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Utility of high performance liquid chromatography/electrospray/mass spectrometry of polar lipids in specifically Per-^{13}C labeled Gram-negative bacteria DA001 as a tracer for acceleration of bioremediation in the subsurface

Cory A. Lytle [a], Mark E. Fuller [b], Ying dong M. Gan [a], Aaron Peacock [a], Mary F. DeFlaun [b], Tullis C. Onstott [c], David C. White [a,d,*]

[a] *Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575, USA*
[b] *Envirogen, Inc., Princeton Research Center, 4100 Quakerbridge Road, Lawrenceville, NJ 08648 USA*
[c] *Department of Geosciences, Princeton University, Princeton, NJ 08544, USA*
[d] *Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37983, USA*

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Abstract

Specific fatty acids from phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) recovered from a per ^{13}C-labeled bacteria can be detected in environmental samples and used as measures of bacterial transport in the subsurface. Detection of palmitic acid (16:0) and oleic acid (18:1) at m/z 271 (255 + 16) and 299 (281 + 18) as negative ions in PG and PE separated by high performance liquid chromatography (HPLC) and detected after up-front collisionally induced dissociation (CID) utilizing electrospray (ES) mass spectrometry (MS) provided sufficient sensitivity and specificity for detection in the presence of the indigenous microbiota. Application of tandem mass spectrometry (MS/MS) in the multiple reaction monitoring (MRM) was use to monitor selected transitions. MRM can increase the sensitivity so that polar lipids recovered from cell densities currently at about 10^4 cells/sample can be detected. This technology provides a non-intrusive mechanism for monitoring the distribution of bacteria added to accelerate in situ bioremediation of subsurface sediments.

Keywords: Environmental detection of bacteria; Per ^{13}C-labeled fatty acids; Electrospray ionization; Phospholipids; Phosphatidylglycerol; Phospatidylethanolamine; High performance liquid chromatography/electrospray ionization/mass spectrometry

1. Introduction

Tracing bacterial movement through subsurface sediments has been problematical, as the bacterial tracer must be marked so it can be readily differentiated from the in situ microbes and must be detected not only with great sensitivity and specificity but also within the environmental constraints of the pro-

* Corresponding author. Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575, USA. Tel.: +1-423-974-8001; fax: +1-423-974-8027.
E-mail address: MILIPIDS@AOL.COM (D.C. White).

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gram. In the current environmental programs of the Natural and Accelerated Bioremediation Research Program (NABIR) of the Office of Science at the United States Department of Energy (DOE), the bacteria to be used as tracers must be isolated from the particular site and not contain radioactive or genetically engineered tracers as biomarkers (DeFlaun et al., 1997; Onstott et al., 2000). Both radioactive labeling (Harvey, 1997) and the engineering of reporter genes such as green fluorescent protein (GFP) (Burlage, 1997) provide sufficient sensitivity/specificity but violate the environmental constraints. Several techniques meeting the environmental constraints have proved successful in field experiments thus far (DeFlaun et al., 2000). Bacteria can be stained with fluorescent stains that remain inside the cell envelopes and do not fade or alter the external surface polymers that might be involved with cell adhesion to the solid matrices in the subsurface (Fuller et al., 2000a). They can be detected with automated microscopy so efficiently as to be cost effective. Another technique utilizes whole-cell quantitative polymerase chain reaction (PCR) that detects specific amplification of the DNA of the tracer organism. The amplification is readily detected with multiple well plates (Kovacik and Holben, 2000). This specific technique overcomes the time, costs and differential amplification problems associated with DNA recovery and PCR amplification from the environment. A ferrographic separation method has also proved successful in tracking bacteria in the field (Zhang and Johnson, 1999a,b). Specific antibodies sited on 50 nm paramagnetic beads, with recovery in focused strong magnetic fields allowed detection of DAPI (4′,6-diamino-2-phenylindole) stained bacteria at concentrations of 10^3 cells/l.

Two additional methods utilize mass labeling (^{13}C) of the tracer bacteria. In one system ground water membrane filter retentates is recovered and the total organic carbon of the retentates is analyzed as ^{13}C-carbon dioxide after pyrolysis (DeFlaun et al., 1997; Holben and Ostrom, 2000). This system does not define the specificity of the localization of the ^{13}C other that it is a part of the groundwater particulate organic carbon (POC). Herein, we report the specific detection of per ^{13}C-labeled negative ions from the palmitic and oleic acids from the two polar lipids of the bacteria.

^{13}C-labeling in biological materials has been difficult to detect, since differentiation of $^{12}C + {}^{1}H$ (12.00000 + 1.007825 = 13.0078) from ^{13}C (13.003345) requires high-resolution mass spectrometry that is expensive and insensitive (Barrie and Prosser, 1996). The problem of differentiating ^{13}C from $^{12}C^{1}H$ is overcome by measuring ^{13}C as ^{13}C–CO_2 (Grossman, 1997). This technique requires isotope ratio mass spectrometry and in the combustion of the POC destroys the identity of the specific components from which the ^{13}C was originally incorporated.

Herein, we report the detection of the negative ions of two fatty acids from the specific polar lipids containing 16 and 18 extra mass units that are sufficiently unique to be tracers of the specific molecules from the per ^{13}C-labeled bacteria.

2. Materials and methods

2.1. Materials

Synthetic phospholipid standards, PG (P0514) and PE (P9137), were purchased from Sigma (St. Louis, MO). Standards were prepared at 10 ppm in methanol and diluted accordingly. All solvents were HPLC grade. Water was obtained from an in-house source of millipore water (Millipore). Piperidine (10,409-4) was purchased from Aldrich (Milwaukee, WI). Narrow Channel Artificial Groundwater (NCAGW) was developed to reflect the Oyster, VA, site groundwater chemistry, and contains (mg/l): $Ca(NO_3)_2 \cdot 4H_2O$, 70; $MgSO_4 \cdot 7H_2O$, 60; $NaHCO_3$, 60; $CaCl_2 \cdot 2H_2O$, 29; $CaSO_4 \cdot 2H_2O$, 25; KNO_3, 10; NaH_2PO_4, 0.4. NCAGW is prepared by adding the chemicals to autoclaved distilled/deionized water and adjusting the pH to 6.0 with 2 N HC1. NCAGW is then sterilized by filtration through 0.2 μm cellulose acetate filter units when necessary.

2.2. Isolation and growth of bacteria

An indigenous aquifer organism was isolated from the DOE/NABIR Oyster, VA, field site. The test facility is located in a surficial aquifer site just South of Oyster, VA, on the DelMarVa peninsula (DeFlaun

et al., 1997; Fuller et al., 2000b). A Gram-negative rod was selected from colonies recovered from NCAGW and designated DA001. DA001 was identified as a *Comamonas* species using 16 S rRNA fingerprinting (similarity index (SI) = 0.965). The organism was selected to be the most non-adsorbent to the soil matrix and therefore the most mobile in the subsurface (DeFlaun et al., 1997). Adhesion-deficient variants of DA001 were selected using a standardized adhesion assay employing Oyster site sediment (DeFlaun et al., 1990, 1997, 1999). DA001 was grown in a nitriloacetic acid-free basal salts medium (BSM), (Hareland et al., 1975) with 0.2% (w:v) $^{13}CH_3{}^{13}COONa$ (99 at.%, Cambridge Isotope Laboratories, Andover, MA, USA) as the sole carbon source. The cells were grown in sealed 50 ml tubes held horizontally and shaken at 150 rpm at room temperature (20–25°C) for 18–24 h (OD at 550 nm of about 1.0). Cells were harvested by centrifugation at 3400 rpm for 20 min at 4°C and resuspended in NCAGW, re-centrifuged, resuspended twice. Cells were the starved at 15°C in NCAGW with shaking for 48–72 h. PLFA analysis showed no differences in patterns between the cells harvested prior to washing and after starvation at 15°C.

Cells were examined for clumping in the Leica TCS-4D confocal scanning laser microscope and stained with acridine orange and live/dead stains as described for direct microscopic counting (Phiefer et al., 1999).

2.2.1. Intact core bacterial transport experiments

Intact sediment cores (70 cm long × 7.2 cm ID) were collected from both the vadose and saturated zone during an excavation in Oyster, VA, in May 1999. Core orientation was parallel to the in situ groundwater flow. Intact cores were prepared and operated as described elsewhere (DeFlaun et al., 1997; Fuller et al., 2000b) with slight modifications to tubing size and configuration as described in DeFlaun et al. (2000). The final height of sediment after core preparation was 50 cm. DA001 cells were labeled with $^{13}CH_3 \cdot {}^{13}COONa$ (99 at.%; Isotec, Miamisburg, OH, USA) in BSM, washed, and stained with CFDA/SE (Fuller et al., 2000a). Three hundred milliliters of NCAGW (equal to one-half of the pore volume (PV) of the sediment), containing approximately 7×10^7 cells were introduced into core NC7-2 (vadose zone core), followed by a continuous injection of Oyster site groundwater. DA001 cell concentrations in the core effluent fractions collected at 20-min intervals were determined by plating, epifluorescent direct counts of CFDA/SE-stained cells, microplate spectrofluorometry. After 28 PV of groundwater had passed through the sediment, the core was drained, frozen and split (DeFlaun et al., 1997). A template consisting of 5 squares by 28 squares (each square 1.6 × 1.6 cm) was used as a guide to subsample the sediment. The sediment under each grid space was transferred to a weighed plastic centrifuge tube, the tube was weighed again, and the weight of tube plus sediment recorded. The sediment in the tube was thoroughly homogenized using a metal spatula, and the gravimetric water content of selected samples along the length of the core was determined. All the samples within the first 15 cm of the influent end of the core, and selected samples from the remainder of the core, were plated for DA001 colony forming units, onto R2A plates in triplicate, after being serially diluted in phosphate buffered saline. The dilutions were also examined for epifluorescent direct counts of CFDA/SE-stained cells. Samples for microscopy were prepared as for CFU, except the sediment suspension was allowed to settle 300 s before a sample was removed and filtered onto black polycarbonate membranes for direct counting.

2.3. Recovery of the polar lipids

The lyophilized bacteria and the soil samples were extracted by a modified Bligh and Dyer one-phase chloroform/methanol/phosphate buffer and the lipids fractionated into general lipid classes (neutral lipid, glycolipid, and polar lipid) by silicic acid column chromatography as previously described (White and Ringelberg, 1998). A portion of the polar lipids was transesterified by mild alkaline methanolysis and the fatty acid methyl esters (FAMEs) analyzed by capillary gas chromatography/mass spectrometry (GC/MS) (Ringelberg et al., 1988). The polar lipids were initially analyzed by HPLC/ES/MS (Lytle et al., 2000) and dilution series and core samples were analyzed by HPLC/ES/MS/MS.

Table 1
Phospholipid fatty acid methyl esters from DA001 determined by GC/MS after transesterification of the total polar lipids. (Fatty acids are designated as numbers of carbon atoms: number of double bonds with the number of carbons from the hydrocarbons or ω end of the molecule. Cy indicates a cyclopropane ring)

FAME	% of Total	^{12}C *m/z*	Per-^{13}C *m/z*
16:0	21	270	286
16:1ω7c	27	268	284
Cy 17:0	17	282	299
18:1ω7c	29	296	314
19:1	6	310	329

2.4. Detection of per ^{13}C-labelled 16:0 and 18:1 in DA001 in soil bacterial polar lipids

The detection, after up-front CID, of the polar lipids from the per ^{13}C labeled DA001 was analyzed in 10 times the biomass of the total polar lipid content of a surface soil sample that had been previously analyzed by GC/MS.

2.5. HPLC

Separation of the phospholipids was carried out on an HP 1100 HPLC (Agilent Technologies, Sunnyvale, CA). The mobile phase consisted of 95/5 (methanol containing 0.002% piperidine/water v/v). A HAISIL HL (Higgins Analytical, Mountain View, CA) column, 30 mm × 1 mm × 5 μ was used for the separation. The column flow rate was 40 μl/min, while 0.2% piperidine in methanol was added post column at a flow rate of 10 μl/min by Harvard "33" Dual Syringe Pump (Harvard Apparatus, Holliston, MA).

2.6. Mass spectrometry

Electrospray mass spectrometry (ES/MS) was performed using both a VG Platform II single quadrupole MS and a Sciex API 365 triple quadrupole MS. Instrument tuning was performed utilizing a synthetic PG standard. Calibration of the Platform II was performed in the negative ion mode with a solution of sodium iodide/cesium iodide. Samples were introduced into the Platform II ES source at a total flow rate of 60 μl/min. The electrospray capillary was operated at −2.78 kV. The counter electrode was operated at 0.41 kV. The cone was set to −80 V, while the skimmer lens offset was set to 5 V. The source was operated at 100°C. The calibra-

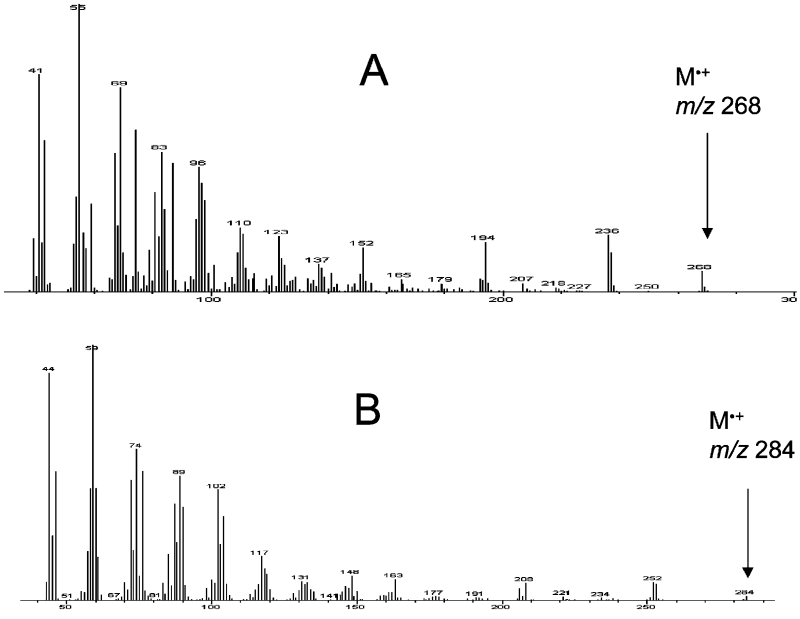

Fig. 1. Electron impact mass spectra of the methyl esters (16:1) of (A) authentic DA001 sample (B) DA001 grown with ^{13}C-acetate as sole carbon source.

Table 2
Negative ions of PLFA generated up-front collision-induced dissociation (CID) in the ESI of PG and PE recovered from per ^{13}C-labeled DA001. Two unique negative ions at m/z 271 and 299 are identified. (Fatty acids are designated as numbers of carbon atoms: number of double bonds with the number of carbons from the hydrocarbon or ω end of the molecule. Cy indicates a cyclopropane ring)

PLFA	^{12}C m/z	Per-^{13}C m/z	Possible overlaps
16:1	253	269	^{12}C-17:0 = 269
16:0	255	271	2 mass units > ^{12}C-17:0, unique ^{12}C 18:6 not known
Cy 17:0	267	284	^{12}C-18:0 = 283
18:1	281	299	^{12}C-20:6 not known, 2 mass units > ^{12}C-19:0, unique
19:1	295	314	^{12}C-21:5 = 315; ^{12}C-21:6 = 313 not known

tion of the API 365 was performed using a mixture of polypropylene glycols in the negative ion mode. Samples were infused into the atmospheric pressure ionization source at a flow rate of 5 μl/min. The

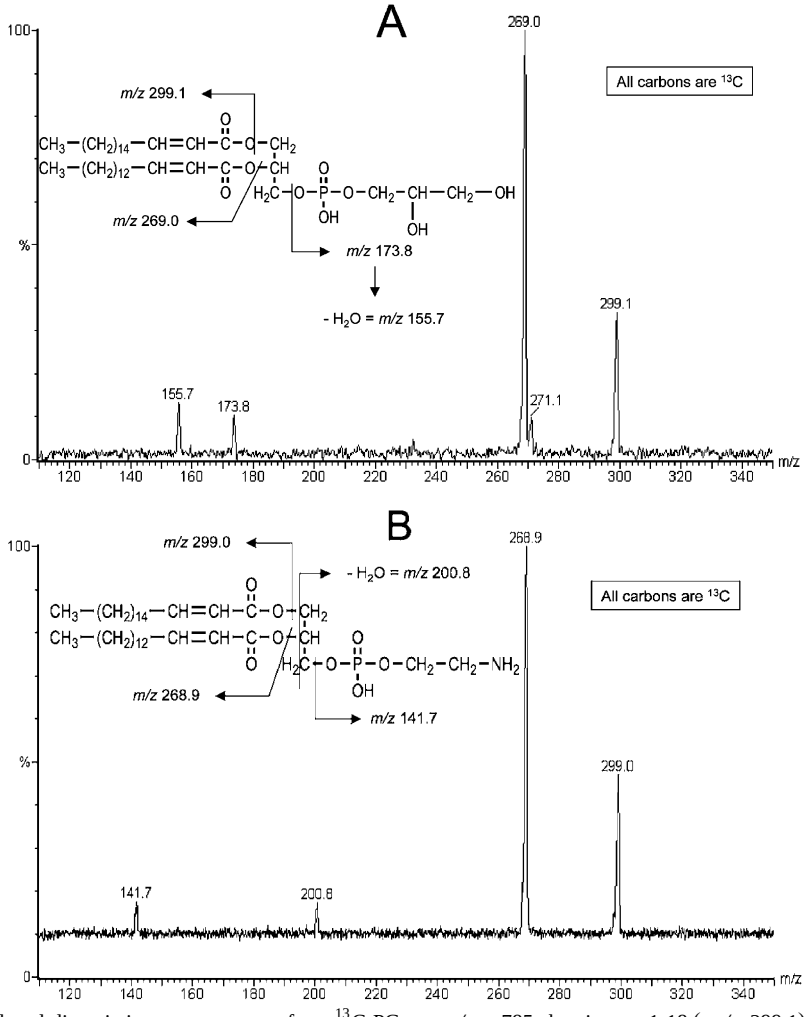

Fig. 2. Collisionally induced dissociation mass spectra of per ^{13}C-PG, at $m/z = 785$ showing *sn*-1 18:(m/z 299.1), *sn*-2 16:(m/z 269.0), glycerol-P (m/z 173.8) and glycerol-P minus H_2O(m/z 155.7) (A); and per ^{13}C-PE at $m/z = 754$ showing *sn*-1 18:1(m/z 299.0), *sn*-2 16:1(m/z 268.9), P-ethanolamine (m/z 141.7), and glycerol P-ethanolamine minus H_2O (m/z 200.8) (B). All carbons are ^{13}C.

TurboIon spray voltage was optimized at −4.4 kV, the entrance potential was optimized at 9.5 V and the declustering potential was optimized at −51 V. The collision energy was optimized at 51 V.

3. Results

3.1. Polar lipid fatty acids of DA001

DA001 contains a simple polar lipid fatty acid composition with 4 PLFA comprising 94% of the total fatty acids (Table 1). The fatty acids are identified as fatty acid methyl esters by their electron impact GC/MS spectra typical of FAMEs (Fig. 1). The FAMEs derived from the per ^{13}C-grown cells have the $[M]^+$ ions expected for their retention indices.

3.2. Detection of unique negative ions of PLFA per ^{13}C-labeled DA001

When detected as negative ions in the MS after up-front CID, the fatty acids of DA001 have the following m/z: 16:1 m/z 253 (+16 = 269); 16:0 m/z 255 (+16 = 271); cy 17:0 m/z 267 (+17 = 284); 18:1 m/z 281 (+18 = 299); 19:1 m/z 295 (+19 = 314) as seen in Table 2 (fatty acids are designated as number of carbon atoms: number of double bonds with the number of carbons from the hydrocarbon or ω end of the molecule; Cy indicates a cyclopropane ring). Of the negative ions formed from the polar lipids, three are not ideal unique tracers as they could overlap several isomers. Per ^{13}C-16:1 at m/z 269 is equivalent to ^{12}C-17:0; per ^{13}C-cy 17:0 at m/z 284 is too close to ^{12}C 18:0 at m/z 283; per ^{13}C-19:1 m/z 314 is too close to 21:5 m/z 315 (^{12}C-21:6 m/z 313 is not known to exist). Two per ^{13}C-PLFA representing 21% and 29% of the total PLFA of DA001 provide unique m/z masses: 16:0 at m/z 271 requires two additional mass units greater than ^{12}C-17:0 at m/z 269; per ^{13}C-181:1 at m/z 299 would require a ^{12}C-20:6 not known to exist or two additional mass units in ^{12}C-19:0 (at m/z 297). These are PLFA with unique m/z when detected as negative ions derived from phospholipids of PG and PE.

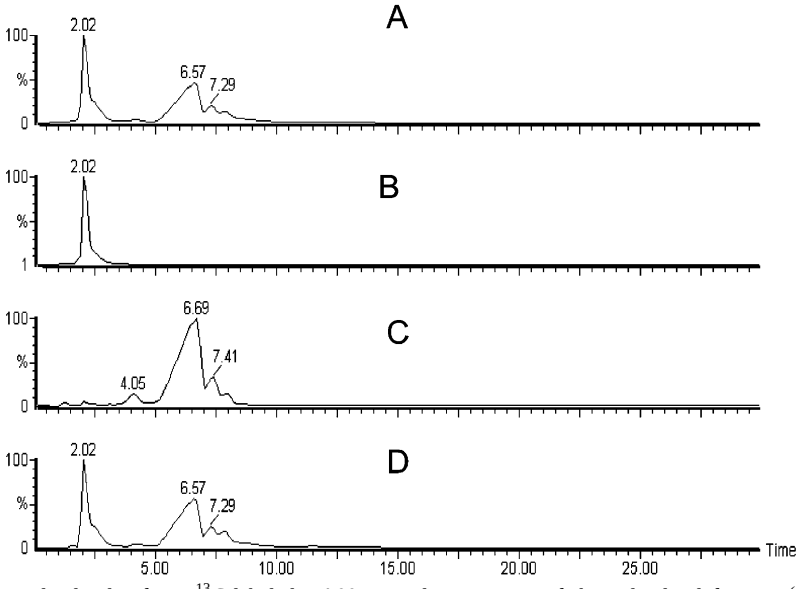

Fig. 3. Detection of the polar lipids of per ^{13}C-labeled DA001. Total ion current of the polar lipid fraction (panel A); extracted ion chromatogram (EIC) of $m/z = 174$ for P-glycerol indicating the PG molecules (panel B); EIC at $m/z = 142$ for P-ethanolamine showing the PE molecules (panel C); EIC at $m/z = 299$ of per ^{13}C-18:1 showing the presence of this fatty acid in all the polar lipid components of per ^{13}C-labeled DA001 (panel D).

3.3. Detection of components of intact PG and PE in per ^{13}C-labeled DA001

Detection of specific intact lipids (of ^{13}C grown DA001) was performed by isolating the parent ions in the first quadrupole of a tandem MS with collisionally induced dissociation (CID) in the second quadrupole followed by scanning the product ions in the third quadrupole shows all components of the specific lipids. In Fig. 2A, the product ions of parent negative ion m/z 785 gave ions at m/z 299 and m/z 269 representing the *sn*-1 (18:1) and *sn*-2 (16:1) fatty acids, respectively. P-glycerol ($-H_2O$) at m/z 153 ($+3 = 156$) and the P-glycerol at m/z 171 ($+3 = 174$). Note the decreased response of *sn*-1 from *sn*-2 and the lower still responses of the polar head groups that are typical of these responses to phospholipids in their electrospray mass spectra (Lytle et al., 2000; Fang and Barcelona, 1998; Smith et al., 1995). In Fig. 2B, the parent negative ion m/z 754 gave *sn*-1 for 18:1 at m/z 299, *sn*-2 at m/z 269 and P-ethanolamine at m/z 140 ($+2 = 142$) and glycerol P-ethanolamine minus H_2O at m/z 196 ($+5 = 201$).

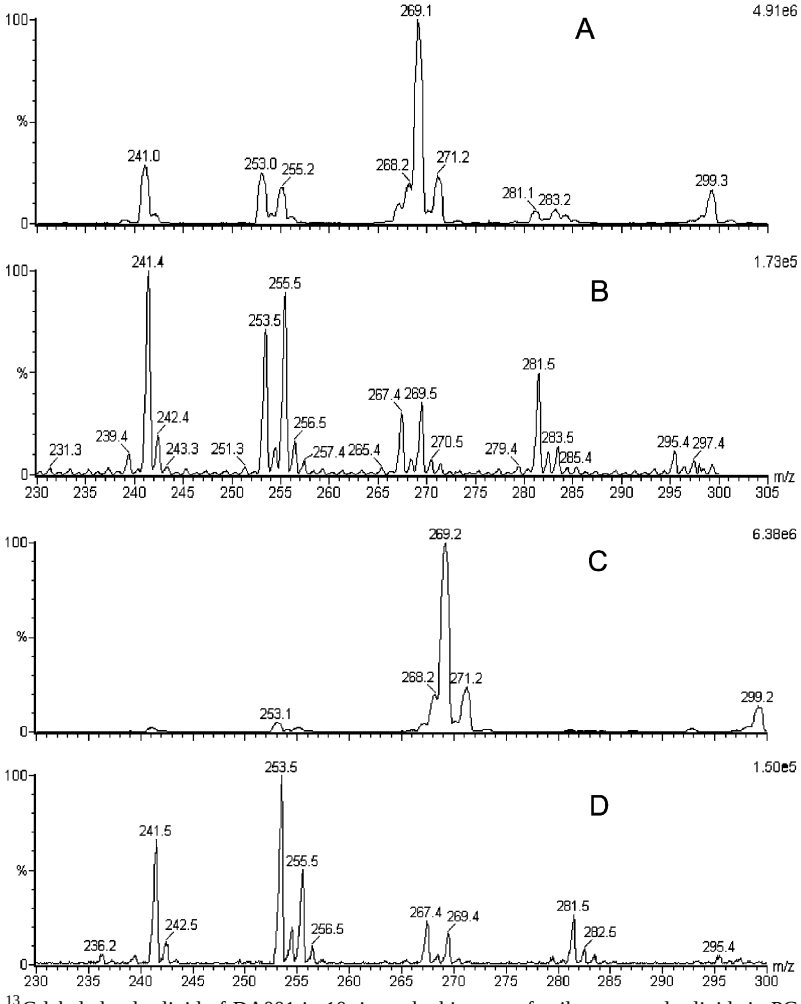

Fig. 4. Detection of per ^{13}C-labeled polar lipid of DA001 in 10 times the biomass of soil extract polar lipids in PG molecules (panel A vs. control panel B with no added DA001) and in PE (panel C with per ^{13}C-labeled lipids vs. control panel D with no added DA001).

3.4. Distribution of unique fatty acid negative ions in per ^{13}C-labelled DA001 PG and PE

The unique PLFA of per ^{13}C 18:1 is distributed in all the lipids of DA001 (Fig. 3). In Fig. 3A, the total ion current from the HPLC/ES/MS of the polar lipids of DA001 are illustrated. There are three major lipids detected. In Fig. 3B, the extracted ion current after up-front CID for m/z 174 for the P-glycerol indicate the first peak represent the total PG content of the bacteria. In Fig. 3C, two major components and one later eluting component are PE molecules as indicated by the presence of m/z 142 for p-ethanolamine $(+2)$ after up-front CID. In Fig. 3D, the presence of per ^{13}C-18:1 detected at m/z 299 after up-front ID in all PG's and PE's of DA001 grown in ^{13}C is established.

3.5. Detection of per ^{13}C-labelled 16:0 and 18:1 in DA001 in soil bacterial polar lipids

The detection, after up-front CID, of the polar lipids from the per ^{13}C labeled DA001, diluted in 10 times the biomass the polar lipid content of a surface soil sample is illustrated in Fig. 4. In Fig. 4A, the PG fraction of the soil polar lipid mixed with the per ^{13}C-labeled bacteria is scanned showing the presence of m/z 299 and m/z 271. These negative ions are not present in the sample of soil without added per ^{13}C-labeled lipids (panel B). Fig. 4 (panel C) shows the presence of m/z 271 and 299 in the PE fraction lipid with the per ^{13}C-labeled bacteria added to soil in contrast to the un-inoculated polar lipids from soil in panel D.

3.6. Detection of phospholipid 18:1 in per ^{13}C-labeled DA001 in Oyster, VA, soil column eluate

Effluent fractions were pooled together to represent 33 samples (Fig. 5). MRM was used to monitor the transition from m/z $754 \rightarrow 299$. No per ^{13}C labeled 18:1 polar lipid was detected in the first four pooled effluent fractions. Per ^{13}C labeled polar lipid begins to elute in the fifth fraction and reaches its maximum in the seventh fraction. A calibration curve was generated from a dilution series of per ^{13}C labeled DA001. The MRM response for the transition $754 \rightarrow 299$ was then used to determine the number of per ^{13}C-labeled cells detected in the effluent fractions from DA001 (Table 3).

3.7. Sensitivity of the detection of 18:1 in per ^{13}C-labeled DA001

Starved DA001 was suspended in NCAGW and diluted in a series of 10-fold dilutions and the sus-

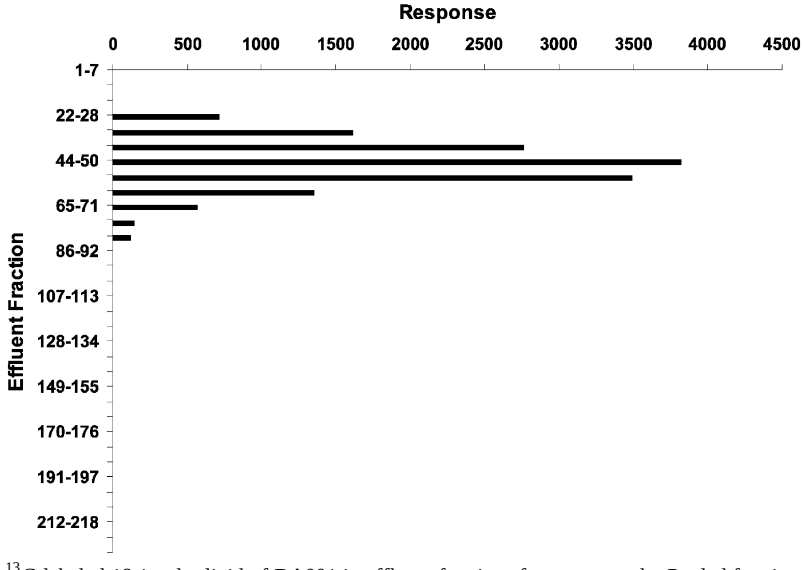

Fig. 5. Detection of per ^{13}C-labeled 18:1 polar lipid of DA001 in effluent fractions from core study. Pooled fractions vs. MRM response for transition m/z $754 \rightarrow 299$.

Table 3
Number of per ^{13}C-labeled 18:1 cells detected in effluent fractions

Pooled fractions	Per ^{13}C-labeled 18:1 detected ($\times 10^6$)
22–28	1.20
29–36	2.68
37–43	4.60
44–50	6.37
51–57	5.82
58–64	2.25
65–71	9.48
72–78	2.42
79–85	2.02

pensions counted. Minimal clumping was observed. With extraction and HPLC/ES/MS with up-front CID of PG and PE the limits of quantitation (LOQ) was 9×10^6 cells, the limits of detection (LOD) was 3×10^6 cells. With HPLC/ES/MS/MS with product ion scans the LOQ was 2×10^5 cells and LOD was 6×10^4 cells. With HPLC/ES/MS/MS utilizing MRM of the transition from m/z 754 → 299 the LOQ was 6×10^4 and the LOD was 2×10^4 cells. With concentration of the sample and nano electrospray, the sensitivity should increase without loss in specificity.

4. Discussion

There are great advantages in the analysis of phospholipids. Phospholipids make up a major part of cell membranes. Lipids are both concentrated and purified by the solvent extraction process. The phospholipids have a negative charge at alkaline pH, and therefore, are ideal for electrospray ionization and an ES compatible, rapid, capillary high performance liquid chromatography (HPLC) system has been developed (Lytle et al., 2000). This provides enormous sensitivity. Up-front collisionally induced dissociation (CID) in the atmospheric pressure source produces negative ions of the ester-linked fatty acids at the *sn*-1 and *sn*-2 position and the phosphate ester at the *sn*-3 position generating negative ions of the polar head groups of the phospholipids. Negative ions are more specific to particular molecular structures than are positive ions when analyzing phospholipids. The mass spectrometry can be further exploited if tandem quadrupole mass spectrometry (TQMS) is utilized. In TQMS selection of specific parental ions for dissociation in the CAD chamber and specific product ion monitoring can increase the sensitivity over single quadrupole scanning with up-front CID by 250 times resulting in subfemtomole/μl detection (Lytle et al., 2000). The PLFA defined in this study as unique when generated in per ^{13}C-labeled cells, 16:0 and 18:1, are among two of the most common found in bacteria and microeukaryotes. Consequently, a multitude of organisms could be used as tracers. Protozoa for example could be fed the per ^{13}C-labeled bacteria for several generations.

Phospholipids provide additional insights in the subsurface. Their metabolic lability to endogenous and exogenous phospholipase make phospholipids excellent indicators for viability in the determination of biomass and the conditions of cell lysis (Ringelberg et al., 1997). They also provide insight into community composition (White et al., 1996), nutritional/physiological status (White, 1995), and in defining end points for bioremediation (White et al., 1998). This technology provides an opportunity to not only trace bacteria added for acceleration of in situ remediation but to understand possible metabolic interactions with the resident microbiota.

5. Conclusion

Herein, we establish that it is possible to unequivocally detect the presence of per ^{13}C-labeled DA001 in the presence of 10 times the soil bacterial lipids and when 10^7 intact ^{13}C-labeled DA001 bacteria are added to a soil column based on the detection of specific ester-linked fatty acids in the PG and PE of the polar lipid extract using HPLC/ES/MS detection after up-front CID. With HPLC/ES/MS/MS utilizing MRM, the detection limit is currently in the range of 10^4 bacteria per sample. Because the lipid extraction represents a purification as well as a concentration, and since electrospray is concentration dependent, improvements in the chromatography with micro flow rates and capillary HPLC could further increase the sensitivity without loss of specificity or speed. Both 16:0 and 18:1 are nearly ubiquitous among microbes. Therefore, if the organism to be

traced can be grown to saturation in ^{13}C, the techniques described herein can provide a specific tracer of that microbial cell in the complex environment of subsurface sediments.

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