance of Actinobacteria and *Bradyrhizobium* in the 'light' fractions has been previously reported following substrate amendment and was linked to enhanced SOM degradation and priming (Bernard *et al.* 2007). According to Fontaine *et al.* (2003) the fast-growing r-strategist that initially utilise the substrate will be superseded by slow-growing K-strategists able to decompose recalcitrant organic compounds (Fontaine *et al.* 2003). Although this was not proven here, *Bradyrhizobia* are oligotrophic slow-growing bacteria whilst Actinobacteria are known for their ability to degrade recalcitrant material. There was a marked decrease in the relative abundance of Acidobacteria and α-proteobacteria (*Nitrosomonas*) and this has been observed before (Bernard *et al.* 2007). Explanations include the possibility that glucose may have induced changes in the soil microenvironment and community structure due to enhanced heterotrophic activity leading to reduced oxygen availability and a community dominated by facultative and anaerobic microorganism (Griffiths *et al.* 1999). Also, with accelerated microbial growth there is higher potential for antagonism through either competition for resources or by the secretion of antimicrobial metabolites (Griffiths *et al.* 1999). Finally, these oligotrophs could be sensitive to high osmotic stress (Griffiths *et al.* 1999) and their uptake mechanisms may have become swamped at the higher glucose concentrations.

Conclusion

This study demonstrates that the response of soil bacteria following amendments with low level pulses of glucose is restricted to a few specific taxa affiliated with *Bacillus, Pseudomonas*, *Burkholderia* and *β*-proteobacteria. The amount of glucose added to soil determines the magnitude of the bacterial response. Trace or small glucose amounts were accompanied by an increase relative abundance of *Bacillus* and *Burkholderia* whilst at higher glucose levels *Pseudomonas* dominated the community. This is probably due to more energy being available for growth. Glucose amendment also impacted indirectly on the community by increasing the abundance of *Bradyrhizobium* and Actinobacteria whilst reducing the abundance of *Nitrosomonas* and Acidobacteria in the 'light' fractions. This probably reflects changes in substrate availability and microbial interactions. Overall, these results show that RNA-RIP can be used successfully to study the fate of labile C substrates, such as glucose in soil.

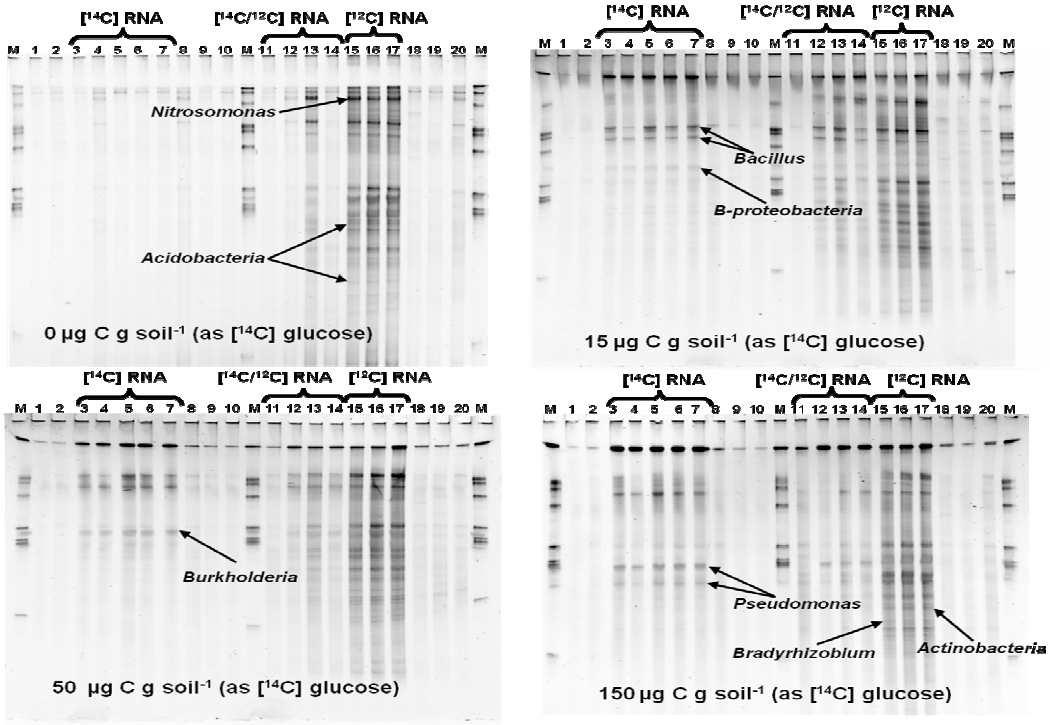

Figure 1. RT-PCR-DGGEgels comparing the bacterial community profiles recovered from first 20 fractions along the RNA-SIP density gradient after 36 h of centrifugation. RNA was extracted from soil in microcosms amended with different concentrations of [^{14}C] glucose (0, 15, 50, and 150 µg C /g soil) after 4 days of incubation. The 'heavy' fractions (3-7) containing the [^{14}C] labelled RNA, the 'light' fractions (15-17) containing the [^{12}C] unlabelled RNA and the 'intermediate' fractions (11-14) containing [^{14}C/^{12}C] labelled RNA. M indicates the marker lane and numbers represent fraction number. Arrows indicate the bands that were excised from the gel, reamplified and sequenced.

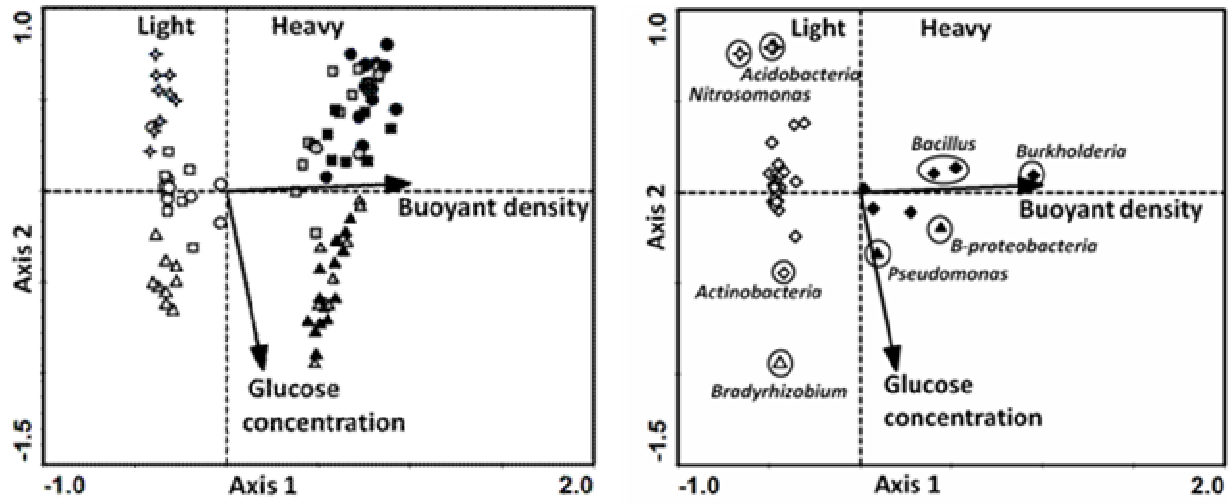

Figure 2a) Biplot from canonical correspondence analysis (CCA) showing the change in bacterial community composition (as judged by DGGE banding patterns) along the buoyant density gradient and with increasing [^{14}C] glucose concentration [first two axes account for 48% of total variance]. Points on the graph represent the bacterial communities recovered from 'heavy' fractions (3-7) containing [^{14}C] labelled RNA (Black■), 'intermediate' fractions (11-14) containing [^{14}C/^{12}C] labelled RNA (Grey ■) and 'light' fractions (15-17) containing [^{12}C] unlabelled RNA (White □) over the first 20 RNA-SIP fractions. The different concentrations of [^{14}C] glucose 0 (star ✦), 15 (circle ●), 50 (square ■) and 150 (triangle ▲) µg C/g soil were assigned accordingly. Arrows represent the explanatory variables [Buoyant density and glucose enrichment concentration] and the direction of the arrow indicates increasing density or concentration

2b) Biplot shows the distribution of bacterial taxa along the same density buoyant gradient. Points represent the bacterial taxa recovered from 'heavy' fractions (3-7) containing [^{14}C] labelled RNA (Black◆) and 'light' fractions (15-17) containing [^{12}C] unlabelled RNA (White ◇). The taxa were assigned to groups based on their abundance at different glucose concentration: taxa only found in the control samples (✦), taxa found in all [^{14}C] glucose concentration amendments (◇/◆) and taxa more abundant in 150 µg C /g soil amendment (△/▲).

References

Bernard L, Mougel C, Maron P-A, Nowak V, Lévêque J, Henault C, Haichar FZ, Berge O, Marol C, Balesdent J, Gibiat F, Lemanceau P, Ranjard L (2007) Dynamics and identification of soil microbial populations actively assimilating carbon from 13C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. *Environmental Microbiology* **9,** 752–764.

De Nobili M, Contin M, Mondini C, Brookes PC (2001). Soil microbial biomass is triggered into activity by trace amounts of substrate. *Soil Biology & Biochemistry* **33,** 1163–1170.

Fontaine S, Mariotti A, Abbadie L, (2003) The priming effect of organic matter: a question of microbial competition? *Soil Biology and Biochemistry* **35,** 837–843.

Griffiths BS, Ritz K, Ebbelwhite N, Dobson G (1999). Soil microbial community structure: effects of substrate loading rates. *Soil Biology and Biochemistry* **31,** 145–153.

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and RNA-based microbial community composition. *Applied and Environmental Microbiology* **66,** 5466-5491.

Schneckenberger K, Demin D, Stahr K, Kuzyakov Y (2008) Microbial utilization and mineralization of [14C] glucose added in six orders of concentration to soil. *Soil Biology & Biochemistry* **40,** 1981–1988.

Whiteley AS, Manefield M, Lueders T (2006) Unlocking the 'microbial black box' using RNA-based stable isotope probing technologies. *Current Opinion in Biotechnology* **17**, 67-71.

Whiteley AS, Thomson B, Lueders T, Manefield M (2007) RNA stable-isotope probing. *Nature Protocols* **2**, 838-844.

Wu J, Brookes PC, Jenkinson DS (1993) Formation and destruction of microbial biomass during the decomposition of glucose and ryegrass in soil. *Soil Biology and Biochemistry* **25,** 1435–1441.