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The behaviour of low molecular weight organic carbon-14 containing compounds in contaminated groundwater during denitrification and iron-reduction.

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Abstract

Aqueous low molecular weight organic carbon-14 (^{14}C) substances can be formed by the oxidation of carbide and impurities within nuclear fuel cladding. During reprocessing and interim storage ^{14}C-labelled organic compounds may leak to the shallow subsurface environments at nuclear facilities where denitrifying and iron reducing zones can exist. ^{14}C-labelled organic compounds (acetate, formate, formaldehyde and methanol) were used as electron donors in microcosm experiments, under both denitrification and iron reduction, using glacial outwash sediments and groundwater composition representative of the Sellafield nuclear reprocessing site, UK. In denitrifying microcosms, <6% of the initial ^{14}C-DOC remained 15 days after injection into the microcosm irrespective of the electron donor; with concurrent $^{14}CO2$ (g) production. Lack of removal in sterile controls suggests that ^{14}C-organics were metabolised by microorganisms. Under iron-reducing conditions both ^{14}C-carboxylates were removed from solution rapidly, but some formaldehyde and methanol remained in solution 32 days after injection into the microcosm so there is potential that a proportion of ^{14}C-formaldehyde and ^{14}C-methanol may persist for longer in subsurface environments.

Keywords: Radiocarbon; Contaminated land; Groundwater; Organic carbon; Anaerobic microbial utilization;

1. Introduction

Contamination of groundwater is common at nuclear sites where historic leaks of radionuclides to subsurface environments, including carbon-14 (^{14}C), technetium-99 (^{99}Tc), strontium-90 (^{90}Sr) and uranium-238 (^{238}U), are co-located with contaminants such as nitrate (from nitric acid) and organic acids. Such groundwater contamination has been recorded at nuclear licensed sites including Sellafield, UK (Thorpe et al., 2012; Law et al., 2010; McKenzie and Armstrong-Pope, 2010), Oak Ridge, TN, USA (Edwards et al., 2007 ; Istok et al., 2004; McBeth et al., 2007), San Juan

Shiprock, NM, USA (Finneran et al., 2002) and Hanford, WA, USA (Singleton et al., 2005). These sites are often typified by their high nitrate and in some cases significant naturally occurring iron(III) oxyhydroxide phases that create a variety of redox conditions in the subsurface including localised reducing zones and can exist at a wide range of pH, although circumneutral pH dominate at most sites (Fredrickson et al., 2004; Istok et al., 2004; Begg et al., 2007; Edwards et al., 2007; McBeth et al., 2007; McKenzie and Armstrong-Pope, 2010; Law et al., 2010; Stamper et al., 2012; Sellafield Ltd., 2016).

Iron reducing conditions may also be deliberately engineered at nuclear sites by biostimulation (where an electron donor is added to groundwater to stimulate the indigenous microbial community and create a reducing environment; Lloyd and Renshaw, 2005), as the radionuclides Tc and U can be reduced to their insoluble, low-valence forms under such geochemical conditions (Lloyd et al., 2000; Burke et al., 2005; Burke et al., 2010; Senko et al., 2005; Morris et al., 2008). Biostimulation enhances microbial respiration and therefore increases the production of HCO_3^- which may increase the pH and promote supersaturation of carbonate mineral phases including siderite and calcite (Monger et al., 1991, Parmar et al., 2000; Roden et al., 2002), both of which have the potential to incorporate inorganic ^{14}C as $^{14}CO_3^{2-}$.

^{14}C is produced at each stage of the nuclear fuel cycle. It is of concern due to its long half-life of 5730 ±40 a (Godwin, 1962), its ability to bioaccumulate (Begg et al., 1992; Cook et al., 1998; Yim and Caron, 2006) and its position as a key radionuclide in safety assessment for geological disposal (NDA, 2012). Inorganic ^{14}C behaviour in groundwater environments is influenced mainly by pH and the availability of divalent cations (Krauskopf and Bird, 1995, Boylan et al., 2017). However, there is concern that ^{14}C-labelled low molecular weight organic (LMWO) substances, which are predicted to form under geological disposal conditions (Wieland and Hummel, 2015), may also form in intermediate waste storage facilities due to the corrosion of activated fuel and fuel cladding (Kaneko et al., 2002; Wieland and Hummel, 2015). During interim storage at nuclear sites, such as within silos at the Sellafield nuclear reprocessing site, UK, reducing conditions may occur due to the lack of oxygen penetration with increasing depth (Kaneko et al., 2002; Wieland and Hummel, 2015).

Corrosion under completely oxic or reducing conditions are expected to produce predominantly $^{14}CO_2$ and $^{14}CH_4$, respectively (Wieland and Hummel, 2015), but redox stratification within a waste storage facility would also allow the formation of ^{14}C-labelled LMWO substances with intermediate oxidation states. The four compounds expected to form at significant concentrations due to the corrosion of fuel cladding are acetate, formate, formaldehyde and methanol (Kaneko et al., 2002; Wieland and Hummel, 2015). These compounds can be utilised by microorganisms under reducing conditions (Lovley and Phillips, 1988; Ferry, 1990; Garrido et al., 2001; Daniel et al., 1999), and acetate consumption has been recorded at nuclear contaminated sites such as Rifle, CO, USA (Anderson et al., 2003) and Sellafield, UK (Newsome et al., 2014, Thorpe et al., 2012), however little work has been undertaken to elucidate the behaviour of ^{14}C-labelled LMWOs.

Equations for the oxidation of these four LMWO substances are shown below under denitrifying conditions (Equations 1-12) and under iron reducing conditions (Equations 13-15). All of these reactions convert organic carbon in to inorganic forms.

Acetate	$CH_3COO^- + 4NO_3^- \rightleftharpoons 4NO_2^- + HCO_3^- + CO_2 + H_2O$	Equation 1
	$CH_3COO^- + 2NO_2^- \rightleftharpoons N_2O + HCO_3^- + CO_2 + H_2O$	Equation 2
	$CH_3COO^- + 4N_2O \rightleftharpoons 4N_2 + HCO_3^- + CO_2 + H_2O$	Equation 3
Formate	$HCOO^- + NO_3^- \rightleftharpoons NO_2^- + HCO_3^-$	Equation 4
	$HCOO^- + 2NO_2^- \rightleftharpoons N_2O + HCO_3^-$	Equation 5
	$HCOO^- + N_2O \rightleftharpoons N_2 + CO_2 + H_2O$	Equation 6
Formaldehyde	$CH_2O + 2NO_3^- \rightleftharpoons 2NO_2^- + CO_2 + H_2O$	Equation 7
	$CH_2O + 2NO_2^- \rightleftharpoons N_2O + CO_2 + H_2O$	Equation 8
	$CH_2O + N_2O \rightleftharpoons N_2 + CO_2 + H_2O$	Equation 9
Methanol	$CH_3OH + 3NO_3^- \rightleftharpoons 3NO_2^- + CO_2 + 2H_2O$	Equation 10
	$CH_3OH + 2NO_2^- \rightleftharpoons N_2O + CO_2 + 2H_2O$	Equation 11
	$CH_3OH + 2N_2O \rightleftharpoons 2N_2 + CO_2 + 2H_2O$	Equation 12

Acetate	$CH_3COO^- + 8FeOOH + 15H^+ \rightleftharpoons 8Fe^{2+} + 2HCO_3^- + 12H_2O$	Equation 13
Formate	$HCOO^- + FeOOH + H^+ \rightleftharpoons Fe^{2+} + HCO_3^- + H_2O$	Equation 14
Formaldehyde	$CH_2O + 4FeOOH + 8H^+ \rightleftharpoons 4Fe^{2+} + CO_2 + 7H_2O$	Equation 15
Methanol	$CH_3OH + 8FeOOH + 18H^+ \rightleftharpoons 8Fe^{2+} + CO_2 + 15H_2O$	Equation 16

This study aims to address the behaviour of ^{14}C-labelled LMWOs in near-field reducing groundwater environments at the circumneutral pH which dominates many nuclear contaminated sites, for example Sellafield site, UK, where pH is most commonly reported between 6 and 7 (Sellafield Ltd., 2016).

The specific objectives of this study were: 1) to investigate the behaviour of four ^{14}C-LMWO substances (acetate, formate, formaldehyde and methanol) in circumneutral aqueous environments in contact with sediment under denitrification and iron-reducing conditions; 2) to establish the extent of transformation of organic ^{14}C to $^{14}CO_2(g)$ and to quantify the amount of ^{14}C retained in the organic and inorganic fractions in solid; 3) to determine any changes to the active microbial population after incubation with the organic compounds under denitrifying or iron-reducing conditions; and 4) to assess the implications of these processes for the fate ^{14}C in dissolved organic carbon (DOC) in the shallow subsurface environments.

2. Materials and Methods

2.1 Sediment

Near surface sediment was collected from the bank of the River Calder, Cumbria, UK (Lat 54°26.3'N, Long 3°28.2'W) in July 2016. This sediment is representative of the glacial/fluvial quaternary deposits underlying the UK Sellafield nuclear reprocessing site (Law et al., 2010; Wallace et al., 2012). Sediment was collected in sterile HDPE bags and stored at 4°C. Prior to use the soil was sieved and the <2mm fraction retained for use.

2.2 Bioreduction microcosms

Sediment microcosms were prepared with 10 ±0.1 g of wet sediment mixed with 100 ±1 mL of a synthetic groundwater media representative of groundwater in the region around Sellafield (Wilkins et al., 2007) in sterile 120 mL glass serum bottles (Wheaton Scientific, U.S.). Two artificial groundwater compositions were used, A) unamended (used for iron-reducing conditions) and B) high nitrate, which was amended with sodium nitrate to produce a final nitrate concentration of 10 mM L^{-1}(used for denitrifying conditions) (Table 1). Groundwater was filtered (0.2µm), sparged with N_2(g) and pH adjusted after deoxygenation prior to addition to microcosm. The microcosm headspaces were sparged with N_2(g) before sealing with butyl rubber septa and crimps. Experiments were run in triplicate with the exception of sterilised control microcosms which were single experiments.

After the microcosms were established, they were incubated in the dark to allow the desired redox conditions to develop (7 days for denitrifying microcosms and 28 days for iron-reducing microcosms). After incubation the desired LMWO (acetate, formate, formaldehyde or methanol) was spiked into the microcosms through the butyl rubber septa. The spike consisted of the non-labelled LMWO at a final concentration of 10^{-5} M and ^{14}C-labelled LMWO at a final concentration of 5 x 10^{-7} M (producing a final ^{14}C activity of 100 Bq ml^{-1} in each bottle). Formate and acetate were added as a sodium salt, and acetate was C^2 labelled (methyl group) (ARC Ltd., USA). After the initial incubation period, the sterile control microcosms were autoclaved for 30 minutes at 120°C prior to spiking with ^{14}C compounds.

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

Compound	g/L in DIW
KCl	0.006
$MgSO_4.7H_2O$	0.0976
$MgCl_2.6H_2O$	0.081
NaCl	0.0094
*$NaNO_3$	0.868

*$NaNO_3$ only added to high nitrate solution composition

2.3 Geochemical methods

Fe(II) in the solid fraction was determined using an acid extraction based on the method of Lovley and Philips (1986) whereby the amount of Fe(II) in the solid is expressed as a percentage of the total 0.5 M HCl extractable Fe present in the sediment. Approximately 0.1 g of sediment was added to 5 mL of 0.5 M HCl and left for 60 minutes, followed by colorimetric assay for Fe(II) and total Fe using the ferrozine method (Viollier et al., 2000: in the total Fe assay extracted Fe(III) is reduced to Fe(II) by addition of hydroxylamine HCl). At circumneutral pH Fe(III) has limited solubility and so total Fe in the porewater is assumed to be the measured Fe^{2+} concentration. The limit of detection for this method is ~0.35 μM. pH was determined using a Thermo Scientific Orion benchtop multimeter and electrodes calibrated daily at pH 4, 7 and 10. Nitrate and nitrite concentrations were determined colorimetrically using a continuous segmented flow analyzer (III) (SEAL Autoanalyzer 3HR, SEAL, UK), which measures nitrite and total nitrite (by reduction of nitrate to nitrite). Nitrate reduction is achieved using a copper-cadmium reactor column. Nitrate is then calculated as the difference between the two values. Aqueous ^{14}C-DOC was measured by mixing 800 μL of filtered (0.2 μm) sample with 10 mL of EcoScintA liquid scintillation fluid (PerkinElmer) on a Packard Tri-Carb 2100 TR (count time = 10 min; energy window = 4-156 keV; detection limit: 20 CPM; counting efficiency: 80%). EcoScintA is assumed to be a measure of ^{14}C-DOC only as it is unable to capture ^{14}C-DIC efficiently (<1%). ^{14}C-DIC is not measured using an alternative method

(see Boylan et al., 2017 for method details) due to interference with ^{14}C-DOC. Gaseous $^{14}CO_2$ was measured by withdrawing a 10 mL gas sample directly from the microcosm headspace and bubbling it through 2 mL Carbo-Sorb E prior to mixing with 10 mL of PermaFluor E liquid scintillation fluid. All liquid scintillation samples were dark adjusted for 24 hours before counting on the liquid scintillation counter (Ahn et al., 2013; Boylan et al., 2018).

2.4 ^{14}C associated with sediment

Inorganic and organic ^{14}C associated with sediment (TIC and TOC) were measured in a two-step process where the sediment is first acidified and then oxidised, and the CO_2 gas evolved in each step is captured by Carbo-Sorb E, mixed with PermaFluor E and quantified by liquid scintillation counter (procedure adapted from Magnusson et al., 2007, full method in supporting information, Section S1). This method is 85-95% efficient at recovering ^{14}C (as measured by Magnusson et al., 2007).

2.5 Microbiology

Eleven soil samples were selected for next-generation sequencing; one from each system (four organic compounds under two different redox conditions) and three unamended samples (sediment collected at the field site and frozen on the same day). DNA was extracted from soil samples (~0.5g) using the Fast DNA spin kit for soil (MP Bio). DNA fragments in the size range 3 kb to ~20 kb were isolated on a 1% agarose "1x" Tris-borate-EDTA (TBE) gel stained with ethidium bromide for viewing under UV light (10x 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 (1ng/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 V4 hyper-variable region of

the 16S rRNA gene. Pooled amplicons were paired-end sequenced on the Illumina MiSeq platform (2x250 bp). Illumina adapter sequences were removed, and the trimmed reads were processed using the UPARSE pipeline (Edgar, 2013) within the USEARCH software (version 9.2) on a Linux platform. Operational taxonomic units (OTUs) were defined by minimum of 97% sequence identity between the putative OTU members. OTUs were assigned to taxonomic groups using the online Ribosomal Database Project (Wang et al., 2007), using a confidence value of 0.7 to give a reasonable trade-off between sensitivity and error rate in the taxonomy prediction.

Statistical analysis was performed to determine the bacterial diversity. In this study 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 number of 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).

3. Results

3.1 Sediment characterisation

Sediment from the Calder Valley has been extensively characterised in previous studies (e.g Law et al., 2010; Wallace et al., 2012). Briefly, the sediment is a poorly sorted sandy loam, with the fine fraction dominated by quartz, albite, microcline, chlorite and mica (see previous study by Boylan et al., 2018 for XRD spectra). The approximate particle composition was 53% sand, 42% silt and 5% clay with and average TOC of 0.56 ±0.08 wt. % (Law et al., 2010), sediment pH was 5.5.

3.2 Microcosm geochemical conditions

Geochemical conditions in the microcosms were measured within one hour of establishment (see Table 2) prior to any amendment (i.e. nitrate addition) to establish pH and concentration of iron and nitrate/nitrite at the start of incubation.

Table 2 Geochemical conditions measured in unaltered microcosms within one hour of establishment.

Geochemical conditions prior to amendments	
pH	5.1-6.6
Fe(II) (as % of total Fe in solid)	0.5 ±0.5%
Fe^{2+} in solution (μM)	<0.35 μM*
Nitrate (mM)	0.8 ±0.01
Nitrite (mM)	0.08 ±0.01

* LOD = Limit of detection (0.35 μM)

3.3 The behaviour of ^{14}C-labelled LMWO compounds under denitrifying conditions

For all denitrification experiments the nitrate was added on day 0, and the microcosms were then incubated for a week to allow denitrifying conditions to develop prior to spiking with the ^{14}C-LMWO substances on day 7. In the acetate experiments the percentage of aqueous ^{14}C-DOC decreased from 100% on day 7 to 2% at the 8 day sample point (i.e. 98% acetate was removed from solution within 24 hrs; see Figure 1a). It then remained at 3 ±2% for the duration of the experiment (14 days). The percentage of ^{14}C-DOC in solution in the formate experiment decreased from 100% on day 7 to 1% at the 8 day sample point, and remained at 3 ±2% for the duration of the experiment (14 days). The concentration of ^{14}C-formaldehyde reduced to 6% by day 14 and remained there until the end of the experiment (day 22). In the methanol experiments the percentage of ^{14}C-DOC in solution decreased from 100% on day 7 to 30% by day 14, with 6% remaining in solution at the end of the experiment (on day 22) (see Supporting information, Figure S1 for complete data set, 7 to 22 days).

All pH values remained circumneutral throughout the duration of the experiments ranging from a maximum value of pH 7 to a minimum of pH 5.5 (days 7- 22; Figure 1b).

In all experiments nitrate concentrations measured on day 7 were 1±0.4 mM, and over the duration of the experiment all concentrations reduced to 0.5±0.3 mM. Nitrite concentrations measured at the addition of LMWO were between 2.1 and 2.6 mM for all experiments. All showed an increase in nitrite concentration between 48 and 72 hours after LMWO addition with concentrations of 3.3; 3.0; 2.6; and 2.2 mM for acetate, formate, formaldehye and methanol respectively. At the final sample point all nitrite concentrations had decreased (2.3; 2.0; 2.2; 1.5 mM respectively).

The amount of Fe(II) in solid fraction remained below 12% for all electron donors for the duration of the experiments with no increasing/decreasing trend in concentration recorded in the experiments. Aqueous Fe^{2+} measurements were <0.35 μM and so below limit of detection for all experiments.

In the control experiments the pH for the acetate experiments was 5.1 on day 7, increasing to 5.8 at the end of the experiment (day 14). For all other electron donors the pH remained relatively constant at 5.6 ±0.2 after inoculation with the LMWO (day 7-14). The percentage of ^{14}C in solution remained at 99 ±1% throughout the experiment for each electron donor. The amount of Fe(II) in solid fraction remained below 10% for each electron donor and the amount of Fe in solution was below the limit of detection. The amount of nitrate in solution was measured at 2.2 ±0.3 mM for all electron donors and the amount of nitrite remained almost constant at 0.5 mM after inoculation with the LMWO (see Supporting information Figure S3).

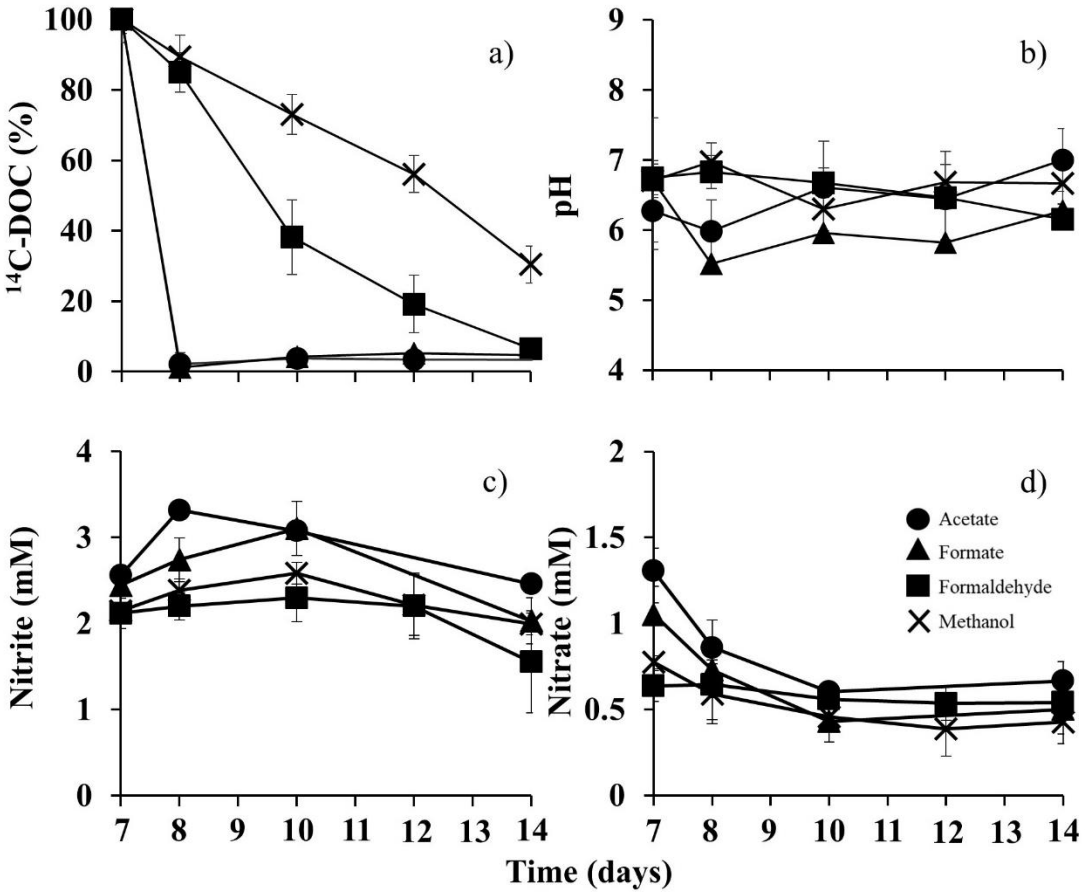

Figure 1 Experimental results of the denitrification experiments a) percentage of ^{14}C-DOC in solution; b) pH; c) concentration of nitrite in solution; d) concentration of nitrate in solution (10mM $NaNO_3$ was added on day 0). The ^{14}C organic compounds were injected on day 7. The acetate and formate experiments were terminated on day 14, but the formaldehyde and methanol experiments were terminated on day 22 (full dataset reported in S.I., Figure S1). Error bars denote one standard deviation of triplicate measurements; where not shown error bars are less than the size of the symbol used.

3.4 ^{14}C speciation at the end of denitrification experiments

The distribution of ^{14}C between the organic aqueous fraction, inorganic and organic solid fractions (TIC and TOC) and CO_2(g) in the headspace at the end of the microcosm experiments is reported in Table 3. There was measurable $^{14}CO_2$(g) in the headspace of all the experiments, with the

lowest value of 25.7% of the initial ^{14}C spike in the acetate experiment and the maximum recovery at 72.5% of the initial ^{14}C spike from the methanol experiment. Very little ^{14}C from any of the LMWOs is retained in any solid phase (TIC or TOC) under denitrifying conditions (highest retention was observed with formaldehyde where 7.3±5.2% of the original ^{14}C activity was recovered from both inorganic and organic fractions).

Table 3 Percentage of ^{14}C in organic aqueous fraction, the inorganic and organic solid fractions and the headspace at the end of the denifrifying experiments. All measurements were based on one sample; where error measurement is recorded this corresponds to the standard deviation of analytical replicates taken from an individual gas wash bottle. DIC – dissolved inorganic carbon; TIC – total solid inorganic carbon; TOC – total solid organic carbon.

	Acetate 14d	**Formate 14d**	**Formaldehyde 22d**	**Methanol 22d**
^{14}C-DOC	3.8%	2.4%	7.9%	8.3%
$^{14}CO_2$(g)	25.7%	49.9%	70.3%	72.5%
^{14}C-TIC	1.0 ±1.5%	2.6 ±4.0%	3.6 ±2.5%	1.6 ±2.4%
^{14}C-TOC	3.0 ±0.1%	3.0 ±4.1%	3.7 ±2.7%	1.7 ±2.6%
Sum	33.5%	58%	85.5%	84.2%
Balance (by difference)*	66.5%	42%	14.5%	15.8%

***** Balance (by difference) is attributed to the ^{14}C-DIC fraction which is not measured.

3.5 The behaviour of ^{14}C-labelled LMWO compounds under iron-reducing conditions

The iron reducing microcosms were initially incubated for 28 days to permit iron reducing conditions to develop. The ^{14}C-LMWO substance was then added on day 28, and the active phase ran from day 28 to either day 35 (acetate and formate) or day 60 (formaldehyde and methanol). The nitrate and nitrite concentrations prior to the incubation were 0.8 ±0.08 mM and 0.08 ± 0.01, respectively. At this point 0.5% of the acid extractable iron in the solid phase was Fe(II), and the concentration of Fe in solution was below the limit of detection (0.35 μM) (see Table 2).

In the acetate experiments the percentage of the initial ^{14}C-LMWO spike that remained in solution decreased from 100% on day 28 to 5% on day 35 (Figure 2a). In the formate experiments the decrease was from 100% on day 28 to 3% on day 35. In the formaldehyde experiment the decrease was from 100% on day 28, to 75% on day 35, and finally to 11% on day 60. Similarly, in the methanol experiment the decrease was more gradual from 100% on day 28 to 19% on day 60 (see Supporting information, Figure S2 for complete data set).

The pH of all experiments remained between pH 6.9 and pH 5.5 for the duration of the experiments. In the acetate experiment 9.4% of the acid extractable iron in the solid phase was Fe(II) on day 28 and by the end of the experiment (day 35) 14.5% of the acid extractable iron in the solid phase was Fe(II). The formate experiment exhibited a slightly greater increase in the proportion of the acid extractable iron in the solid phase that was Fe(II) (it increased from 7.6% on day 28 to 20% on day 35). The formaldehyde and methanol experiments exhibited similar patterns to the acetate and formate experiments, but the greater test durations meant that 32.7% and 29% of the acid extractable iron in the solid phase that was Fe(II) by day 60 (Figure 2c).

On day 28 aqueous Fe^{2+} was measured in all the active microcosms (1.0, 1.0, 0.7 and 0.4 μM, in the acetate, formate, formaldehyde and methanol systems, respectively). It varied slighty between day 28 and the end of each test without clear patterns but was always between 0.4 and 1.5 μM (Figure 2d). Nitrate concentrations remained below 0.2 mM in every system throughout the duration of the experiment, nitrite concentrations were all below 0.1 mM for the duration of the experiment.

In the control experiments the pH for all the systems remained relatively constant at 5.6 ±0.2 for the duration (day 28-35). The percentage of the ^{14}C LMWO in solution remained at 100 ±2% throughout the experiment in all four systems. At the point of ^{14}C LMWO addition (day 28) between 13 and 20% of the acid extractable iron in the solid phase was Fe(II), and the aqueous Fe concentration was between 0.84 and 1.9 μM. The nitrate concentration was <0.06 mM in all the controls and the nitrite concentration was 0.02 mM (see Supporting information, Figure S4).

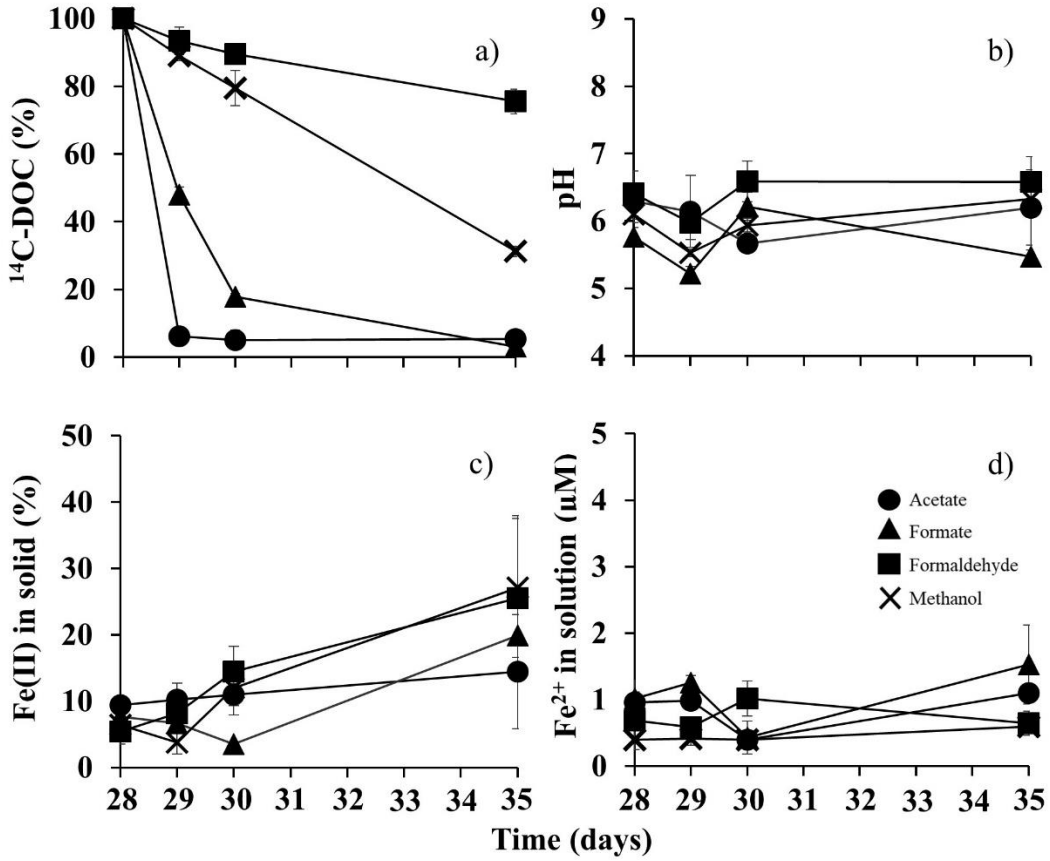

Figure 2 Experimental results of the iron-reducing experiments a) percentage of ^{14}C-DOC in solution; b) pH; c) fraction of Fe(II) in solids; d) Fe^{2+} in solution. The ^{14}C organic compounds were injected on day 28. The acetate and formate experiments were terminated on day 35, but the formaldehyde and methanol experiments were terminated on day 60 (full dataset reported in S.I., Figure S2). Error bars denote one standard deviation of triplicate measurements; where not shown error bars are less than the size of the symbol used.

3.6 ^{14}C speciation at the end of the iron-reducing experiments

The distribution of ^{14}C between the organic aqueous fraction, inorganic and organic solid fractions and the CO_2(g) in the headspace at the end of the iron-reducing microcosm experiments is reported in Table 4. In all the experiments 44.6% to 64.9% of the ^{14}C spike was converted to CO_2(g) in the headspace. Only a small proportion of ^{14}C from any of the LMWOs is retained in any solid

phase under iron-reducing conditions (highest retention was observed with methanol where about 10% of the original ^{14}C activity was recovered from both the inorganic and organic fractions).

Table 4 Percentage of ^{14}C in the organic aqueous fraction, inorganic and organic solid fractions and the headspace at the end of the iron-reducing experiments. All measurements were based on one sample; where error measurement is recorded this corresponds to the standard deviation of analytical replicates taken from an individual gas wash bottle. DIC – dissolved inorganic carbon; TIC – total solid inorganic carbon; TOC – total solid organic carbon.

	Acetate 35d	Formate 35d	Formaldehyde 60d	Methanol 60d
^{14}C-DOC	5.5%	3.2%	19.3%	11%
$^{14}CO_2$(g)	51.7%	64.5%	44.6%	64.9%
^{14}C-TIC	2.3 ±2.4%	5.0 ±0.2%	1.5 ±2.3%	5.1 ±0.3%
^{14}C-TOC	5.2 ±0.1%	2.8 ±2.4%	4.9 ±0.1%	5.2 ±0.4%
Sum	64.6%	75.5%	70.3%	86.2%
Balance (by difference)*	35.4%	24.5%	29.7%	13.8%

***** Balance (by difference) is attributed to the ^{14}C-DIC fraction which is not measured.

3.7 Microbial community composition

Illumina MiSeq analysis gave >100,000 paired-end reads per sample after quality control. The eleven samples of this study were part of a combined pool of 8,763,897 million paired-end reads which passed the chimera check and these were clustered in to OTUs (>97% sequence identity) in the UPARSE pipeline and assigned to taxonomic groups. OTUs classified as archaea (8% of non-chimeric reads) and bacteria which were not classified at phylum level with a confidence of >0.7 (41% of non-chimeric reads) were excluded from further analysis. This resulted in 7073 OTUs in the eleven samples which were classified to bacteria phylum with a confidence greater than 0.7 which were used to characterise the impact of ^{14}C-labelled electron donors on microbial populations under varying redox conditions.

Taken together the samples contained bacteria from 11 phyla that individually represented more than 1% of the total population (the "major" phyla). The unaltered sediment samples contained between 10 and 11 individually identified phyla at more than 1% of the total population. Acidobacteria was the most abundant phylum (28±2% of the reads), followed by Proteobacteria (27±2%), Actinobacteria (10±3%) and Verrucomicrobia (10±3%; Figure 3). This microbial community composition was similar to those described in previous studies using sediment collected from the same site (Geissler et al., 2011; Thorpe et al., 2012).

Under nitrate reducing conditions for acetate, formate and formaldehyde the number of major phyla was 10. Acidobacteria is the most abundant phylum (28-35%). The relative abundance of Proteobacteria was between 29 and 30%, with the largest relative contribution from the Betaproteobacteria class (11-13%). In the methanol sample the number of major phyla was reduced to 7, 35% of OTUs were associated with the Betaproteobacteria class, with the order of Burkholderiales accounting for 92% of the reads. 98% of the Gammaproteobacteria were associated with the Xanthomondales order and particularly the Rhodanobacter genus (see Figure 3; Table S1).

The number of major phyla represented under iron reducing conditions was 9 for the acetate and formate systems. The most abundant phylum was Proteobacteria (39 ±1%), Acidobacteria (24%) and Actinobacteria (13 ±1%). The number of phyla represented in the formaldehyde system was 8. The most abundant of which was Proteobacteria (61%), followed by Acidobacteria (11%) and Actinobacteria (9%). In the methanol system there were 9 major phyla. The most abundant of these was Proteobacteria (52%), followed by Acidobacteria (19%) and Planctomycetes (5%) (see Figure 3).

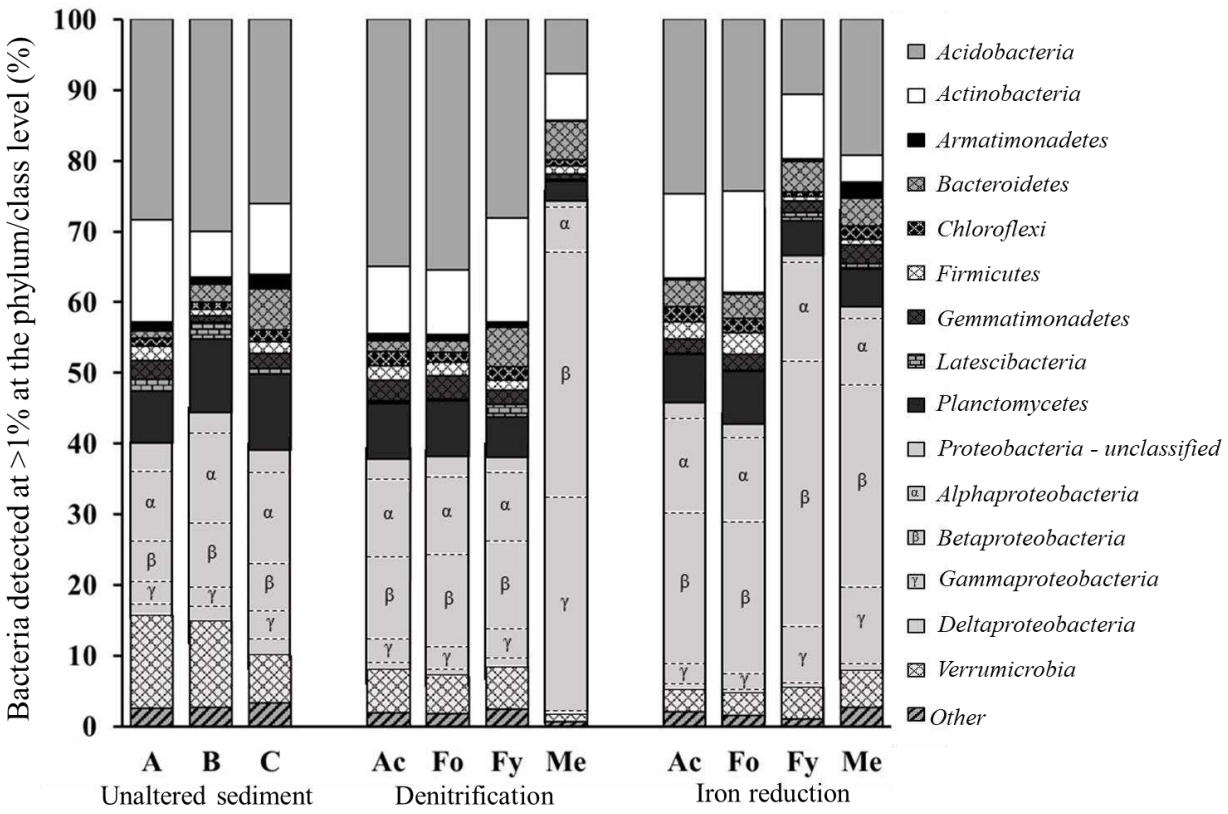

Figure 3 Bacterial phylogenetic diversity within the unaltered sediment (A-C) and under denitrification and iron reduction (Ac- acetate; Fo- formate; Fy- formaldehyde; Me-methanol). Phyla with relative abundance less than 1% of the community are grouped as "Other". Dashed line denotes classes of Proteobacteria descending as follows: unclassified; α; β; γ; δ.

The OTU richness (D_0^{α}) for each sample is shown below in Table 5. The average richness for the unaltered sediment was 5790 OTUs, but this decreases by between 31% and 44% under both reducing conditions. On average there are 75% fewer common species under reducing conditions than in the unaltered sediment (D_1^{α} for the unaltered sediment was 922 ±336 OTUs, whereas it was between 105 and 360 OTUs under the reducing conditions). Similarly there were 75% fewer dominant species (D_2^{α} for unaltered sediment is 218 ± 114, whereas it was between 17.9 and 68.8 under reducing conditions). As common and dominant OTUs accounted for more than 78% and 48% of total sequence reads in all the samples, the decrease in the number of common and dominant OTUs in the reducing systems represented a shift towards fewer, but more abundant OTUs under these conditions.

Table 5 Alpha-diversity measurements, D_0^{α}, species richness, D_1^{α}, exponential of Shannon entropy, D_2^{α}, inverse of the Simpson index for samples under denitrification (Ac- acetate; Fo- formate; Fy- formaldehyde; Me-methanol).

	Unaltered sediment				**Denitrification**				**Iron reduction**			
	Time after addition of LMWO				7d	7d	15d	15d	7d	7d	32d	32d
	Total incubation time				14d	14d	22d	22d	35d	35d	60d	60d
	A	B	C	Average	Ac	Fo	Fy	Me	Ac	Fo	Fy	Me
D_0^{α}	5640	6590	5150	5790 ±598	3820	3950	3400	3520	3840	3460	3230	3510
D_1^{α}	495	1320	960	922 ±336	360	342	256	105	348	280	210	126
D_2^{α}	80.8	360	212	218 ±114	68.8	64.6	53.7	17.9	66.6	53.9	53.7	52.3

4. Discussion

4.1 The behaviour of aqueous ^{14}C-LMWO substances during denitrification

After the period of incubation it is assumed that the concentration of organic matter remaining in the unaltered sediment would be minimal, consequently at the point of addition of the LMWOs they would act as the primary source of electron donor in each system. In the acetate and formate microcosms LMWO removal from solution occurred concurrently with nitrate reduction (Figure 1), and resulted in $^{14}CO_2$ accumulation in the microcosm headspace. As removal was not observed in the sterile systems, it was most likely a microbial process. Oxidation of non-fermentable organic substrates, such as acetate, formate, formaldehyde and methane (metabolism) by organotrophic bacteria must be coupled to the reduction of an electron acceptor which was most probably nitrate. Formaldehyde and methanol were removed from solution less rapidly than acetate and formate, and their removal continued after the nitrate concentration stabilized during a period when the nitrite concentrations decrease. This suggests their removal may have been coupled to nitrite reduction as well as nitrate reduction. The concentration of nitrate at the point of LMWO addition varies from 1.3

and 0.64 mM for the four systems, this was probably due to different rates of nitrate reduction occurring in each microcosm, however the removal of ^{14}C-LMWOs in each system suggests that denitrification continued and the variation in concentration did not affect the utilisation of the various LMWO compounds.

At phylum level, the microbial populations of the denitrification experiments using acetate, formate and formaldehyde were similar to those of the unaltered sediment, with ~30% of OTUs belonging to the Proteobacteria, this corresponds with previous work under oxic conditions where no changes were identified when LMWOs were added at 10 μM concentration (Boylan et al., 2018). Only the denitrification experiments using methanol exhibited a significant change in the bacterial population. The number of major phyla in this sample reduced from 11 in unaltered sediment to 7, with the Proteobacteria dominating the.community More than half of the total reads were from the Betaproteobacteria (35%) and Gammaproteobacteria (30%) classes of Proteobacteria. The increase in the Gammaproteobacteria was principally due to an increase in sequences from the Xanthomondales order, and specifically the genus Rhodanobacter (this genus contains facultative anaerobes capable of denitrification; Prakash et al., 2012). It was present in all the samples from the denitrification system, but was much increased in the methanol sample and has been identified at other nuclear contaminated sites as having an important role in denitrification (e.g. Oak Ridge, TN, USA, Green et al., 2012). The increase in the number of Betaproteobacteria was principally due to an increase in sequences from the order Burkholderiales which contains many methylotrophic representatives (i.e. species that can reduce compounds containing a single carbon; Kalyuzhnaya et al., 2008).

4.2 The behaviour of aqueous ^{14}C-LMWO substances under iron reducing conditions

The percentage of Fe(II) in the solid fraction was increasing prior to injection of the LMWO (when the LMWO on day 28 was injected Fe(II) was 5 to 10 times the level found in the unaltered microcosms), and both the nitrate and nitrite concentrations were very low throughout the duration of the experiments, which together indicate that iron-reducing processes were established prior to addition of ^{14}C-labelled electron donors in these tests. At the end of all experiments more than 44% of ^{14}C was recovered as $^{14}CO_2$(g), whereas no ^{14}C-DOC removal from solution was observed in control

experiments (see supporting information Figures S3-S4), suggesting microbial utilisation is occurring in all systems, however the rate of ^{14}C removal varied between the LMWOs. The carboxylates (acetate and formate) were rapidly removed from solution with around 5% remaining on day 35, whereas more than 10% of the ^{14}C-formaldehyde and ^{14}C-methanol remained in solution on day 60. This suggests that the ^{14}C-formaldehyde and ^{14}C-methanol are most likely to be mobile in subsurface environments under iron-reducing conditions relative to denitrifying conditions.

Although all measured ^{14}C concentrations in solid fraction are low, there was a slightly higher percentage of ^{14}C-TOC under iron reducing conditions compared to denitrification. In the TIC fraction 5% of the total ^{14}C was retained in both the formate and methanol systems, possibly in carbonates such as siderite which can precipitate under microbially induced iron-reducing conditions; Thorpe et al., 2012; Wieland and Hummel, 2014. A simple PHREEQC calculation suggests that siderite can be supersaturated under iron reducing conditions at pH > 6.7 if there is sufficient Fe(II) and carbonate (see supporting information, S5), therefore siderite precipitation was possible in the methanol system as the pH reached 6.9. Some ^{14}C was also retained in the TOC fraction for all electron donors, which suggests retention either through sorption reactions or assimilation.

Under iron-reducing conditions the phylogenetic composition of the microbial community was different to that of the unaltered sediment with all samples showing an increase in the relative abundance of Betaproteobacteria class (7.2 ±1.5% for unaltered compared with 27.3 ±6.6% for iron-reducing). All systems showed an increase in the proportion of reads associated with the order Burkholderiales which includes species able to utilise both single C organic compounds and those containing C-C bonds (Kalyuzhnaya et al., 2008; Chistoserdova et al., 2009). The largest increase was associated with the formaldehyde sample where more than 34% of total reads belong to this order. There was a decrease in the number of dominant OTU's (as represented by the D_2^{α} value) between theelectron donors (acetate> formate> formaldehyde> methanol), but an increase in the proportion of reads assigned to these OTUs, from a minimum of 48% for acetate to 73% for methanol.This indicates a shift towards bacterial populations with a smaller number of more abundant OTUs as the electron donor becomes more reduced and suggests that acetate and formate can be used by a wider

range of bacteria than formaldehyde or methanol.. A similar pattern is seen replicated in the denitrifying experiments, although to a lesser degree (minimum of 48% for acetate to a maximum of 65% for methanol).

4.3 Implications for persistence of ^{14}C-containing LMWO compounds in anaerobic subsurface environments

^{14}C-labelled carboxylates (acetate and formate) do not persist in aqueous form under denitrification and iron-reducing conditions in sediment which has an active microbial population as a rapid transformation from organic to inorganic ^{14}C occurs. The rate of ^{14}C-formaldehyde and ^{14}C-methanol oxidation under both denitrification and iron reducing conditions was slower than for the acetate and formate which may suggest that fewer microbes are adapted to use these substrates (this is supported by the lower diversity values). Retention of ^{14}C by inorganic and organic solids was minimal across all electron donors and redox conditions (<6% of original activity) suggesting that in subsurface environments there will be little retention in the solid fraction at circumneutral pH. However both the inorganic and organic solid phase association will be affected by changes in groundwater pH (Boylan et al., 2017; Gu and Schulz, 1991; Krauskopf and Bird, 1995; Sposito, 1989) with the potential to increase the ^{14}C retention. These results imply that aqueous ^{14}C will be most persistent in groundwater as ^{14}C-containing alcohol and aldehyde compounds under iron reducing conditions, but as even these compounds are slowly utilised, ^{14}C-LMWOs are unlikely to persist in shallow sub-surface environments in the long term.

5. Conclusions

This study shows that across most redox and electron donor systems ^{14}C-LMWO substances are removed rapidly from solution. The production of inorganic ^{14}C is attributed to microbial utilisation of the ^{14}C-LMWO substances, which in subsurface environments would increase the ^{14}C associated with the dissolved inorganic pool. The retention in solid phase is minimal in both organic and inorganic phases reaching a maximum of ~5% of total ^{14}C activity with sorption of the organic species limited at circumneutral pH values as the anion exchange capacity is restricted (Gu and

Schulz, 1991; Sposito, 1989). The indigenous microbial population in this study represent a diverse mix of phyla which are ubiquitous in terrestrial environments and are likely to be similar to those found in the shallow subsurface of nuclear sites, e.g. Sellafield reprocessing site, UK. They are able to utilise ^{14}C-labelled carboxylate electron donors (acetate and formate) rapidly under reducing conditions, formaldehyde and methanol are both utilised quickly under denitrification conditions, but under iron-reducing conditions both ^{14}C-formaldehyde and ^{14}C-methanol may persist for longer in subsurface environments and be transported with groundwater flow.

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Declarations of interest

None.

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