Variable effects of labile carbon on the carbon use of different microbial groups in black slate degradation

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Abstract

Weathering of ancient organic matter contributes significantly to biogeochemical carbon cycles over geological times. The principle role of microorganisms in this process is well recognized. However, information is lacking on the contribution of individual groups of microorganisms and on the effect of labile carbon sources to the degradation process. Therefore, we investigated the contribution of fungi, Gram-positive and Gram-negative bacteria in the degradation process using a column experiment. Investigations were performed on low metamorphic black slates. All columns contained freshly crushed, sieved (0.63–2 mm), not autoclaved black slates. Two columns were inoculated with the lignite-degrading fungus Schizophyllum commune and received a culture medium containing 13C labeled glucose, two columns received only this culture medium and two control columns received only water.

The total mass balance was calculated from all carbon added to the slate and the CO2 and DOC losses. Phospholipid fatty acids (PLFA) were extracted to investigate microbial communities. We used both the compound specific 14C and 13C signal of the PLFA to quantify carbon uptake from black slate carbon, whereas PLFA of Gram-negative bacteria used only 8% of carbon from the slates. PLFA of Gram-negative bacteria and fungi were both mostly activated by the glucose addition. The added Schizophyllum did not establish well in the columns and was overgrown by the indigenous microbial community. Our results suggest that especially Gram-positive bacteria are able to live on and degrade black slate material. They also benefit from easy degradable carbon from the nutrient broth. In natural environments priming due to root exudates might consequently enhance weathering.

1. INTRODUCTION

Weathering of ancient organic material, together with the burial of recent organic matter controls the C and O cycle on geological time scales (Berner, 1987; Petsch and Berner, 1998; Bouchez et al., 2010). About 90% of organic matter (OM) (15,000 × 1018 g C) resides in shales and other sedimentary rocks (Berner, 1989). Under subsurface conditions organic carbon in sedimentary rocks is recalcitrant to degradation, but exposure of this organic material to oxygenated surfaces can result in OM loss and release of CO2. It is estimated that weathering of black shales causes 12% of annual CO2 flux from oxidative weathering of sedimentary rocks (Jaffe et al., 2002).

Besides abiotic processes (Chang and Berner, 1998, 1999), microorganisms can contribute to weathering of ancient organic material. The uptake of organic carbon by microbes consumes O2 and releases CO2 and therefore
has implication on the global atmospheric carbon balance. Degradation of black thermally immature shale organic matter by microorganisms was first shown by Petsch et al. (2001b), who enriched microbial biomass on cultures containing only shale organic matter. Microorganisms growing on these cultures assimilated 74–94% of radiocarbon-free material. The uptake of ancient carbon from soils and sediments by microorganisms is generally accepted (Rethemeyer et al., 2004; Kramer and Gleixner, 2006; Kuzyakov et al., 2009). However, so far it is not well understood during rock weathering which microbial groups e.g. Gram-positive bacteria, Gram-negative bacteria or fungi are responsible for the degradation and how this process can be controlled.

In general, weathering of black shales results in a loss of aliphatic carbon (Petsch et al., 2001a), whereas aromatic carbon, which needs stronger oxidative enzymes like peroxidases or laccases, remains in the shales. The organic matter of low metamorphic slates contains mainly aromatic compounds. With increasing level of biodegradation, however, aromatic compounds are also decomposed (Volkman et al., 1984). This suggests the importance of fungi that produce these enzymes in the degradation process. Evidently, Wengel et al. (2006) demonstrated in a batch experiment that release of dissolved organic carbon from slates and charcoal was enhanced by the activity of the basidiozyme Schizophyllum commune. In general, S. commune is known to degrade polycyclic aromatic hydrocarbons (Matsubara et al., 2006), TNT (Bayman and Radkar, 1997) and textile dyes (Abadulla et al., 2000). Lignolytic fungi like S. commune create extracellular radical producing enzymes with very low substrate specificity that even degrade complex polymers like lignite and coal under aerobic conditions (Hofrichter et al., 1997a,b, 1999). Consequently, the inoculation of shales and slates with S. commune should increase slate weathering and the uptake of slate derived carbon. Gram-positive and Gram-negative bacteria are also known to degrade organic macromolecules (Vicuna, 1988; Zimmermann, 1990; Perestelo et al., 1996). Differences between both groups are based on the chemical and physical properties of their cell walls. Gram-positive bacteria have a high amount of peptidoglycan and abundant teichoic acids in their cell walls. Gram-negative bacteria have only a thin peptidoglycans layer and no teichoic acids. Actinomycetes, which are related to Gram-positive bacteria, are able to colonize solid surfaces due to their mycelial structure. Many bacterial strains, especially actinomycetes, have been shown to depolymerise lignin (Ball et al., 1989; Berrocal et al., 1997), but their ability to mineralize lignin is limited compared to fungi (Buswell and Odier, 1987; Ball et al., 1989; Godden et al., 1992). Gram-negative bacteria are generally considered to grow fast on easily degradable organic matter (Burke et al., 2003; Lockie et al., 2004) whereas Gram-positive bacteria are more related to soil derived carbon (Fierer et al., 2003; Kramer and Gleixner, 2008), which can contain ancient organic matter (Rethemeyer et al., 2005; Kramer and Gleixner, 2006).

Labile organic substrates like glucose can be used to stimulate microbial activity and to induce so called priming. Priming effects are changes in the turnover of organic matter caused by moderate treatment of the substrate e.g. microorganisms can use more refractory organic carbon after addition of labile organic matter (Hamer and Marschner, 2002) or they are activated by the added substrate, strongly increase their numbers and finally target more remaining refractory substrates if labile organic matter is completely consumed (De Nobili et al., 2001; Kuzyakov and Bol, 2006). For a review regarding priming effects see Kuzyakov et al. (2000).

Phospholipid fatty acids (PLFA) analyses combined with compound specific stable isotope and radiocarbon measurements are well established to determine the carbon uptake in microorganisms (Petsch et al., 2001a; Kramer and Gleixner, 2006). PLFA are generally used to compare the structure of microbial communities (Frostegård et al., 1993a,b; Bossio and Scow, 1998). Phospholipids are a major part of the cell membrane and after cell death they are rapidly degraded. This ensures that only viable microorganisms are investigated (White et al., 1979; Tunlid and White, 1992). Due to differences in chemical structure, some PLFA can be indicative of specific microbial groups like bacteria or fungi (Zelles, 1997, 1999; Báth and Anderson, 2003). Branched chain (br) fatty acids generally are associated with Gram-positive bacteria (Lechevalier and Lechevalier, 1988; Zelles, 1997), Gram-negative bacteria are characterized by having monounsaturated fatty acids (MUFA) (Zelles, 1997) and the polyunsaturated fatty acid (PUFA) 18:2ω6,9 is a marker for fungi (Frostegård and Báth, 1996). Straight chain saturated fatty acids are equally distributed among groups (Zelles, 1997). Additionally, 13C labeling (Pombo et al., 2002; Abraham and Hesse, 2003; Kramer and Gleixner, 2006) or the use of natural 13C signals of carbon substrates is a common method to determine carbon sources of microorganisms.

14C decays with a half-life of 5730 years. Organic material older than ~60,000 years contains no detectable 14C. Carbon of black slate consequently is radiocarbon free and makes this carbon pool fossil. If any radiocarbon free material is incorporated by microbes, this will be directly reflected by the 14C content of PLFA (Rethemeyer et al., 2005; Slater et al., 2006; Kramer and Gleixner, 2006). With the natural 13C and 14C labeling we can discriminate between the effects glucose has on microbial growth and to which extent microbial groups are able to assimilate black slate organic matter. The study aims to answer the following questions:

1. What are the relative roles of fungi. Gram-positive and Gram-negative bacteria in black slate degradation?
2. Can the inoculation with lignite-degrading fungi enhance the degradation of black slate?
3. Does priming with glucose enhance microbial weathering?

2. METHODS

2.1. Site description and sampling

Black slate material was taken from a mining waste dump in Schmiedefeld/Thuringian Forest (50°32′ N, 11°12′ E) with an age of approximately 50 yrs. The area was a former mining district, where alum, vitriol of iron-
and copper, sulfur and earth colors were produced. Black slates from the lower graptolite shale formation (Lower Silurian age) outcrop in Variscan deformed anticlines of East and South Thuringia, Germany. Slates are low-grade metamorphic rocks that form from shale under pressure at temperatures of a few hundred degrees. It consists besides organic carbon of illite, illite-smectite, chlorite, muscovite, quartz, pyrite/marcasite and minor amounts of opaline silica and phosphates and carbonates. Coalification varies between vitrinite reflectance <3% and >6% Rm (Kunert, 1999). Dark-colored unweathered slates contain a wide range (6–23% of Corg) whereas the strongly weathered (light-colored) slates contain a much lower range and organic matter content (0.2–2% of Corg) (Fischer et al., 2009). Slates used in our experiment were dark gray and contained 2.7% organic carbon. Pyrolysis experiments have indicated that the black slate organic matter consists mainly of non-pyrolysable material and contains only minor potions of pyrolysable material (Fischer et al., 2009). The small low-temperature signal was caused by molecules containing CH3 groups (bitumen fraction, 80–300 °C) and the CO2 release between 300 and 800 °C results from the presence of carboxylic groups in the kerogen macromolecule.

Untreated black slate material was sieved to sand size between 0.63 and 2 mm to create a well-aerated pore space. During sieving coarse roots, plant and animal residuals were extracted by hand. Thus, not all modern carbon could be removed as can be seen from 14C values of black slate (see Section 3.5). Plant material can be either too small to be picked out by hand or plant-derived DOC can be adsorbed on the slate surfaces.

Black slate carbon content was measured from ball-milled subsamples by elemental analysis before incubation (Elementar Analysensysteme GmbH, Hanau, Germany).

2.2. Microorganisms and growth conditions

S. commune was obtained from the strain collections at the Institute of Microbiology, University of Jena. S. commune (strain 4–40) was cultured on minimal medium agar plates (20 g/l glucose, 2 g/l asparagine, 1 g/l K2HPO4, 0.5 g/l KH2PO4, 0.5 g/l MgSO4?7H2O, 100 μl/l thiamine dichloride, 15 g/l agar). Pre-grown cultures were made from 1 cm2 of S. commute that was added to 300 ml of a liquid minimal medium. The flasks were shaken for 7 days with 140 rpm at 28 °C. The pre-cultures were centrifuged at 4000 rpm and washed twice with sterile distilled water. 20 ml of microbial suspension (approximately 1 g microbial biomass) was added to 200 ml of a medium containing 10 g/l glucose, 0.5 g/l asparagine, 0.5 g/l K2HPO4, 0.25 g/l KH2PO4, 0.2 g/l MgSO4?7H2O, 0.01 g/l FeSO4?7H2O, 100 μl thiamine dichloride (column medium). The pH was adjusted to 6.3 with 5 N NaOH. Two hundred milliliters of medium per column was used for inoculation.

2.3. Column setup

The columns contained 1.5 kg (wet weight) of non-auto-claved black slate material with a grain size of 0.63 to 2 mm. Columns were made of PVC 30 cm high and had a diameter of 10 cm. Columns were mounted on buchner-funnels (Brand, Germany) filled with Teflon filter material (Stockhausen, Germany) with a pore size of 70 μm. Two columns were inoculated with fungus S. commune using the column medium described above, two columns received only the column medium and two control columns received only sterile deionised water (Fig. 1). After initial feeding and inoculation, only sterile deionised water was added to the columns except on day 122, when 200 ml of the column medium but without glucose was added. The addition proved that no nutrient limitation was present. Columns were watered with sterile deionised water through the upper part every four to five days when the water content dropped below 20%. The water content was adjusted to about 20–25% to avoid drying and allow oxygen exchange. Water outflow only occurred when new water was supplied. The water content was monitored with Theta probes (Type ML2X, Cambridge, England) connected to a data logger (Campbell Scientific, Inc., USA) and evaluated by the datalogger support software ‘PC208W’ (Campbell Scientific, Inc., USA). The columns were incubated for 183 days in climate chambers at 28 °C and 60% air humidity in darkness.

2.4. Total carbon loss from the columns

The carbon loss was calculated from gaseous and liquid carbon. CO2 efflux was measured to determine microbial respiration. For CO2 measurements, the Vaisala-Sensor GMP343 (measurement range 0–4000 ppm, accuracy 2%) (Vaisala, Finland) was installed in a headspace-chamber (PVC, 16.5 cm high, dia 10 cm) which could be attached to the columns. Inside the headspace, a fan was used to ensure mixing of the air. CO2 efflux was calculated as concentration increase with time in an isolated volume. The sensor makes approximately one measurement per second and was mounted on the columns for around 10 min. Results were sent to the post-processing program ‘RS232Log’ (Jena, Germany). Linear regression on the set of measurements was used to determine the emission rate. CO2 measurements were performed on days 1, 2, 3, 4, 6, 8, 10, 12, 15, 18, 22, 25, 31, 37, 44, 51, 74, 93, 114, 122, 124, 152, 183. The total CO2 efflux during the 183 day period was estimated from a potential fit to the data of daily measurements, except for the control columns, where no trend in the results was observed and consequently all daily measurements were averaged.

The effluent was collected and analyzed for dissolved organic carbon (DOC) and glucose content from the medium. After sampling, the effluent was filtered through membrane filters with a pore size of 0.45 μm. Aqueous samples were taken on days 3, 8, 19, 39, 84 and 141. DOC was determined using a high TOC analyzer (detection limit 0.5 mg/l, precision for samples between 0.5 and 10 mg/L C < 5%) (Elementar Analysensysteme GmbH, Hanau, Germany). Glucose content was measured with the ion chromatograph ICS-3000 ( Dionex, Sunnyvale, USA). To determine the amount of carbon that was produced the total carbon loss from the columns was calculated using following equation:
Total $C_{\text{loss}} = C_{\text{respiration (183 day)}} + C_{\text{DOC-loss}} - C_{\text{glucose-loss}}$.  

where $C_{\text{respiration (183 day)}}$ is the respiration rate in mg C per column over the 183 day period, $C_{\text{DOC-loss}}$ the amount of total DOC and $C_{\text{glucose-loss}}$ the total amount of glucose content measured in the effluent during the incubation period.

Priming related to the added C (glucose, asparagine) was calculated using the following equation:

$$\text{Priming [\%]} = \left( \frac{\text{Total } C_{\text{loss}}}{\text{Total } C_{\text{input}}} \right) \times 100.$$  

where total $C_{\text{loss}}$ is obtained by equation 1 and total $C_{\text{input}}$ is the total amount of carbon (glucose and asparagine) that was added to the columns.

Statistical evaluation was performed with Microsoft Office Excel 2003 (Microsoft, USA). For samples with only two replicates per treatment average deviation was calculated.

2.5. Examination of the microbial communities

One kilogram slate material was extracted according to the method established by Bligh and Dyer (1959) as described earlier by Kramer and Gleixner (2006). Briefly, lipids were extracted from black slates by adding a mixture of chloroform, methanol and a 0.05 M phosphate buffer (pH 7.4) to the untreated column slate material. After the initial extraction, lipids were partitioned into neutral lipids (with chloroform), glycolipids (with acetone) and phospholipids (with methanol) using silica-filled solid phase extraction columns. The phospholipids were subjected to mild alkaline hydrolysis and methylation. The fatty acid methyl esters (FAMEs) were separated from OH-substituted FAMEs and unsaponifiable lipids using NH$_2$ columns. In the last step unsubstituted ester-linked FAMEs are separated with Ag-impregnated SCX columns into saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. The separation into saturated, monounsaturated and polyunsaturated fatty acids before GC analysis is essential to have a sufficient peak separation for reliable $\delta^{13}$C measurements and preparative fraction collection for $^{14}$C measurements. The FAMEs were quantified with a GC-FID (HP-6890 series-GC, Palo Alto, USA) using an ultra 2 column (50 m length $\times$ 0.32 mm I.D.; 0.52 µm film thickness) and He as carrier gas. The temperature program started at 140 °C (1 min isotherm). Then the temperature rose by 2 °C min$^{-1}$ to 270 °C. This temperature was held for 6 min followed by a final heating rate of 30 °C min$^{-1}$ to 320 °C, which was held for 3 min. PLFA were quantified based on integration of peak areas, using co-injected PLFA nonadecanoic acid-methyl ester (19:0) as internal standard.

Peak identification and peak purity were investigated using an ion trap mass spectrometer (GCQ, Thermoquest, Germany). Databases of the National Institute of Standards and Technology (NIST), Wiley 6.0 databases and an in-house database were used to identify PLFA. In addition reference substances were used as described earlier (Kramer and Gleixner, 2006). The specific PLFA were grouped into four microbial communities. Among 35 determined fatty acids the following were used as general microbial marker: 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0. Gram-negative bacteria were indicated by all measured monounsaturated and cyclic fatty acids. Gram-positive bacteria were represented by measured i/a15:0, br16:0, i/a, 10Me, br17:0, br18:0 and br19:0 fatty acids, whereas the 10Me17:0 fatty acid is a marker for the Gram-positive actinomycetes. PLFA 18:2$\alpha_{6,9}$ was used as a marker for fungal biomass.

Total bacterial biomass was estimated from the sum of PLFA i15:0, a15:0, 15:0, br16:0, 16:1o9, 16:1o7, i7:0, a17:0, cy17:0, 17:0, 10Me17:0, br18:0, 18:1o7 and cy19:0 using the conversion factor of 1.4 $\times$ 10$^{-17}$ mol bactPLFA/cell (Frostegård and Báath, 1996) and 20 fg biomass C/cell (Báath, 1994) to calculate bacterial carbon. Fungal biomass was calculated using the conversion factor 11.8 mol 18:2o6,9/g biomass-C (Klammer and Báath, 2004).

Statistical analyses were performed with SPSS Version 16.0 (SPSS Inc., Chicago, USA). Standard deviation of PLFA was calculated from two independent replicates per treatment and three independent extractions per replicate. Principal component analysis (PCA) of PLFA data was
carried out to investigate treatment effects. Significant differences of means for microbial groups in the different treatments were judged by analysis of variance (ANOVA) and Tukey's test.

2.6. Stable isotope measurements

The δ¹³C values of black slates and glucose were determined by an elemental analyser (EA 1110, CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (IRMS) (DELTAplusXL, Finnigan MAT, Bremen, Germany). Solid samples were weighted according to the carbon content into tin capsules (1 mg black slate, 0.11 mg glucose) and combusted in an oxygen stream at 1150 °C. The δ¹³C values were determined on evolved CO₂, which was transferred to the IRMS. Standard deviation for analytical uncertainties of bulk samples was 0.22±0.05.

Compound specific determination of δ¹³C values of individual FAME was performed by an IRMS (DELTAplusXL, Finnigan MAT, Germany). Column and temperature programs were the same as described above for PLFA measurements. All δ¹³C values are averages of three measurements. Results were corrected for the methylation reagent (Kramer and Gleixner, 2006). Isotope ratios are expressed as δ¹³C in per mill [‰]. Values are relative to the reference standard V-PDB using NBS 22 (Coplen et al., 2006).

The median for standard deviation of δ¹³C values of PLFA is about −0.5 and ranged between −0.04 and −2.

The contribution of glucose derived carbon in percent was calculated based on the isotopic shift (δ¹³C values) between microbial PLFA of the different treatments relative to the isotopic difference of glucose and black slate by the following equation (Eq. (3)) (Gleixner et al., 2002; Kramer and Gleixner, 2006):

\[
\text{Glucose uptake [%]} = \left( \frac{\delta^{13}C_{\text{PLFA,medium-fed columns}} - \delta^{13}C_{\text{PLFA,control columns}}}{\delta^{13}C_{\text{glucose}} - \delta^{13}C_{\text{black slate}}} \right) \times 100
\]

where \( \delta^{13}C_{\text{PLFA,medium-fed columns}} \) is the isotope ratio of the phospholipid fatty acid obtained from these columns, \( \delta^{13}C_{\text{PLFA,control columns}} \) is the isotope ratio of the phospholipid fatty acid from the control columns, \( \delta^{13}C_{\text{glucose}} \) that of glucose added to the columns and \( \delta^{13}C_{\text{black slate}} \) that of black slate used in the experiment. Due to fractionation in δ¹³C in different lipids the PLFA of the different treatments have to be used in the numerator.

2.7. Bulk and compound specific ¹⁴C measurements

¹⁴C content of black slate material and glucose were measured at the Max Planck Institute for Biogeochemistry in Jena with accelerator mass spectroscopy 3MV AMS (High Voltage Engineering Europe) (Steinhof et al., 2004). Approximately 1 mg carbon per sample was weighted into tin capsules and combusted by an elemental analyser (EA/NA 1110 NC 2500, Thermo Quest Italia S.p.A., Italy). The CO₂ evolved during dry combustion was reduced to graphite by heating H₂ and CO₂ in the presence of iron powder at 650 °C. The graphite was pressed into targets and measured with the AMS. Detection limit is about 0.2 pMC (percent modern carbon). Standard deviation is based on either statistical error of all measurements or on standard deviation of several runs of black slate and glucose sample.

¹⁴C measurements of FAME were performed at UC Irvine KCCAMS laboratory (Southon and Santos, 2004). We needed between 10 and 30 μg of carbon for the radiocarbon measurement, so we injected known ¹⁴C FAME standards into the preparative capillary gas chromatograph (PCGC) and trapped and processed them as we did our samples to determine corrections for contamination during purification procedures (data not shown). Other tests were used to determine corrections for the sample combustion and graphitization procedures (Santos et al., 2007a; Kramer et al., 2010). Since these corrections are size dependent, we used different numbers of injections to obtain different amounts of our standard PLFA (approximately 10, 20, 30 μg). We measured the ¹⁴C content of individual FAME (16:0, microbial marker) and grouped FAME (i/a, 10Me 17:0 – Gram-positive marker and 16:1o9, 17:1 (1,2,3), 18:1o9, 18:1o7 – Gram-negative marker) extracted and isolated from the column experiment. FAME used for ¹⁴C analysis are composites made from the two column replicates. We had to combine compounds from Gram-positive and Gram-negative marker to get enough carbon to measure ¹⁴C. Compounds combined for ¹⁴C measurements were co-eluted into the same trap. FAME were separated using a GC (Agilent-6890 series GC, Agilent Technologies, Santa Clara, USA) coupled to a preparative fraction collector (PFC) (Gerstel, Germany). The column used was a DB-5 ms (30 m length × 0.53 mm I.D.; 0.5 μm film thickness) with ultrapure helium as carrier gas. The initial temperature was 140 °C (1 min isotherm). Then temperature rose by 5 °C min⁻¹ to 220 °C, followed by a heating rate of 2 °C min⁻¹ to 230 °C and a final heating rate of 20 °C min⁻¹ to 320 °C, held for 3 min. The reisolated samples were quantified with the GC-FID using the same column and temperature program as described above.

Isolated compounds were dried and placed into combustion tubes. Combustion and graphitization of FAME was performed at the UCI Keck Carbon Cycle AMS facility (Southon et al., 2007a, 2007b). In the first step, carbon dioxide was produced by combustion at 550 °C in evacuated quartz tubes in the presence of CuO and silver wire. CO₂ was reduced to graphite with hydrogen over pre-baked iron powder at 450 °C for samples <30 μg and at 550 °C for samples ~1 mg. Graphite was pressed into targets and mounted on wheels for ¹⁴C analysis. The ¹⁴C signal of the FAME had to be corrected for the single carbon atom added to each molecule during derivatisation (Kramer and Gleixner, 2006).

¹⁴C data are expressed in percent fraction modern carbon (FMC) relative to the ¹⁴C activity of the oxalic acid standard and corrected to δ¹³C of −25‰ using values measured in AMS.

The carbon used in the medium-fed columns can be assumed to be mixtures of two endmembers clearly distinguished by their δ¹³C and δ¹⁴C values. The first endmember is glucose (δ¹³C = −11.1‰, δ¹⁴C = 1.6074 FMC). The second endmember represents black slate carbon (δ¹³C = −28.5‰, δ¹⁴C = 0.079 FMC). Based on the differences in ¹⁴C values
between microbial PLFA using glucose and black slate it was possible to calculate the proportion of black slate derived carbon in the microbial PLFA (Eq. (4)). The contribution of black slate derived carbon in percent was calculated based on the isotopic shift ($\Delta^{14}C$ values) between microbial PLFA from treatments fed with the culture medium and modern $^{14}C$ values relative to the isotopic difference of glucose and black slate using the following equation.

\[
\text{Black slate uptake} \% = \frac{\delta^{14}C_{\text{PLFA,medium-fed column}} - \delta^{14}C_{\text{glucose}}}{\delta^{14}C_{\text{black slate}} - \delta^{14}C_{\text{glucose}}} \times 100
\]

\[(4)\]

whereas $\delta^{14}C_{\text{PLFA,medium-fed column}}$ is the $^{14}C$ content of the specific phospholipid fatty acid, $\delta^{14}C_{\text{glucose}}$ the $^{14}C$ content of the glucose used and $\delta^{14}C_{\text{black slate}}$ the $^{14}C$ content of the black slate. Because we have no data for PLFA received only a modern carbon source the $^{14}C$ value of glucose was used in the enumerator. Values were used in fraction modern carbon (FMC).

3. RESULTS

3.1. Respiration measurements

Respiration rates in the columns differed considerably between columns that obtained a culture medium and control columns that only received water (Table 1). The respiration rates in control columns with water varied between 0.23 and 3.29 mg C-CO$_2$ d$^{-1}$, which resulted in an averaged carbon flux of 258.1 mg C-CO$_2$ over the 183 day period (Fig. 2). Initial CO$_2$ effluxes of up to 277 mg C-CO$_2$ over the 183 day period was highest from the control-medium columns (74 mg C ± 0.8, Table 1) and declined to former magnitudes two days later indicating that no mineral limitation was present.

3.2. Liquid carbon loss

Treatment with a growth medium strongly affected the DOC loss from the columns. Control columns lost only 2.1 mg DOC (±0.2) and no glucose was measured in the effluent, while control medium columns lost 118 mg DOC (±26) and Schizophyllum columns 220 mg DOC (±14) and both also lost glucose. Therefore, DOC exports were corrected for the glucose loss. The corrected DOC loss over the 183 day period was highest from the Schizophyllum columns (122 mg C ± 5.1). It was about 50 mg C larger than in the control-medium columns (74 mg C ± 0.8, Table 1)

3.3. Microbial communities

Total amount of PLFA depended strongly on carbon and nutrient supply from the medium. The PLFA concentration was lowest in the control columns (270 ng PLFA g$^{-1}$ dw, ±13, Table 1). The detected amounts represent the indigenous microflora of the non-sterile, surface-sampled black slates. Feeding of the columns with a culture medium resulted in considerable higher amounts of PLFA, whereas only minor differences were observed between both medium-fed treatments. PLFA concentrations were 1387 (±38) ng PLFA g$^{-1}$ dw in the control-medium columns

Table 1

|                      | Control | Control-medium | Schizophyllum |
|----------------------|---------|----------------|--------------|
| Total C$_{\text{added}}$ [mg C] | 0       | 836            | 836          |
| C$_{\text{gh}}$ loss [mg C]       | 0       | 44             | 98           |
| Respiration [mg C]          | 258 (53) | 1270 (109)     | 1177 (88)    |
| DOC loss [mg C]             | 0       | 118 (26)       | 220 (14)     |
| DOC-C$_{\text{gh}}$ loss [mg C] | 2 (0.3) | 74 (0.8)       | 122 (5)      |
| Total carbon loss [mg C]    | 260 (52) | 1344 (108)     | 1299 (93)    |
| Priming [%] related to controls | 212 (32) | 216 (28)       |              |
| Priming [%] related to C$_{\text{added}}$ | 161 (10) | 155 (9)        |              |
| PLFA [ng g$^{-1}$ dw]       | 270 (13) | 1387 (38)      | 1218 (80)    |
| Bacterial cells g$^{-1}$ dw | 4.0 × 10$^7$ (2.6 × 10$^6$) | 1.8 × 10$^8$ (2.5 × 10$^6$) | 1.7 × 10$^8$ (1.0 × 10$^6$) |
| Bacterial carbon µg g$^{-1}$ dw | 0.8 (0) | 3.7 (0.1)      | 3.3 (0.2)    |
| Fungal carbon µg g$^{-1}$ dw | 0.9 (0.1) | 11.7 (1.6)     | 5.8 (0.8)    |
| Metabolic quotient [mg C-CO$_2$ h$^{-1}$/g microbial carbon] | 24 (2.6) | 39 (0.8)       | 67 (1.4)     |

Average deviation based on column replicates in parentheses; dw, dry weight
and 1218 (±80) ng PLFA g⁻¹ dw in the Schizophyllum columns. The amount of PLFA converted to bacterial cells equals 4.0 × 10⁷ (±2.6 × 10⁶) bacterial cells in the control columns per g dw. This corresponds to 0.8 (±0.05) μg bacterial carbon per g dw in the control columns. The amount of the fungal marker corresponded to 0.86 (±0.1) μg fungal carbon per g dw. In the control-medium and Schizophyllum columns bacterial cells varied between 1.6 × 10⁷ (Schizophyllum 1) and 1.9 × 10⁷ (control-medium 2) per g dw, which corresponded to 3.1 and 3.7 μg bacterial carbon per g dw. Fungal carbon differed between both treatments. Surprisingly, more fungal biomass was found in the control-medium columns compared to columns inoculated with S. commune. According to abundance of 18:2ω6:9 fungal carbon accounted for 11.7 (±1.6) μg per g dw in the control-medium columns and 5.8 (±0.8) μg per g dw in the Schizophyllum columns.

Furthermore, principal components analysis (PCA) revealed a shift in microbial community structure between the different treatments, indicating a clear response of microbial communities to culture medium addition. According to the principal component analysis, the first principal component accounted for 63% of the variation. The control columns were separated on this axis from both other treatments. PC2 explained only 15% of the variation. This axis separated the control medium columns from the control and Schizophyllum columns. The most abundant PLFA in all columns was 16:0 (Table 2). Their relative abundance reached 17.7 mol% of total PLFA in the control columns but declined to 11.5 mol% in the control-medium and 10.6 mol% in the Schizophyllum columns. Second abundant PLFA in the control columns was 10Me17:0 (7.9%) indicative for Gram-positive actinomycetes, whereas in the medium-fed columns 18:1ω9, indicative for Gram-negative bacteria, was second abundant. ANOVA revealed significant differences between treatments if microbial groups were compared separately. PLFA representing Gram-positive bacteria dominated microbial communities in all columns. There were only weakly significant differences between the treatments (p = 0.036). Feeding with a culture medium increased the relative amount of fatty acids representing Gram-negative bacteria and to some extent of fungi. The concentration of PLFA representing Gram-negative bacteria was lowest in control columns (21.6 mol%, as percentages of total), and significantly higher in other medium-fed columns (control-medium 30.9 mol% and S. commune 28.7 mol%, p < 0.01 for comparison between control and both medium-fed treatments, respectively). The abundance of fungal PLFA was low in all columns but increased relative to controls (1.0 mol%) in the control-medium columns (2.7 mol%) and Schizophyllum columns (1.5 mol%), with significant differences between the control-medium columns and both other treatments.

3.4. Stable isotope analysis

The two applied carbon sources differed widely in δ¹³C values. The bulk δ¹³C value of black slate was −28.8‰ (±0.5), whereas the C4-glucose had a δ¹³C value of −11.1‰ (±0.2). The different ¹³C signatures of black slate and glucose facilitate to identify the uptake of different carbon sources into microbial biomass. Compound specific ¹³C analysis of individual PLFA revealed differences between the medium-fed columns and the control columns. The isotopic signatures of PLFA from control columns were in general close to the black slate isotopic signal (Table 3). In both medium-fed columns the isotopic signature shifted towards the glucose derived isotopic signature. The differences in ¹³C values of PLFA between control and medium-fed columns were used to determine the glucose uptake into microbial biomass (Eq. (3)). Calculations are based on the assumption that there is 100% black slate uptake in the control columns. The glucose uptake in both medium-fed treatments was the highest in fungal PLFA and into PLFA representing Gram-negative bacteria. A glucose uptake into PLFA representing fungal biomass of ~82% in the control-medium columns and ~83% in the Schizophyllum columns was calculated. PLFA of Gram-negative bacteria benefited from glucose addition by an uptake of 39% (control-medium) and 53% glucose (Schizophyllum), respectively, whereas the Gram-positive markers incorporated about 36% in both medium-fed treatments.

3.5. Radiocarbon measurements

Black slate was nearly radiocarbon free having only 0.079 (±0.00062) fraction modern C, whereas glucose was
modern (1.0674 ± 0.0022; fraction modern C). A minimum amount necessary for reliable 14C measurements was found to be 10 μg (data not shown). The three samples fulfilling the requirements had a fraction modern carbon value of 0.8486 (±0.0618) for PLFA representing Gram-positive bacteria (i/a, 10Me 17:0, control-medium columns), 0.8931 for general microbial marker (±0.0682, 16:0, Schizophyllum columns) and 0.9904 (±0.0542) for Gram-negative marker (16:1x9, 17:1 (1,2,3), 18:1x9, 18:1x7, Schizophyllum columns. Accordingly, we calculated carbon use from the slate in the order of 22, 18, and 8%, respectively (Eq. (4)).

4. DISCUSSION

We were able to demonstrate for the first time the impact of different microbial groups on degradation of low metamorphic black slate in the presence of a labile carbon source. To demonstrate this we combined a mass balance approach using carbon budgets and compound specific 13C and 14C analysis of PLFA. Contributions from degradation of black slate were evident from all three approaches. Especially results of 14C analysis imply that Gram-positive bacteria were best adapted to black slate degradation. Moreover, Gram-positive bacteria dominated the microbial community in all columns indicating that they might be best adapted to live in black slate environments. The 10Me17:0 fatty acid, a marker for Gram-positive actinomycetes, was found in relatively high amounts in all columns. Feeding with a culture medium increased the amount of Gram-negative bacteria in the medium-fed columns. Former investigations showed that Gram-negative bacteria are more dependent on the input of fresh plant derived organic matter (Kramer and Gleixner, 2006; Habekost et al., 2008), which is easily degradable like glucose, whereas Gram-positive bacteria have been found to use fossil organic carbon from soils (Kramer and Gleixner, 2006).

Table 2
Amounts of identified PLFA of all treatments after incubation period in mol% g⁻¹ dw (dry weight). Standard deviation in parentheses. Standard deviation is calculated from two replicates per treatment and three individual extractions per replicate.

|          | Control | Control medium | Schizophyllum |
|----------|---------|----------------|--------------|
| 14:0     | Microbes | 2.38 (0.30)    | 1.87 (0.12)  | 1.90 (0.11) |
| 15:0     | Microbes | 1.26 (0.16)    | 1.27 (0.13)  | 1.27 (0.13) |
| 16:0     | Microbes | 17.71 (0.41)   | 11.47 (0.71) | 10.59 (0.71) |
| 17:0     | Microbes | 1.31 (0.18)    | 1.32 (0.11)  | 1.26 (0.13) |
| 18:0     | Microbes | 3.54 (0.22)    | 2.94 (0.14)  | 2.87 (0.04)  |
| 20:0     | Microbes | 1.16 (0.22)    | 1.00 (0.07)  | 1.05 (0.13)  |
| i15:0    | Bacteria (G+) | 5.32 (0.37)   | 4.56 (0.34)  | 5.27 (0.30)  |
| a15:0    | Bacteria (G+) | 4.07 (0.58)   | 4.23 (0.25)  | 5.88 (0.16)  |
| br16:0   | Bacteria (G+) | 5.30 (0.42)   | 5.37 (0.31)  | 5.76 (0.19)  |
| br16:0   | Bacteria (G+) | 1.22 (0.10)   | 1.26 (0.18)  | 1.26 (0.18)  |
| 10Me17:0 | Bacteria (G+) | 7.92 (1.22)   | 5.64 (0.34)  | 4.93 (0.15)  |
| br17:0   | Bacteria (G+) | 2.01 (0.05)   | 1.33 (0.07)  | 1.22 (0.14)  |
| i17:0    | Bacteria (G+) | 6.06 (0.16)   | 6.06 (0.39)  | 5.96 (0.22)  |
| br17:0   | Bacteria (G+) | 2.54 (0.22)   | 3.82 (0.21)  | 4.36 (0.19)  |
| a17:0    | Bacteria (G+) | 5.02 (0.13)   | 4.68 (0.42)  | 4.72 (0.34)  |
| br17:0   | Bacteria (G+) | 4.80 (0.45)   | 2.54 (0.25)  | 2.41 (0.05)  |
| br18:0   | Bacteria (G+) | 0.69 (0.09)   | 0.96 (0.08)  | 1.03 (0.14)  |
| br18:0   | Bacteria (G+) | 1.11 (0.20)   | 1.82 (0.11)  | 1.87 (0.09)  |
| br19:0   | Bacteria (G+) | 4.20 (0.15)   | 2.09 (0.13)  | 1.89 (0.08)  |
| br19:0   | Bacteria (G+) | 0.47 (0.07)   | 1.33 (0.11)  | 1.39 (0.12)  |
| br19:0   | Bacteria (G+) | 0.49 (0.09)   | 1.33 (0.06)  | 1.59 (0.10)  |
| cy19:0   | Bacteria (G-) | 1.95 (0.25)   | 1.53 (0.07)  | 1.41 (0.10)  |
| cy19:0   | Bacteria (G-) | 6.25 (0.74)   | 3.99 (0.30)  | 4.97 (0.22)  |
| 15:1     | Bacteria (G-) | 0.67 (0.05)   | 1.34 (0.07)  | 1.23 (0.13)  |
| 16:1     | Bacteria (G-) | 1.29 (0.09)   | 1.19 (0.13)  | 1.19 (0.13)  |
| 16:1o9   | Bacteria (G-) | 2.87 (0.39)   | 2.94 (0.36)  | 2.64 (0.13)  |
| 16:1o7   | Bacteria (G-) | 1.78 (0.20)   | 2.32 (0.21)  | 2.51 (0.12)  |
| 16:1     | Bacteria (G-) | 1.38 (0.08)   | 1.73 (0.09)  | 2.09 (0.14)  |
| 17:1     | Bacteria (G-) | 0.96 (0.06)   | 1.36 (0.05)  | 1.35 (0.14)  |
| 17:1     | Bacteria (G-) | 0.87 (0.07)   | 1.03 (0.07)  | 0.79 (0.39)  |
| 18:1o9   | Bacteria (G-) | 3.96 (0.41)   | 8.59 (1.75)  | 7.53 (0.76)  |
| 18:1o7   | Bacteria (G-) | 1.48 (0.17)   | 1.71 (0.11)  | 1.97 (0.10)  |
| 20:1     | Bacteria (G-) | 1.64 (0.11)   | 1.64 (0.11)  | 1.02 (0.11)  |
| 18:2o6,9 | Fungi     | 1.02 (0.27)   | 2.69 (0.91)  | 1.52 (0.32)  |
| Sum      | Microbes  | 27.35 (0.62)   | 19.86 (1.17) | 18.94 (0.47) |
|          | Gram (+)  | 50.03 (1.94)   | 46.98 (2.36) | 49.54 (1.14) |
|          | Gram (−)  | 21.60 (1.60)   | 30.85 (2.92) | 28.69 (0.97) |
|          | Fungi     | 1.02 (0.27)   | 2.69 (0.91)  | 1.52 (0.32)  |
Compound specific stable isotope and 14C analysis support the findings of PLFA distribution. As mentioned before, membrane lipids of microorganisms have a 13C signature related to the 13C signature of their diet. However, even when grown on a single carbon source the 13C signature of the same PLFA can vary considerably between different strains of bacteria and fungi or different PLFA of the same strain can respond differently to the same carbon source (Abraham et al., 1998). The average isotopic composition of PLFA from control columns is within the range of 3–5‰ relative to slate organic matter (−28.8‰). PLFA of medium-fed columns showed 13C signatures closer to the glucose signal, but they were depleted in 13C relatively to glucose. The depletion relative to glucose in the medium-fed columns supports the findings that slate carbon was assimilated and indicated that substrate use varied among microbial groups. Different offsets between microbial groups of control and medium-fed columns were measured and indicated that substrate use varied among microbial groups. Generally, Gram-positive bacteria had lower 13C values and glucose uptake compared to PLFA of Gram-negative bacteria and fungi of the same medium-fed treatment especially in the Schizopyllum columns (Table 3 and Fig. 3). The lower glucose uptake could be indicative for the uptake of more black slate derived carbon into these particular fatty acids representing Gram-positive bacteria. Fungi were most enriched in 13C (Fig. 5) and thus, assimilated the largest proportion of glucose. Calculations for glucose uptake in the medium-fed columns are based on the assumption that the slate carbon uptake in the controls is 100%. The 14C measurements of black slate material suggest that 7.9% of modern carbon was present and might contribute to microbial growth in the control columns. During sieving coarse roots, plant and animal residuals were extracted by hand. However, not all modern carbon could be removed as can been seen from the 14C results could be removed as can been seen from the 14C results. Radiocarbon measurements of fatty acids of column microorganisms indicated that some black slate derived carbon assimilated by microorganisms after the addition of...
The highest uptake of radiocarbon free material was found in PLFA associated with Gram-positive bacteria, corroborating the fact that Gram-positive bacteria are better adapted to the degradation of recalcitrant organic material than Gram-negative bacteria. Uptake of recalcitrant parts of organic matter with low $^{14}$C concentrations into Gram-positive bacteria also represented by br17:0 fatty acids were observed by Kramer and Gleixner (2006). The amount of $^{14}$C found in fatty acids representing Gram-negative bacteria was considerably higher than the amount found in Gram-positive bacteria. This is in accordance with the fact that we found less Gram-negative bacteria especially in the columns were no glucose was added and that Gram-positive bacteria were more depleted in $^{13}$C than Gram-negative bacteria and fungi. This is also supported by observation from Rethemeyer et al. (2004, 2005) who found high $^{14}$C levels of monounsaturated fatty acids (MUFA, 16:1, 17:1, 18:1) in fossil-carbon rich soil horizons.

It is established that white-rot fungi are most efficient in the degradation of recalcitrant organic matter like lignite (Catcheside and Mallett, 1991; Hofrichter et al., 1997a). Kerogen consists of a mixture of resistant marine and terrestrial organic macromolecules. The structure of kerogen fractions within black slate suggests that similar components comparable to lignite are present which might also be degradable by fungal enzymatic activity. We assumed that fungi might be more numerous in the columns. However, the amount of fungi was relatively low in all columns. Active growth and the formation of large mycelia are required for a success-
Microbial use of black slate carbon

ful colonization of the environment. Studies have shown that the activity of inoculated white-rot fungi was improved by the addition of glucose and mineral salts in non-sterile soils (Morgan et al., 1993). A primary carbon source is also required to establish biodegradation of recalcitrant compounds. However, the preferences of white-rot fungi to colonize wood might limit their capability to live in soil containing environments. Thus, other organic substrates like straw and wood shavings might have been a better carbon sources to stimulate fungal growth (Morgan et al., 1993).

Our results suggest that only a small amount of fungi benefits most from glucose, which is also confirmed by results from litter degradation (Rubino et al., 2009).

We did not find any influence of inoculation with the fungi S. commune on respiration rate and total amount of PLFA. The lack of inoculated fungal growth in these non-autoclaved columns could be explained by the inability of the fungus to compete with the indigenous soil microorganisms (Radtk et al., 1994; in der Wiesche et al., 1996; Martens and Zadrazil, 1998; Andersson et al., 2000). Surprisingly, the amount of fungal carbon was even higher in the control-medium columns compared to the Schizophyllum columns. The growth of bacteria antagonistic to S. commune might have been stimulated by the culture medium and might have also negatively affected the indigenous fungal population in these columns. However, DOC loss was stimulated in Schizophyllum columns compared to other columns. Martens and Zadrazil (1998) observed that non-colonizing fungi started to disintegrate resulting in the accumulation of water which diffuses into the soil. Products of mycelium decay from non-colonizing S. commune might have stimulated the higher DOC loss from the Schizophyllum columns compared to the control-medium columns.

Carbon input is considered to be the limiting factor for microbial biomass. The microbial community composition and the amount of microorganisms in the columns depended on the availability of glucose. Glucose as labile carbon source is easily degradable and supports the growth of microorganisms as can be seen from the increased numbers of PLFA and the higher CO2 efflux rates from the medium-fed columns. However, the abundance of PLFA indicates a small microbial population. Compared to natural oxygenated environments, the PLFA concentration was low (Burke et al., 2003; Habekost et al., 2008). PLFA concentrations in the columns were also lower than PLFA concentration measured by Petsch et al. (2003) in a core section (28.9 nmol g⁻¹), which contained nearly 100% slate organic matter. However, compared to investigations performed by Colwell et al. (1997) and Ringelberg et al. (1997) the columns showed a 100- to 500-fold greater PLFA concentration than detected in slates and sandstones of deep subsurface sediments with similar organic matter sources, but under anoxic conditions. The limited supply of easy degradable carbon resulted in a low level of microbial biomass and activity in the columns. Carbon in the control columns was only provided by black slate or atmospheric CO2 deposition for chemoautotrophic organisms. Results of gaseous and liquid carbon loss indicate that addition of a culture medium resulted in a higher loss of carbon derived from black slates. Carbon loss was enhanced in the medium-fed columns compared to control columns. Additionally, if the total carbon loss in the medium-fed columns was related to the added carbon (glucose, asparagine) it could be shown that the amount of carbon released from these columns exceeded the amount of added carbon by 60% (Table 1). The observed positive priming effect is related to enhanced degradation of black slate derived carbon, since the additionally released C-CO2 exceeded the amount of added carbon introduced with the culture medium. However, plant and animal debris might also contribute to the enhanced carbon release. Positive priming for black carbon in soils as a result of glucose addition was also shown by Hamer et al. (2004) and Kuzyakov et al. (2009).

The observed positive priming effect relative to controls is similar to effects measured by Hamer et al. (2004) for charred black carbon. The studies by Hamer et al. (2004) and Kuzyakov et al. (2009) suggested cometabolic decomposition. This suggest that microorganisms are not dependent on black slate use as carbon and energy source, but enzymes produced by microorganisms are able to degrade black slate. The combined results from compound specific 13C and 14C analysis suggest that different microbial groups use different carbon sources for their growth. Our 13C results suggest that especially Gram-positive bacteria are able to use black slate derived carbon as carbon source and incorporate into microbial biomass. Gram-positive bacteria might even benefit from a priming effect. A glucose uptake of ~33% indicated by 13C values coincided with a relatively high black slate uptake as can be seen for the PLFA marker for Gram-positive bacteria (i, a, 10Me17:0br) and microorganisms (Figs. 4 and 5). In contrast the higher glucose uptake in Gram-negative bacteria resulted in almost no black carbon uptake. Results can have implications on degradation processes in natural environments. Stockpiles are exposed to the earth’s surfaces for decades and are now partly overgrowing by plants and forests. Such conditions may facilitate microorganisms to grow on black slate surfaces and accelerate degradation processes depending on the input of fresh organic material.

In conclusion we could demonstrate:

1. The biodegradability of low grade metamorphic black slates was demonstrated using a column experiment. Microbial degradation of high molecular weight substances must therefore be considered as a process affecting organic matter in sediments and rocks when active microorganisms are present.
2. We observed positive priming as a result of glucose addition. A moderate glucose uptake corresponded with a relative high black slate uptake.
3. Taken together results from PLFA, stable isotope and radiocarbon measurements, the results emphasize that especially Gram-positive bacteria are able to utilize black slate derived carbon. Gram-positive bacteria might have advantages to colonize solid surfaces and to degrade complex biopolymers.
4. The inoculation with the fungus S. commune did not affect degradation of black slates in our experiment, probably because the inherited microbial population outcompeted the fungus.
5. The significant difference in $^{14}$C content of fossil geological systems compared to modern carbon sources can be used to investigate microbial carbon uptake and cycling.

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