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Behaviour of carbon-14 containing low molecular weight organic compounds in contaminated groundwater under aerobic conditions

Aislinn A. Boylan[a,1], Douglas I. Stewart[b], James T. Graham[c], Ian T. Burke[a,*]

[a] School of Earth and Environment, University of Leeds, Leeds, LS2 9JT, UK
[b] School of Civil Engineering, University of Leeds, Leeds, LS2 9JT, UK
[c] National Nuclear Laboratory, Sellafield, Cumbria, CA20 1PG, UK

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ABSTRACT

Short chain carbon-14 (^{14}C) containing organic compounds can be formed by abiotic oxidation of carbides and impurities within nuclear fuel cladding. During fuel reprocessing and subsequent waste storage there is potential for these organic compounds to enter shallow subsurface environments due to accidental discharges. Currently there is little data on the persistence of these compounds in such environments. Four ^{14}C-labelled compounds (acetate; formate; formaldehyde and methanol) were added to aerobic microcosm experiments that contained glacial outwash sediments and groundwater simulant representative of the Sellafield nuclear reprocessing site, UK. Two concentrations of each electron donor were used, low concentration (10^{-5} M) to replicate predicted concentrations from an accidental release and high concentration (10^{-2} M) to study the impact of the individual electron donor on the indigenous microbial community in the sediment. In the low concentration system only ~5% of initial ^{14}C remained in solution at the end of experiments in contact with atmosphere (250–350 h). The production of $^{14}CO_2$(g) (measured after 48 h) suggests microbially mediated breakdown is the primary removal mechanism for these organic compounds, although methanol loss may have been partially by volatilisation. Highest retention of ^{14}C by the solid fractions was found in the acetate experiment, with 12% being associated with the inorganic fraction, suggesting modest precipitation as solid carbonate. In the high concentration systems only ~5% of initial ^{14}C remains in solution at the end of the experiments for acetate, formate and methanol. In the formaldehyde experiment only limited loss from solution was observed (76% remained in solution). The microbial populations of unaltered sediment and those in the low concentration experiments were broadly similar, with highly diverse bacterial phyla present. Under high concentrations of the organic compounds the abundance of common operational taxonomic units was reduced by 66% and the community structure was dominated by Proteobacteria (particularly Betaproteobacteria) signifying a shift in community structure in response to the electron donor available. The results of this study suggest that many bacterial phyla that are ubiquitous in near surface soils are able to utilise a range of ^{14}C-containing low molecular weight organic substances very rapidly, and thus such substances are unlikely to persist in aerobic shallow subsurface environments.

1. Introduction

Low molecular weight organic (LMWO) substances have long been considered a potential source of future carbon-14 (^{14}C) release from deep subsurface environments due to the predicted accumulation of $^{14}CH_4$ in underground repositories (Jefferies, 1990; Jackson and Yates, 2011; Limer et al., 2011, 2013; Marshall et al., 2011). Now there is concern that corrosion of activated fuel and fuel cladding may form a range of LMWO substances as a by-product (Wieland and Hummel, 2015) providing a source for it's potential release to shallow subsurface environments from storage ponds. ^{14}C is a known contaminant of the nuclear reprocessing plant at Sellafield, Cumbria, UK (Sellafield Ltd., 2016) and is of concern as a radioactive contaminant due to its long half-life (5730 ± 40a, Godwin, 1962) and its bioavailability (Bracke and Müller, 2008; Baston et al., 2012). The formation of ^{14}C occurs at each stage of the nuclear power generation process (Eabry et al., 1995) from the parent isotopes nitrogen-14 (^{14}N), oxygen-17 (^{17}O) and carbon-13 (^{13}C), especially due to the presence of ^{14}N impurities in components of the fuel and fuel cladding. During fuel reprocessing the fuel and cladding (e.g. steel encapsulation, Mg-alloy) are stored in large

* Corresponding author.
E-mail address: I.T.Burke@leeds.ac.uk (I.T. Burke).
[1] Present address: EPFL ENAC IIE EML, CH C2 392 (Bâtiment CH), Station 6, CH-1015 Lausanne, Switzerland.

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water filled ponds, often as a short term measure prior to decanning and reprocessing (NDA, 2014; Morozov et al., 2016; Sellafield Ltd., 2016). At the free surface of such ponds oxic conditions are expected whereby ^{14}CO formed from carbides in the fuel cladding would be expected to oxidise to $^{14}CO_2$ (McCollom and Seewald, 2007). At greater depth the oxygen penetration is minimal and corrosion of readily oxidised metals, such as magnesium, uranium and iron, leads to chemically reducing conditions forming within storage ponds (Equations (1)–(3)).

$$Mg + 2H_2O \rightleftharpoons Mg(OH)_2 + H_2 \quad (1)$$

$$U + 2H_2O \rightleftharpoons UO_2 + 2H_2 \quad (2)$$

$$Fe + 2H_2O \rightleftharpoons Fe(OH)_2 + H_2 \quad (3)$$

This potential redox stratification, with oxic conditions at the pond surface becoming more reducing with depth, would allow a variety of soluble ^{14}C-containing organic compounds (^{14}C-DOC) to form within the storage ponds (Kaneko et al., 2002). Organic forms of ^{14}C such as formaldehyde and methanol are also present within in nuclear waste due to their formation within pressurised water reactors (Matsumoto et al., 1994; Vance et al., 1995; Petit et al., 2013). In the past leaks have occurred in storage silos/ponds in Canada and the UK, (Evenden et al., 1998; Killey et al., 1998; Bird et al., 1999; Marshall et al., 2015; Sellafield Ltd., 2016), at Sellafield reprocessing site this has led to groundwater contamination from organic acids and pH variance across site (Law et al., 2010; Thorpe et al., 2012) which may affect the microbial populations within the subsurface.

A range of ^{14}C-containing LMWO substances can form from oxidation of carbide in spent fuel and corrosion of the fuel cladding (Fig. 1). The most abundant will be CO_2 and CH_4, whose concentrations are expected to be orders of magnitude higher than any other ^{14}C-containing molecules produced (Wieland and Hummel, 2015), but CO_2 and CH_4 are not expected to persist in shallow subsurface environments. At circumneutral pH $^{14}CO_2$ speciates as $H^{14}CO_3^-$ in water, and isotopic exchange reactions with atmospheric $^{12}CO_2$ can rapidly deplete ^{14}C-dissolved inorganic carbon (DIC) concentrations in storage ponds that are open to atmosphere (see Boylan et al., 2017 for full discussion of ^{14}C-DIC behaviour in surface and groundwater environments). $^{14}CH_4$ has very low solubility at surface pressures and temperatures (Clever and Young, 1987) and is therefore expected to strongly partition to the gas phase and is assumed to be present as either pore gas or is released and diluted in atmosphere. Thus acetate, formate, formaldehyde and methanol have been identified as the main chemical forms for aqueous, organic ^{14}C released to groundwater by leaks from reprocessing waste storage ponds (Kaneko et al., 2002; Wieland and Hummel, 2015). The presence of these different highly soluble ^{14}C-DOC molecules in groundwater is potentially a source for aqueous ^{14}C release and transport in the shallow subsurface at nuclear sites.

In the shallow subsurface LMWO molecules can be metabolised by many of the diverse range of microbes found in these environments. Balanced equations for the use of the four most common ^{14}C-DOC compounds produced from the nuclear fuel cycle during aerobic metabolism are shown below (Equations (4)–(7); Lovley and Phillips, 1988; Ferry, 1990). All of these reactions convert organic carbon into inorganic forms (Equations (4) and (5) indicate that oxidation of acetate and formate produce bicarbonate suggesting that these reactions have the potential to increase groundwater pH). Further, carboxylates and similar LMWO molecules can sorb to, or become incorporated into soil particulates, which may also contribute to their low natural aqueous concentrations (0.1–1000 μM) in aquifers (van Hees et al., 2002; Fischer and Kuzyakov, 2010).

Acetate $$CH_3COO^- + 2O_2 \rightleftarrows HCO_3^- + CO_2 + H_2O \quad (4)$$

Formate $$2HCOO^- + O_2 \rightleftarrows 2HCO_3^- \quad (5)$$

Formaldehyde $$HCHO + O_2 \rightleftarrows CO_2 + H_2O \quad (6)$$

Methanol $$2CH_3OH + 3O_2 \rightleftarrows 2CO_2 + 4H_2O \quad (7)$$

This study examines the fate of ^{14}C added as acetate, formate, formaldehyde and methanol in aerobic groundwater systems. The specific objectives were: 1) to investigate the behaviour of aqueous, organic ^{14}C in contact with sediment and atmosphere; 2) to establish the extent of the oxidation of organic ^{14}C to $^{14}CO_2(g)$; 3) to assess the potential for organic and inorganic ^{14}C accumulation in sediment; 4) to determine which part of the indigenous microbial community found in the sediment favour the organic compounds under aerobic conditions; and 5) to assess the implications of these processes for ^{14}C-DOC release and migration in shallow, oxic subsurface environments.

2. Materials and methods

2.1. Sediment

Sediment was collected from the River Calder valley near Calder Bridge, Cumbria, UK (Lat. 54°26.3′N, Long. 3°28.2′W) in August 2015. This sediment is representative of the glacial/fluvial superficial deposits that underlie the neighbouring Sellafield nuclear reprocessing site, UK (Wallace et al., 2012; Law et al., 2010). Sediment was collected in HDPE plastic containers before being transferred to sterile HDPE bags and stored at 4 °C. All experiments were started within three months to ensure that the microbial community remained representative of the unaltered sediment. Prior to use the fresh sediment was sieved to retain < 2 mm fraction. X-ray powder diffraction (Cu K-alpha radiation) using a Bruker D8 Advance XRD was used to characterise the sediment mineralogy. Sediment pH was measured at collection site using standard methodology (ASTM, 2006).

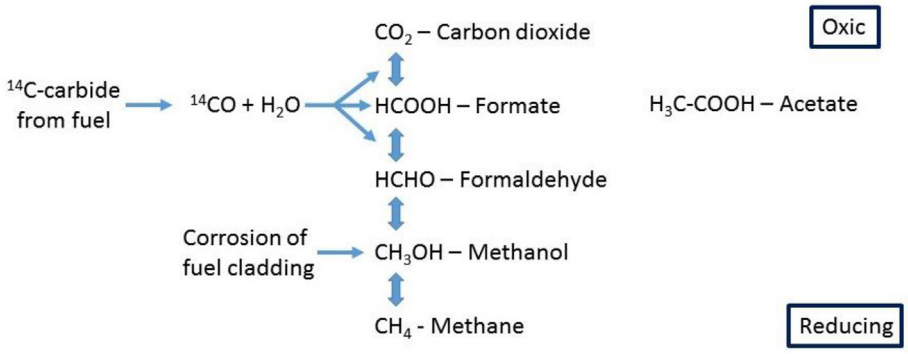

Fig. 1. The formation of organic ^{14}C compounds in the nuclear fuel cycle under varying redox conditions (McCollom and Seewald, 2007; Kaneko et al., 2002; Wieland and Hummel, 2015).

Table 1
Solution composition for synthetic groundwater, modified from Wilkins et al. (2007).

Compound	g/L in DIW
KCl	0.006
$MgSO_4{\cdot}7H_2O$	0.098
$MgCl_2{\cdot}6H_2O$	0.081
$NaNO_3$	0.028
NaCl	0.0094
$NaHCO_3$	0.24
pH	8.3

2.2. ^{14}C-DOC partitioning under aerobic incubation (open flasks)

Triplicate experiments were established in 500 mL glass Erlenmeyer flasks using 5 g of sediment and 50 ± 0.5 mL of synthetic groundwater. The composition of the synthetic groundwater (Table 1) is based on the groundwater present in similar sediments at the nearby Low Level Waste Repository (LLWR) (Wilkins et al., 2007). Flasks were left to equilibrate overnight (minimum 12 h), and then one of four organics (sodium acetate, sodium formate, formaldehyde or methanol) were added at two concentrations: low (10^{-5} M) or high (10^{-2} M). The specific DOC concentrations were chosen to represent the low levels predicted to be present in nuclear wastes (10^{-5} M; Kaneko et al., 2002; Wieland and Hummel, 2015) and a significantly elevated concentration (10^{-2} M) that would have greater potential to alter the indigenous microbial population (to potentially elucidate the active microbial community in each system). A small quantity of a ^{14}C-labelled version of the same organic compound (50 µL of 4.6×10^{-7} M solution to give an activity of 100 Bq ml^{-1} in experiments; ARC Ltd. USA) was added at the same time (the methyl, or C^2, carbon of acetate was labelled). Solution-only control experiments were also established at 10^{-5} M to assess any potential losses to atmosphere of the organic compounds studied (glassware was sterilised at 160 °C for 2 h, solutions were 0.2 µm filtered).

To ensure oxygen was available, sterile flasks were stoppered with foam bungs and incubated on an orbital shaker (Stuart SSL1; 30 rpm; 16 mm orbit) at room temperature in the dark. Periodically 1 mL of solution was removed and centrifuged for 5 min at 14,000*g* in 1.5 mL microcentrifuge tubes (particle size cut-off < 200 nm, Gimbert et al., 2005). After centrifugation the supernatant was analysed for ^{14}C-DOC by liquid scintillation counting on a Packard Tri-Carb 2100TR (0.8 mL sample, 10 mL PerkinElmer EcoScint A scintillation fluid; count time = 10 min; energy window = 4–156 keV; detection limit: 20 CPM; counting efficiency: 80%). ^{14}C-DIC is not measured due to interference from ^{14}C-DOC. Samples were stored for a 24 h period prior to counting. Solution pH was also periodically measured in experiments using a Thermo Scientific Benchtop multimeter and electrodes calibrated daily at pH 4, 7 and 10.

At the end of the experiment sediments from two flasks from each triplicate set were separated by filtration (Whatman 2), washed with DIW and air-dried for further solid phase analysis detailed below. Sediment from the third flask of each triplicate were put in to 50 mL centrifuge tubes and the supernatants were removed. The tubes were immediately placed in a −20 °C freezer for microbial DNA analysis.

2.3. Short term ^{14}C-DOC partitioning in aqueous fraction (closed bottles)

Triplicate 50 ± 0.5 mL experiments were set up in 125 mL serum bottles using 5 g of sediment and synthetic groundwater. The open bottles were left to equilibrate with atmosphere overnight before addition of either sodium acetate, sodium formate, formaldehyde or methanol. The concentrations of the organic compounds were set to 10^{-2} M and 10^{-5} M and spiked with ^{14}C-labelled organic compounds (50 µL of 4.6×10^{-7} M solution with an activity of 100 Bq ml^{-1}; acetate was C^2 labelled). The experiments were then sealed with butyl rubber stoppers and aluminium crimps, but the large air headspace (~75 mL) ensured aerobic conditions were maintained in the short-term. Aqueous, organic ^{14}C activity and pH was measured after 48 h as previously described. A second set of closed experiments were run using the same method to quantify the amount of $^{14}CO_2$(g) in the headspace. A 10 mL gas sample was withdrawn from the headspace and bubbled through 2 mL of Carbo-Sorb E. This was then mixed with 10 mL of PermaFluor E and left for at least 24 h prior to counting to remove interference from chemiluminescence (Ahn et al., 2013).

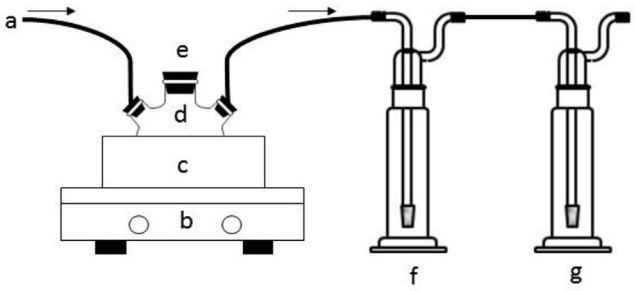

Fig. 2. Apparatus for the wet acidification and oxidation of sediment: a) N_2(g) inlet line; b) stirrer hotplate; c) water bath; d) 250mL round bottom flask with three inlets; e) sample and reagent addition; f) 125 mL gas washing tube with frit (porosity grade 0) containing 100 mL of Carbo-Sorb E for ^{14}C capture and g) 125 mL gas washing tube with frit (porosity grade 0) containing 100 mL of 1 M NaOH acting as a safety bottle to prevent the release of $^{14}CO_2$(g). Arrows represent the direction of N_2(g) flow through the apparatus.

2.4. Amount of ^{14}C associated with total inorganic carbon (TIC) and total organic carbon (TOC) in sediment from the aerobic incubation experiments

Method is based on studies by Magnusson (2007) and Ahn et al., 2013, recovery of standard was 85–98% for ^{14}C. Known activities of ^{14}C-labelled LMWOs were analysed in this study with recovery of 85–95%. Two gas washing bottles (Drechsel bottle, 125 mL, QuickFit) were attached in series to the reaction vessel (250 mL round bottom flask with three inlets, QuickFit) via the gas outlet line (Fig. 2); the first gas washing bottle contained 100 mL of Carbo-Sorb E (a high capacity $^{14}CO_2$(g) absorber compatible with the counting cocktail Permafluor E +), the second contained 100 mL of 1 M NaOH. A sample of ~1 g dry weight sediment (recovered at the end of the 10^{-5} M experiments described above) was placed in the reaction vessel with a magnetic stirrer. The carrier gas (N_2) flow rate was 40 mL/min. A two-step method of analysis was used to differentiate between the inorganic carbon fraction (carbonates, CO and CO_2) by reducing the pH to pH 3 and organic (hydrocarbons and organic acids) by adding an oxidant to the mixture. First a volume of 20 mL 2 M H_2SO_4 was added to the reaction vessel which resulted in a pH ~3. The vessel was then purged for 30 min with N_2 and mixed with a magnetic stirrer. Following acid treatment of the sediment the gas washing bottles were exchanged for duplicates. In the second step 20 mL of 5% potassium persulfate and 4 mL of 4% $AgNO_3$ was added to the reaction vessel and heated to 80–90 °C. Further additions of $K_2S_2O_8$ and $AgNO_3$ at the same concentration and volume were made after one and two hours of reaction. The system was left to react for a further hour to give a total reaction time of 3 h. Triplicate samples of 1 mL were collected from that gas washing bottles that contained Carbo-Sorb E (Magnusson, 2007). Each 1 mL Carbo-Sorb E sample was mixed with 10 mL of PermaFluor E and were left to dark adjust for 24 h prior to counting on the liquid scintillation counter.

2.5. DNA extraction and sequencing of the V4 hyper-variable region of the 16S rRNA gene

Bacterial DNA was extracted from eleven sediment samples. These

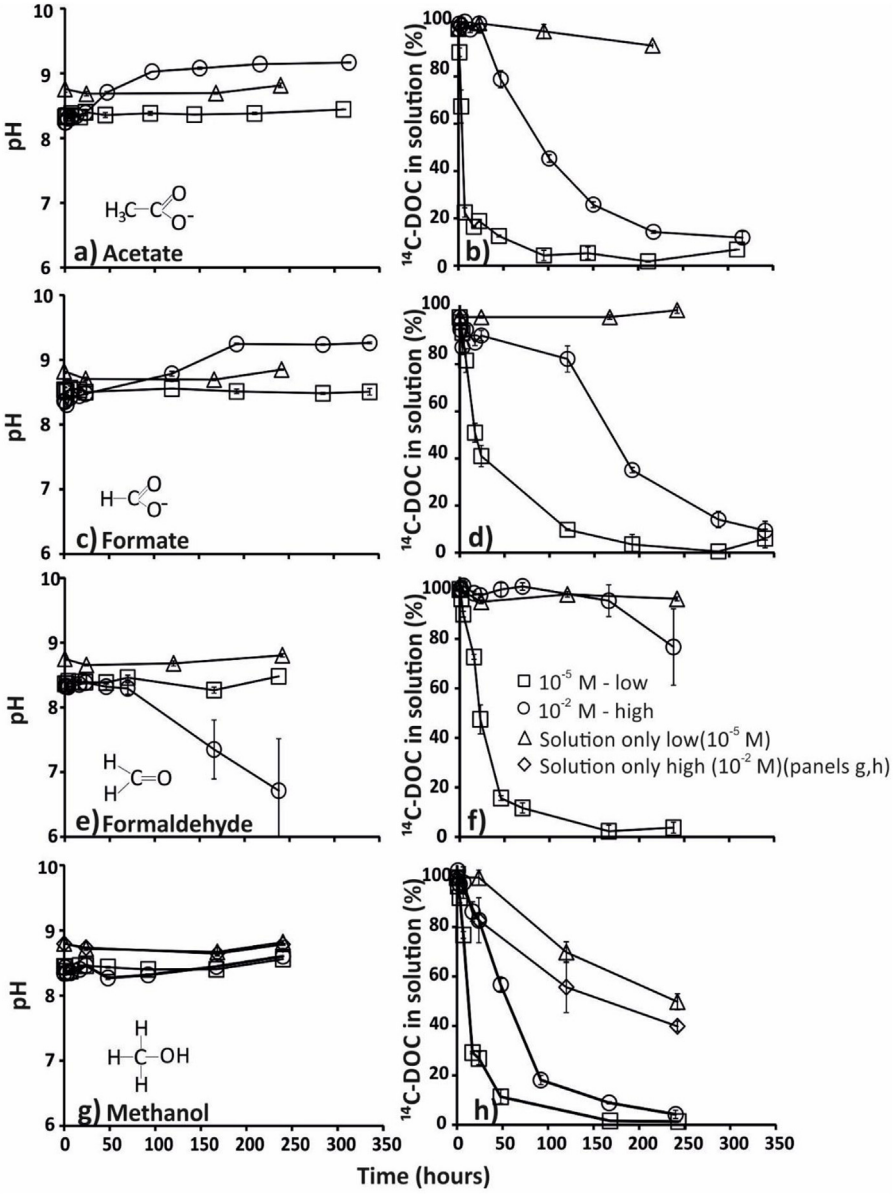

Fig. 3. Experimental results from open flask experiments show the evolution of pH and ^{14}C-DOC activity for the first 250 h in (a, b) ^{14}C-acetate amended experiments; (c, d) ^{14}C-formate amended experiments; (e, f) ^{14}C-formaldehyde amended experiments and, (g, h) ^{14}C-methanol amended experiments. Error bars denote one standard deviation of triplicate measurements; where not shown, error bars are less than the size of the symbols used.

consisted of one sample from each of the 10^{-2} M LMWO systems and one from each of the 10^{-5} M LMWO systems (see Section 2.3) and three samples of the unaltered sediment that was frozen on the day of collection. DNA was extracted from samples (~0.5 g of damp sediment) using the Fast DNA spin kit for soil (MP Biomedicals, USA). DNA fragments larger than 3 kb were isolated on a 1% agarose "1×" Tris-borate-EDTA (TBE) gel stained with ethidium bromide for viewing under UV light (10× TBE solution supplied by Invitrogen Ltd., UK). The DNA was extracted from the gel using a QIAquick gel extraction kit (QIAGEN Ltd, UK); final elution was by 1/10th strength elution buffer (unless explicitly stated, the manufacturer's protocols supplied with all kits employed were followed precisely). DNA concentration was quantified fluorometrically using a Qubit dsDNA HS Assay (Thermo Fisher Scientific Inc., USA).

DNA samples (1 ng/μL in 20 μL aqueous solution) were sent for sequencing at the Centre for Genomic Research, University of Liverpool, where Illumina TruSeq adapters and indices were attached to DNA fragments in a two-step PCR amplification targeting the 16S rRNA gene (Caporaso et al., 2011). Pooled amplicons were paired-end sequenced on the Illumina MiSeq platform (2 × 250 bp) generating ~9 M clusters of data. Illumina adapter sequences were removed, and the trimmed reads were processed using the UPARSE pipeline (Edgar, 2013) within the USEARCH software (version 9.2; Edgar, 2010) on a Linux platform. Overlapping paired end reads were merged prior to quality filtering and relabelling. After dereplication, clustering, chimera filtering and singletons removal was performed simultaneously on the dataset. Operational taxonomic units (OTUs) were defined by minimum of 97% sequence identity between the putative OTU members (Newsome et al., 2014). OTUs were allocated to a taxon using UTAX command and the RDP database. A confidence value of more than 0.7 was required for classification to balance sensitivity and error rate in the prediction (OTUs not classified to the level of phylum, or classified as archaea were excluded from subsequent analysis). The entire set (~9 M reads) was then allocated to the OTUs and reported in the OTU table with the taxonomy and abundance of the OTUs.

Statistical analysis was performed to determine the bacterial

diversity. In this paper the alpha diversity was defined using Hill numbers, D_q, (Hill, 1973; Jost, 2006). Hill numbers define the biodiversity as the reciprocal mean of proportional abundance and compensate for the disproportionate impact of rare taxa by weighting taxa based on abundance, the degree of weighting is controlled by the index q where increasing q places progressively more weight on the high-abundance species in a population (Hill, 1973; Jost, 2006, 2007; Kang et al., 2016). D_0 is the unweighted Hill number and is equal to the species richness. D_1 is a measure of the common species and is equivalent to the exponential of Shannon entropy. D_2 is a measure of the number of dominant species and is equivalent to the inverse of Simpson concentration (Hill, 1973; Jost, 2006, 2007). Non-metric Multi-Dimensional Scaling (NMDS) was used to graphically represent the similarity between bacterial assemblages after exposure to different concentrations of LMWO substances. This was done in an optimised two dimensional space using the Bray-Curtis dissimilarity matrix. NMDS was carried out in the package 'vegan' (Oksanen et al., 2013) in RStudio v 99.9.9 (RStudio Team, 2015). The microbial community data were input as a matrix of the relative abundance of each OTU in each sample.

3. Results

3.1. Sediment characterisation

Sediment from the sampling location has been extensively characterised (e.g. Law et al., 2010; Wallace et al., 2012). Law et al., 2010, describe the sediment as poorly sorted sandy loam with an approximate particle composition of 53% sand, 42% silt, 5% clay. Field observations in this study confirm that the sediment is predominantly fine sand and silt with most/all particles < 2mm. XRD spectroscopy (see Figure S1) shows that the sediment is dominated by quartz, with some albite and microcline and minor amounts of chlorite and mica. The sediment pH was 5.5.

3.2. The partitioning of ^{14}C-labelled organic compounds under aerobic conditions (open flasks)

The synthetic groundwater had a pH value of 8.7. Addition of sediment to the synthetic groundwater resulted in a solution pH of ~8.3. The pH values of the solution-only experiments and those containing sediment and low concentration (10^{-5} M) LMWO substances did not change significantly over the experimental timescale (which ranged from 238 to 340 h).

The pH values of the high concentration (10^{-2} M) acetate and formate experiments both increased from ~pH 8.3 to ~ pH 9.2 at the end of the experiments. Conversely the pH value of the high concentration formaldehyde experiment decreased from ~8.3 to ~7.7. Whereas the pH value of the high concentration methanol system remained steady at 8.3 (Fig. 3a, c, e & g).

There was rapid and almost complete (> 95%) removal of ^{14}C from the aqueous phase in all the low concentration LMWO experiments (Fig. 3b, d, f & h). No significant removal (< 10%) was found in the corresponding solution only experiments, except for the methanol systems where ^{14}C removal was observed in both control experiments (10^{-5} and 10^{-2} M). The sediment recovered at the end of the 10^{-5} M experiments retained only small proportion of the ^{14}C within either the TIC (3 ± 3% to 12 ± 6%) or TOC (2 ± 2% to 5 ± 2%) fractions (Table 2).

There was also ^{14}C removal in all the high concentration LMWO experiments, although there was a lag period at the start of the tests with the carboxylates and formaldehyde before removal was detected, and the removal was contemporaneous with a change in the solution pH (pH increased with carboxylate removal but decreased with formaldehyde removal). In the experiments with the carboxylates and formaldehyde ^{14}C removal was incomplete after 10 days (particularly formaldehyde where on average there was only 25% removal was observed with large variation between replicates). The high concentration methanol experiment lacked an identifiable lag phase, and ~95% removal was observed after 10 days. Also, the initial rate of ^{14}C removal was similar to that observed in the equivalent control, suggesting that methanol volatility may have contributed to removal (see discussion 4.3).

3.3. Short term ^{14}C-DOC partitioning in aqueous fraction (closed bottles)

These short term experiments had a large headspace and were run for only 48 h to ensure that oxygen in the headspace was not exhausted, to be comparable with the initial part of the corresponding open flask tests described above. Comparison of ^{14}C-DOC in the closed bottles with that in the open flask tests (Fig. 4) indicates that parallel experiments at both high and low concentrations behaved similarly with all experiments recording similar values. Values of ^{14}C-DOC for closed experiments were all within one standard deviation of the open experiment results from the closest time point except for ^{14}C-methanol. At high concentration 81 ± 1% of ^{14}C-methanol remained in solution in closed experiments, however 57 ± 3% remained in solution for open experiments. This corresponds with the earlier suggestion that methanol volatility may have contributed to removal as more ^{14}C-methanol remains in solution when there is a closed headspace (see section 4.3).

Analysis of gas sampled from the head space of a second set of closed bottle experiments indicates that a measurable fraction (8 ± 3%–18 ± 2%; Table 3) of the ^{14}C-DOC added to the experiments had been converted into $^{14}CO_2$ (g) in the first 48 h.

Combining the ^{14}C activities recovered for the aqueous and gaseous phase does not account for the total ^{14}C activity added to the original samples. The balance is likely to be DIC, which was not measured (in open systems ^{14}C is rapidly lost to atmosphere by isotope exchange, but in the closed systems $^{14}CO_2$ will have equilibrated across the aqueous and gaseous and solid phases; Boylan et al., 2017). The measurement of $^{14}CO_2$(g) is consistent across three organics, formate; formaldehyde; and methanol (17 ± 11%, 16 ± 4% and 18 ± 2% respectively), but slightly lower for the acetate system (8 ± 3%). This corresponds with the results shown in Table 2 where a higher percentage of ^{14}C from acetate oxidation is retained in the solid fraction as carbonate (12 ± 6%) and associated with organic C (5 ± 2%) which may suggest that there has been assimilation of ^{14}C in to microbial biomass.

3.4. Microbial community analysis

Sufficient DNA was recovered for high-throughput amplicon

Table 2
Percentage recovered of organic and inorganic bound^{14}C in sediment and^{14}C-DOC at the end of the low concentration experiments (10^{-5} M).

	Acetate 316 h	Formate 340 h	Formaldehyde 238 h	Methanol 240 h
^{14}C-TIC	12.3 ± 5.9%	3.0 ± 3.5%	4.0 ± 1.7%	6.1 ± 2.8%
^{14}C-TOC	4.9 ± 1.8%	4.6 ± 3.3%	1.7 ± 1.8%	1.9 ± 1.9%
^{14}C-DOC	6.8 ± 0%	6.0 ± 3.9%	3.7 ± 2.2%	1.3 ± 0.1%
Balance (by difference)	76 ± 7.7%	86.4 ± 10.7%	90.6 ± 5.7%	90.7 ± 4.8%

*Balance (by difference) refers to ^{14}C-DIC and $^{14}CO_2$(g) fractions which are not measured.

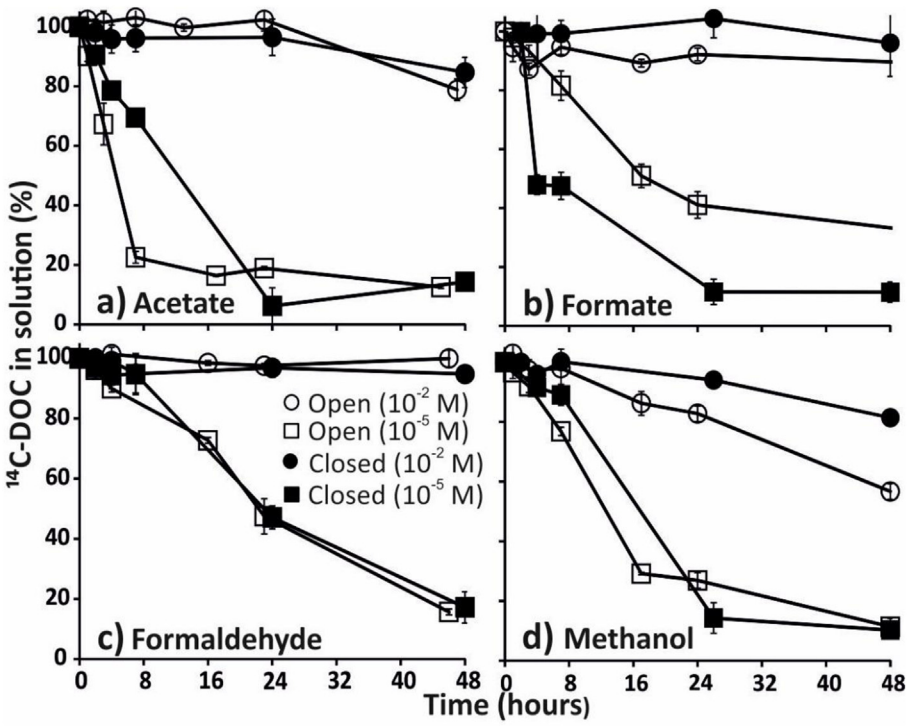

Fig. 4. Data from both open flask (replotted from Fig. 3) and corresponding closed bottle experiments for: (a) ^{14}C-acetate amended experiments; (b) ^{14}C-formate amended experiments; (c) ^{14}C-formaldehyde amended experiments; and, (d) ^{14}C-methanol amended experiments. Error bars denote one standard deviation of triplicate measurements; where not shown, error bars are less than the size of the symbols used.

sequencing from ten of the eleven samples chosen for analysis (the high concentration formaldehyde system yielded insufficient DNA; recovery was attempted from the replicate with the lowest formaldehyde removal from solution). For these ten samples the Illumina MiSeq run yielded > 100,000 paired-end reads for each sample after quality control. The ten samples in this study were part of a combined pool of 8,763,879 million paired-end reads from the sequencing run that passed the chimera check and were used to identify the characteristic OTUs. The combined pool was clustered into OTUs (> 97% sequence identity) in the UPARSE pipeline, and assigned to taxonomic groups. OTUs classified as archaea (4% of non-chimeric reads) and bacteria which were not classified at phylum level with a confidence of > 0.7 (23% of non-chimeric reads) were excluded from further analysis. This resulted in 7792 OTUs classified to bacteria phylum with a confidence greater than 0.7 which were used to characterise the bacterial populations in the samples from this study.

OTUs classified at the level of a phylum with a confidence > 0.7 were grouped to identify phyla that represented > 1% of the total number of pair-end reads from of this study. There were 11 phyla that represented more than 1% of the total population (Fig. 5). At least 10 of these 11 phyla represented > 1% of the population of each of the unaltered sediment samples. 10 ± 1 of these phyla represented > 1% of the population of each of the low concentration LMWO samples, whereas only 7 ± 1 of these phyla represented > 1% of the population of each of the high concentration LMWO samples. The most abundant phyla in the unaltered sediment were Acidobacteria (28 ± 2% of the reads), Proteobacteria (28 ± 1%) and Planctomycetes (10 ± 1%). The low concentration system showed a similar distribution (Acidobacteria, 34 ± 6%; Proteobacteria, 31 ± 5%; Planctomycetes, 8 ± 2%). These results are similar to the microbial communities found in previous studies of sediment collected at the same site (Geissler et al., 2011; Thorpe et al., 2012) and similar to the communities found on Sellafield nuclear waste reprocessing site (Newsome et al., 2014). By contrast, the distribution of OTUs between phyla varied with amendment in the high concentration experiments. In the high acetate system the most abundant phyla were Proteobacteria (64%) and Verrucomicrobia (14%) with Acidobacteria only making up 7% of the population. Within the phylum Proteobacteria, Betaproteobacteria (32% of the overall relative abundance) and Alphaproteobacteria (17% of the overall relative abundance) were the most abundant classes. The high formate system was dominated by Proteobacteria (45%) and Acidobacteria (23%). Within the phylum Proteobacteria the most abundant classes were Betaproteobacteria (26% of the overall relative abundance) and Alphaproteobacteria (14% overall relative abundance). In the high methanol system the most abundant phylum is Proteobacteria (71%), followed by Acidobacteria (13%). Within Proteobacteria over 63% of the overall relative abundance is associated with the class Betaproteobacteria.

The OTU richness (D_0^{α}) for each sample is shown in Table 4. The average richness for the unaltered sediment is 6300 ± 350 OTUs. The values for richness for the low concentration systems all lie within one standard deviation of the unaltered sediment value. The high concentration systems show slightly lower richness. The species richness takes no account of the relative abundance of the OTUs. The number of common species (D_1^{α}) in the unaltered sediment is 895 ± 219 OTUs. The D_1^{α} values for the low concentration systems are all within one standard deviation of this value. However there are fewer common species in the high concentration systems (D_1^{α} varied between 87 OTUs for methanol and 597 OTUs for formate). D_2^{α} for the unaltered

Table 3
Concentration of organic^{14}C in aqueous pool and gaseous pool (as CO_2(g)) at 48 h sampling point in low concentration experiments.

	Acetate 48 h	Formate 48 h	Methanol 48 h	Formaldehyde 48 h
^{14}C-DOC	28 ± 3%	52 ± 5%	44 ± 3%	48 ± 1%
$^{14}CO_2$(g)	8 ± 3%	17 ± 11%	16 ± 4%	18 ± 2%
Balance (by difference)[a]	64%	31%	40%	34%

[a] Balance (by difference) refers to the ^{14}C-DIC, ^{14}C-total organic carbon (TOC) and ^{14}C-total inorganic carbon (TIC) fractions which are not measured.

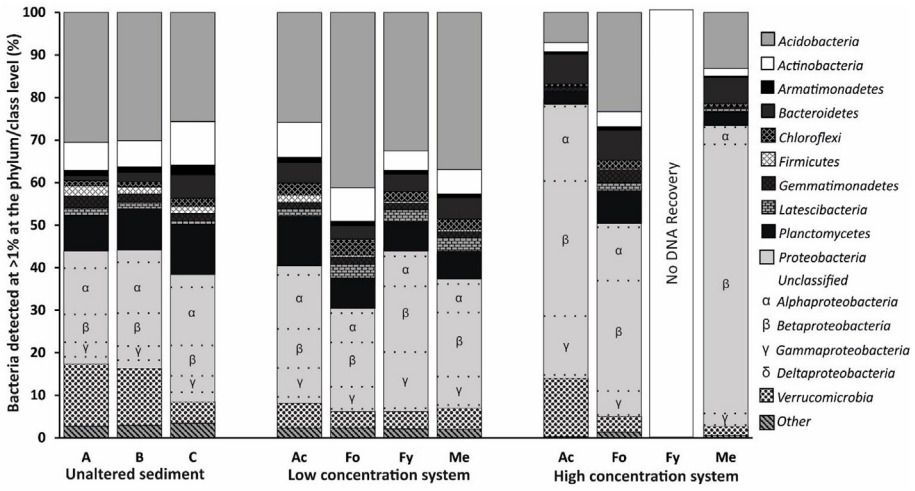

Fig. 5. Bacterial phylogenetic diversity within unaltered sediment (A–C); high concentration systems and low concentration systems (Ac- acetate; Fo-formate; Fy-formaldehyde; Me-methanol). Phyla with relative abundance less than 1% of the total population are grouped as "Other". Dashed line denotes classes of Proteobacteria descending as follows: unclassified; α; β; γ; δ.

sediment is 194 ± 91 OTUs, the D_2^{α} values for the low concentration systems are all within one standard deviation of this value. There are fewer dominant species in the high concentration systems where D_2^{α} ranged from 7 OTUs for methanol to 107 OTUs for formate. Common OTUs accounted for > 74% of total sequence reads in all samples, with dominant OTUs accounting for > 50% of total reads. The decrease in number of common and dominant OTUs in the high concentration system therefore means that there were fewer, but more abundant OTUs in the high concentration systems compared with the low concentration systems and unaltered sediment.

In the two-dimensional NMDS representation of the OTU frequencies in the ten samples (see Fig. 6) the unaltered sediment shows variation, although two samples have clustered together (UnA, B) sample C shows some dissimilarity. The low concentration samples (ACL, FOL, FYL, MEL) generally cluster very closely together and tend to lie midway between the unaltered and high concentration systems. The high concentrations are not clustered; the high formate system (FOH) is relatively close to the low concentration samples, whereas acetate and methanol (ACH, MEH) are further away from the unaltered and low concentration systems, and from each other. NMDS is an indirect gradient analysis which in this case optimises ordination based on a dissimilarity matrix. The analysis suggests that even at low concentrations there are changes to the bacterial population which are only reflected when examining individual species frequencies.

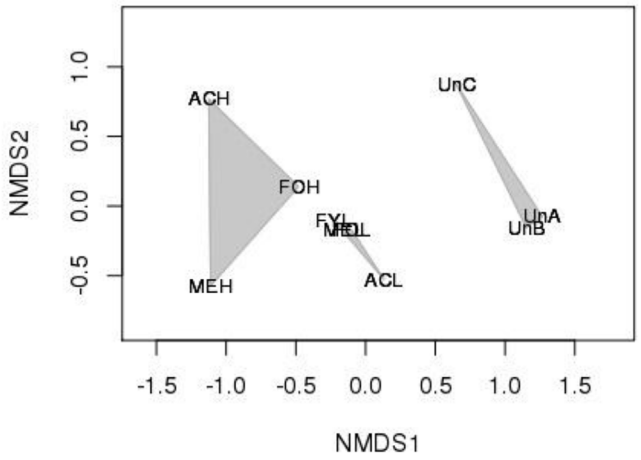

Fig. 6. Two-dimensional non-metric multidimensional scaling (NMDS) ordination for differences in the bacterial community composition distribution based on Bray-Curtis distances of the community (OTUs) by site/sample matrix. The stress value associated with this two dimensional representation is < 0.05 which suggests reasonable fit with the data. ACH: Acetate, high concentration; FOH: Formate, high concentration; MEH: Methanol, high concentration; ACL: Acetate, low concentration; FOL: Formate, low concentration; FYL: Formaldehyde, low concentration; MEL: Methanol, low concentration; UnA: Unaltered sediment (a); UnB: Unaltered sediment (b); UnC: Unaltered sediment (c).

4. Discussion

4.1. Fate of simple carboxylates in aerobic sediment-water systems

More than 90% of the added ^{14}C-acetate and ^{14}C-formate was removed from solution after 5 days in experiments amended with 10^{-5} M carboxylates. As no ^{14}C removal is recorded in equivalent solution-only experiments, it is inferred that microbial utilisation was responsible for the removal. This inference is supported by the production of $^{14}CO_2$(g) during the first 48 h in the closed-system experiments. Although the high concentration tests exhibited a lag phase lasting between 1 and 4 days with no significant ^{14}C removal observed it is likely that the indigenous microbial population utilised these electron donors at the same rate as the low concentration tests, but due to the higher overall concentration of LMWO substances, this had little impact on the proportion of the initial ^{14}C-organic remaining in solution. Thus the

Table 4
Alpha-diversity measures, D_0^{α}, species richness, D_1^{α}, exponential of Shannon entropy and concentration, D_2^{α}, inverse of the Simpson concentration. Ac – Acetate, Fo – Formate, Fy – Formaldehyde, Me – Methanol. Values displayed to 3 significant figures.

	Unaltered sediment			Average	Low concentration system				High concentration system			
	A	B	C		Ac	Fo	Fy	Me	Ac	Fo	Fy	Me
D_0^{α}	6110	6790	5990	6300 ± 350	6640	6010	6050	6130	4610	3770	n.a[a]	5660
D_1^{α}	616	918	1150	895 ± 219	939	891	903	1040	246	597	n.a[a]	87
D_2^{α}	108	153	320	194 ± 91	236	196	179	257	63	107	n.a[a]	7

[a] n.a. = not available.

increase in the rate of ^{14}C-organic consumption after 4 days may reflect a shift in the microbial population towards species that can metabolise the carboxylate present. The pH data from these tests support this as there were significant changes in solution pH for both acetate and formate, consistent with the respiration reactions given above (Equations (4) and (5)).

The TOC results show that there is a modest amount of ^{14}C associated with the organic solid fraction in both systems (< 5% of original activity added). For acetate this may be due to incorporation of the methyl group into microbial biomass, however formate assimilation in to microbial biomass is unlikely as the carboxyl group is less likely to be assimilated than other functional groups (Fischer and Kuzyakov, 2010). The anionic exchange capacity of the sediment is restricted at circumneutral pH (Sposito, 1989; Gu and Schulz, 1991) and is unlikely to account for this retention, however, sorption can occur between formate and bacterial cell walls. Cell walls are known to become increasingly negatively charged with increasing pH which causes the deprotonation of organic acids such as formate. The anionic formate can then react with metal complexes associated with the cell walls (Schultze-Lam et al., 1996; Fein et al., 1997) leading to sorption of formate and retention in the TOC phase due to its association with the biomass. Acetate shows a higher retention in TIC compared to formate, according to Equations (4) and (5) both of these organic compounds will produce bicarbonate when utilised, however acetate produces both bicarbonate and CO_2. At the pH of these experiments a proportion of the CO_2 produced may also speciate as bicarbonate (Langmuir, 1997). This increase in aqueous bicarbonate concentration allows adsorption and precipitation reactions to occur which lead to the accumulation of ^{14}C in the solid inorganic phase (Boylan et al., 2017), this assumption is supported by PHREEQC modelling (see SI, section 2) which suggests that calcium carbonate minerals including calcite and dolomite would be supersaturated in this system. These precipitation reactions may be particularly likely at higher pH (> 8) as can be found in alkali leachate plumes which exist underneath nuclear reprocessing facilities (Marshall et al., 2015; Parry et al., 2011).

The phylogenetic composition identified for the unaltered and low concentration sediments are broadly similar to previous results on unaltered sediment from the same location with Acidobacteria and Proteobacteria consistently identified as major constituents (Geissler et al., 2010; Thorpe et al., 2012). The microbial communities in the high concentration carboxylate systems both differed from the unaltered sediment and low concentration systems. Diversity was reduced, with a reduction of 73% and 34% in the number of common OTUs (for acetate and formate respectively) compared to the unaltered sediment community. There was an increase in the relative abundance of the Betaproteobacteria class for both electron donors, with the most abundant order being Burkholderiales in both systems. This has been identified previously in Sellafield site sediments (Newsome et al., 2014) and is an order that contains a number of methylotrophic bacteria which are able to utilise both C1 compounds (i.e. those which do not contain a C-C bond) and a variety of multi-carbon compounds (Kalyuzhnaya et al., 2008).

4.2. Fate of simple aldehydes in aerobic sediment-water systems

In the low concentration (10^{-5} M) system, more than 95% of ^{14}C-formaldehyde was removed from solution after 7 days whereas no removal was recorded in solution-only experiments. Also, in the closed-bottle experiments, $^{14}CO_2$(g) was measured in the headspace of the low concentration microbially active experiments after 48 h. This suggests that, like the carboxylate systems, microbially mediated breakdown of ^{14}C-formaldehyde removal from solution. In the high concentration system, the percentage of ^{14}C-formaldehyde in solution remained high after 250 h (~75%) with a large variation between replicates. This suggests that a high concentration of formaldehyde may be toxic to some microbes (Manchee et al., 1983; Trevors, 1996; Weir et al., 1996). This is supported by poor DNA recovery from the sediment suggesting there were lower cell numbers in the 10^{-2} M formaldehyde after 10 days than in any of the other systems.

4.3. Fate of simple alcohols in aerobic sediment-water systems

In contrast to the other systems studied, up to 50% of ^{14}C-methanol was lost after 10 days in the solution-only experiments performed at both high and low concentrations (10^{-2} and 10^{-5} M). This suggests that some ^{14}C-methanol was lost directly from solution by volatilisation even in the lower concentration experiments, but there is no evidence of volatilisation in the formaldehyde system which has a higher vapour pressure. However a study by Green and Vener (1955), suggests that a small addition of formaldehyde to water results in mixtures which have volatility similar to pure water, this result is not replicated in methanol-water mixtures and so may account for the lack of volatilisation seen in the formaldehyde experiments. Methanol removal was faster in the microbially active experiments than in the solution-only experiments, particularly in the low concentration system, suggesting that some ^{14}C-methanol removal was associated with microbial metabolism. Methanol is ubiquitous in subsurface environments and is commonly utilised as an electron donor due to its high solubility in water (Madigan et al., 2014). Microorganisms which are capable of utilising methanol as a carbon source are therefore frequently identified in subsurface environments and the results of this study suggest that ^{14}C-methanol at low concentration is rapidly degraded by microorganisms. This is supported by the measurement of $^{14}CO_2$(g) in the headspace of low concentration closed-bottle experiments after 48 h (use of methanol during aerobic respiration produces CO_2; Equation (7)). Also there was a shift in the microbial population of the high concentration system to a community dominated by Betaproteobacteria, particularly species from the order Methylophilales (~55% of total reads). One particular OTU of the family Methylophilaceae is responsible for approximately 40% of total reads, many species of Methylophilaceae have been shown to utilise methanol (and other C1 compounds) (Anthony, 1982; Doronina et al., 2014).

4.4. Implications for the fate of simple ^{14}C-organics in the shallow subsurface environment

The electron donors considered in this study, especially formate and acetate, are rapidly utilised by microbes. Three of these electron donors contain single carbon atoms that are not readily assimilated by microbes (Johnson and Quayle, 1964), so most will be oxidised to CO_2 which will either be retained as aqueous HCO_3^-, precipitated as solid carbonates, or released as CO_2(g). Measurable quantities of $^{14}CO_2$(g) were found in all the sealed low concentration systems after 48 h, which is consistent with organic matter oxidation during microbially mediated aerobic respiration. If the indigenous community of Calder Bridge soil is representative of that which underlies the Sellafield nuclear reprocessing site, UK, this suggests that this microbial community is able to utilise a range of ^{14}C-LMWO substances, removing them from solution rapidly in low concentration systems. There was a change in the indigenous microbial populations when exposed to high concentration conditions, allowing bacterial orders such as Methylophilales to dominate the community structure. For acetate, formate and methanol this shift in population structure still results in rapid removal from solution of the ^{14}C-LMWOs.

For carboxylates and aldehydes, it would be expected that increasing amounts of $^{14}CO_2$ would be produced over time as more ^{14}C-organics are utilised (Ishii et al., 2015). In surface environments this $^{14}CO_2$ would undergo isotope exchange with the much larger stable isotope CO_2(g) pool in the atmosphere, and thus be quickly diluted and dispersed. In solutions in good contact with atmosphere, ^{14}C-methanol and possibly other ^{14}C-labelled alcohols (e.g. ethanol, propanol) can be lost by volatilisation and thus there is the potential to form gaseous ^{14}C

(Hoch, 2014). However, any ^{14}C-methanol would be highly diluted in atmosphere, and is likely to be oxidised in a number of days to $^{14}CO_2$(g) (Grosjean, 1997). In subsurface environments there is only limited connectivity to atmosphere and therefore microbial respiration (and the associated inorganic ^{14}C generation) is likely to be responsible for the ^{14}C-methanol removal.

In subsurface environments $^{14}CO_2$ produced by microbial metabolism will be retained in the aqueous phase as part of the DIC pool due to the limited connectivity to atmosphere and high partial pressure of CO_2(g) in pore gas phase. In this instance its behaviour would be governed by pH and the availability of divalent cations to form carbonate minerals as discussed in previous studies (Langmuir, 1997; Inskeep and Bloom, 1985; van Geen et al., 1994; Hodkin et al., 2016; Boylan et al., 2017). The low concentration experiments (10^{-5} M) are indicative of anticipated concentrations in solutions that are in contact with fuel cladding wastes (Wieland and Hummel, 2015), therefore, it is clear that if released into aerobic sedimentary environments, aqueous ^{14}C-labelled LMWO substances are unlikely to persist in groundwater for more than a few days.

5. Conclusions

This study shows there is minimal persistence of ^{14}C-labelled LMWO substances in solution in the presence of sediment with an active microbial population under aerobic conditions. Therefore the primary fate of the ^{14}C-LMWO substances released to shallow subsurface is to be converted to ^{14}C-DIC by indigenous soil microorganisms. Generally there is very little ^{14}C retention in the solid fraction, the only exception to this is acetate where modest retention in the organic (5%) and inorganic (12%) fractions was observed (see Table 2), suggesting that limited amounts of longer chain organic molecules may be retained in solid fraction. The indigenous microbial community detected in the sediment samples in this study represent a diverse mix of bacterial phyla which are ubiquitous in terrestrial environments and are therefore likely to be similar to those found in the subsurface below Sellafield nuclear reprocessing site (Geissler et al., 2011; Thorpe et al., 2012; Newsome et al., 2014). In high concentration systems there is evidence for increasing dominance of Betaproteobacteria, with the order Burkholderiales being most abundant with acetate and formate electron donors and Methylophilales being the most abundant when methanol was the electron donor. Any ^{14}C-LMWO substances, particularly C1 compounds, are therefore unlikely to persist or bioaccumulate in their organic forms when in contact with microbial populations (i.e. subsurface environments) where they are rapidly utilised and transformed to $^{14}CO_2$.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvrad.2018.06.016

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