Journal of Environmental Management 188 (2017) 246–254

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Elucidating carbon sources driving microbial metabolism during oil sands reclamation

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form
22 October 2016
Accepted 13 November 2016
Available online 14 December 2016

Keywords:
Reclamation
Oil sands
Composite tailings (CT)
Compound specific radiocarbon analysis (CSRA)
Microbes
Phospholipid fatty acids (PLFA)

ABSTRACT

Microbial communities play key roles in remediation and reclamation of contaminated environments via biogeochemical cycling of organic and inorganic components. Understanding the trends in *in situ* microbial community abundance, metabolism and carbon sources is therefore a crucial component of effective site management. The focus of this study was to use radiocarbon analysis to elucidate the carbon sources driving microbial metabolism within the first pilot wetland reclamation project in the Alberta oil sands region where the observation of H_2S had indicated the occurrence of microbial sulphate reduction. The reclamation project involved construction of a three compartment system consisting of a freshwater wetland on top of a sand cap overlying a composite tailings (CT) deposit. Radiocarbon analysis demonstrated that both dissolved and sediment associated organic carbon associated with the deepest compartments (the CT and sand cap) was primarily fossil ($\Delta^{14}C = -769$ to -955‰) while organic carbon in the overlying peat was hundreds to thousands of years old ($\Delta^{14}C = -250$ to -350‰). Radiocarbon contents of sediment associated microbial phospholipid fatty acids (PLFA) were consistent with the sediment bulk organic carbon pools (Peat: $\Delta^{14}C_{PLFA} = -257$‰; Sand cap $\Delta^{14}C_{PLFA} = -805$‰) indicating that these microbes were using sediment associated carbon. In contrast, microbial PLFA grown on biofilm units installed in wells within the deepest compartments contained much more modern carbon that the associated bulk carbon pools. This implied that the transfer of relatively more modern carbon was stimulating the microbial community at depth within the system. Correlation between cellular abundance estimates based on PLFA concentrations and the $\Delta^{14}C_{PLFA}$ indicated that the utilization of this more modern carbon was stimulating the microbial community at depth. These results highlight the importance of understanding the occurrence and potential outcomes of the introduction of relatively bioavailable carbon to mine wastes in order to predict and manage the performance of reclamation strategies.

1. Introduction

It is estimated that mining activities produce a total volume of 7125 Mt/year of tailings worldwide across all extractive industries (Mudd and Boger, 2013). Many reclamation landscapes designed to manage these materials involve waste materials high in organic compounds as well as sulphur and iron constituents that are important terminal electron acceptors for organic carbon degradation, so proper design and material placement with an understanding of the microbial biogeochemical cycling is required. Microbial biogeochemical cycling is an important component of the functioning of any ecosystem, including mine waste deposits. The extent and impact of this cycling in any given system is determined by the abundances, carbon sources and metabolisms of in situ microbial communities. For instance, microbial carbon cycling can remove organic contaminants from a system via mineralization during cellular metabolic activities (e.g. Megharaj et al., 2011). Numerous studies have assessed the role of these biodegradation processes in determining the fate and transport of organic compounds (Essaid et al., 2015). This microbial biogeochemical cycling can also be associated with the mobilization or generation of undesirable metabolic products, such as the hydrogen sulphide (H_2S) generated by sulphate reducing bacteria (SRBs) (Muyzer and Stams,

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http://dx.doi.org/10.1016/j.jenvman.2016.11.029
0301-4797/

2008). The sources and cycling of the inorganic reactants that are involved in redox cycling, such as sulphate concentrations in the case of the activities of SRBs, have likewise been well studied (Vile et al., 2003; Wu et al., 2013, 2011). However, in the latter cases, the organic carbon pool is often not well characterized, despite its role as the electron donor driving redox cycling.

The pool of organic carbon present in an environmental system is often complex and comprised of a wide range of compounds with a wide range of bioavailabilities. In many systems, such as soils, the biological cycling of organic compounds has been related to their age, with recent biogenic compounds cycling quickly and residual recalcitrant compounds cycling slowly based on the radiocarbon ages of soil carbon fractions and respired CO_2 (Trumbore, 2009). The development of techniques allowing direct assessment of the radiocarbon content of microbial cellular constituents such as phospholipid fatty acids (PLFA) enables a new perspective on such studies (Mahmoudi et al., 2013a, 2013b; Petsch et al., 2001; Slater et al., 2005; Whaley-Martin et al., 2016). Using this approach, the biogeochemical cycling of petroleum hydrocarbons has been shown to range from very rapid utilization and degradation by the in situ microbial community (Mahmoudi et al., 2013b) to very little degradation of the petroleum hydrocarbons due to the preferential utilization of natural organic matter (Mahmoudi et al., 2013a; Slater et al., 2005). Management of mine waste materials often involves high levels of both recalcitrant organic material and redox sensitive species that have been excavated from below ground. Since waste materials management often occurs at the ground surface, there is a considerable potential for exchange of materials and microbial communities derived from surface ecosystems with the wastes being managed, intentionally or not. This mixing has the potential to drive significant changes in biogeochemical cycling. In particular, the introduction of recently produced, bioavailable organic matter may stimulate microbial biogeochemical cycling of redox reactive species to a far greater extent than the recalcitrant organic matter associated with the mine waste materials. Such stimulation may drive generation of metabolites that require management, such as H_2S or mobilized metal constituents. Understanding the interplay between the surface environment surroundings and mine wastes is thus crucial in developing effective management strategies.

The large volume of oil sands processed by extraction plants in Alberta's oil sands mines results in some of the largest tailings facilities in the world (COSIA, 2014), holding a volume of tailings waste exceeding 700 million m^3 (Dominski, 2007). These significant volumes highlight the critical need to understand the biogeochemical process associated with the material. Reclamation of oil sands tailings is made more challenging than in other resource sectors because the waste exists as fluid fine tailings (FFT) with very slow sedimentation and consolidation rates (COSIA, 2014). One approach used to manage FFT is to amend it with gypsum to reduce the double diffusive layer around the fine sized (<44 μm) clay minerals that are present and then combine with sand. After dewatering, the resulting more consolidated mixture of sand, residual bitumen, clay fine and gypsum is referred to as composite tailings (CT).

Syncrude is currently undertaking the first pilot scale wetland reclamation project in the Alberta Oil Sands Region. A goal of this project was to construct the initial conditions to allow the development of a fen wetland above a deposit of CT over time. This wetland watershed was constructed on a sand-capped CT deposit and is a permanently reclaimed area that will contribute to the final closure landscape as committed to in regulatory approvals (Wytrykush et al., 2012). This pilot watershed research facility will provide data to support future large-scale wetland reclamation projects and address challenges associated with tailings reclamation providing important insight for the management of oil sands tailings specifically and for reclamation efforts more broadly. Early in the wetland construction process (2009) H_2S gas was episodically detected associated with surface dewatering wells, suggesting the occurrence of microbial sulphate reduction within the underlying CT and/or sand cap materials. Hot water extraction of sand removes 88–95% of bitumen (Masliyah et al., 2004) and the residual bitumen organic carbon present in CT is highly recalcitrant, so that microbial sulphate reduction was assumed to be limited by access to labile organic carbon. However, the observation of H_2S generation raised the question whether introduction of younger, relatively more labile organic carbon from the developing wetland was stimulating microbial sulphate reduction within the system (Reid and Warren, 2016). The objectives of the current study were to elucidate the carbon sources being utilized by the in situ microbial communities within the fen/CT system. Specifically, we investigated whether inputs of more modern, more bioavailable organic carbon sources from the surface environment were driving increased microbial activity and stimulating H_2S production.

This study focussed on both un-reclaimed (no reclamation soil cover placed) and reclaimed (reclamation soil cover placed) CT. For the purposes of this study the reclamation project was divided into three “compartments”. The deepest compartment was the ~35 m of CT that had been deposited in Syncrude's East In Pit. The intermediate compartment was a ~10 m “sand cap” that had been placed over the CT deposit. The uppermost compartment consisted of a 0.5 m layer of clay overlain by a 0.5 m layer of peat salvaged from mine advancement. The peat layer was planted with local plant species and flooded in order to establish the initial conditions for peat forming wetlands to develop over time (Fig. 1). Characterization of microbial carbon sources and abundances was achieved by collecting samples that represented each compartment (surface peat, sand cap, and CT) of the system using a range of approaches. This included surface collection of solid matrix materials where accessible, installation of biofilm units in monitoring wells within the sand cap and CT compartments, and direct drilling to sample the CT deposit at an adjacent unreclaimed site. The abundance of microbial biomass within each compartment was determined via phospholipid fatty acid (PLFA) analysis. PLFA degrade within days to weeks after cell death (Harvey et al., 1986; White et al., 1979) and thus represent the viable bacterial and microeukaryotic community at a site. Concurrent radiocarbon values of potential carbon sources (total organic carbon (TOC), residue after solvent extraction (extracted residue: EXT-RES), and dissolved organic carbon, (DOC)), combined with compound specific radiocarbon analysis (CSRA) of PLFA enabled determination of microbial carbon source utilization by these microbial communities. This approach is based on the fact that petroleum hydrocarbons are millions of years old and thus contain no significant ^{14}C ($\Delta^{14}C = -1000‰$) while organic carbon recently produced from the atmosphere will have modern levels ($\Delta^{14}C$ = ~55‰ (Turnbull et al., 2007)). Since $\Delta^{14}C$ is normalized to a $\delta^{13}C$ of −25‰ during data handling to remove the effects of biosynthetic fractionation (Stuiver and Polach, 1977), the $\Delta^{14}C$ of microbial phospholipids can be directly compared to potential carbon sources to apportion the extent of their utilization. This approach has been used to directly identify microbial carbon sources during intrinsic (Ahad and Pakdel, 2013) and engineered bioremediation (Cowie et al., 2010, 2009), as well as the lack of significant degradation of recalcitrant petroleum hydrocarbons in the presence of more labile carbon sources (Mahmoudi et al., 2013a; Slater et al., 2005). Recently, this technique has been used to identify the role of inputs of relatively modern, bioavailable organic carbon in driving release of arsenic into shallow groundwater in Bangladesh (Whaley-Martin et al., 2016). However, to our knowledge, this is the first time that it has been applied to address carbon sources driving microbial cycling at a site where mine

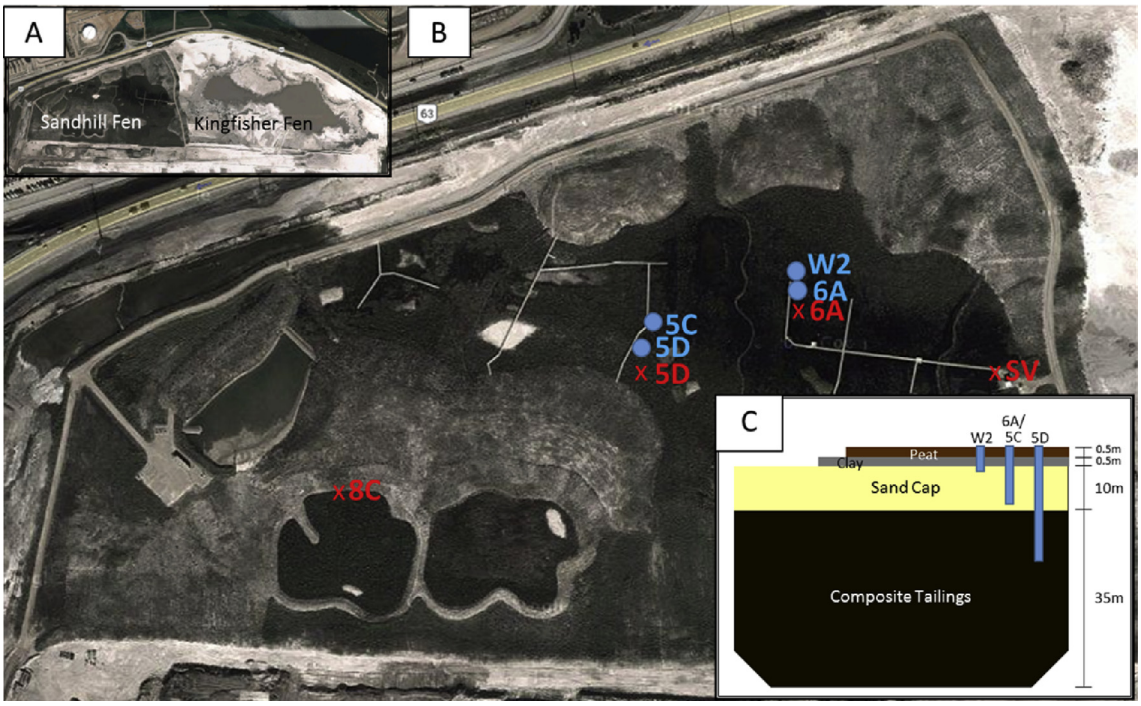

Fig. 1. Map of the study site. A) Sandhill Fen reclamation site and adjacent un-reclaimed Kingfisher Fen composite tailings deposit; B) Sandhill Fen with surface sampling locations (red X) and well locations (blue circle); C) cross section of layers in (not to scale), and sampling well depths. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

wastes are being managed. This insight into the interplay between more modern, bioavailable organic carbon and older, recalcitrant carbon provided by this study will not only inform the management of oil sands tailings, but also other situations where mine wastes containing both recalcitrant organic matter and reduced redox sensitive species occur.

2. Methods and materials

2.1. Site description

Sandhill fen is a full-scale pilot wetland reclamation project located in Syncrude's East In Pit (57°02'23.6″N 111°35'30.0″W, Fort McMurray, AB), a previously mined area which has since been filled with for CT and tailings sand (Fig. 1) (Wytrykush et al., 2012). The site consists of three major compartments: 1) the CT deposit; 2) the overlying sand cap; and 3) the surface peat/clay and fen system. Prior to 2009, ~35 m of CT had been hydraulically deposited in the East In Pit. Beginning in 2009, 10 m of tailings sand (downstream of the bitumen extraction process) was hydraulically placed on top of the nominally 35 m of CT. After tailings deposition, reclamation activities began. Placement of soil layers from stockpiles was done with trucks and dozers. Clay placement (0.5 m) in the fen footprint began in the winter of 2009. In early 2011, 0.5 m of peat material recently salvaged from a nearby site was placed on top of the clay layer. This peat was then seeded with fen vegetation (Wytrykush et al., 2012). In May 2012, the fen was flooded with fresh water from the Mildred Lake Reservoir to establish conditions for a freshwater wetland. Sets of samples were collected over the course of wetland construction and establishment (July 2011, May 2012, August 2012, November 2012, December 2012, July 2013, September 2013 and June 2014). Specific samples collected at each time varied based on the materials that were accessible and the addition of new sampling approaches, particularly the biofilm units which were collected in September 2013 and June 2014.

2.2. Surface fen sample collection

Surface samples were collected from a number of sites (Fig. 1) during various stages of fen construction from depths of 10–30 cm below the surface. Sampling of solid materials was restricted to the upper 50 cm of the fen system. Initially solid material samples were collected from exposed sand cap, however after the clay and peat placement, surface samples primarily consisted of peat material. Sand cap samples were collected opportunistically from surface exposures when available. These surface samples allowed assessment of the microbial community abundance and carbon sources initially in the sand cap and then in the developing fen. Surface samples were collected from two sites, 6A and sump vault (SV) at all time points, and at selected time points from sites 8C and 5D (Fig. 1). Solid material samples were collected using a metal shovel and sterile silicone spatula and subsequently sealed in sterile Whirlpak (Nasco) bags. All tools were sterilized with 70% ethanol immediately prior to use. All field samples were kept on ice until they could be frozen and stored at −20 °C.

2.3. Solid CT sample collection

As described elsewhere (Reid and Warren, 2016), solid CT samples were collected from depth via coring of the CT deposit at the adjacent Kingfisher Fen site (Fig. 1). The Kingfisher site overlies the same CT deposit as the Sandhill fen, but was accessible to drilling as the proposed second test of the dry reclamation approach at this site had not yet been initiated. Kingfisher fen CT (KFCT) samples were obtained in December 2012 using an amphibious track-mounted drill platform. Samples were collected using core tubes with a diameter of 50 mm and extruded in 2 m increments into sterile Whirlpak bags (Nasco) within an Atmosbag

glove bag (Sigma Aldrich) filled with nitrogen gas. After the Whirlpak bags were sealed, they were kept on ice until they could be frozen and stored at −20 °C.

2.4. Biofilm unit collection

In order to assess microbial carbon sources at depth at the Sandhill Fen site, "biofilm units" were deployed in monitoring wells that accessed the surface, sand cap, and CT compartments of the fen system. "Biofilm units" consisted of perforated Teflon (Chemours) tubes packed with pre-combusted glass wool to provide a large surface area for microbial growth. Prior to emplacement, biofilm units were cleaned via immersion in a series of hexane, acetone and methanol, to ensure they were sterile and carbon free. A number of different wells were sampled that collected material at different depths between the CT and peat surface. Well W2 was screened at 2 m depth and sampled as close as possible to the interface of the peat and the sand cap. Wells 6A and 5C were screened at a depth of 8 m such that they sampled the bottom of the sandcap. Well 5D was screened at 16 m, within the underlying CT compartment. Biofilm units were suspended at the bottom of wells over two time periods: July-Sept 2013 and Sept 2013-June 2014. Upon collection, biofilm units were sealed inside sterile Whirlpak bags and kept on ice until they could be frozen and stored at −20 °C. Detailed descriptions of the aqueous geochemistry of each compartment have been previous reported in Reid and Warren (2016). Briefly, pH varied between 6.49 and 8.68, consistent with previously reported ranges in Syncrude tailings ponds (Fedorak et al., 2003; Holowenko et al., 2000). Temperature as measured during summer campaigns was 10.26–20.53 °C, showing no clear pattern with depth. Dissolved oxygen saturation at the surface (<0.5 m depth) was 62–107%, and 0% at all lower depths.

2.5. Bulk analyses: dissolved organic carbon (DOC), total organic carbon (TOC), and extracted residue (EXT-RES)

Water samples that corresponded to the biofilm unit deployments were collected for DOC analysis in Sept 2013 and June 2014 using polyethylene tubing and an inertial lift (Waterra) pump system. Samples were collected and analyzed as per Reid and Warren (2016). Briefly, wells were purged of ~3x well volume before collecting sample water into precombusted glass bottles. Samples were kept on ice until they could be frozen and stored at −20 °C. DOC concentrations were measured as per Reid and Warren (2016) by filtering water through a syringe-driven 0.7 μm GF/F glass microfiber filter unit (GE Life Sciences), and analyzing the filtrate with a Shimadzu TOC-L Total Organic Carbon Analyzer with an autosampler ASI-L using the 680 °C combustion catalytic oxidation method recommended by the manufacturer (Mandel Scientific). Standard error for DOC concentrations was <4% (Table S2). DOC samples for radiocarbon analysis were thawed and vacuum filtered through pre-combusted glass microfiber 0.7 μm filters (Whatman grade GF/F), then freeze-dried using a 4K BT XL-105 desktop model VIRTIS freeze dryer, leaving behind solid material that was treated with 1 N $HCl_{(aq)}$ and dried overnight at 60 °C to remove carbonate in the samples. This was repeated until no fizzing was visible on addition of acid (maximum 3 times). The resulting carbonate free residues were analyzed as described below.

Samples for TOC analysis were dried in an oven overnight at 60 °C. In order to analyze the non-solvent extractable organic carbon, solid residues after solvent extraction were also dried in an oven overnight at 60 °C. These samples were analyzed as "EXT-RES" and represented the organic carbon in the solid samples not removed by solvent extraction. This operational definition is based on White et al. (2005). Organic carbon concentrations were obtained by measuring total carbon (TC) and total inorganic carbon (TIC) with a Shimadzu TOC-L Total Organic Carbon Analyzer with a solid sampler SSM-5000A attachment (Mandel Scientific), according to the manufacturer protocol, and subtracting TIC values from TC values. Standard error of replicate TOC measurements was <4% (Table S1) for peat and sand samples, and 20% for KFCT. TOC and EXT-RES samples for ^{14}C analysis were treated with 1 N $HCl_{(aq)}$ and dried overnight at 60 °C to remove carbonate in the samples. This was repeated until no fizzing was visible on addition of acid (maximum 3 times). The resulting carbonate free residues were analyzed as described below.

2.6. Phospholipid fatty acid (PLFA) extraction

Phospholipid fatty acid concentrations were determined using the modified Bligh and Dyer method (Bligh and Dyer, 1959), with solvent in a ratio of 1:2:0.8 dichloromethane (DCM): methanol (MeOH): 0.1 M phosphate buffer. For solid material, between 47 and 174 g wet sediment (equivalent to 16–53 g dry weight) were submerged in solvent overnight, while biofilm units were submerged in their entirety overnight. Solvent was filtered through pre-rinsed 1.5 μm pore filter paper (Whatman 934 AH), then phase separated with milliQ ultrapure water. The organic fraction was collected as the total lipid extract (TLE) and subsequently concentrated using a rotary evaporator (Brinkmann) and separated into three fractions (f_1: DCM, f_2: acetone, f_3: MeOH) by gravity column chromatography with fully activated silica gel (Aldrich; particle size 63–200 μm, pore size 0.7–0.85 cm^3/g). The methanol fraction (f_3 containing PLFA) was evaporated to dryness under a gentle stream of ultrahigh purity nitrogen. PLFA were then converted to fatty acid methyl esters (FAMEs) by mild alkaline methanolysis using isotopically characterized MeOH, and the resulting FAMEs were purified by secondary silica gel chromatography.

PLFA concentrations were analyzed using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 single quadrupole mass spectrometer. Chromatographic separation was performed using DB-5MS column (30 m × 0.25 mm × 0.25 μm film thickness) with an initial temperature of 50 °C (1 min) with the following ramps: 20 °C/min to 130 °C, 4 °C/min to 160 °C, 8 °C/min to a final temperature of 300 °C (5 min). PLFA were identified using retention times and fragmentation patterns via comparison to the Bacterial Acid Methyl Ester (BAME) Mix (Supelco). PLFAs were quantified using commercially available external standards and reproducibility was better than ±5%.

All solid samples were analyzed in duplicate for PLFA concentrations but due to limited sample material biofilm samples could not be processed in duplicate. Lipid concentrations are reported on a mass basis of PLFA normalized to the total mass of solid sample used (ng/g) or total quantified moles for the biofilm units. PLFA concentrations were used to generate estimates of cellular abundances based on a mean conversion factor of 4.0×10^4 cells/pmol PLFA (Green and Scow, 2000).

2.7. Radiocarbon analysis of PLFAs, TOC, EXT-RES and DOC

The ^{14}C content of PLFAs in solid samples was determined using two types of sample preparation techniques on the same sample, which were confirmed to be consistent by testing a subset of samples with both approaches (details in SI). In the first method, bulk PLFA extracts were purified and concentrated using preparative capillary gas chromatography (PCGC) per Slater et al. (2005). Bulk PLFAs were separated from any other carbon potentially present in the PLFA fraction on an Agilent gas chromatograph (DB-5 60 m × 0.53 mm x 0.25 μm film thickness) interfaced with a Gerstel

preparative fraction collector (PFC) system. Using this system, samples were repeatedly injected and material from selected retention times was trapped in prebaked Pyrex U-tubes at temperatures below freezing. Collected PLFAs were transferred into prebaked GC autosampler vials using 1 mL of dichloromethane. Samples were then rerun on the GC/MS to ensure sample purity. The second method of ^{14}C analysis of PLFA measured the ^{14}C content of entire PLFA containing fraction after silica gel purification without any chromatographic separation. This approach was utilized for some solid samples and for the biofilm units. Of the four tests compared this way, three were the same within analytical error (i.e. a difference of less than 40‰: See supporting information). One sample was slightly outside of analytical error (difference of 83‰) however, this difference was minor in comparison to the variations between samples in the study and thus was not considered an issue during data interpretation.

Radiocarbon content of PLFA, EXT-RES, DOC and TOC samples were analyzed at one of the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) facility at Woods Hole Oceanographic Institute, the W.M. Keck Carbon Cycle Accelerator Mass Spectrometry lab at University of California Irvine, or the Center of Applied Isotope Studies (CAIS) at the University of Georgia. All samples were transferred to prebaked 6 mm quartz tubes and converted to CO_2 using closed tube combustion. The resulting CO_2 was purified, quantified and reduced to graphite using standard procedures. Samples processed at NOSAMS and CAIS also reported the ^{13}C content of the combusted CO_2.

The reported ^{14}C data is in $\Delta^{14}C$ notation expressed in ‰, which is the deviation of a sample from the 95% activity in 1950 A.C. of Natural Bureau of Standards (NBS) oxalic acid 1 standard normalized to a $\delta^{13}C$ value of −25‰ (Stuiver and Polach, 1977). $\Delta^{14}C$ values of the PLFAs were corrected during data processing for the addition of menthol carbon during transesterification. The measurement uncertainty for $\Delta^{14}C$ of TOC, EXT-RES and DOC is the AMS reported error. The reported uncertainty of the $\Delta^{14}C$ PLFA was one standard deviation of the mean or the conventional error for PLFA analysis which was assumed to be ±20‰ and includes uncertainty from the AMS measurements as well as sample preparation and handling.

2.8. Statistical analyses

Data sets were too small to assume normal distributions, therefore non-parametric statistical analyses were carried out using IBM SPSS 22.0 software. The Mann-Whitney *U* Test (Mann and Whitney, 1947) was used when investigating the difference between two means, and the Kruskal-Wallis test (Kruskal and Wallis, 1952) was used when comparing >2 means.

3. Results and discussion

3.1. Bulk carbon pools

3.1.1. Solid materials

Total organic carbon concentrations were generally higher in the surface peat samples (mean 146 ± 115 mg/g (n = 7), range 18–301 mg/g) than in the underlying sand (mean 18 ± 18 mg/g (n = 4), range 7.7–45.5 mg/g) (Table S1). Mean TOC concentrations of 8.7 mg/g in the sand were consistent with KFCT samples reported in Warren et al. (2015), which ranged from 7.5 to 12.2 mg/g (n = 5). Radiocarbon contents of the bulk carbon phases varied between compartments with the most modern (highest $\Delta^{14}C$) occurring in the surface peat and most ancient (lowest $\Delta^{14}C$) in the sand and CT. The $\Delta^{14}C$ of TOC and extracted residues were very similar for the peat samples (mean $\Delta^{14}C_{TOC}$ of −349 ± 104‰ (n = 7); mean $\Delta^{14}C_{EXT\text{-}RES}$ of −298 ± 73‰ (n = 7) (Table 1, Fig. 2). $\Delta^{14}C_{EXT\text{-}RES}$ values were within error or more modern than corresponding $\Delta^{14}C_{TOC}$ with one exception in September 2013. These results indicated a consistent source for these carbon pools that is hundreds to thousands of years old, as would be expected for peat deposits (Zoltai, 1991). The minor variation between the TOC and EXT RES carbon pools is likely due to sample heterogeneity and is not considered to be significant with the resolution of this study.

In contrast to the peat samples, radiocarbon contents of the bulk carbon phases present in the sand samples had high proportions of fossil carbon, with one exception. Sand $\Delta^{14}C_{TOC}$ had a mean value of −866 ± 185‰ (n = 8) and sand $\Delta^{14}C_{EXT\text{-}RES}$ had a value of −687 ± 194‰ (n = 5) (Table 1, Fig. 2). The relatively large variation in these mean sand $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{EXT\text{-}RES}$ values was driven by one sample from August 2012, which was not as depleted in ^{14}C as the other samples with a $\Delta^{14}C_{TOC}$ of −423‰ and $\Delta^{14}C_{EXT\text{-}RES}$ of −360‰. If these outlier values are removed the mean $\Delta^{14}C_{TOC}$ of the sand is −929 ± 49‰ and the mean $\Delta^{14}C_{EXT\text{-}RES}$ is −769 ± 74‰. Given that the August 2012 $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{EXT\text{-}RES}$ values were both within the range of values observed for surface peat samples (Table 1, Fig. 2), the most obvious interpretation is that this sample has been influenced by the presence of the peat and is not representative of the sand compartment. This sample was thus removed from further data comparisons. In all cases, the sand $\Delta^{14}C_{EXT\text{-}RES}$ was more modern than the $\Delta^{14}C_{TOC}$. For both peat and sand, the fact that the EXT-RES contained younger carbon than the TOC indicates that the carbon removed during solvent extraction contained fossil carbon. Finally, the $\Delta^{14}C_{TOC}$ of the two KFCT samples collected at 4 and 32 m depths in the CT deposit that underlies the Sandhill fen contained the highest proportion of fossil carbon (−955 ± 46‰) (Table 1, Fig. 2).

The proportion of fossil carbon in the sand and CT compartments can be assessed via an isotopic mass balance between petroleum carbon ($\Delta^{14}C = -1000$‰) and the isotopic compositions of potential end member sources (Equation (1)). The two most applicable end members for this comparison are modern atmospheric carbon or peat derived carbon. Equation (1) is formulated to determine the fraction of petroleum carbon as compared to atmospheric inputs. Replacing the $\Delta^{14}C_{atmosphere}$ with $\Delta^{14}C_{peat}$ enables the same comparison for the other potential end member source.

$$f(petroleum) = \frac{\Delta^{14}C_{measured} - \Delta^{14}C_{atmosphere}}{\Delta^{14}C_{petroleum} - \Delta^{14}C_{atmosphere}} \quad (1)$$

If it is assumed that the modern carbon inputs to the CT occurred during the filling and settling activities that formed the CT deposit (which is open to atmospheric deposition and has potential for some occurrence of surface growth), then inputs of carbon with modern values representative of the last 30 years would be expected. Using a $\Delta^{14}C$ of 55‰ (Turnbull et al., 2007) to represent such inputs, the isotopic mass balance indicates a maximum contribution of 4% modern carbon (96% fossil C; Table 2). If inputs to the CT system were derived from detrital inputs from soil erosion or dust, the $\Delta^{14}C$ of these inputs might be expected to be representative of the surrounding peat landscapes. Using the mean $\Delta^{14}C$ of the peat EXT-RES measured in this study as representative of such modern inputs, the isotopic mass balance indicates a slightly greater maximum contribution of 6% peat carbon (94% fossil; Table 2). Thus, while there is evidence of the presence of some relatively modern carbon within the CT, these inputs are negligible and 94–96% of the carbon in the CT is fossil, consistent with being petroleum derived.

In the sand samples both $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{EXT\text{-}RES}$ showed evidence of greater modern carbon inputs. $\Delta^{14}C_{TOC}$ values

Table 1
Mean $\Delta^{14}C$ for samples from each compartment in per mil (‰). Reported variance is ± one standard deviation of the mean or the conventional error for PLFA measurement (20‰), whichever is larger.

	Sample type	$\Delta^{14}C \pm SD$ (‰)	Number of Samples
Surface sand (outlier removed)	TOC	−929 ± 49	7
	EXT-RES	−769 ± 74	4
	PLFA	−913 ± 20	3
Surface sand (with outlier)	TOC	−866 ± 185	8
	EXT-RES	−687 ± 194	5
	PLFA	−805 ± 215	4
Surface peat	TOC	−349 ± 104	7
	EXT-RES	−298 ± 73	7
	PLFA	−257 ± 70	7
KFCT	TOC	−955 ± 46	2
Depth samples	W2 DOC (2 m)	−250 ± 28	2
	W2 Biofilm PLFA (2 m)	−217 ± 20	2
	5C/6A DOC (8 m)	−838 ± 118	6
	6A Biofilm PLFA (8 m)	−406 ± 20	1
	5D DOC (16 m)	−903 ± 35	2
	5D Biofilm PLFA (16 m)	−721 ± 86	2

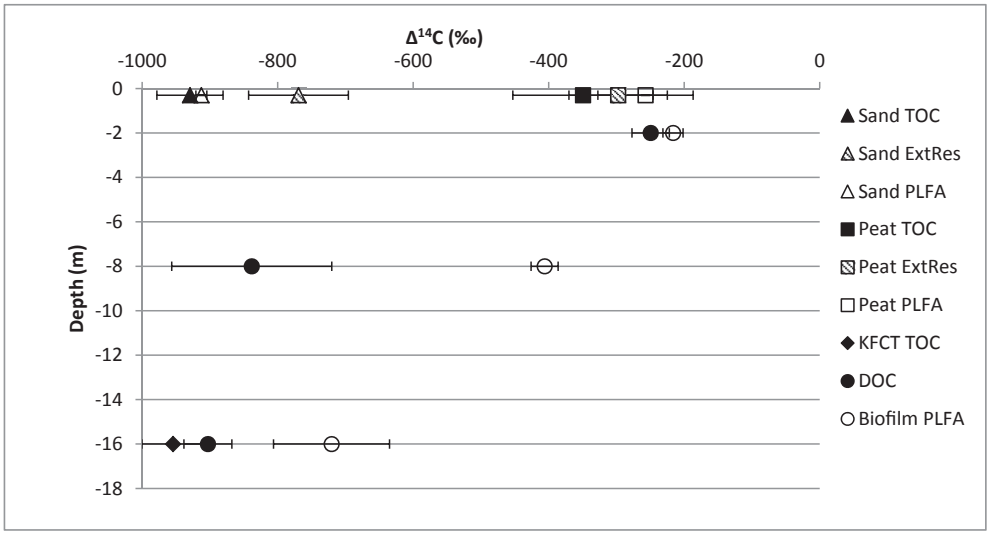

Fig. 2. Mean radiocarbon signatures of total organic carbon (TOC), extracted residue (EXT-RES), dissolved organic carbon (DOC) and PLFA from each compartment. Error bars are one standard deviation around the mean or the conventional error for PLFA measurement (20‰), whichever is larger. Note sand values are calculated after removal of the outlier value (Aug 2012).

Table 2
Mass-balance calculated contributions of possible carbon sources to measured samples.

	Fraction petroleum (−1000‰)	
	vs. Modern C (+55‰)	vs. Mean peat C (−298‰)
Sand TOC	0.93	0.90
Sand EXT-RES	0.78	0.67
Sand PLFA	0.92	0.88
KFCT TOC (16 m)	0.96	0.94
	Fraction DOC at depth	
	vs. Modern C (+55‰)	vs. DOC from 2 m (−250‰)
2 m PLFA	0.89	–
8 m PLFA	0.52	0.27
16 m PLFA	0.81	0.72

of −929 ± 49‰ were comparable to those observed for the CT. Using the same isotopic mass balance approach (Eq. (1)), the relative contribution of modern carbon at this depth in the sand is limited to 7–10% input of either modern or peat age carbon respectively (93% or 90% fossil; Table 2). Interestingly, the $\Delta^{14}C_{EXT\text{-}RES}$ values indicated that the non-extractable carbon pool contains relatively more modern carbon than the TOC pool. In this case, an isotopic mass balance with the modern atmosphere or peat derived carbon indicates a maximum potential contribution of 22–33% (78%–67% fossil; Table 2) in the EXT-RES. Given the fact that these samples were collected from the surface of the sand cap material, either of these sources would be reasonable. Notably, the majority of the carbon in the sand was still fossil, consistent with being petroleum derived.

3.1.2. Dissolved organic carbon (DOC)

DOC concentrations in the monitoring wells where biofilm units were deployed were generally consistent with depth ranging from a mean of 61 mg/L at 2 m depth to 72 mg/L at 8 m depth and 70 mg/L at 16 m depth (Reid and Warren, 2016). These values were consistent with mean DOC values from other samples at the site that ranged from 21 to 127 mg/L. There was no apparent trend in DOC concentrations with either depth or location. The $\Delta^{14}C_{DOC}$ varied with depth. At 2 m depth in the peat compartment the $\Delta^{14}C_{DOC}$ had a mean value of −250 ± 28‰ (n = 2). This decreased to

a mean $\Delta^{14}C_{DOC}$ = −838 ± 118‰ (n = 6) at 8 m depth near the bottom of the sand cap and further to a $\Delta^{14}C_{DOC}$ of −903 ± 35‰ (n = 2) at 16 m within the CT (Table 1, Fig. 2). At all depths, the $\Delta^{14}C_{DOC}$ was within the range of variability observed for the bulk sedimentary carbon sources at each depth, indicating that the bulk OC pools were the source of the DOC, consistent with previous studies that used radiocarbon contents to identify the source of DOC and its relationship to bulk organic matter pools (Evans et al., 2007; Kalbitz and Geyer, 2002; Whaley-Martin et al., 2016).

3.2. PLFA – microbial carbon sources

3.2.1. PLFA based microbial abundances and carbon sources

PLFA concentrations were higher in the peat ($2.4 \times 10^4 \pm 1.4 \times 10^4$ ng/g, n = 12) as compared to the sand ($1.4 \times 10^3 \pm 1.3 \times 10^3$ ng/g, n = 8) and the KFCT, which was orders of magnitude lower, ranging from 45 to 82 ng/g at five depths (Reid and Warren, 2016). The Mann-Whitney *U* Test shows that PLFA concentrations in the sand samples are significantly different from those in the peat samples ($p = 1.034 \times 10^{-4}$). Due to the high variation within extraction replicates, results from non-parametric statistical testing show that variations in PLFA concentration within each group (6A sand, 6A peat, SV sand, SV peat) do not achieve statistical significance and can be attributed to natural variation and/or heterogeneity within the sites. Cellular abundances, as calculated from the PLFA concentration, were 2.9×10^9 cells/g for the peat samples, 1.6×10^8 cells/g for the sand samples, and 6.0×10^6 cells/g for the KFCT samples. PLFA distributions and stable carbon isotopic compositions ($\delta^{13}C$) of carbon pools and PLFA did not show any insightful variations between samples/sites and are thus not shown.

3.2.2. CSRA of microbial lipids in peat, sand cap, and biofilm units

In general, the $\Delta^{14}C_{PLFA}$ of the solid material samples tracked the $\Delta^{14}C$ of the bulk carbon phases. The mean $\Delta^{14}C_{PLFA}$ of the peat samples (−257 ± 70‰, n = 7) was within error of the $\Delta^{14}C_{EXT\text{-}RES}$ but was slightly more positive than the $\Delta^{14}C_{TOC}$. If individual sample pairs were considered, the $\Delta^{14}C_{PLFA}$ were within error (±20‰, n = 4) or contained more modern carbon (n = 3) than the corresponding EXT-RES, while the TOC generally contained a slightly greater component of fossil carbon. Thus it appears that the microbial community is utilizing carbon derived from the bulk organic matter of the peat, which involves relatively modern carbon.

In the sand samples, the $\Delta^{14}C_{PLFA}$ was −805 ± 215‰ (n = 4) when the August 2012 sample was included. Exclusion of this outlier sample which shows influence of peat derived carbon inputs gave a mean $\Delta^{14}C_{PLFA}$ of −913 ± 8‰ (n = 3), though for further data analysis the uncertainty on this value was assumed to be the ±20‰ associated with the analysis. With the outlier removed, the $\Delta^{14}C_{PLFA}$ was the same within error as the $\Delta^{14}C_{TOC}$ and more negative than the $\Delta^{14}C_{EXT\text{-}RES}$. This indicates that the PLFA are derived from the TOC pool and that the microbes are not utilizing the EXT RES pool despite the presence of more modern C. However, it must be noted that all of the carbon in the sand cap (TOC and EXT RES) is primarily fossil in nature and so the EXT RES pool may still be expected to be highly recalcitrant.

The PLFA extracted from the biofilm units represents microbial growth that occurred over the period of their installation. While cell density cannot realistically be estimated, the $\Delta^{14}C_{PLFA}$ of the biofilm units directly reflects the carbon source being utilized by the newly grown microbial biomass. At 2 m (Well W2) the $\Delta^{14}C_{PLFA}$ of the biofilm units was −217 ± 20‰ (n = 2), within error of the $\Delta^{14}C_{PLFA}$ of the surface peat samples and the $\Delta^{14}C_{DOC}$ at that depth. Thus the aqueous microbial community and the community associated with the solid substrates were using the same carbon sources. It is possible that the PLFA at this depth contain a mixture of modern carbon inputs combined with inputs from the DOC pool. In this case, a modern component with $\Delta^{14}C$ of +55‰ would have contributed a maximum of 11% of the carbon in the PLFA (DOC contribution 89%; Table 2). However, this input is relatively minor and as there was no direct evidence of modern carbon in this system, the simplest explanation is that the microbial community was utilizing the peat derived carbon pool.

In contrast, the $\Delta^{14}C_{PLFA}$ was more modern than the corresponding DOC and bulk carbon phases in the sand cap and CT compartments. The greatest indication of bacterial utilization of more modern carbon within the available pool occurred at 8 m depth in the sand cap. The draining of water between the sand and wetland layers, and pumps flushing water through deeper layers of the site, have caused complex flow in the sand cap that may have encouraged downward movement of surface carbon (Reid and Warren, 2016). In the sand cap, the average $\Delta^{14}C_{PLFA}$ was 430‰ higher than the $\Delta^{14}C_{DOC}$, indicating a much greater input of more modern, high $\Delta^{14}C$ carbon. This observation is somewhat surprising given that the $\Delta^{14}C_{DOC}$ indicated that DOC was derived from the bulk TOC of the sand cap. However, as the abundances of the microbial community are small relative to the DOC concentrations, even relatively small inputs of more modern carbon may be able to support microbial community activity. Assuming that the most likely source of more modern carbon to the microbial community at this depth is the fen system that had been installed above, the potential inputs can be calculated by isotopic mass balance by adjusting Equation (1). If the two end members are assumed to be the local DOC at 8 m and DOC at 2 m (representing peat-derived surface carbon), the mass balance indicates 73% of the microbial carbon is peat derived (27% DOC at depth; Table 2). Again, the possibility of modern inputs being transported to 8 m depth and utilized exists. If so, the contribution of modern carbon (+55‰) to the microbial PLFA decreases to approximately 50%. Again, however, given that none of the carbon pools at any depth, including the PLFA pool, indicated inputs of modern atmospheric carbon, this scenario must be considered less likely. Overall therefore, the evidence indicates that the carbon used by the microbial community at 8 m depth is more than 70% derived from peat-based surface sources despite the DOC pool being consistent with the bulk TOC of the sand compartment.

Within the CT, the biofilm $\Delta^{14}C_{PLFA}$ again contained more modern carbon than the corresponding DOC (difference of 180‰), however, to a much lesser extent than in the sand cap. As in the sand cap, this more modern carbon may have been derived from inputs via the DOC pool, and there is evidence of very little presence of modern carbon within DOC pool at 16 m (>96% fossil derived, Table 2). Isotopic mass balance between the $\Delta^{14}C_{DOC}$ at 16 m and peat equivalent carbon as defined by $\Delta^{14}C_{DOC}$ at 2 m indicates PLFA is comprised of 28% peat-derived carbon (72% from DOC at depth; Table 2). If the higher $\Delta^{14}C$ carbon is instead modern carbon with $\Delta^{14}C$ of 55‰, the contribution is 19% modern.

3.3. Implications to microbial community distribution

The results of this study demonstrate that while the microbial community present at depth in the system was still using fossil carbon derived from either TOC or petroleum hydrocarbons, they were also using large proportions of more modern carbon, likely derived from the surficial peat, during their growth. This effect is most notable in the sand cap interface between the surface fen construction and the CT. Preferential metabolism of younger carbon over petroleum hydrocarbons has been reported in oil sands tailings ponds (Ahad and Pakdel, 2013), other petroleum contaminated

sites (Mahmoudi et al., 2013a, 2013b; Slater et al., 2005), and marine systems (Cherrier et al., 1999; Pearson et al., 2001). The results presented here indicate the preferential microbial uptake of higher $\Delta^{14}C$ carbon available as only a minor component in the DOC being transported to sand and CT layers. Such preferential uptake of more modern carbon highlights the importance of modern carbon inputs in supporting microbial activity at depth. If the microbial utilization of this more modern carbon component is rapid due to its limited availability or greater bioavailability, it would be removed from the system quickly, keeping the $\Delta^{14}C_{DOC}$ value low and comparable to the bulk TOC.

This influence of the age (as an indicator of recalcitrance) of carbon sources being utilized by the in situ microbial community is borne out by the distribution of cellular abundances within the system. The lowest cellular abundances were found in the KFCT samples at 6×10^6 cells/gram (Fig. 3, Table S2). These cellular abundances are on the low end that is expected for subsurface systems. Sand cap cellular abundances were almost two orders of magnitude higher (1.6×10^8 cells/g), at the higher end of expectations for subsurface systems. Finally, peat cellular abundances were the highest (2.9×10^9 cells/g) which is consistent with biomass in sediment of natural peat wetlands (D'Angelo et al., 2005; Del Rio, 2004; Golovchenko et al., 2007). PLFA concentrations varied significantly ($p = 1.034 \times 10^{-4}$) between compartments and were correlated with the $\Delta^{14}C_{PLFA}$, with an exponential relationship with an r^2 of 0.87 (Fig. 3 A). Conversely, the exponential relationship between PLFA concentration and TOC concentration had an r^2 of 0.17 (Fig. 3 B), suggesting that the impact of age of input carbon on cellular abundances can be far greater than indicated by input carbon concentrations alone.

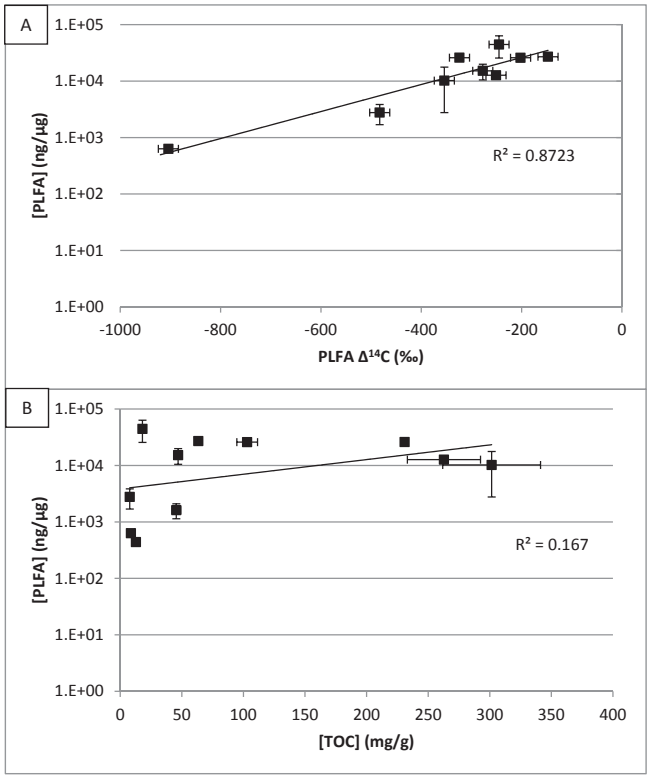

Fig. 3. PLFA concentrations from surface samples plotted against A) $\Delta^{14}C_{PLFA}$. Vertical error bars are 1 standard deviation, horizontal error bars 20‰; B) total organic carbon concentration. Vertical error bars are 1 standard deviation, horizontal error bars one standard error. Trend lines are exponential.

4. Conclusion and implications to site management

Collectively, the insights generated by these results indicate that the presence of even minor contributions of more modern carbon can strongly stimulate microbial community activity. In this study, even though the DOC in the sand cap had a maximum of 14% contribution of peat derived carbon (or only 9% of modern carbon), the PLFA of the microbial community at this depth was comprised of more than 70% peat age equivalent carbon (or 53% modern carbon). Given that the cellular abundances in the sand cap were approaching levels expected for surface systems (10^8 cells/g) and that cellular abundances in the sand cap were correlated with the radiocarbon content of the carbon they were utilizing as reflected by the $\Delta^{14}C_{PLFA}$, this demonstrates that a relatively small input of modern carbon can be an important driver of cellular abundance and by extension microbial activity. Since there is evidence that much of the microbial activity within the sand cap is related to sulphur cycling (Reid and Warren, 2016), these observations imply that these inputs of more modern carbon can greatly contribute to the generation of metabolites such as H_2S that represent a potential management hazard at this site. These observations can also be generalized beyond this site to other mine waste management settings where bioavailable carbon could potentially be transported into zones containing redox reactive species. Prior to their extraction, the biogeochemical cycling of redox reactive species present in mined materials may be expected to be minimal due to low availabilities of bioavailable carbon in subsurface systems. However, reclamation of waste materials often occurs on land surfaces in close proximity to, or integrated within, surface ecosystems, as was the case for the constructed wetland reclamation system in this study. This proximity of bioavailable carbon and redox reactive species can result in a reactive interface driven by microbially-mediated biogeochemical cycling. One possible outcome of the influx of labile, more modern carbon to such as system would be stimulation of the breakdown of petroleum compounds via the priming effect (Fontaine et al., 2004, 2003; Kuzyakov et al., 2000) which would be a benefit to site management. However, increased levels of biogeochemical cycling may also induce generation of unexpected metabolic products, such as H_2S or mobilized metals. The results of this study demonstrate that the impact of inputs of younger, more bioavailable carbon to geochemically reactive zones can result in stimulation of microbial biogeochemical cycling. Given the range of potential outcomes associated with this increased cycling, understanding the occurrence and extent of such stimulation is an important issue in predicting the performance of reclamation activities.

Acknowledgements

The authors thank Syncrude Canada Limited Environmental Research Group for logistical support during field sampling campaigns. We thank J. Kirby for technical support in the lab and M. Reid, K. Kendra and T. Colenbrander-Nelson for sample collection. This research was supported by funding from Syncrude Canada Limited and the Natural Sciences and Engineering Research Council of Canada (CRDPJ 403361-10).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2016.11.029.

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