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Unfavorable environmental conditions: Consequences for microbial metabolism and C stabilization in soil

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Ezekiel K. Bore

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Director: Prof. Dr. Achim Dohrenbusch

Evaluation committee:

Prof. Dr. Michaela Dippold, Department of Biogeochemistry of Agroecosystems, University of Goettingen, Germany (1st reviewer)

Prof. Dr. Sandra Spielvogel, Department of Soil Science, University of Kiel, Germany (2nd reviewer)

Prof. Dr. Andreas von Tiedemann, Department of Crop Sciences, University of Goettingen, Germany (3rd reviewer)

Prof. Dr. Yakov Kuzyakov, Department of Soil Science, University of Goettingen, Germany (4th reviewer)

To my wife – Florence, children: Cyprian, Michelle, Michaela and to God

Summary

Soil microorganisms are primary drivers of biogeochemical cycles, making them an important link between pedosphere and atmosphere. Soil microbes control the carbon (C) transfer from terrestrial ecosystem to the atmosphere via the decomposition of soil organic matter (SOM). Thus, soil microbes have the power to geo-engineer the climate through mineralization of C and nitrogen (N) compounds into greenhouse gases. However, soil microorganisms are frequently exposed to unfavorable conditions either naturally or anthropogenically. For every unfavorable condition, some microorganisms have been found to not only tolerate the conditions, but also often require the conditions to perform their functions. Therefore, understanding adaptation mechanisms enabling microorganisms to survive and function under the unfavorable conditions is essential predicting the response of nutrients and C cycle to diverse expressions of global change.

Position-specific ^{13}C and ^{14}C labeling and compound-specific analysis were applied as the main methodological approach to study microbial adaptations to unfavorable conditions and mechanisms underlying C stabilization in soil. The unfavorable conditions were: 1) subzero temperatures, 2) respiration inhibition by toxicants, and 3) nutrient limitation induced by sorption in soil. Incorporation of ^{13}C or ^{14}C into CO_2, bulk soil, microbial biomass and phospholipid fatty acids (PLFA) was quantified to identify adaptation mechanisms with the aid of metabolic tracing.

Subzero temperatures induced a switch from pentose phosphate pathway (PPP) to glycolysis. ^{13}C incorporated into microbial biomass was 3-fold higher at -5 than +5 °C, which points to a synthesis of intracellular compounds such as glycerol and ethanol in response to freezing. Even at -20 °C, less than 0.4% of ^{13}C was recovered in dissolved organic C (DOC) after one day, demonstrating complete glucose uptake by microorganisms. Consequently, we attributed the 5-fold higher extra- than intracellular ^{13}C to secreted antifreeze compounds. This suggests that with decreasing temperature, intracellular antifreeze protection is complemented by extracellular mechanisms to avoid cellular damage by crystallizing water. These mechanisms reflect the general response of microbial groups in soil. To understand the effect of freezing on individual microbial group in soil phospholipid fatty acid (PLFA) analysis was performed. Based on these results, a strong increase of mono-

unsaturated fatty acids and corresponding ^{13}C incorporation at -5 °C was attributed to 1) desaturation within existing fatty acid chains, and 2) *de novo* synthesis of PLFA. On contrary, ^{13}C incorporation into short-chain branched fatty acids was dominant at -20 °C. This reflects adaptation of microbial membranes to subzero temperatures.

A part from freezing, microbial activity can be hampared by toxicant exposure due to human activities. Despite exposure, CO_2 is persistently released from soils. To determine the origin and understand the mechanism underlying such persistent CO_2 release, soil microorganisms were exposed to sodium azide (NaN_3) as model toxicant inhibiting respiration. Respiration inhibition prevented ^{13}C incorporation into PLFA and decreased total CO_2 release. However, ^{13}C in CO_2 increased by 12% compared to control soils due to an increased use of glucose for energy production. The allocation of glucose-derived carbon towards extracellular compounds, demonstrated by a 5-fold higher ^{13}C recovery in bulk soil than in microbial biomass, suggests the synthesis of redox active substances for extracellular disposal of electrons to bypass inhibited electron transport chains within the cells. PLFA content doubled within 10 days of inhibition, demonstrating recovery of the microbial community. This growth was largely based on recycling of metabolically expensive biomass compounds, e.g., alkyl chains, from microbial necromass. The bypass of intracellular toxicity by extracellular electron transport permits the fast recovery of the microbial community.

Toxicants are not the only limitation in soils. Soil is full of C; however, this C is not always avilabe because 70-100% is found in close association with organic and mineral fractions in soil. This means that soil microorganism suffer starvation induced by nutrient sorption in soil. To identify metabolic adaptations of soil microbes to nutrient limitation induced by sorption, we tracked transformation of sorbed alanine. Sorption of alanine decreased the initial mineralization peak by ≈80% compared to free alanine. Consequently, a 4-fold incorporation of ^{14}C into microbial biomass was induced by sorption. Additionally, C-2 and C-3 of sorbed alanine remained in equal proportion in soil until day 3 contrary to free alanine in which significantly lower C-2 was incorporated than C-3. Even more vivid, an increased incorporation of C-2 into microbial biomass under sorption reveals a decrease of C flux through the citric acid cycle. Therefore, use of sorbed substrate shifts microbial metabolism towards a higher C use in anabolism, resulting in increased carbon use efficiency (CUE).

In addition to changes in microbial metabolic activity, shift in microbial community structure to compensate for the loss of more sensitive populations also occurs under unfavorable conditions. The fungal/bacterial PLFA ratio revealed a shift towards bacteria at subzero temperatures and in presence of respiration inhibiting toxicants. However, susceptibility of fungal populations to toxicant was short-lived compared to subzero temperatures. This suggests that fungi are more resilient to toxicants than perturbations imposed by freezing.

Changes in microbial metabolic activity and community structure due to changes in environmental conditions also influence ecosystem-level C, energy and nutrient flows, especially considering that C derived from labile compounds such as sugars have been shown to persist longer in soil than those from compounds of high recalcitrance such as lignin. Tracking glucose and ribose-derived C under long-term field conditions revealed that different mechanisms underlie their persistence. The persistence of glucose-derived C was mainly dominated by recycling. On contrary, stabilization in non-living SOM characterized the persistence of ribose-derived C. Therefore, even with the same class of labile compounds – the monosaccharides, the mechanisms responsible for C stabilization differ. Thus, stabilization of C in soil is largely influenced by metabolic transformation of the compound – affected not only by the compound class and its metabolic pathway, but also by the environmental conditions to which the microorganism is adapting its metabolism.

Thus, for every condition investigated, some microorganisms have shown tolerance, implying that the limit of life on Earth is far from being well defined. Combining positions-specific labelling with compound-specific analysis revealed such mechanisms allowing microbes to survive and function under unfavorable conditions. Microorganisms induce a suite of not only metabolic and physiological changes, but also shifts in microbial community structure in response to unfavorable conditions. The changes in microbial metabolic activity influences C stabilization in soil. Knowledge on these adaptation strategies and their implications for C fluxes in crucial in predicting changes in C cycles induced by various phenomena of global change.

Zusammenfassung

Bodenbiogeochemische Stoffkreisläufe werden primär durch Bodenmikroorganismen angetrieben, was diese zu einem wichtigen Bindeglied zwischen der Pedo- und der Atmosphäre macht. Bodenmikroben kontrollieren den Kohlenstoff (C)-transfer von terrestrischen Ökosystemen zur Atmosphäre durch die Zersetzung von organischer Bodensubstanz (OBS). Daher haben Bodenmikroben die Macht, das Klima durch die Mineralisierung von C- und Stickstoff (N)-verbindungen zu Treibhausgasen zu „geoingeeren". Allerdings sind Bodenmikroorganismen häuftig widrigen Bedingungen ausgesetzt, welche entweder natürlich vorkommen oder vom Menschen verursacht werden. Für jeden widrige Bedingung gibt es widerrum Mikroorganismen, welche diese nicht nur tolerieren, sondern sie sogar benötigen um ihre Funktionen auszuführen. Um die Änderungen von Nährstoff- und C-Kreisläufen unter globalem Wandel vorherzusagen ist es daher essenziell, Adaptionsmechanismen, welche es Mikroorganismen erlauben unter widrigen Bedingungen zu überleben und funktionieren, zu verstehen.

Positionsspezifische ^{13}C und ^{14}C Markierung und substanzspezifische Analysen wurden als Hauptmethoden angewandt, um mikrobielle Anpassungen an widrigen Bedingungen, sowie die zugrunde liegenden C-Stabilisierungsmechanismen im Boden zu erfassen. Die widrigen Bedingungen waren: 1) Temperaturen unter 0 °C, 2) Respirationsinhibierung durch Giftstoffe, und 3) Nährstofflimitierung durch Sorption an Bodenpartikel. Einbau von ^{13}C oder ^{14}C in CO_2, Gesamtboden, mikrobielle Biomasse und Phospholipid-Fettsäuren wurde quantifiziert um mit Hilfe von „metabolic Tracing" die Adaptionsmechanismen aufzudecken.

Temperaturen unter 0 °C induzierten einen Wechsel vom Pentose-Phosphat-Weg (PPP) zu Glycolyse. ^{13}C-Einbau in mikrobielle Biomasse war bei -5 °C 3-fach höher als bei +5 °C, was auf die Synthese interzellulärer Bestandteile wie Glycerol oder Ethanol als Reaktion auf Frost hindeutet. Sogar bei -20 °C wurde nach einem Tag weniger als 0,4% des ^{13}C im gelösten organischen C (DOC) gefunden. Dies demonstriert die komplette Aufnahme von Glukose durch Mikroorganismen. Den 5-fach höheren Gehalt an extra- im Vergleich zum intrazellulären ^{13}C führten wir daher auf ausgeschiedene Frostschutzsubstanzen zurück. Dies legt den Schluss nahe, dass mit sinkender Temperatur interzellulärer von extrazellulärem Frostschutz unterstützt wird um Zellschäden durch kristallisierendes Wasser zu vermeiden.

Ein starker Anstieg des Gehalts von einfach ungesättigten Fettsäuren und damit verbundener höherer Einbau von ^{13}C bei -5 °C wurde durch 1) Desaturierung von existierenden Fettsäureketten und 2) Neubildung von PLFA erklärt. Im Gegensatz dazu überwog bei -20 °C der Einbau von ^{13}C in kurzkettige, verzweigte Fettsäuren. Dies zeigt einen Adaptionsmechanismus der mikrobiellen Membranen an Temperaturen unter 0 °C auf.

Respirationsinhibierung durch Natriumazid (NaN_3) verhinderte den Einbau von ^{13}C in PLFA und verminderte den gesamten CO_2-Ausstoß. Der Gehalt von ^{13}C in CO_2 war jedoch im Vergleich mit den Kontrollböden um 12% erhöht, da die aufgenommene Glukose verstärkt zur Energieerzeugung benutzt wurde. Die 5-fach höhere ^{13}C-Wiederfindung im Boden im Vergleich zur mikrobiellen Biomasse legt nahe, dass glukosebürtiger C zur Produktion von extrazellulären Substanzen genutzt wurde. Die Produktion von redoxaktiven Substanzen zur extrazellulären Beseitigung von Elektronen würde es den Zellen erlauben, die inhibierten Elektronentransportketten zu umgehen. Der Gehalt an PLFA verdoppelte sich zehn Tage nach der Inhibierung, was eine Erholung der mikrobiellen Gemeinschaft demonstriert. Der beobachtete Wachstum basierte dabei zum Großteil auf dem Recycling von metabolisch teuren Biomassekomponenten, z.B. Alkylketten, von mikrobieller Nekromasse. Das Umgehen von interzellulären Giftstoffen durch den Transport von Elektronen außerhalb der Zellen erlaubt eine schnelle Erhohlung der mikrobiellen Gemeinschaft.

Sorption von Alanin verminderte das initiale Mineralisierungsmaximum um ≈80% im Vergleich zu freiem Alanin. Der Einbau von sorbiertem Alanin in die mikrobielle Biomasse war vier Mal höher als von freiem Alanin. Zusätzlich dazu waren C-2 und C-3 von sorbiertem Alanin an Tag 3 noch gleichermaßen im Boden vorhanden, wärend C-2 von freiem Alanin schon deutlich weniger eingebaut war als C-3. Noch deulicher wird der Unterschied zwischen freiem und sorbiertem C-2, wenn mikrobielle Biomasse betrachtet wird. Dies deckt einen verringerten Fluss durch den Zitronensäurezyklus auf. Daher verändert die Nutzung von sorbiertem Substrat die metabolen Wege hin zu einem verstärkeren anabolen Einsatz, womit eine höhere Kohlenstoffnutzungseffizienz (CUE) einher geht.

Zusätzlich zu den Änderungen der mikrobiellen Stoffwechselaktivität findet unter widrigen Bedingungen eine Anpassung der mikrobiellen Gemeinschaft statt, um den Verlust von sensitiveren Populationen zu kompensieren. Das Verhätnis von pilzlichen zu bakteriellen PLFA zeigte, dass durch niedrigere Temperaturen und respirationsinhibierende Giftstoffe

eine stärkere bakteriell geprägte Gemeinschaft entsteht. Die Nachteile für die pilzlichen Mikroorganismen, welche durch Giftstoffe ausgelöst wurden waren allerdings von kürzerer Dauer als diejenigen der niedrigen Temperaturen. Gegenüber von Giftstoffen scheinen Pilze also eine größere Wiederstandskraft zu besitzen als gegenüber Frost.

Änderungen des mikrobiellen Stoffwechsels und der Struktur der mikrobiellen Gemeinschaft als Reaktion auf sich ändernde Umweltbedingungen haben auch einen Einfluss auf C-, Energie- und Nährstoffflüsse auf der Ökosystemebene, besonders in Anbetracht der Tatsache, dass gezeigt wurde, dass C aus labilen Quellen wie Zuckern im Boden länger erhalten bleiben kann als Substanzen mit hoher Rekalzitranz wie Lignin. Die Verfolgung von glukose- und ribosebürtigem C unter Langzeitfeldbedingungen zeigte dabei, dass der Persistenz verschiedene Mechanismen zu Grunde liegen können. Die Persistenz von glukosebürtigem C konnte zum Großteil durch Recycling erklärt werden. Im Gegensatz dazu charakterisierte die Stabilisierung in nicht-lebender OBS die Persistenz von ribosebürtigem C. Sogar innerhalb einer Stoffklasse labiler organischer Stoffe – den Monosacchariden – unterscheiden sich also Stabilisierungsmechanismen. Daher wird deutlich, dass die Stabilisierung von C im Boden zum Großteil von metabolen Transformationen abhängt, welche nicht nur von der Substanzklasse und ihren Stoffwechselwegen bedingt wird, sondern auch von den Umweltbedingungen an welche die Mikroorganismen ihren Metabolismus angepasst haben.

Für jede untersuchte Bedingung haben Mikroorganismen Toleranz gezeigt, was darauf hindeutet, dass die Grenzen für Leben auf der Erde alles andere als gut definiert sind. Die Kombination von positionsspezifischer Markierung mit substanzspezifischen Analysen deckte die Mechanismen auf, welche es Mikroben erlauben unter widrigen Bedingungen zu überleben und funktionieren. Mikroorganismen verwenden eine Reihe von metabolen und physiologischen Anpassungen, aber auch Änderungen in der Zusammensetzung der mikrobiellen Gemeinschaft, als Antwort auf widrige Bedingungen. Die Anpassungen der mikrobiellen Stoffwechselaktivität beeinflust die C-Stabilisierung im Boden. Daher ist das Wissen über diese Anpassungsstrategien und ihre Auswirkungen auf C-Flüsse entscheidend für die Vorhersage von Änderungen der C-Kreisläufe, welche durch verschiedene Ausprägungen des globalen Wandels induziert werden.

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Table of contents

List of Figures

List of Tables

Abbreviations

ANOVA	analysis of variance
C	carbon
Cd	cadmium
CEC	cation exchange capacity
CFE	chloroform fumigation extraction
Cu	copper
CUE	carbon use efficiency
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
EA	elemental analyzer
EOC	extractable organic carbon
FAME	fatty acid methyl esters
GC-MS	gas chromatography mass spectrometer
Hg	mercury
HSD	honest significance difference
LMWOS	low molecular weight organic substances
MRT	mean residence time
N	nitrogen
PLFA	phospholipid fatty acids
RNA	ribonucleic acid
SOC	soil organic carbon
SOM	soil organic matter
TOC	total organic carbon
TN	total nintrogen

1. Extended summary

1.1. Introduction

1.1.1. Role of microorganisms and their metabolism in soils

Soil is a key environmental resource, supporting a wide range of ecosystem functions and services. Understanding soil biogeochemistry is essential in managing ecosystem services provided by soil such as water quality, soil fertility and mitigating climate changes through reduced C inputs and feedbacks (Schmidt et al., 2011). Soils store at least thrice as much carbon (C) in soil organic matter (SOM) as in either atmosphere or terrestrial vegetation (Parry, 2007). Although most soil C is derived from plant material (Koegel-Knabner, 2002), almost the entire proportion pass through microbial biomass before being transformed into SOM (Miltner et al., 2012; Liang et al., 2017). Microorganisms secrete extracellular enzymes (exoenzymes) into soil, which degrade plant and microbial residues mainly by depolymerization of macromolecules, producing soluble substrates. The solubilized substrates can be taken up by plants or utilized by microbes for energy production (ATP), biosynthesis of various cellular components and ultimately release of CO_2 as a by-product (Gunina and Kuzyakov, 2015). SOM is thus one of the factors controlling the atmospheric CO_2 concentration and its proper management can help in mitigating climate change and enhancing global food security (Lal, 2004).

1.1.1.1. Impacts of environmental disturbances on microbial functioning

Soil microbes are the crucial link between the pedosphere and the atmosphere. Microorganisms control the transfer of C from terrestrial ecosystem to the atmosphere via the decomposition of SOM. Thus, microbes directly control the atmospheric CO_2 concentrations. However, soil microbes are exposed to global change factors, which include temperature fluctuations (Allison and Treseder, 2008; Frey et al., 2008), altered nutrient supply (Demoling et al., 2008; Allison et al., 2010), and moisture availability (Hawkes et al., 2011). Additionally, anthropogenic perturbations such as fertilization, use of pesticides or soil contamination with heavy metals from mines, industrial, agricultural and technological applications also have detrimental effects on soil microorganisms. Such unfavorable conditions disrupt normal functioning of the biomolecules, which may cause cell death (Howland, 1998). Therefore, scientists tend to assume that there are strict boundaries to the

biosphere imposed by terrestrial life's requirement for a rather narrow and specific range of environmental conditions (Rampelotto, 2010). However, recent studies show that life is not always as sensitive as we might have imagined, and that the limits of life on Earth are far from being well defined (Rampelotto, 2010). Previous notions of what is or not a hostile environment have turned out to be erroneous. For every unfavorable environmental condition, some microorganisms have shown not only to tolerate the conditions, but also often require the conditions to function. Accordingly, microbes occupy every conceivable ecological niche, meaning that they must be having physiological adaptation mechanisms to survive and remain functional or they will die (Schimel et al., 2007). However, these adaptation strategies may create physiological cost at organism level and can alter the active microbial community composition, create shifts in ecosystem-level C, energy and nutrient flows (Schimel et al., 2007). Therefore, understanding adaptation strategies enabling microorganisms to survive and function under fluctuating environmental conditions is important for predicting the response of nutrients and C cycles to diverse expressions of global change.

1.1.1.2. Metabolic pathways reconstruction based position-specific labeling

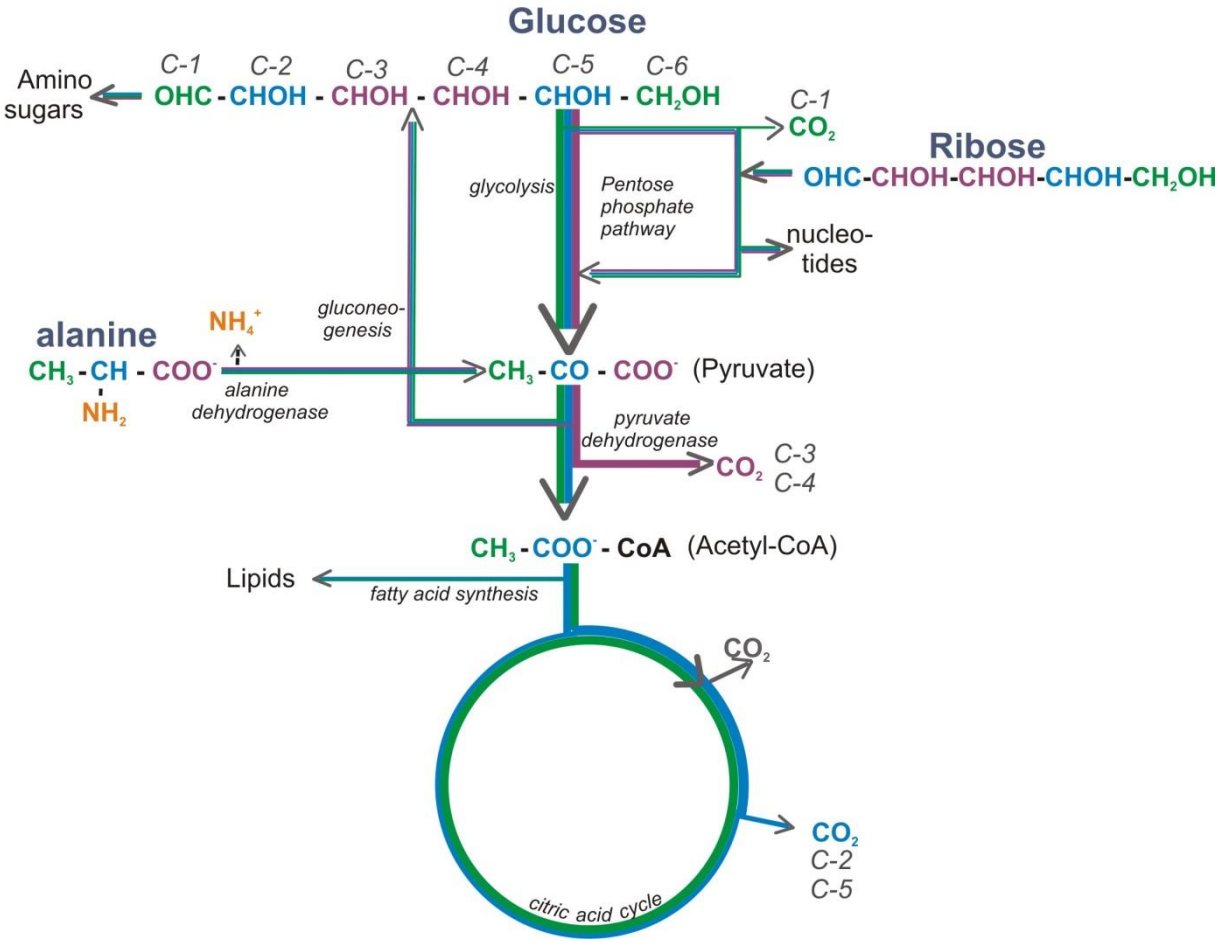
Knowledge of metabolic pathways through which organic substances are transformed in soil is crucial in unraveling these mechanisms. Thus, metabolic tracing is an invaluable tool for identifying alterations in microbial transformation of organic substances under unfavorable conditions (Scandellari et al., 2009; Dijkstra et al., 2011b; Dippold et al., 2014). To track changes in metabolic pathways during microbial transformation of organic substances in soil under fluctuating environmental conditions, use of labeled substances is crucial. Uniformly labeled substances have been used to study net SOM transformations under steady state conditions. Unfortunately, uniformly labeled substances only offer quantitative information, without giving insights into the mechanisms driving the fluxes. The advent of position-specific labeled substances revolutionized the perception on microbial metabolism as an integral component of SOM transformation. Use of position-specific labeled substances allows fate of individual molecular positions to be determined (Scandellari et al., 2009; Dijkstra et al., 2011b; Apostel et al., 2015), permitting detailed reconstruction of metabolic pathways and *de novo* formed products (Dippold and Kuzyakov, 2013). This technique

provides a toolbox required to elucidate changes in microbial metabolism brought about by altered environmental conditions. Therefore, combining uniform and position-specific labeled substances avails not only quantitative, but also qualitative information on microbial metabolism under investigated conditions.

Studies tracking the fate of individual substrate C atoms by position-specific labeling have been used to characterize and model microbial metabolic pathways under ordinary conditions (Dijkstra et al., 2011b). In this thesis, microbial metabolism under disturbed conditions in laboratory was unraveled using uniform and position-specific ^{13}C labeled glucose and ^{14}C labeled alanine. Consequences of fluctuating environmental conditions on microbial metabolism and C stabilization in the field were explored using uniform and position-specific ^{13}C labeled glucose and ribose. Sugars are the main components of low molecular weight organic substances (LMWOS) (Gunina and Kuzyakov, 2015). Sorption and other interactions with SOM are nearly irrelevant for sugars, because they neither have charged functional groups nor hydrophobic molecular parts. Thus, their fate is mainly determined by microbial utilization (Gunina and Kuzyakov, 2015). Among the sugars, glucose is the most ubiquitous and abundant substrate for microorganisms in soil (Fischer et al., 2007; Kuzyakov, 2010), making it a potent substrate for metabolic tracing. Ribose is also a potent substrate for metabolic tracing because of existence of strong coupling between hexose and pentose uptake by microorganisms (Baumann and Baumann, 1975; Nobre et al., 1999). Amino acids are also important components of LMWOS because they couple the C and nitrogen (N) cycles (Dippold and Kuzyakov, 2013). In top soil, amino bound N constitutes 7 – 50 % of total N (Stevenson, 1982; Gardenas et al., 2011). Among the amino acids, alanine is the most abundant in dissolved organic matter (Fischer et al., 2007). Besides, other physical factors such as sorption are important for amino acid transformation in soil. Thus, alanine was included as potent candidate for metabolic tracing also due to its entry point into metabolism – the pyruvate dehydrogenase level, where the two main metabolic pathways (glycolysis and citric acid cycle) are linked. To understand the role of metabolic tracing in SOM dynamics, transformation pathways of glucose, ribose and alanine are discussed in detail.

Using position-specific ^{13}C labeled glucose and pyruvate, Dijkstra et al. (2011b) developed a numeric model, which reconstructs the partitioning of glucose between glycolysis, the

pentose phosphate pathway (PPP) and biomass production as summarized in extended summary (ES) figure 1. Glucose C-3 and C-4 positions are completely oxidized to CO_2, while the remaining positions C-1/C-2 and C-5/C-6 in C_2-moieties will have a ratio of 1:1 after glycolysis. Whereas in PPP, C_2-moeties of positions C-2, C-3 and C-5, C-6 will have a ratio of 2:3 and position C-1 and C-4 are respired completely. Ribose is transformed via PPP in the same step as glucose after oxidation of C-1 position (ES figure 1) (Caspi et al., 2008). For alanine, oxidative deamination to pyruvate by alanine hydrogenase occurs before entering metabolism at the interface between glycolysis and the onset of the citric acid cycle (ES figure 1) (Caspi et al., 2008; Keseler et al., 2009; Dippold and Kuzyakov, 2013).



ES Figure 1: *Basic metabolism of glucose, ribose and alanine in heterotrophic organisms. Colored arrows indicate the fate of individual molecular positions.*

With this metabolic background knowledge, we investigated microbial metabolism under three unfavorable environmental conditions in laboratory and mechanisms of C stabilization in soil under long-term field conditions. The unfavorable conditions were 1) subzero

temperatures, 2) presence of respiration-inhibiting toxicants, and 3) sorption of substrates in soil.

1.1.2. Tracing microbial metabolism in unfavorable environments

1.1.2.1. Microbial metabolism at subzero temperatures

During winters, photosynthesis is nearly non-existent and respiration is 3 – 7 % of the mid-summer intensity (Panikov et al., 2006). Thus, high-altitude and latitude ecosystems, which can be net sink during summer, are often net source of CO_2 to the atmosphere on an annual basis (Oechel et al., 1997). Respiration of cold-adapted microbes has been suggested to contribute significantly to the CO_2 emission in these ecosystems (Oechel et al., 1997; Panikov et al., 2006). However, molecular and biochemical adaptations responsible for microbial freeze tolerance remain unclear. This knowledge gap hampers the estimation of C balances and ecosystem feedback responses. Thus, better understanding of microbial community dynamics and consequences on C and N mineralization in ecologically representative subzero temperatures is needed to accurately model greenhouse gas release from microbial activity in frozen soils.

Most of the studies on metabolism at low temperatures are based on isolated pure cultures from permafrost or use permafrost samples with inherently adapted microbes (Rivkina et al., 2000; Mykytczuk et al., 2013; Tuorto et al., 2014). However, subzero temperatures are also relevant to soils in temperate zones that freeze during winter. In frozen state, significant greenhouse gases are released from temperate soils, which make these soils a good analog for what we might expect from permafrost (Nikrad et al., 2016). The importance of these soils in global C cycling while frozen and their vulnerability to thawing, calls for a better understanding of how microbes in these soils will contribute to global C feedback under climate change (Nikrad et al., 2016). Therefore, mechanisms allowing microbes to survive and remain functional in temperate frozen soils were covered within the scope of this thesis.

1.1.2.2. Microbial metabolism in soil exposed to respiration-inhibiting toxicants

Microbial activity can also be altered by human activities such as fertilization, use of pesticides or soil contamination by heavy metals from mines, industrial, agricultural and technological application. However, mechanisms pertaining persistent CO_2 release from soils

exposed to toxicants (Voroney and Paul, 1984; Trevors, 1996) or contaminated with heavy metals (Bond et al., 1976; Ausmus et al., 1978; Fliessbach et al., 1994) remain unclear. Understanding the mechanisms responsible for CO_2 emission under such disturbances is important for predicting the response of nutrient and C cycles to future anthropogenically induced environmental changes (Schimel, 2013).

In this study, NaN_3 was used as model toxicant, inhibiting respiratory chains in a similar manner as pesticides such as phosphines or heavy metals such as mercury (Hg) and copper (Cu). Paradoxically, azide addition increased CO_2 efflux from soil in previous studies (Rozycki and Bartha, 1981; Trevors, 1996). The CO_2 emitted from soil following microbial metabolic inhibition was previously ascribed to active oxidative extracellular enzymes (EXOMET) already present in soil or released from dead cells (Maire et al., 2013). However, since this study solely traced catabolism, the ultimate source of the emitted CO_2 could not be definitively concluded. We therefore assessed microbial catabolic and anabolic processes with an aim of establishing the origin and underlying mechanisms of persistent CO_2 release from soils exposed to toxicants inhibiting respiratory chains.

1.1.2.3. Microbial metabolic adaptations to nutrient limitation induced by sorption

Beside the conditions disrupting metabolic processes in general, low substrate availability might be an additional factor limiting microbial performance in a certain environments. Many organic substances such as amino acids possess functional groups that allow sorption onto organic and mineral fractions to occur in soil. Sorption, being one of the processes stabilizing C in soil (Guggenberger and Kaiser, 2003; Lützow et al., 2006; Koegel-Knabner et al., 2008), can subsequently be a main process limiting microbial access to their growth substrate. However, this effect varies strongly with soil type, minerology, experimental approach and functional group of amino acids (Jones and Hodge, 1999; Dippold and Kuzyakov, 2013). Sorption can occur via ion exchange of positively charged amino group, by ligand exchange of carboxyl group(s) and by hydrophobic interactions with alkyl groups. Despite this information, the effect of sorption on microbial metabolism and consequently, the mechanisms behind the C stabilization are not fully understood.

Microbial utilization of free amino acids shows that metabolization occur mainly intracellularly after uptake by transport system (Hosie and Poole, 2001; Dippold and Kuzyakov, 2013). Uptake kinetics of single amino acids revealed that microbial uptake outcompete sorption in soil (Fischer and Kuzyakov, 2010). Lack of strongly sorbed amino acids in ecosystems suggests that sorption does not completely protect amino acids from biodegradation (Stevenson, 1982; Dippold et al., 2014). Indeed, sorption strength negatively correlates to microbial utilization of amino acids (Jones and Hodge, 1999). Thus, sorption is a key driver regulating the fate of amino acids. Sorption of alanine on mineral particles decreased microbial uptake and shifted metabolic pathways to a higher flux through the anabolism (Dippold et al., 2014). Whether these effects exist in soil, how it affects individual microbial groups and the resulting consequences on C stabilization remain unclear. Therefore, a study to bridge this knowledge gap was designed.

1.1.3. Persistence of labile C in terrestrial ecosystem

Persistence of labile C in soil may be attributed to functional groups, which allows interaction with organic and mineral fractions in soil as well as modifying microbial metabolism. The main source of bulk SOM is plant biomass, which is a complex conglomerate of different polymers and many monomeric compounds (Thomson, 1993). This conglomerate undergoes oxidative and hydrolytic degradation by enzymes and microorganisms, which may be accompanied by secondary structural changes (Stevenson, 1994). Any change in size and decomposition rates of soil C pools will potentially alter the atmospheric CO_2 concentration. Thus, it is important to know more about mean residence time (MRT) of C in physical and chemical fractions of soils in order to predict and model the behavior of soil C with respect to climate change (Houghton et al., 1998). This is particularly important because soil C dynamics in multiple SOM pools are related to origin and nature of carbonaceous compounds, with MRTs estimated to range from days to thousands of years (Gleixner et al., 2002). Molecular structure has long been thought to determine long-term decomposition rate of organic substances in soil (Schmidt et al., 2011). However, labile C atoms like those of sugars have been shown to persist longer in soil than those of chemical compounds of high recalcitrance such as lignin (Gleixner et al., 2002; Amelung et al., 2008; Schmidt et al., 2011). Therefore, mechanisms controlling C stabilization in soil still remain

elusive, which calls for better understanding in order to initiate new approaches (e.g. agricultural management practices) geared towards C sequestration.

Sugars are the most abundant labile compounds in the biosphere because they constitute structural components of most living biomass (Koegel-Knabner, 2002). Sugars are not only utilized by microorganisms as building block for various cellular components, but also as an energy source (Gunina and Kuzyakov, 2015). Additionally, sugars play an important role in aggregate formation. Polysaccharides act as glue, binding both mineral and organic particles, resulting in microaggregate formation (Six et al., 1999). To better understand the mechanisms underlying persistence of labile compounds' C atoms in soil, the fate of glucose and ribose as model sugars were assessed under long-term field conditions.

In this thesis, ^{13}C and ^{14}C position-specific labeling was applied as the main methodological approach to study microbial metabolism under unfavorable environmental conditions and C stabilization mechanisms. In combination with uniform labeling, position-specific labeling allowed identification of microbial adaptations to environmental stress in laboratory studies. Consequences of fluctuating environmental conditions on microbial metabolism and C stabilization under long-term field studies could also be elucidated. Therefore, position-specific labeling is a unique tool enabling transformation pathways of organic substances to be studied in detail as a key step in resolving the mystery of the fate of C in soil, which remains a key question to be answered in order to understand the mechanisms regulating the belowground C cycle. The findings as well as implications are summarized in the subsequent chapters.

1.1.4. Objectives

The main objective of this thesis was to identify microbial metabolic adaptations to unfavorable environmental conditions using position-specific ^{13}C and ^{14}C labeling and the resulting consequences on C stabilization in soil. The specific objectives were:

1. To identify mechanisms enabling microbes to survive and function under frozen soil conditions:
    a. reconstruct the metabolic pathways and predict the resulting metabolic adaptation mechanisms
    b. assess the effects of freezing on microbial community structure and identify modifications of alkyl chains in membrane phospholipids
2. To investigate microbial metabolic adaptations to respiration-inhibiting toxicants
3. To elucidate the consequences of C-limitation induced by sorption of organic substances on the microbial metabolism
4. To reveal the mechanisms underlying the persistence of labile compounds' C atoms in terrestrial ecosystems

1.2. Experiments and methods

1.2.1. Laboratory experiments

The soils were collected from agriculturally used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 a.s.l.) with a mean annual temperature of 7 °C, and a mean annual precipitation of 874 mm. The soil had a pH (KCl) of 4.88, a pH (H_2O) of 6.49, a total organic C (TOC) and total N (TN) content of 1.77% and 0.19%, respectively. Cation exchange capacity (CEC) was 13 $cmol_C\ kg^{-1}$. The soil was collected from 0-10 cm, sieved to 2 mm, and stored at 5 °C until use for incubation studies.

1.2.1.1. Microbial metabolism in soil at subzero temperatures

The soils were incubated in airtight screw-cap glass microcosms with a base layer of quartz sand. About 80 g samples of soil were transferred to sample rings and installed on ceramic plates above the quartz sand. To equalize densities, an equal pressure was applied to all soils. The soils were rewetted to field capacity by adding 10 ml of water to the underlying sand and stored at +5 °C for 2 days. Thereafter, the microcosms were preconditioned at +5, 5 and 20 °C for 24 hours. These temperatures were chosen to simulate 1) average annual soil temperature at the sampling site (control), 2) average winter temperature, and 3) lowest temperatures in some winters on this site and common deep freezer storage temperatures, respectively. Four position-specifically ^{13}C-labeled isotopomers of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4, and ^{13}C-6), uniformly ^{13}C-labeled (U-^{13}C) and unlabeled glucose (natural abundance background) were applied to the soils in separate microcosms with four replicates of each. With four fine tip pipettes, 5 ml of 2.55 mM glucose solutions were applied on soil surface. This C concentration was less than 5% of microbial biomass C and therefore, does not alter microbial community structure (Kuzyakov, 2010). Cups with 5 ml of 1 M NaOH were placed in each microcosm to trap CO_2. To depress the freezing point of the traps at -20 °C, 20% (w/v) of NaCl was dissolved in NaOH. The microcosms were sealed and incubated at the respective temperatures.

1.2.1.2. Microbial metabolism in soil with respiration-inhibiting toxicants

Incubation was setup as described in section 1.2.1.1. Two treatments comprising of microbial activity inhibition with sodium azide (NaN_3) and control soils (without NaN_3) were prepared. Four position-specific ^{13}C-labeled isotopomeres of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4 and

^{13}C-6), uniformly ^{13}C-labeled (U-^{13}C) and non-labeled glucose (natural abundance background) were applied to the soil in separate microcosms with four replicates each and three sampling dates resulting in 144 individual microcosms. With four fine tip pipettes, 5 mL of 2.55 mM glucose solution was applied onto the soil surface in each microcosm. Glucose solution for the treated soil contained 1 mM NaN_3 to sustain inhibition. Cups filled with 5 ml of 1 M NaOH were placed into each microcosm to trap CO_2. Microcosms were sealed and incubated at 5 °C in the dark.

1.2.1.3. Effects of sorption on microbial transformation of alanine

The experiment consisted of sorbed and free alanine. To produce soil with sorbed tracer, solutions of alanine labeled with ^{14}C on each of the three positions (^{14}C-1, ^{14}C-2, and ^{14}C-3) were added to soil sterilized by γ-radiation (10 h at 53 kGy) at synergy Health (Radeberg, Germany). About 10 g of the soil with sorbed alanine was added to about 70 g of non-sterilized soil at the beginning of the incubation. In the free Alanine treatment, the same amount of position-specific labeled alanine as in sorbed treatment was applied to approximately 80 g of soil in each microcosm. During the 10 days of incubation, incorporation of ^{14}C from alanine into CO_2, extractable organic carbon, microbial biomass and soil was analyzed.

1.2.2. Field experiment

The field experiment was set up in the same site where soil for the laboratory experiments was collected (see section 1.2.1). Briefly, a 12 × 12 m field was divided into four quadrants, each representing a replicate. Columns, each with diameter of 10 cm and height of 13 cm were installed in the quadrants to a depth of 10 cm. Four position-specifically ^{13}C-labeled isotopomers of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4, and ^{13}C-6), two position-specifically ^{13}C-labeled isotopomers of ribose (^{13}C-1, ^{13}C-5), uniformly ^{13}C-labeled (U-^{13}C) and unlabeled glucose and ribose were applied to the soils in separate columns in the quadrants, organized in randomized block design. 10 ml of 0.09 mM C monosaccharide solutions (glucose and ribose) were applied to the soil at five points in the columns via syringes to allow uniform distribution. This C concentration was chosen to simulate the natural sugar pool in soil solution (Fischer et al., 2007). To prevent leaching of the added monosaccharides due to rainfall, a roof was erected over the plot for the first 10 days. Since the columns had no

vegetation growth, monosaccharide uptake was solely microbial. Soils in the columns were destructively sampled and weighed after 3, 10, 50, 250, 430 and 800 days.

1.2.3. Analytical methods

1.2.3.1. Quantification of CO_2 and $\delta^{13}C$ values

0.4 ml of each CO_2 trap was diluted with ultrapure water in the ratio 1:10. CO_2 content was then measured with a non-dispersive infrared (NDIR) gas analyser (TOC 5050, Shimadzu Corporation, Kyoto, Japan). $SrCO_3$ was precipitated from the remainder of the CO_2 traps using $SrCl_2$. After drying the precipitates at 60 ˚C, bulk $\delta^{13}C$ values were determined by weighing 1-2 g of each sample into a tin capsule and measuring with a Flash 2000 Elemental Analyser coupled by a ConFlo III interface to a Delta V advantage IRMS (all Thermo Fisher, Bremen, Germany). ^{13}C content in CO_2 was calculated according to a mixing model (Gearing et al., 1991)

1.2.3.2. Radiochemical analysis

Respired and released CO_2 from combustion of soil samples were trapped in NaOH. ^{14}C in NaOH and microbial biomass extracts were measured by scintillation counter after mixing with a scintillation cocktail.

1.2.3.3. Quantification of ^{13}C in soil and microbial biomass

Microbial biomass extracted by chloroform-fumigation extraction (CFE) (Wu et al., 1990) and soil samples were analysed for $\delta^{13}C$ values after freeze drying. The measurements were performed with elemental analyzer (Eurovector, Milan, Italy) coupled by a ConFlo III interface to a Delta V advantage IRMS (both units from Thermo Fisher Scientific, Bremen, Germany). ^{13}C incorporation was calculated according to a mixing model (Gearing et al., 1991).

1.2.3.4. ^{13}C-PLFA analysis

^{13}C incorporated into phospholipid fatty acids (PLFA) was determined by compound-specific analysis. This entails extraction of polar lipids according to Bligh-and-Dyer approach (Frostegård et al., 1991; Gunina et al., 2014). The lipids were purified by liquid-liquid and solid phase extraction chromatography then hydrolyzed into free fatty acids. This was

followed by derivatization of free fatty acid to fatty acid methyl esters (FAMEs). ^{13}C incorporated into PLFAs was quantified by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and mixing models with the respective background treatments as a reference.

1.3. Results and Discussion

1.3.1. Overview of main results of the studies

Objectives and main conclusions of individual studies are presented in ES Table 1.

ES Table 1: objectives and main conclusions of the studies undertaken

Study	Objectives	Main conclusions
Study 1: Microbial metabolism in soil at subzero temperatures: Adaptation mechanisms revealed by position-specific ^{13}C labeling.	• To reconstruct microbial metabolic pathways at subzero temperatures • Elucidate microbial metabolic adaptations to freezing	• Metabolic pathway switched from PPP to glycolysis at subzero temperatures • Intracellular antifreeze mechanisms at -5 °C were complemented by extracellular mechanism at -20 °C
Study 2: Structural and physiological adaptations of soil microorganisms to freezing revealed by position-specific labelling and compound-specific ^{13}C analysis	• To identify adaptations of microbial phospholipids to freezing • Investigate freezing effects on microbial community structure	• Mono-unsaturated PLFAs were abundant at -5 °C, but a shift to saturated short-chain branched fatty acids dominated at -20 °C. • Microbial community structure shifted towards bacteria at suzero temperatures
Study 3: Soil microorganisms can overcome respiration inhibition by coupling intra- and extracellular metabolism: ^{13}C metabolic tracing reveals the mechanisms.	• Identify origin of CO_2 in soils with respiration inhibiting toxicants • Investigate microbial metabolic adaptations to respiration inhibition	• CO_2 release from soils with respiration inhibiting toxicants was largely due to intracellular metabolism • Glucose-derived ^{13}C was mainly used by microorganisms for energy

		production and syntheses of extracellular electron transport compounds to bypass respiration inhibition • Growth during recovery period was based on the recycling of necromass compounds
Study 4: Sorption of alanine changes microbial metabolism in addition to availability	• Determine the effects of sorption on microbial metabolism • Reveal the mechanisms of C stabilization induced by sorption	• Sorption decreased initial mineralization of alanine by ≈80% • Sorption induced a decrease in C flux through the citric acid cycle, causing a higher allocation of C to anabolism than catabolism
Study 5: Persistence of sugar-derived C in soil is controlled by the initial use of the C positions in the microbial metabolism	• Identify metabolic pathways of monosaccharide transformations under field conditions • Reveal mechanisms underlying persistence of C derived from labile compounds in soil	• Glycolysis, PPP and gluconeogenesis occurred simultaneously during sugar transformations • The persistence of glucose-derived C was mainly dominated by recycling while stabilization in non-living SOM characterized the persistence of ribose-derived C

1.3.2. Microbial activity in unfavorable environments

Individually, microbes certainly emit small amounts of C into the atmosphere, but on global scale, the abundance of microorganisms that mineralize compounds in soil into greenhouse

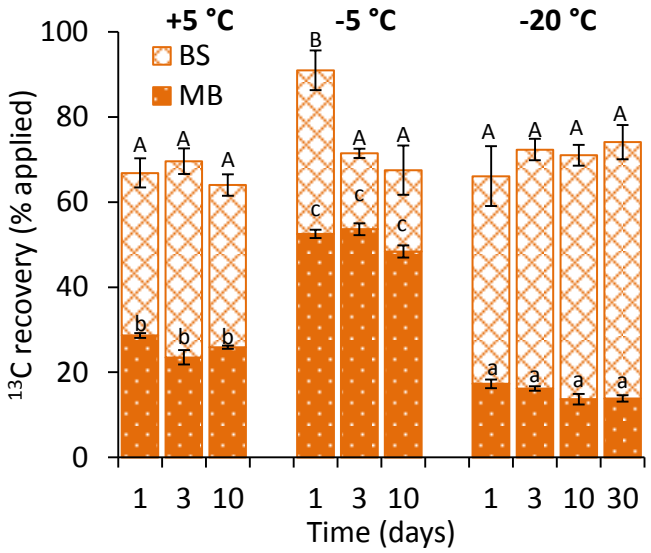
gases gives them the power to geo-engineer the climate (Nikrad et al., 2016). However, there are many potential conditions and cases under which microbes are exposed to unfavorable conditions in soils and some of them have been studied in detail than others. Within the scope of this thesis, I selected representative unfavorable conditions to be able to observe a large spectrum of metabolic adaptation strategies by tracing via position-specific isotope labeling. Consequently, freezing, which is not only an abiotic environmental factor limiting the kinetics of many processes but also expose microbes to physical challenges in their environment was selected. Secondly, toxicant directly inhibiting one specific microbial pathway i.e. redox equivalent regeneration was applied and subsequent mechanisms enabling microorganisms to overcome this inhibition was elucidated. Thirdly, microbial adaptation to limited nutrient supply was simulated by sorption, which is a common process limiting nutrient accessibility in soil. These three unfavorable conditions cover a broad range of adaptation strategies discussed in detail in the following sections.

1.3.2.1. Microbial metabolism at subzero temperatures

In frozen state, significant amount of greenhouse gases are released from temperate soils, making these soils a good analog for what we might expect from permafrost (Nikrad et al., 2016). The importance of these soils in global C cycling while frozen and their vulnerability to thawing, calls for a better understanding of how microorganisms in these soils will contribute to global C feedback. To identify microbial adaptations to freezing, soils were incubated with position-specific ^{13}C labelled glucose at +5 (control), -5 and -20 °C.

The ^{13}C recovery pattern in CO_2 showed high oxidation of the C-1 position at +5 °C, revealing that glucose was predominantly catabolized via the pentose phosphate pathway (Dijkstra et al., 2011c; Apostel et al., 2015). On the contrary, metabolic behaviours completely switched over to a preferential respiration of the glucose C-4 position at -5 °C and this behaviour was still weakly visible at -20 °C. High ^{13}C recovery from the C-4 position reflects glucose transformation via glycolysis (Dijkstra et al., 2011c; Apostel et al., 2015). To understand the cause of the switch, we assessed ^{13}C incorporation into microbial biomass.

^{13}C incorporation into microbial biomass at -5 °C was 2 and 3 times higher than at +5 and -20 °C respectively, and this incorporation made up more than two-thirds of the total glucose-derived ^{13}C recovered in bulk soil (ES Figure 2).



ES Figure 2: *^{13}C recovery (mean ± SEM, n = 4) of uniformly labeled glucose in bulk soil (BS) and extractable microbial biomass (MB), 1, 3, 10 and 30 (-20 °C) days after application at +5, -5 and -20 °C. Significant effects (p<0.05) of temperature and time and on recovered ^{13}C in BS are indicated by upper case letters above the error bars, and in extractable MB by lower case letters.*

Recently, ^{13}C incorporation into microbial DNA was not detected after 1 month at subzero temperatures (Tuorto et al., 2014). This suggests that glucose utilization by microorganisms at -5 °C was mainly intracellular metabolic responses to freezing and was rapidly activated. Intracellularly, vast majority of prokaryotic cells contain no unbound water (Coombe, 2002). Hence, deleterious effects of freezing to these microbes, include, but are not limited to osmotic imbalance. Water is not a limiting factor at -5 °C the solute concentration increase in unfrozen volume, depressing the freezing point depression (Brooks et al., 1997; Price, 2000). Hence, microorganisms invest energy in the synthesis of intracellular compounds that minimize effects of osmotic stress brought about by freeze-dehydration. Freeze-dehydration alters cell membrane ultrastructure and membrane bilayer fusion and causes organelle disruption (Mazur, 2004). Glycerol and ethanol were recently identified as abundantly synthesized compounds at -4 °C (Drotz et al., 2010). These compounds act as cryoprotectants and are compatible solutes against freeze-dehydration (Block, 2003). The synthesis of these compounds is consistent with glucose transformation via glycolysis, which is confirmed by the high ^{13}C recovery from C-4 in CO_2. Glycerol therefore limits intracellular damage caused by freezing, hence maintaining microbial activity and CO_2 production at subzero temperatures (Drotz et al., 2010).

We obtained lowest ^{13}C incorporation into microbial biomass at -20 °C (1.3 and 3 times lower than at +5 and -5 °C, respectively) (ES Figure 2). Nonetheless, the ratio of extracellular to microbial biomass ^{13}C in soil was highest (ratio 5:1) at -20 °C (ES Figure 2). This high ratio could not be attributed to the non-metabolized glucose that remained in the soil because less than 0.4% of applied ^{13}C was recovered in DOC at day 1. The high ^{13}C recovery in bulk soil compared to microbial biomass at -20 °C suggests that microorganisms utilized glucose for the formation of extracellular antifreeze compounds. To resist physical, environmental and biological stress, and to survive in diverse ecological niches, microorganisms form biofilms. These biofilms are mainly composed of exopolysaccharides (EPS) secreted by microbes. Recently, culture-based studies revealed EPS secretion at -15 °C by bacterial cells (Mykytczuk et al., 2013). In addition to cryoprotection, the high polyhydroxyl content of EPS lowers the freezing point and ice nucleation temperatures (Nichols et al., 2005; Qin et al., 2007; De Maayer et al., 2014). Sugars units forming the EPS are synthesized via the pentose phosphate pathway. This underlines the greater relevance of the pentose phosphate pathway (oxidation of C-1) at -20 °C than at -5 °C. Nonetheless, higher oxidation of C-4 than C-1 at -20 °C reveals that intracellular antifreeze protection is also crucial at this temperature.

Freezing also constraints severely membrane fluidity (Russell et al., 1995; Rodrigues and Tiedje, 2008) and functioning of membrane bound enzymes (Berry and Foegeding, 1997). Beside, shifts in community structure would be expected to compensate for the loss of more sensitive population members, when microorganisms are subjected to freezing. Consequently, PLFA analysis was conducted to investigate adaptations of microbial phospholipids to freezing and the effects on community structure. Gram-negative PLFAs were abundant in soil incubated at -5 °C. This was accompanied by a twofold increase in ^{13}C incorporation into gram-negative PLFAs. In a recent study, the proportion of phospholipids containing recently-synthesized, ^{13}C-containing compounds, and average degree of unsaturation of the corresponding acyl chains, was also found to be higher at -4 °C than at temperatures above 0 °C (Drotz et al., 2010). Increase in both PLFA content and ^{13}C incorporation can only be explained by a parallel occurrence of two processes: 1) Desaturation within existing fatty acid chains (Nichols and Russell, 1996; Russell, 2002) and 2) *de novo* synthesis of PLFA, which means an increase in their abundance in response to freezing. In contrast, gram-positive bacteria incorporated 2 times higher ^{13}C into their PLFAs

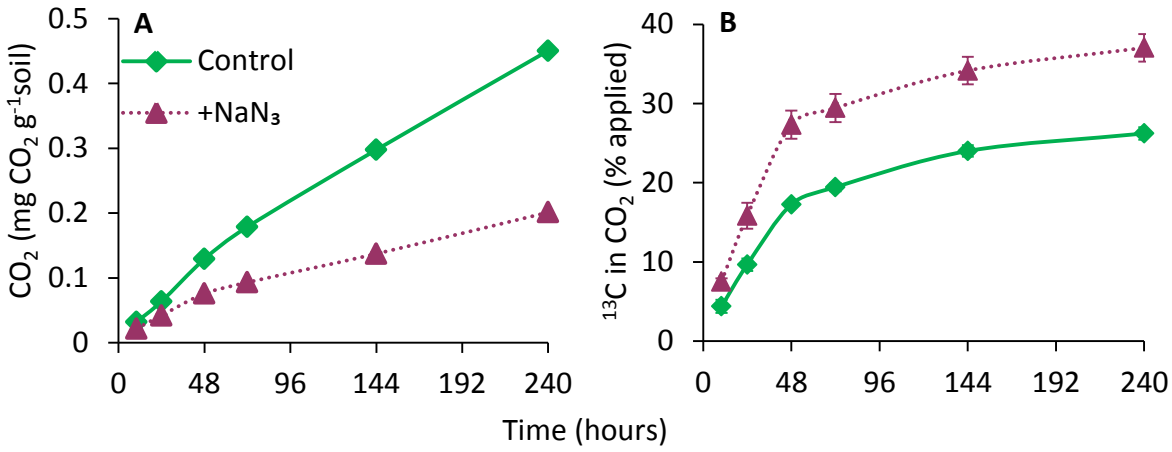
at -20 °C than at -5 and +5°C without a significant increase in their fatty acid contents. The lower the temperature, the more of their PLFAs were characterized by shorter *iso*- and *anteiso*- branching, which requires their *de novo* synthesis from short-chain branched fatty acid precursors (Russell, 2002). This reflects adaptation of microbial membranes to respective subzero temperatures. Moreover, the fungal/bacterial PLFA ratio was 1.5 times lower at subzero temperatures than at +5°C, reflecting a shift in microbial community structure towards bacteria.

Therefore, freezing induced 1) a switch in metabolic pathway from PPP to glycolysis, 2) a change in antifreeze mechanisms from intracellular at -5 °C to extracellular at -20 °C, and 3) modifications of PLFAs from mono-unsaturated alkyl chains at -5 °C to short-chain branched fatty acids at -20 °C, and 4) a shift in microbial community structure towards bacteria.

1.3.2.2. Microbial activity in soils with respiration-inhibiting toxicants

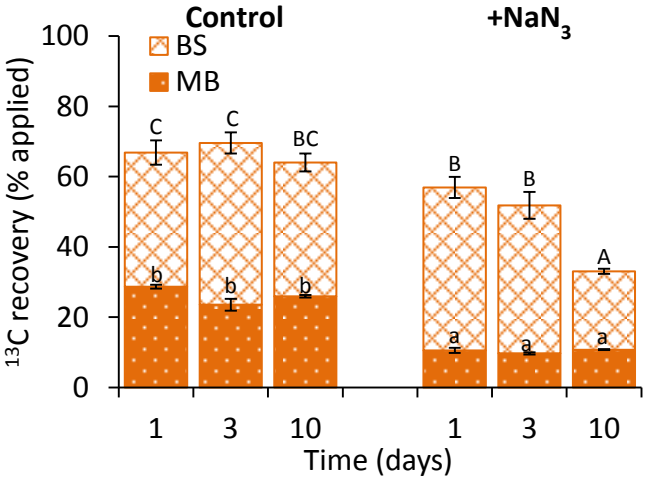
Toxicant exposure is another factor limiting microbial activity in soil. Management practices in agricultural soils such as weed and pest control often involve use of chemicals. These chemicals may create physiological cost at organism level that can alter the active microbial community composition, create shifts in ecosystem-level C, energy and nutrient flows (Schimel et al., 2007). Often, CO_2 is persistently released from soils exposed to toxicants (Voroney and Paul, 1984; Trevors, 1996) or contaminated with heavy metals (Bond et al., 1976; Ausmus et al., 1978; Fliessbach et al., 1994). The mechanisms responsible for CO_2 emission under such disturbances remain unclear. To unravel these mechanisms, NaN_3 (used as model toxicant inhibiting respiration) was incubated in soil with position-specifically ^{13}C-labeled glucose and ^{13}C was quantifed in CO_2, bulk soil, microbial biomass and phospholipid fatty acids (PLFA).

Ten days after glucose application, total CO_2 respired from azide-treated soil was 50% lower compared to control (ES Figure 3A). Position-specific ^{13}C recovery in CO_2 followed a classical pattern characteristic of high pentose-phosphate pathway activity, with C-1 > C-4 > C-2 > C-6, irrespective of inhibition. However, ^{13}C recovery in CO_2 was 12% higher in inhibited soil compare d to control (ES Figure 3B).



ES Figure 3: *Cumulative CO_2 (mean± SE) (**A**) and ^{13}C (mean± SE) recovered in CO_2 (**B**) from uniformly labeled glucose respired during 10 days in control and NaN_3 treated soils.*

Persistent CO_2 emission from soils with minimized microbial activity has previously been attributed to release of active oxidative extracellular enzymes (EXOMET) from dead organisms (Maire et al., 2013). To prove or reject the relevance of EXOMET compared to cellular metabolism, investigation of intracellularly formed metabolites (i.e. de-novo formed microbial biomass) was conducted. One day after respiration inhibition, more than 9% of the applied ^{13}C was recovered in microbial biomass (ES Figure 4).



ES Figure 4: *^{13}C recovery (mean ± SE) from uniformly labeled glucose in bulk soil (BS) and extractable microbial biomass (MB), 1, 3 and 10 days after application in control and azide-exposed soils. Significant effects ($P<0.05$) of NaN_3 addition and time according to Tukey Honest Significance Difference (Tukey HSD) post-hoc test in BS are indicated by upper case letters above the error bars, while MB by lower case letters.*

Recovery of ^{13}C in microbial biomass reflects survival of some microbes and contradicts the EXOMET theory. Therefore, we conducted PLFA analysis to identify microbial groups that survive inhibition. Ten days after inhibition, ^{13}C incorporation into PLFA of each microbial

group was almost negligible. However, fatty acid content associated with each microbial group doubled within ten days, attaining similar levels as in the control soils. Azide inhibits electron transfer in non-phosphorylating submitochondrial particles at cytochrome oxidase and catalase (Keilin, 1936) resulting in cell asphyxiation (Winter et al., 2012). Continued respiration after inhibition, without incorporation of glucose-derived ^{13}C into PLFAs despite doubling PLFA contents raised two questions: 1) How do microorganisms manage intracellular respiration without electron acceptors (NAD^+ or $NADP^+$), which cannot be regenerated after azide inhibition? 2) How do microorganisms grow without incorporating glucose into their PLFA?

Soil microorganisms can temporarily use either humic substances to shuttle electrons towards positively charged soil minerals (e.g. Fe^{3+} and Mn^{2+}) or excrete reduced metabolites like quinones or phenolic compounds (Vempati et al., 1995; Newman and Kolter, 2000; Pentrakova et al., 2013). Extracellular electron transfer by excretion of reduced metabolites could explain the high ^{13}C recovery in the bulk soil compared to microbial biomass during the first 3 days following inhibition. Increase in PLFA content during the ten days reflects recovery of the microbial community. Resources for this growth were recycled from the large pool of microbial necromass resulting from NaN_3 addition (Dippold and Kuzyakov, 2016). Recycling of resources by microbes was reflected by significant drop of ^{13}C in bulk soil between day 3 and 10 (ES Figure 4). Therefore, ecosystems are self-regulating systems that evolve mechanisms of self-repair and their biological populations are adapted to resist and recover from environmental fluctuations (EFSA, 2016).

1.3.2.3. Metabolic adaptations to sorption induced substrate limitation

In addition to toxicant exposure, soil microorganisms can suffer starvation because 70 – 100% of C in SOM exists as complexes sorbed to mineral or organic surfaces in soil (Lützow et al., 2006; Schmidt et al., 2011). Sorption was frequently investigated in the context of C stabilization, with the increased persistence of sorbed SOM being interpreted as physico-chemical stabilization effect of the surface interactions. Nonetheless, how the use of sorbed substrates affects microorganisms and whether this effect interacts with the physico-chemical effects on soil surfaces remain unclear. Consequently, alanine, the most abundant amino acid in soil and easily sorbed to mineral and organic fractions due to charged

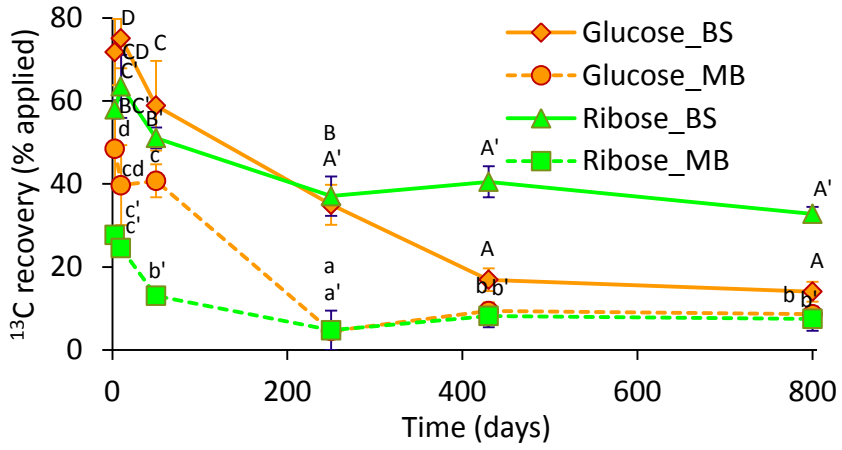
functional groups (Fischer and Kuzyakov, 2010; Dippold et al., 2014) was used to shed more light on sorption effects on microbial metabolism.

The initial mineralization peak of sorbed alanine was delayed 2-fold compared to free alanine. The delay in sorbed C mineralization could be attributed to decreased accessibility, which promotes C stabilization (Dungait et al., 2012). Position-specific ^{14}C labelled alanine revealed a shift in metabolism induced by sorption. For sorbed alanine, C-2 and C-3 remained in equal proportion in soil until day 3 unlike the free alanine in which significantly higher C-3 than C-2 was incorporated. This implies that less C from sorbed alanine was used for energy production in the citric acid cycle, or that C from sorbed alanine was predominantly fed into slower cycling microbial pools. To confirm the possibility of these scenarios, we assessed ^{14}C incorporation into microbial biomass. Incorporation of sorbed alanine C-2 into microbial biomass was 4 times higher than that of free alanine on day 1. This reveals a decrease of C flux through the citric acid cycle (Caspi et al., 2008). Microorganisms increase investment in microbial biomass formation pathways when C accessibility decreases (Dippold et al., 2014). Therefore, sorption induced a higher anabolic use of C in soil as already observed with pure minerals (Dippold et al., 2014). Thus, in addition to reduced accessibility, sorption increases fluxes of substrates to microbial biomass, which can have positive feedback on C stabilization in soil, as large amount of biomass is formed in close proximity to sorbing surfaces (Miltner et al., 2012).

1.3.3. Stabilization of C derived from labile compounds in soil

The bulk SOM is mainly plant derived, but a large proportion pass through microbial biomass before stabilization (Koegel-Knabner, 2002; Miltner et al., 2012). Consequently, any change in microbial metabolic activity in response to environmental changes will ultimately affect the stability of C in soil. The view that SOM stabilization is dominated by the selective preservation of recalcitrant organic components that accumulate in proportion to their chemical properties is no longer valid (Lützow et al., 2006). Therefore, it is necessary to know more about the residence time of C in physical and chemical fractions of soils to predict and model the behavior of soil C with respect with respect to climate change (Houghton et al., 1998).

The mean residence time (MRT) of uniformly labeled ^{13}C ribose in bulk soil (BS) and extractable microbial biomass (MB) were 3 and 2 times higher than of glucose, respectively. This suggests that ribose and glucose were incorporated into different cellular components. The continuous decrease of glucose-derived ^{13}C in soil (ES Figure 5) was attributed to sustained intra- and extracellular recycling as well as mineralization of the components. After day 250, glucose C positions converged in MB and pathway-specific, positional fingerprint was no longer visible. Existence of decomposing and constructing pathways in parallel within microbial metabolism, leads to a mixing of C positions after several days (Scandellari et al., 2009; Apostel et al., 2015). Therefore, the mechanism controlling the remaining glucose ^{13}C in soil after 250 days can be attributed to recycling within microbial cells and microbial products in extracellular environment.

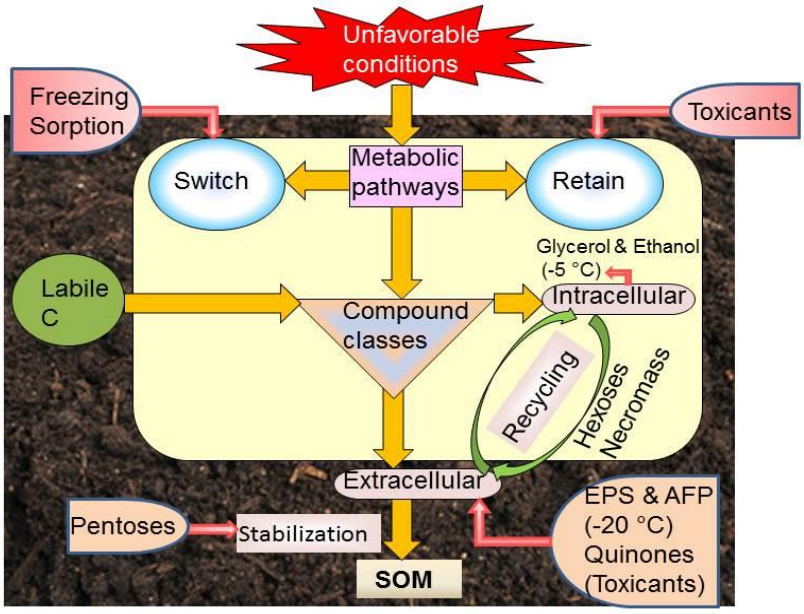


ES Figure 5: *^{13}C incorporation (mean ±SEM) of uniformly labelled monosaccharides into bulk soil (BS) and extractable microbial biomass (MB). Letters above the error bars indicate significant effects ($p<0.05$) of time on ^{13}C recovery (ribose ^{13}C in BS = A', ribose ^{13}C in MB = a', glucose ^{13}C in BS = A and glucose ^{13}C in MB = a).*

Turnover time of individual glucose C position was different from each other irrespective of the pool. Moreover, none of the glucose C position's MRT could be related to turnover time of the entire molecule based on uniform labeling. The differences in turnover time of glucose C positions reveal intensive metabolization and incorporation of modified compounds. A recent study revealed that the metabolite products consist of fast turnover components such as amino acids, sugars, organic acids and alcohols, which forms a minor portion of microbial biomass (Malik et al., 2016). Majority of the components were high molecular weight polymeric compounds of lipid, protein and polysaccharide origin (Hart et al., 2013; Malik et al., 2016). A universal substrate like glucose, which spreads in each

metabolic pathway in cells and can thus be found in each of these compounds as fragments or individual atoms (Apostel et al., 2015).

For ribose, there was a sustained decrease of ^{13}C in soil till day 250 (ES Figure 5). However, ribose-derived ^{13}C remained stable after 250 days, implying stabilization in non-living SOM. One possible direct biochemical ribose C utilization is the formation of ribonucleotide backbone of DNA and RNA (Caspi et al., 2008; Keseler et al., 2009). Persistence of ^{13}C in nucleic acids has previously been observed in other studies (Griffiths et al., 2006; Malik et al., 2015). It is possible that after microbial death, nucleic acids strongly interacts with mineral and organic fractions of the soils, inhibiting their availability for microbial reuse (Lützow et al., 2006). Furthermore, the MRT of ribose C-1 was 2- and 3-fold lower in BS and MB than C-5, respectively, confirming that recycling of ribose occur to a minor extend. Otherwise, if recycling occurred appreciably, we would observe convergence of ribose C-1 and C-5 after some time as in the case of glucose. Therefore, persistence of glucose C in soil was dominated by recycling while stabilization in non-living SOM accounted for persistence of ribose.



ES Figure 6: *Grahical representation of microbial adaptations to unfavourable conditions and mechanisms underlying persistence of labile C.*

1.4. Conclusions

Soil microbes are always exposed to unfavorable environmental conditions naturally or anthropogenically. Understanding mechanisms enabling microbes to survive and function under such conditions will improve prediction and modeling of the fate of soil C and other nutrients with respect to various aspects of global change such as climate change or land use intensification. Thus, informed decisions pertaining climate change mitigation and management of ecosystem services provided by soil can be undertaken.

Applying position-specific labeled substances permitted reconstruction of metabolic pathways under unfavorable conditions. Freezing induced a switch of metabolic pathway from PPP to glycolysis at subzero temperatures. Sorption, which simulated low substrate availability, decreased C flux to citric acid cycle leading to an increased allocation of C towards anabolism.

In addition to switching of metabolic pathways, unfavorable conditions induced synthesis of particular compound classes as adaptation strategy. Intracellularly, microorganism synthesized glycerol and ethanol to protect the cells against freeze-dehydration (ES figure 6). Extracellularly, exopolysaccharides (EPS) and antifreeze proteins (AFP) were synthesized to protect the cells against deleterious effects of ice. Glucose-derived ^{13}C was mainly used by microorganisms for energy production and syntheses of quinones and phenolic compounds as extracellular electron transport substances to bypass respiration inhibition. Furthermore, microorganisms altered the composition of PLFAs in the membranes depending on the temperature. Mono-unsaturated PLFAs were abundant at -5 °C, but a shift to saturated short-chain branched fatty acids dominated at -20 °C. Therefore, microorganisms induce a suite of physiological processes to cope with unfavorable conditions.

Adaptation to unfavorable conditions also shifts microbial community structure to compensate for the loss of more sensitive populations. The fungal/bacterial PLFA ratio was 1.5 times lower at subzero temperatures than at +5°C, reflecting a shift in microbial community structure towards bacteria. Also under respiration inhibition, fungal/bacterial ratio revealed a greater short-term susceptibility of fungal populations. However, a 2-fold increase of the fungi/bacteria ratio to a level equal to control soils within 3 days shows fast

recovery of fungal biomass. This suggests that fungi are more resilient to toxicants than perturbations imposed by freezing.

Understanding the short-term metabolic response of organisms is a prerequisite for predicting the long-term effect of such reactions on C stabilization, which is crucial for estimating C-balances under changing environmental conditions. The view that SOM stabilization is dominated by the selective preservation of recalcitrant organic components is no longer valid. C derived from labile compounds such as sugars have been shown to persist longer in soil than those from compounds of high recalcitrance such as lignin. Therefore, using position-specific labelled glucose and ribose under long-term field conditions, mechanism underlying persistence of C derived from labile compounds was revealed. In a nut shell, C stabilization in soil is a product of metabolic transformation. The persistence of glucose-derived C was mainly dominated by recycling (ES figure 6). On contrary, stabilization in non-living SOM characterized the persistence of ribose-derived C. For alanine, sorption decreased C flux through the citric acid cycle, causing increased incorporation into microbial biomass, with positive feedback on C stabilization. Therefore, the mechanisms responsible for C stabilization are distinct for each group of labile compound. This underscores the relevance of microbes in assigning stabilization characteristics to compounds. Thus, stabilization of C in soil is largely influenced by metabolic transformation, which is controlled by microorganism based on the environmental conditions.

Thus, for every condition investigated, some microorganisms have shown tolerance, implying that the limit of life on Earth is far from being well defined. Combining position-specific labeling and compound-specific analysis revealed mechanisms allowing microorganisms to tolerate or even grow under unfavorable conditions. The mechanistic understanding obtained from these studies will improve estimation of global C-balances and ecosystem feedback responses.

1.5. Contributions to the included manuscripts

This thesis comprises of three published, one submitted and a complete manuscript, which is ready for submission in collaboration with various co-authors. The co-authors listed on the manuscripts contributed as follows:

Study 1: Microbial metabolism in soil at subzero temperatures: Adaptation mechanisms revealed by position-specific ^{13}C labeling

Status: Published in the Frontiers in Microbiology, 2017, 8, 946**.** doi:10.3389/fmicb.2017.00946

Bore, E.: Experimental design, laboratory analyses and drafting of the final manuscript

Apostel, C.: Laboratory experiments execution and data acquisition

Sara, H.: Laboratory experiments execution and data analysis

Kuzyakov, Y.: Reviewing of the manuscript

Dippold, M.: Experimental design and reviewing of the manuscript

Study 2: Structural and physiological adaptations of soil microorganisms to freezing revealed by position-specific labelling and compound-specific 13C analysis

Status: Submitted to European Journal of soil science; date: August 25, 2017.

Bore, E.: Experimental design, execution and drafting of the final manuscript

Sara, H.: Experiment execution, data analyses and drafting of the manuscript

Kuzyakov, Y.: Reviewing of the manuscript

Dippold, M.: Experimental design and reviewing of the manuscript

Study 3: Soil microorganisms can overcome respiration inhibition by coupling intra- and extracellular metabolism: ^{13}C metabolic tracing reveals the mechanisms

Status: published in international society of microbial ecology journal (ISME J), 2017, 11 (6): 1423-1433

Bore, E.: Experimental design, execution and drafting of the final manuscript

Apostel, C.: Experimental execution, sampling and reviewing of the manuscript

Sara, H.: Sampling and data analyses

Kuzyakov, Y.: Experimental design and reviewing of the manuscript

Dippold, M.: Experimental design and reviewing of the manuscript

Study 4: Sorption of Alanine changes microbial metabolism in addition to availability

Status: Published in Geoderma Journal, 2017, 292:128-134

Apostel, C.: Experimental design and execution, sampling, laboratory analyses and drafting of the manuscript

Dippold, M.: Experimental design and reviewing of the manuscript

Bore, E.: Experiments execution, sampling and reviewing the manuscript

Kuzyakov, Y.: Reviewing of the manuscript

Study 5: Persistence of sugar-derived C in soil is controlled by the initial use of the C positions in the microbial metabolism

Status: In preparation

Bore, E.: Laboratory analyses, data analyses, and drafting of the manuscript

Dippold, M.: Experimental design, execution, sampling and reviewing of the manuscript

Kuzyakov, Y.: Experimental design and reviewing of the manuscript

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2. Publications and manuscripts

2.1. Study 1: Microbial metabolism in soil at subzero temperatures: Adaptation mechanisms revealed by position-specific ^{13}C labeling

Ezekiel K. Bore[1*], Carolin Apostel[1,2], Sara Halicki[1], Yakov Kuzyakov[1,2,3], Michaela A. Dippold[1]

[1]Deparment of Agricultural Soil Science, University of Goettingen, Goettingen, Germany

[2]Department of Soil Science of Temperate Ecosystems, University of Goettingen, Goettingen, Germany

[3] Institute of Environmental Sciences, Kazan Federal University, Kazan, Russia

Correspondence:

Ezekiel K. Bore

email: ezekielbore7@gmail.com

Abstract

Although biogeochemical models designed to simulate carbon (C) and nitrogen (N) dynamics in high-latitude ecosystems incorporate extracellular parameters, molecular and biochemical adaptations of microorganisms to freezing remain unclear. This knowledge gap hampers estimations of the C balance and ecosystem feedback in high-latitude regions. To analyze microbial metabolism at subzero temperatures, soils were incubated with isotopomers of position-specifically ^{13}C-labeled glucose at three temperatures: +5 (control), -5 and -20 ^{o}C. ^{13}C was quantifed in CO_2, bulk soil, microbial biomass and dissolved organic carbon (DOC) after 1, 3 and 10 days and also after 30 days for samples at -20 °C. Compared to +5 °C, CO_2 decreased 3- and 10-fold at -5 and -20 °C, respectively. High ^{13}C recovery in CO_2 from the C-1 position indicates dominance of the pentose phosphate pathway at +5 °C. In contrast, increased oxidation of the C-4 position at subzero temperatures implies a switch to glycolysis. A 3-fold higher ^{13}C recovery in microbial biomass at -5 than +5 °C points to synthesis of intracellular compounds such as glycerol and ethanol in response to freezing. Less than 0.4% of ^{13}C was recovered in DOC after one day, demonstrating complete glucose uptake by microorganisms even at -20 °C. Consequently, we attribute the 5-fold higher extracellular ^{13}C in soil than in microbial biomass to secreted antifreeze compounds. This suggests that with decreasing temperature, intracellular antifreeze protection is complemented by extracellular mechanisms to avoid cellular damage by crystallizing water. The knowledge of sustained metabolism at subzero temperatures will not only be useful for modelling global C dynamics in ecosystems with periodically or permanently frozen soils, but will also be important in understanding and controlling the adaptive mechanisms of food spoilage organisms.

Keywords: Psychrophiles, Cryoprotectants, Position-specific labeling, Metabolic pathways, Freeze tolerance.

2.1.1. Introduction

Microbial processes in high-latitude ecosystems with permafrost or frozen soils during winter periods are important contributors to global carbon (C) and nitrogen (N) cycling. Metabolic activity has been detected in soil at temperatures as low as -39 °C (Panikov et al., 2006; Schaefer and Jafarov, 2016) and an absence of subzero temperature limit for microbial metabolism was suggested by Price and Sowers (2004). Although biogeochemical models designed to simulate C and N dynamics in high-latitude ecosystems incorporate extracellular parameters (Schaefer and Jafarov, 2016), molecular and biochemical adaptations responsible for microbial freeze tolerance remain unclear. This knowledge gap hampers the estimation of C balances and ecosystem feedback responses. Consequently, tracing the metabolic pathways through which microorganisms transform organic substances under frozen conditions is crucial for unraveling the metabolic adaptation mechanisms (Scandellari et al., 2009; Dijkstra et al., 2011b; Dippold et al., 2014). Physiological studies have demonstrated that microorganisms remain metabolically active under frozen conditions, even at temperatures below -20 °C (Panikov et al., 2006; Amato and Christner, 2009). A recent study has shown metabolism suggestive of microbial growth (DNA replication) at temperatures down -20 °C (Tuorto et al., 2014). Therefore, a better understanding of how microorganisms circumvent challenges under frozen conditions is crucial. The challenges that organisms experience under frozen conditions include: 1) denaturation and loss of protein flexibility, 2) loss of membrane fluidity, which affects nutrient transport and decreases the activity of membrane-bound enzymes (Chattopadhyay, 2006), and 3) DNA and RNA secondary structures become more stable, inhibiting replication, transcription and translation (D'Amico et al., 2006). Extracellularly, freezing leads to: 1) low water availability and activity (Oquist et al., 2009; Stevenson et al., 2015), 2) low thermal energy, slowing diffusion of nutrients and excreted wastes to and from microorganisms (Hoehler and Jorgensen, 2013), and 3) high solute concentrations causing osmotic imbalance (Lorv et al., 2014). Intrusive ice crystals formed intracellularly and extracellularly can mechanically damage the cells (Gilichinsky et al., 2003). Several studies have been conducted to understand the survival strategies adopted by microorganisms to overcome these challenges. Attributes include the synthesis of cold-adapted enzymes that have high specific activities at low temperature (Herbert, 1989; Berry and Foegeding, 1997; Nakagawa et al., 2003), synthesis of antifreeze proteins that bind to ice crystals, inhibiting their growth and

recrystallization (Fletcher et al., 2001; Holt, 2003; Lorv et al., 2014), and adjustment of membrane composition by synthesis of unsaturated fatty acids to increase fluidity (Berry and Foegeding, 1997; Drotz et al., 2010).

Most of the studies on metabolism at low temperatures are based on isolated pure cultures from permafrost or used permafrost samples with inherently adapted microbes (Rivkina et al., 2000; Mykytczuk et al., 2013; Tuorto et al., 2014). However, subzero temperatures are also relevant to soils in temperate zones that freeze during winter. In frozen state, significant greenhouse gases are released from temperate soils, which make these soils a good analog for what we might expect from thawing permafrost (Nikrad et al., 2016). The importance of these soils in global C cycling while frozen and their vulnerability to thawing, calls for a better understanding of how microbes in these soils will contribute to global C feedback (Nikrad et al., 2016). This study was therefore designed to gain insights into microbial activities in temperate frozen soils. The study is bolstered by the novel method of applying position-specifically labeled substances as metabolic tracers in soil microbiomes (Scandellari et al., 2009; Dijkstra et al., 2011b; Apostel et al., 2015). Glucose has been identified as one of the most suitable candidates for tracing metabolic processes in soil, because it lacks physical and chemical interactions with mineral or organic soil components due to absence of charged functional groups or hydrophobic moieties (Fischer et al., 2010; Apostel et al., 2015). The use of position-specifically labeled glucose permits detailed reconstruction of microbial metabolic pathways and enables conclusions to be drawn on the microbial products formed under frozen conditions (Scandellari et al., 2009; Dijkstra et al., 2011a; Dippold and Kuzyakov, 2013; Apostel et al., 2015). This approach helps to identify the metabolic adaptations for overcoming the challenges under frozen conditions.

Analyses of ^{13}C incorporated from specific glucose positions into soil, microbial biomass, DOC and CO_2 were conducted to identify microbial metabolic adaptations in frozen soil habitats. Poor water availability and low thermal energy limit microbial activity in frozen conditions. To survive these stresses, microorganisms form biofilms composed of exopolysaccharides (EPS). Sugars units forming the EPS are synthesized via the pentose phosphate pathway. Therefore, we hypothesized that the glucose C-1 position will be preferentially oxidized to meet energy demands. Consequently, high ^{13}C recovery from the glucose C-1 position is

expected in CO_2. More of the remaining glucose C positions will be used for EPS synthesis than cellular compounds at subzero temperatures. This would result in higher ^{13}C in the extracellular environment than in microbial biomass from the remaining glucose C positions due to the secreted EPS.

2.1.2. Materials and methods

2.1.2.1. Sampling site

The soils were collected (0-10 cm depth) from agriculturally used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 m a.s.l.) in August 2014, sieved to 2 mm, and stored for 1 day at +5 °C except for samples (n = 3) used to determine dry weight. Mean annual temperature and precipitation at the site are +7 °C and 874 mm respectively. The soil had a pH (KCl) of 4.88, a pH (H_2O) of 6.49, a total organic carbon (TOC) and total nitrogen (TN) content of 1.77% and 0.19%, respectively, and a cation exchange capacity (CEC) of 13 $cmol_C\,kg^{-1}$.

2.1.2.2. Experimental design

Screw-cap glass microcosms (10 cm diameter and height of 12 cm) with a base layer of quartz sand were used for incubations. 80 g samples of soil were transferred to sample rings and installed on ceramic plates above the quartz sand. The soils were rewetted to field capacity by adding 10 ml of water to the underlying sand at +5 °C for 2 days. Thereafter, the microcosms were preconditioned at +5, -5 and -20 °C for 24 hours. These temperatures were chosen to simulate 1) average annual soil temperature at the sampling site (control), 2) average winter temperature, and 3) lowest temperatures in some winters on this site and common deep freezer storage temperatures, respectively. Four position-specifically ^{13}C-labeled isotopomers of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4, and ^{13}C-6), uniformly ^{13}C-labeled (U-^{13}C) and unlabeled glucose (natural abundance background) were applied to the soils in separate microcosms with four replicates of each. 5 ml of 2.55 mM glucose solutions were applied on top of the soil. This C concentration was less than 5% of microbial biomass C and therefore, does not alter microbial community structure (Kuzyakov, 2010). Cups with 5 ml of 1 M NaOH were placed in each microcosm to trap CO_2. 20% (w/v) of NaCl was dissolved in NaOH traps at -20 °C to depress the freezing point. The microcosms were sealed and incubated at the

respective temperatures. NaOH in the vials was exchanged after 10 hours, 1, 2, 3, 6 and 10 days at +5 and -5 °C and in addition after 15, 20 and 30 days at -20 °C. Soil samples were collected after 1, 3, and 10 days at +5 and -5 °C and also after 30 days at -20 °C, to account for the slow processes expected in deeply frozen conditions. 30 g of each sample was immediately subjected to chloroform fumigation-extraction, as described below.

2.1.2.3. Analytical methods

Amount and $\delta^{13}C$ value of CO_2

0.4 ml of each CO_2 trap was diluted 1:10 with ultrapure water, and the CO_2 content was determined with a non-dispersive infrared (NDIR) gas analyzer (TOC 5050, Shimadzu Corporation, Kyoto, Japan). The remainder of the CO_2 in the NaOH traps was precipitated with 5 ml of 0.5 M $SrCl_2$ solution. $SrCO_3$ precipitates were separated by centrifuging four times at 2000 xg for 10 min and washing in between with Millipore water until pH 7 was attained. $\delta^{13}C$ values of the dried $SrCO_3$ (1-2g) were measured with a Flash 2000 elemental analyzer coupled by a ConFlo III interface to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Fisher Scientific, Bremen, Germany). ^{13}C respired from the applied glucose was calculated according to a mixing model equation 1 and 2 (Gearing et al., 1991), where the C content of the background ($[C]_{BG}$) in Eq.1 was determined by Eq.2.

$$[C]_{CO_2} \cdot r_{CO_2} = [C]_{BG} \cdot r_{BG} + [C]_{appG} \cdot r_{appG} \quad (1)$$

$$[C]_{CO_2} = [C]_{BG} + [C]_{appG} \quad (2)$$

where:

$[C]_{CO_2/BG/appG}$ C content of the sample/background/applied glucose (mg C g^{-1}soil)

$r_{CO_2/BG/appG}$ ^{13}C atom %-excess of labeled sample/background/applied glucose (at %)

Quantification of bulk soil C content and ^{13}C signature

Aliquots of soil were freeze dried, ground in a ball mill and 13-15 mg were weighed into tin capsules. ^{13}C isotope measurements were performed with EuroVektor elemental analyzers (HEKAtech GmbH, Wegberg, Germany) coupled by a ConFlo III interface to a Delta Plus XP IRMS (both units from Thermo Fisher Scientific, Bremen, Germany). C recovery from applied glucose was calculated according to Eq. 1 and 2.

^{13}C in microbial biomass and DOC determination

Microbial biomass C was determined by chloroform fumigation-extraction. Two subsamples of 12 g were taken from each soil sample. One set of subsamples was extracted directly, while the other was first fumigated with chloroform for 3 days in a desiccator to lyse microbial cells. 36 ml of 0.05 M K_2SO_4 was used to extract organic C on an orbital shaker for 1.5 h. Samples were centrifuged for 10 min at 2000 rpm and the supernatant was filtered for determination of C concentration (TOC/TIC analyzer, Multi C/N 2100 AnalytikJena, Jena, Germany). About 25 mg (fumigated) and 40 mg (unfumigated) freeze-dried extracts were used for $\delta^{13}C$ determination via EA-IRMS, performed by the same instrument coupling used for bulk soil $\delta^{13}C$ determination. Incorporation of glucose C into fumigated and unfumigated samples was calculated according to Eq. 1 and 2. Microbial biomass C and ^{13}C were calculated by subtracting unfumigated from fumigated values and dividing by an extractability correction factor of 0.45 (Wu et al., 1990). C concentration and ^{13}C recovery in unfumigated samples are DOC and its ^{13}C content, respectively.

2.1.2.4. Statistical analysis

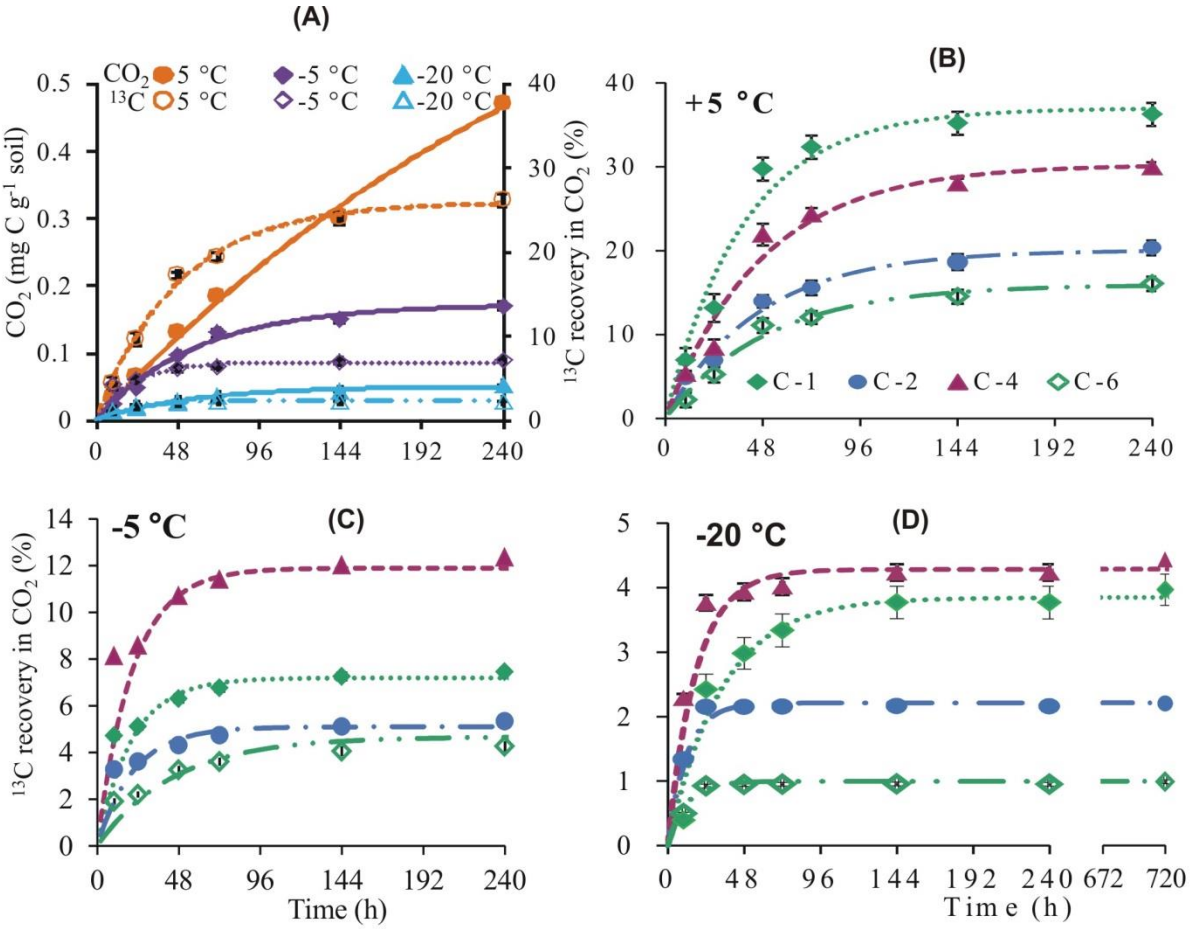
A Nalimov outlier test with 95% significance level was performed on CO_2. ^{13}C incorporation into bulk soil, microbial biomass, and CO_2 were tested for significant differences between the positions, time of incubation and effect of temperature with a factorial analysis of variance (ANOVA). If assumptions of normality and homogeneity of variances with groups were not met, outcomes were validated by a nonparametric Kruskal Wallis ANOVA. Significant differences were determined with Tukey Honest Significance Difference (Tukey HSD) post-hoc test at a confidence level of 95%. Statistical tests were performed with Statistica (version 12.0, Statsoft GmbH, Hamburg, Germany).

2.1.3. Results

2.1.3.1. CO_2 and recovery of incorporated ^{13}C

CO_2 was released down to -20 °C, although cumulatively, there were 3- and 10-fold reductions at -5 and -20 °C, respectively, compared to +5 °C at day 10. Similarly, recovery of glucose-derived ^{13}C in CO_2 at -5 and -20 °C was respectively 19% and 24% lower than +5 °C (Figure 1A). At +5 °C, ^{13}C recovery in CO_2 showed preferential oxidation of the glucose C-1

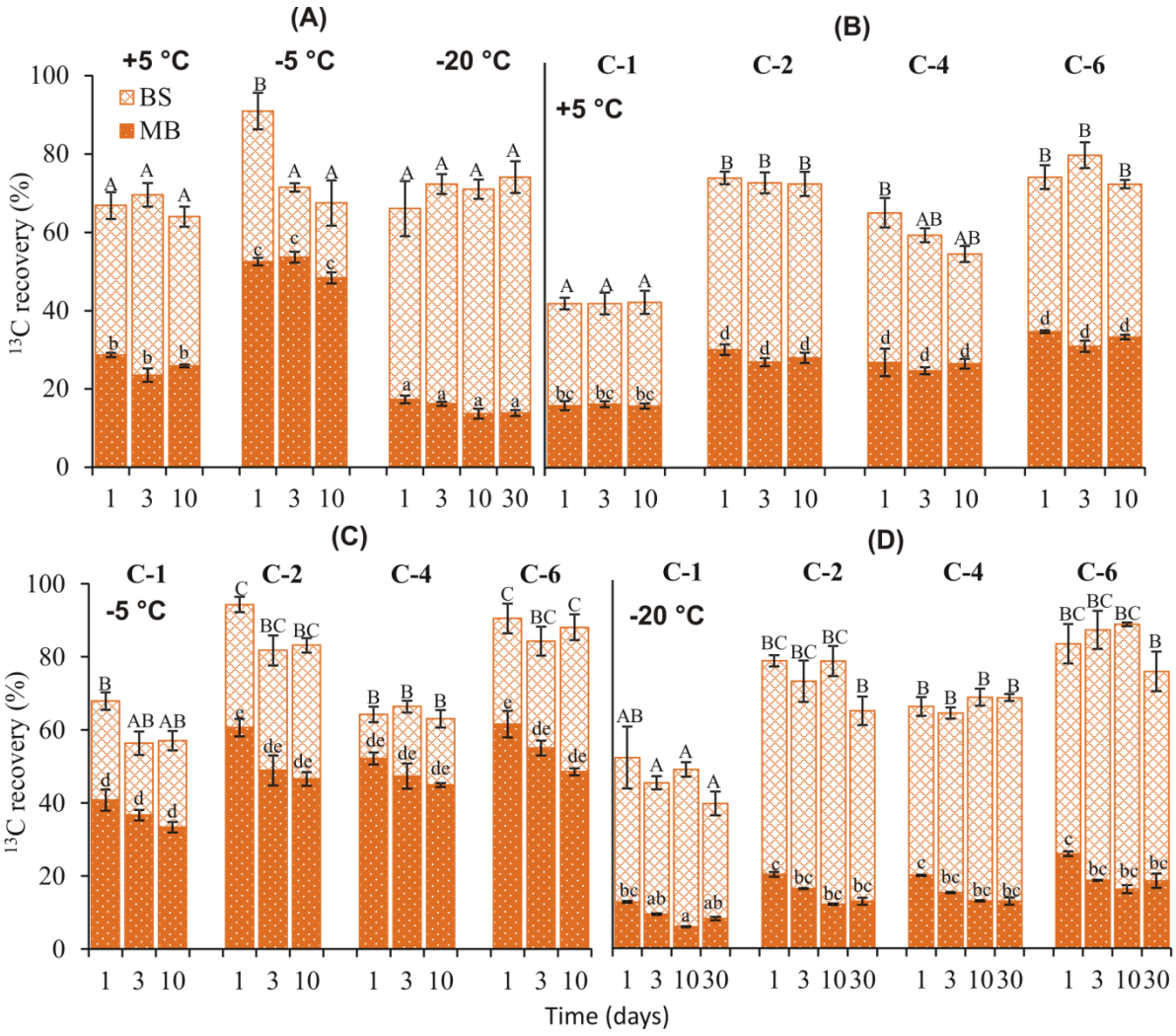
position. In contrast, the glucose C-4 position was the most respired at subzero temperatures. Irrespective of temperature, two phases of glucose C mineralization were observed: 1) high glucose ^{13}C respiration within the first 3 days at 5 °C and 2 days at -5 and -20 °C and 2) low respiration of glucose-derived ^{13}C thereafter (Figure 1B-D).



S1 Figure 1: *Cumulative CO_2 and ^{13}C in CO_2. Cumulative CO_2 (mean ± SEM, n = 4) (solid symbols and continuous lines) and ^{13}C (mean ± SEM, n = 4) (open symbols and broken lines) recovered in CO_2 released from uniformly labeled glucose* ***(A)*** *and cumulative ^{13}C (mean ± SEM, n = 4) recovered in CO_2 released from position-specifically labeled glucose at +5 (**B**), -5 (**C**) and -20 °C (**D**). ^{13}C curves were fitted with nonlinear least-square regressions according to an exponential equation ($cum^{13}C(t) = {}^{13}C_{max}*(1-e^{-kt})$), where cum ^{13}C (t) is the cumulative ^{13}C amount depending on time, $^{13}C_{max}$ is the parametrically determined maximum of ^{13}C, k is the mineralization rate and t is time (parameter estimates in supplementary Table S1). Steven's runs test for the linearized, fitted ^{13}C curves revealed no deviation from linearity (supplementary Table S2). Significant differences ($p<0.05$) between fitted curves are displayed in Supplementary Table S4.*

2.1.3.2. ^{13}C in bulk soil and microbial biomass

At day 1, ^{13}C recovery in bulk soil from uniformly labeled glucose was over 24% higher at -5 °C than at +5 and -20 °C. After the first day, recovery did not differ with temperature (Figure 2A). Similarly, position-specific patterns of ^{13}C recovery in bulk soil were comparable between +5 and -20 °C but differed at -5 °C. At +5 and -20 °C, ^{13}C recoveries in bulk soil from C-2, C-4 and C-6 were significantly higher ($p<0.05$) than C-1 (Figure 2B and D). In contrast, recovery from C-2 and C-6 was significantly higher ($p<0.05$) than from C-1 and C-4 at -5 °C (Figure 2C).



S1 Figure 2: *^{13}C recovery in bulk soil (BS) and microbial biomass (MB). ^{13}C recovery (mean ± SEM, n = 4) from uniformly **(A)** and position-specifically labeled glucose in bulk soil and extractable microbial biomass, 1, 3, 10 and 30 (-20 °C) days after application at +5 (**B**), -5 (**C**) and -20 °C (**D**). Significant effects ($p<0.05$) of temperature, days and individual glucose*

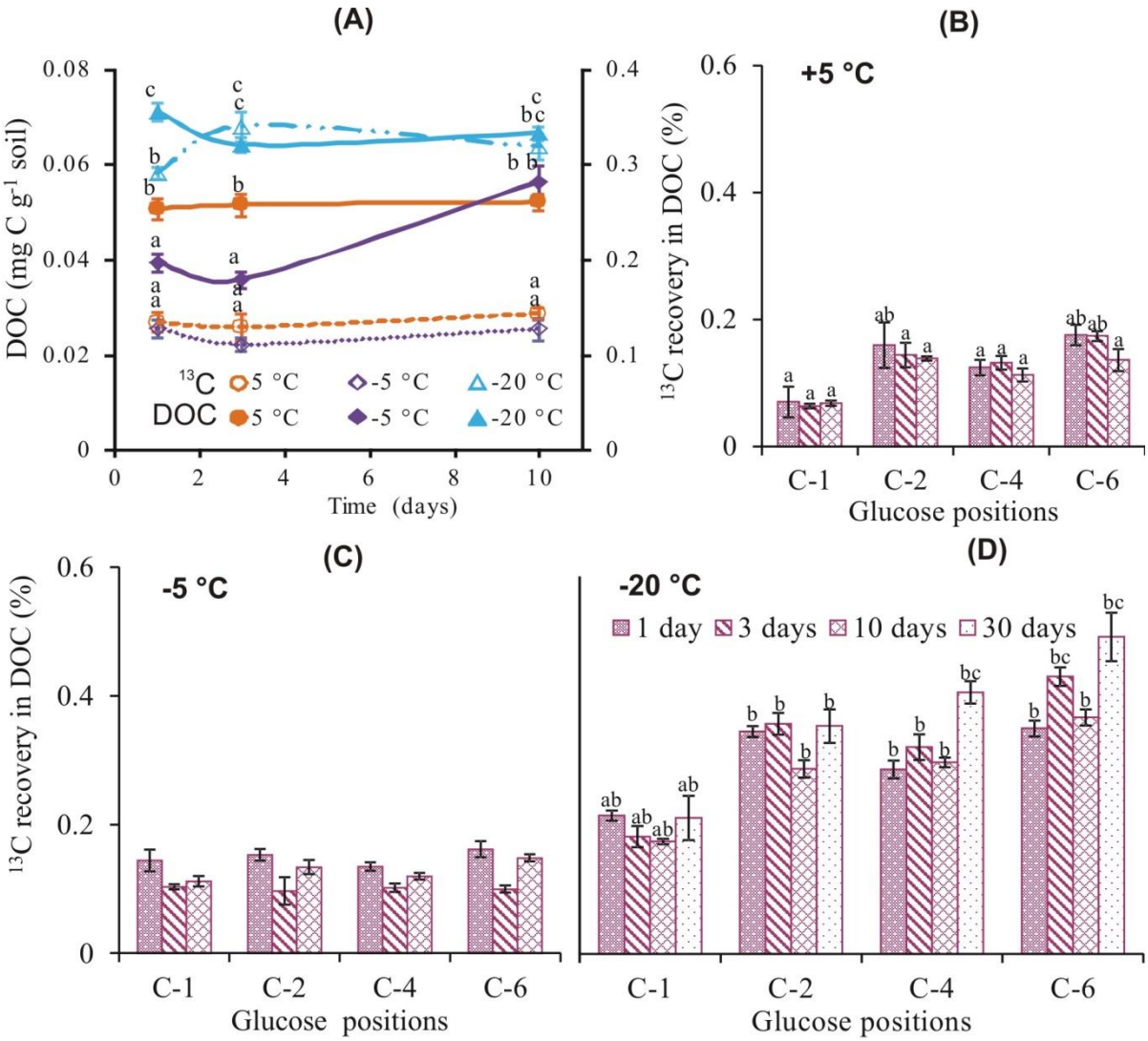
positions in BS are indicated by upper case letters above the error bars, and in extractable MB by lower case letters.

From uniformly labeled glucose, the highest ^{13}C recovery from microbial biomass was obtained at -5 °C and it was 2 and 3 times higher than at +5 and -20 °C, respectively (Figure 2A). The position-specific patterns of ^{13}C incorporation into microbial biomass were not affected by temperature. The ^{13}C recovery in microbial biomass from C-1 was ≈1.7 times lower than C-2, C-4 and C-6 at each temperature (Figure 2B-D). Moreover, recovery from each position did not differ over time at any incubation temperature.

The ratio of extracellular ^{13}C recovery in bulk soil to microbial biomass was highest at -20 °C (factor 3), followed by +5 °C (factor 1.5) and -5 °C (factor 0.6) (Fig. 2A).

2.1.3.3. DOC

Total extractable C in soil was highest at -20 °C, being 1.4- and 2.3-fold higher than +5 °C and -5 °C, respectively (Figure 3A). Whereas DOC did not differ over time at -20 and +5 °C, it increased by a factor of 1.5 between day 3 and 10 at -5 °C, to reach a level similar to +5 °C. ^{13}C recovery in DOC at -20 °C was also 2 times higher than both at -5 and +5 °C (Figure 3A).



S1 Figure 3: *DOC and ^{13}C in DOC. Total DOC (mean ± SEM, n = 4) extracted (solid symbols and continuous lines), ^{13}C recovery (mean ± SEM, n = 4) from uniformily labelled (open symbols and broken lines)* ***(A)*** *and ^{13}C recovery (mean ± SE, n = 4) from glucose positions in DOC at +5 (**B**), -5 (**C**) and -20 °C (**D**). Significant effects (p<0.05) of temperature, days and individual glucose positions in DOC are indicated by letters above the error bars.*

Less than 0.4% of the applied ^{13}C was recovered in DOC on day 1, irrespective of temperature (Figure 3A). The position-specific ^{13}C patterns detected in CO_2 were different to DOC at each temperature. Whereas recoveries in DOC at -5 °C did not differ beween the four glucose positions, the recovery from the glucose C-6 position was twice as high as C-1 at +5 and -20 °C (Figure 3B-D).

2.1.4. Discussion

2.1.4.1. Metabolic pathways revealed by glucose mineralization

Microbial activity has been detected in soil down to -39 °C (Panikov et al., 2006; Schaefer and Jafarov, 2016). This has evoked interest in the adaptive metabolic mechanisms of microorganisms at such extremely low temperatures. At subzero temperatures, low thermal energy is expected to slow down processes such as microbial substrate uptake (Hoehler and Jorgensen, 2013). However, less than 0.4% of applied ^{13}C was recovered in DOC on the first day, irrespective of temperature (Figure 3A) suggesting that all applied glucose was taken up by microorganisms within the first day. These results agree with previous data showing that substrate uptake of highly available substances, such as glucose, are largely independent of temperature (Herbert and Bell, 1977; Schimel and Mikan, 2005). At the end of the incubation period, cumulative CO_2 was 3- and 10-fold lower at -5 and -20 °C than +5 °C respectively (Figure 1A). The results agree with studies showing strong temperature sensitivity of CO_2 production in frozen soils (Monson et al., 2006; Panikov et al., 2006; Drotz et al., 2010). Irrespective of temperature, two phases of glucose C mineralization were observed: Phase 1 with high rates of ^{13}C recovery in CO_2 is consistent with intense glucose mineralization. Phase 2 thereafter, with low ^{13}C recovery rates (Figure 1B and D), reflects mineralization of glucose-derived metabolites (Blagodatskaya et al., 2011). These results contradict those of Drotz et al. (2010), who observed low ^{13}C in CO_2 during phase 1 and high in phase 2. This discrepancy may reflect the soil types used for incubation. While our soils were agriculturally used loamy Luvisols, their soils were forest Podsols from the boreal region sampled from the organic horizon. Cold temperatures were shown to increase concentraion of DOC in these soils (Haei et al., 2010), which implies a high concentration of low molecular weight organic substances and therefore low competition for applied substrate among microbes. In contrast, our agricultural soil was low in organic matter and the competition between microbes for applied glucose was highest immediately after application, leading to a fast uptake and release of glucose-derived CO_2.

The ^{13}C recovery pattern in CO_2 showed high oxidation of the C-1 position in control soils (Figure 1B), and revealed that glucose was predominantly catabolized via the pentose phosphate pathway (Caspi et al., 2008; Dijkstra et al., 2011c; Apostel et al., 2015). Contrary to our hypothesis, metabolic behaviors completely switched over to a preferential

respiration of the glucose C-4 position at -5 °C and this behavior was still weakly visible at -20 °C (Figure 1C and D). High ^{13}C recovery from the C-4 position reflects glucose transformation via glycolysis (Dijkstra et al., 2011c; Apostel et al., 2013). Significant ^{13}C recovery from the C-1 position, especially at -20 °C, implies that, at such very low temperatures the pentose phosphate pathway again plays a greater role than at -5 °C. This shift in metabolic pathways may reflect the need for antifreeze compounds. To confirm this interpretation, we assessed ^{13}C incorporation into microbial biomass.

2.1.4.2. ^{13}C incorporation into microbial biomass

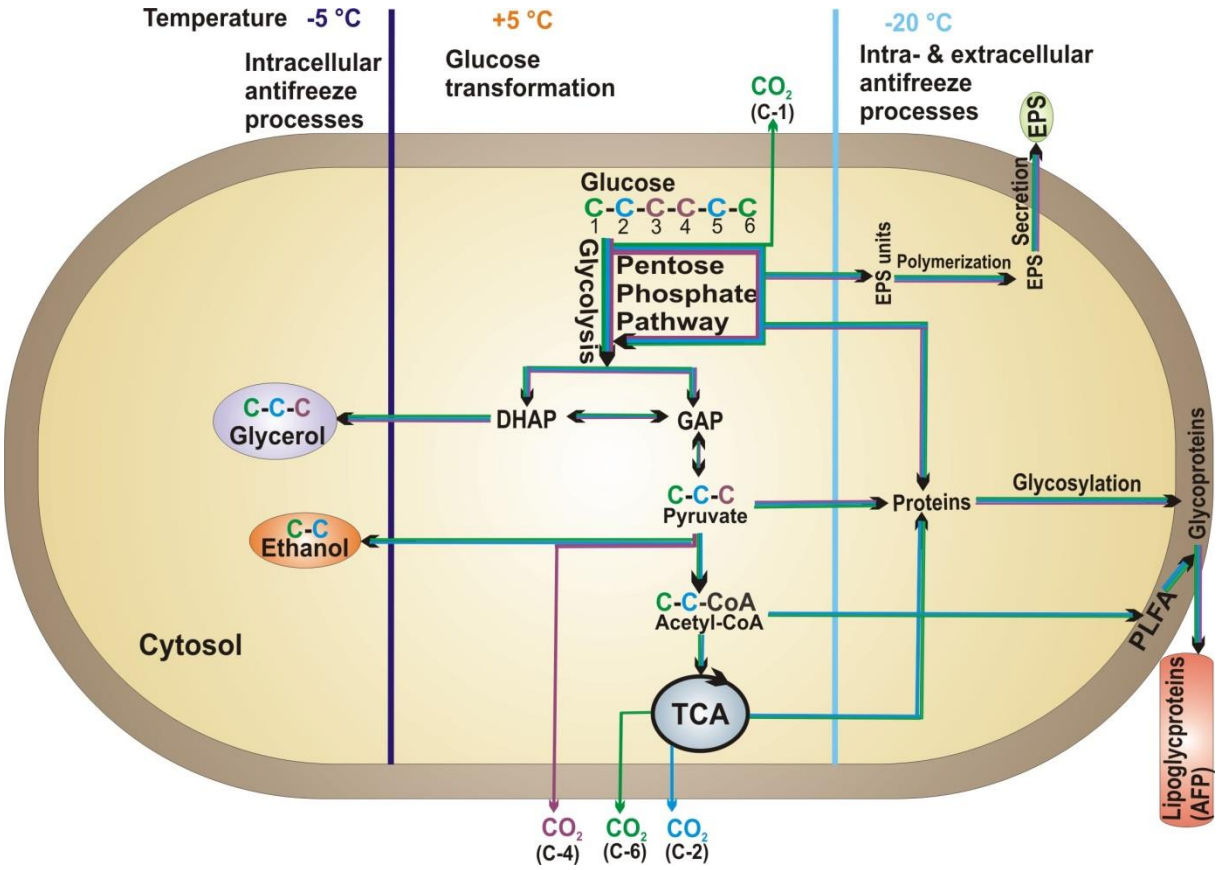
Liquid water in soil is a prerequisite for microbial activity (Oquist et al., 2009). At +5 °C, microbial activity was not constrained by water availability or slow substrate diffusion. The enzymes responsible for organic matter decomposition also remain active at this temperature (Razavi et al., 2015; Razavi et al., 2016). Consequently, this temperature was favorable for glucose use, as reflected by the 4- and 13-fold higher ^{13}C recovery in CO_2 than at -5 and -20 °C, respectively. Nonetheless, this high catabolic use of glucose-derived ^{13}C with increasing temperature did not correspond to anabolic use. The highest ^{13}C incorporation into microbial biomass was recorded at -5 °C. The position-specific ^{13}C recovery patterns in bulk soil and microbial biomass at +5 °C were complementary to the metabolic fluxes observed in CO_2, showing that glucose was predominantly metabolized via pentose phosphate pathway. The dominance of this pathway at similar temperatures under moderate C supply reflects the classical metabolic C allocation observed in previous studies (Dijkstra et al., 2011c). This is triggered by the need for pentose and NADPH for biosynthesis (Fuhrer and Sauer, 2009).

Although water availability, water activity and thermal energy are low at subzero temperatures (Davidson and Janssens, 2006; Oquist et al., 2009; Stevenson et al., 2015), recent studies demonstrated microbial growth down to -15 °C in permafrost isolates (Mykytczuk et al., 2013), -20 °C in permafrost cores (Tuorto et al., 2014) and the microbial metabolism limit was set at -33 °C (Rummel et al., 2014). Therefore, the substantial amount of ^{13}C incorporated into microbial biomass we observed at -5 °C is not surprising. To survive and grow at such extremely low temperatures, microorganisms induce a suite of physiological processes which include both metabolic and biomass adjustments (Wouters et

al., 2000; Drotz et al., 2010). A better understanding of these metabolic and biomass adjustments will be necessary in estimating the role of microorganisms in biogeochemical cycles of frozen soils.

2.1.4.3. Antifreeze adaptation mechanisms

^{13}C incorporation into microbial biomass at -5 °C was 2 and 3 times higher than at +5 and -20 °C respectively, and this incorporation made up more than two-thirds of the total glucose-derived ^{13}C recovered in bulk soil (Figure 2A). In a recent study, ^{13}C incorporation into microbial DNA was not detected after 1 month at subzero temperatures (Tuorto et al., 2014). This suggests that glucose utilization by microorganisms at -5 °C were mainly intracellular metabolic responses to freezing and was rapidly activated. Intracellularly, for the vast majority of prokaryotic cells, contain no unbound water (Coombe, 2002). Hence, deleterious effects of freezing to these microbes, include, but are not limited to osmotic imbalance. Since water is not a limiting factor at -5 °C, due to freezing point depression by increasing solute concentration in unfrozen volume (Brooks et al., 1997; Price, 2000), microorganisms invest energy in the synthesis of intracellular compounds that minimize effects of osmotic stress brought about by freeze-dehydration. This freeze-dehydration alters cell membrane ultrastructure and membrane bilayer fusion and causes organelle disruption (Mazur, 2004). Glycerol and ethanol were recently identified as abundantly synthesized compounds at -4 °C (Drotz et al., 2010). The synthesis of these compounds is consistent with glucose transformation via glycolysis, which is confirmed by the high ^{13}C recovery from C-4 in CO_2. These compounds act as cryoprotectants and are compatible solutes against freeze-dehydration (Block, 2003). Glycerol also interacts with water through hydrogen bonds depressing its freezing point (Lovelock, 1954). Glycerol therefore limits intracellular damage caused by freezing, hence maintaining microbial activity and CO_2 production at subzero temperatures (Drotz et al., 2010). We emphasize that conversion of glucose to alcohols (ethanol and glycerol) does not limit synthesis of other cellular compounds such as phospholipids and proteins. Nonetheless, conversion of glucose to alcohols explains the high glycolysis (C-4) and low pentose phosphate pathway (C-1) fingerprints (Fig. 1C) observed at -5 °C (Figure 4).



S1 Figure 4: *Proposed model of microbial glucose transformation pathways at +5 °C (central compartment) and antifreeze adaptation mechanisms at sub-zero temperatures (-5 °C on the left and -20 °C on the right compartments). Colored arrows correspond to glucose C positions and indicate their fate.*

We obtained lowest ^{13}C incorporation into microbial biomass at -20 °C (1.3 and 3 times lower than at +5 and -5 °C, respectively) (Figure 2A). Nonetheless, the ratio of extracellular ^{13}C to microbial biomass in soil was highest (ratio 5) at -20 °C (Figure 2A). This high ratio could not be attributed to the non-metabolized glucose that remained in the soil because less than 0.4% of applied ^{13}C was recovered in DOC at day 1 (Figure 3A); moreover, extracellular ^{13}C recovery increased again at later time points. The high ^{13}C recovery in bulk soil compared to microbial biomass at -20 °C suggests that microorganisms utilized glucose for extracellular antifreeze compounds. To resist physical, environmental and biological stress, and to survive in diverse ecological niches, microorganisms form biofilms. These biofilms are mainly composed of exopolysaccharides (EPS) secreted by microbes. Recently, culture-based studies revealed EPS secretion at -15 °C by bacterial cells (Mykytczuk et al., 2013). In addition to cryoprotection, the high polyhydroxyl content of EPS lowers the

freezing point and ice nucleation temperatures (Nichols et al., 2005; Qin et al., 2007; De Maayer et al., 2014). A key intermediate linking the anabolic pathway of EPS production and the catabolic pathway of glucose degradation is glucose-6-phosphate. In this step, the C flux bifurcates between the formation of glycolysis products and the biosynthesis of sugar monomers for EPS production via the pentose phosphate pathway (Welman and Maddox, 2003) (Figure 4). Increasing glucose concentration was found to increase EPS production at low temperatures (Cayol et al., 2015). EPS production and secretion potentially explain the relevance of glycolysis and the pentose phosphate pathways, as reflected by preferential oxidation of glucose C-4 followed by C-1 positions (Figure 1D). This would account for the higher extracellular ^{13}C in soil at -20 °C compared to -5 and +5 °C (Figure 2A). The poor extractability of EPS in water (Hughes, 1997) may explain why extracellular ^{13}C was not entirely recovered in DOC at -20 °C. Nevertheless, high ^{13}C recovery in DOC coincided with high absolute DOC at -20 °C (Figure 3A). This is consistent with the results of both Krembs et al. (2002), who found that EPS concentration in ice correlated positively with DOC, and with those of Hentschel et al. (2008), who observed the highest DOC content in forest soil at -13 °C, followed by successively lower values at +5 and -3 °C. This pattern, based on forest soils in a field experiment, agrees perfectly with our observation on agricultural soils in a laboratory experiment (Figure 3A), suggesting that underlying adaptation strategies are rather general. The spectroscopic properties of DOC at -13 °C did not indicate lysis of microbial biomass induced by freezing (Hentschel et al., 2008), which confirm the maintenance of cellular integrity.

In addition to EPS, organisms commonly synthesize antifreeze proteins (AFP) to overcome freezing effects. These AFP have been identified in fungi, bacteria, plants, insects and polar fishes (Holt, 2003). Some bacterial AFP are secreted as lipoglycoproteins or anchored on the outer cell membrane as lipoproteins, stabilizing membrane lipids at low temperatures (Kawahara, 2002). Based on adsorption inhibition, bound AFP inhibit rapid water movement between ice crystals, preventing destabilization of small ice crystal grains and thus minimizing ice recrystallization (Yu et al., 2010). For this reason, 3-8% of water remains unfrozen as thin films coating organo-mineral particles. This protects viable cells sorbed onto their surfaces from mechanical destruction due to intrusive ice crystals growing in frozen soil (Gilichinsky et al., 2003). The unfrozen water provides channels through which nutrients

reach the cells and through which waste products are eliminated by diffusion (Rivkina et al., 2000). The synthesis of extracellular antifreeze proteins further underlines the greater relevance of the pentose phosphate pathway (oxidation of C-1) at -20 °C than at -5 °C (Figure 1D). Increased pentose phosphate pathway activity signifies increased NADPH production, which is required as a source of reduction equivalents in protein and lipid synthesis (Nelson et al., 2008). Moreover, synthesis of hardly-extractable extracellular AFP could also account for the 5-fold higher extracellular ^{13}C recovery in bulk soil than in microbial biomass at -20 °C (Figure 2A).

The position-specific tracing of metabolic pathways in our study revealed that microorganisms induce a suite of temperature-dependent physiological processes to cope with freezing. This enables both catabolic and anabolic processes. These physiological changes reflect microbial resilience, enabling them to inhabit any ecological niche. Whether these responses are accompanied by a microbial community shift or solely reflect phenotypic plasticity remains to be determined.

2.1.5. Conclusions

Position-specific ^{13}C labelling proved to be a valuable tool in understanding the survival strategies of microorganisms at subzero temperatures. Glucose was metabolized by the pentose phosphate pathway at +5 °C and this shifted to glycolysis at subzero temperatures. High ^{13}C recovery within microbial biomass at -5 °C suggests that microorganisms invest energy and resources in intracellular antifreeze metabolites. In contrast, strategies differ greatly at -20 °C, where predominantly extracellular secretion protects the cells.

High-latitude C and N dynamic models such as Simple Biosphere/Carnegie-Ames-Stanford Approach (SiBCASA) focuses on extracellular parameters and only the thawing of permafrost is assumed to increase CO_2 emission (Schaefer et al., 2011; Schaefer and Jafarov, 2016). This increase is likely to be overestimated because permafrost soils – even if completely frozen at low temperatures – exhibit considerable microbial activity. This newly gained information is useful in modelling C dynamics at high altitude and latitude regions with frozen soils, hence, improving the estimation of global C balances and ecosystem feedback responses.

Author Contributions

The concept was contributed by YK and MD, experimental design by EB and MD, data acquisition and analysis by CA and SH, interpretation and drafting by EB and revision including final approval of the version by YK and MD.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Original research

Ezekiel K. Bore*, Carolin Apostel, Sara Halicki, Yakov Kuzyakov, Michaela A. Dippold

Correspondence: Ezekiel K. Bore, ezekielbore7@gmail.com

Table S1. Parameter estimates for the fitted ^{13}C curves

		5 °C		-5 °C		-20 °C	
Position		Estimate	p-value	Estimate	p-value	Estimate	p-value
C-1	13C(max)	36.98275	0	7.18775	0.000000	3.843498	1.82E-12
	k	0.025597	8.65E-09	0.04887	0.000000	0.029554	0.00093
C-2	13C(max)	20.05699	0	5.098329	0.000000	2.211274	0
	k	0.02166	0	0.046749	0.000048	0.083188	2.43E-08
C-4	13C(max)	30.26421	0	11.89662	0.000000	4.28137	0
	k	0.02094	0	0.04826	0.000000	0.05567	1.15E-09
C-6	13C(max)	15.95253	0	4.669367	0.000000	0.995224	0
	k	0.01983	0	0.022271	0.000095	0.087748	3.78E-09

Table S2. Steven's runs test for linearity: Regression analysis parameters for fitted ^{13}C curves (ns= not significant)

Temperature	Position	R^2	$P_{slope\neq0}$	$P_{runs\ test}$	$P_{equlity\ of\ slopes}$	$P_{equality\ of\ intercepts}$
5 °C	C-1	0.5353	0.0001	0.3000	0.3215	0.009379
	C-2	0.5980	< 0.0001	0.3000		
	C-4	0.8295	< 0.0001	0.3000		
	C-6	0.7541	< 0.0001	0.3000		
-5 °C	C-1	0.9281	0.002	0.3000	<0.0001	ns
	C-2	0.7506	0.0256	0.5000		
	C-4	0.9801	0.0012	0.5000		
	C-6	0.6170	0.0641	0.3000		
-20 °C	C-1	0.6316	0.0105	0.0714	0.2489	<0.0001
	C-2	0.5287	0.0929	0.1071		
	C-4	0.6674	0,0072	0.1071		
	C-6	0.5134	0.117	0.0714		

Table S3. Total microbial biomass C (mean ± SE) extracted during incubation

	∑MBC (µg g-1 dry soil)		
Temperature	1	3	10
5 °C	251.36 (6.47)	199.31 (13.90)	231.51 (7.89)
-5 °C	300.76 (6.84)	350.86 (8.04)	237.41 (19.48)
-20 °C	180.45 (10.58)	193.98 (13.46)	167.26 (7.92)

Table S4. Significant differences ($p<0.05$) between the fitted curves for individual temperature are indicated by superscript letters on ^{13}C (max) values.

	Positions			
Temperature	C-1	C-2	C-4	C-6
5 °C	36.98^{d}	20.06^{b}	30.26^{c}	15.95^{a}
-5 °C	7.19^{b}	5.1^{a}	11.9^{c}	4.65^{a}
-20 °C	3.84	2.21	4.28	0.99

2.2. Study 2: Structural and physiological adaptations of soil microorganisms to freezing revealed by position-specific labelling and compound-specific ^{13}C analysis

Ezekiel K. Bore[a,#], Sara Halicki[a,#], Yakov Kuzyakov[a,b,c], and Michaela A. Dippold[a]

[a] *Department of Agricultural Soil Science, University of Goettingen, 37077 Goettingen, Germany*

[b] *Department of Soil Science of Temperate Ecosystems, University of Goettingen, 37077 Goettingen, Germany*

[c] *Institute of Environmental Sciences, Kazan Federal University, 420049 Kazan, Russia*

[#]*Equal contribution*

Corresponding Author:

Ezekiel K. Bore

Department of Agricultural Soil Science

University of Goettingen

Buesgenweg 2

37077 Goettingen

email: ezekielbore7@gmail.com

Tel.: 0551-3922106

Fax.: 0551-3933310

Abstract

The mechanisms enabling survival and functioning of psychrophiles in frozen environments remain unclear. We investigated the adaptations of microbial phospholipids to freezing and the resulting effects on community structure using position-specific labelling and compound-specific ^{13}C analysis. Soils were incubated with ^{13}C labelled glucose at +5 (control), -5 and -20 °C and ^{13}C was quantified in CO_2 and phospholipid fatty acids (PLFA). Total CO_2 decreased 2- and 5-fold at -5 and -20 °C, respectively, compared to +5 °C. ^{13}C recovered in CO_2 showed high oxidation of the C-1 at +5 °C, revealing that the pentose phosphate pathway dominated glucose transformation. However, at subzero temperatures, C-4 was preferentially oxidized, suggesting a switch to glycolysis. The strong increase in abundance of gram-negative PLFA in soil incubated at -5 °C was accompanied by a twofold increase in ^{13}C incorporation into their PLFAs. Increase in both PLFA content and ^{13}C incorporation can only be explained by simultaneous occurrence of: 1) Desaturation within existing fatty acid chains and 2) *de novo* synthesis of PLFA. In contrast, gram-positive bacteria incorporated 2 times higher ^{13}C into their PLFAs at -20 °C than at higher temperatures (-5 and +5°C) without a significant increase in their fatty acid contents. Moreover, the fungal/bacterial PLFA ratio was 1.5 times lower at subzero temperatures than at +5°C, reflecting a shift in microbial community structure towards bacteria. Therefore, microorganisms adapted to freezing by switching metabolic pathway from the pentose phosphate pathway to glycolysis, adjusting phospholipid fatty acids and shifting microbial community structure towards bacteria.

Keywords: Psychrophiles, Phospholipid adaptations, Community structure, Metabolic tracing, Subzero temperatures

Highlights

- **Mechanisms enabling survival and functioning of psychrophiles in frozen environments.**
- **Position-specific labelling and PLFA analysis reveals more about adaptations to freezing.**
- **Phospholipid fatty acids adjust and microbial community shifts in response to freezing.**
- **Microbial metabolism persists at subzero temperatures and influences biogeochemical cycles.**

2.2.1. Introduction

Low temperature environments are the most widely distributed extreme habitats. Consequently, psychrophiles represent the most abundant, diverse and widely distributed extremophiles on earth (D'Amico et al., 2006). Recently, significant CO_2 release was recorded from soils in boreal and tundra ecosystems at subzero temperatures (Panikov et al., 2006). Such processes point towards the significance of psychrophiles in biogeochemical cycling in high-latitude regions. Additionally, psychrophiles and their biomolecules possess enormous potential in biotechnological, biomedical and industrial applications (Huston, 2008; Venketesh and Dayananda, 2008). Despite increased efforts to bioprospect for novel psychrophiles, their cold adaptation mechanisms remain largely unexplored. Using the novel technique of position-specific ^{13}C labelling and phospholipid fatty acid (PLFA) analysis, this study aimed at investigating microbial metabolic responses to subzero temperatures in soils and consequences on microbial community structure.

The perception that microbial metabolic activity ceases when soil temperature falls below 0 °C has changed (Nikrad et al., 2016). Recently, biogenic CO_2 production was detected in frozen soils at temperatures down to -39 °C (Panikov et al., 2006; Schaefer and Jafarov, 2016). Moreover, bacterial growth was determined down to -15 °C in permafrost isolates (Mykytczuk et al., 2013) and -20 °C in permafrost cores (Rivkina et al., 2000; Tuorto et al., 2014). This implies that microbial processes play a significant role in C cycling in high-latitude ecosystems, which harbours 40% of earth's soil C stocks (Noble et al., 2000; Oquist et al., 2009; Tuorto et al., 2014).

These studies are mostly based on arctic soils containing microbes adapted to perpetual cold conditions (Rivkina et al., 2000; Mykytczuk et al., 2013; Tuorto et al., 2014). Microbial processes in temperate soils that experience similar freezing conditions during winter are rarely studied. In the frozen state, significant amounts of greenhouse gases are released from temperate soils, making these soils a good analogue of what is expected in permafrost (Nikrad et al., 2016). Thus, this study was designed to determine the effects of cold temperatures on microorganisms in temperate agricultural soils that are exposed to seasonal winter freezing down to -20 °C. Cold temperatures place severe constraints on cellular functions, including, but not limited to, negative influences on membrane fluidity

(Russell et al., 1995; Rodrigues and Tiedje, 2008) and functioning of membrane bound enzymes (Berry and Foegeding, 1997). When the growth temperature of microorganisms is decreased, fluid-like membrane components become gel-like, preventing normal protein functioning (Beales, 2004). Thus, for cell membranes to be physiologically active under such extreme conditions, acyl chains of the membrane lipids must maintain a liquid-like state to keep the membrane proteins active. Beside such adaptations, shifts in community structure would be expected to compensate for the loss of more sensitive population members, when microorganisms are subjected to freezing. However, such shifts in community structure and the underlying molecular, biochemical and structural changes responsible for freeze tolerance have not been well characterized. For these reasons, position-specific ^{13}C labelling and PLFA analysis was conducted to achieve this aim.

PLFAs are components of microbial cell membranes and are formed by intact and proliferating cells. Using position-specific labelled substances allows tracing of individual molecular positions, permitting reconstruction of metabolic pathways and *de novo* formed products such as PLFAs (Dippold and Kuzyakov, 2013). In most organisms, the main pathways of glucose metabolization are glycolysis and the pentose phosphate pathway (PPP), where different glucose C positions are metabolised to C_2-moieties (Acetyl-CoA), which are subsequently oxidized to CO_2. As described by Dijkstra et al. (2011c), glucose C-3 and C-4 positions are respired completely, while the remaining positions C-1/C-2 and C-5/C-6 in C_2-moieties will have a ratio of 1:1 in glycolysis. Whereas in PPP, C_2-moeties of positions C-2, C-3 and C-5, C-6 will have a ratio of 2:3 and position C-1 and C-4 are respired completely. The C_2-moeties derived from labelled glucose are precursors in PLFA formation and can be detected in a majority of microbial groups' cell membranes (Apostel et al., 2015). Some PLFAs are specific for a certain group of organisms, but many others are non-specific. Characterization of extracted fatty acids enables re-construction of the microbial community structure, while patterns of position-specific ^{13}C incorporation into the specific fatty acids enables tracing the metabolic processes of the respective microbial groups in soil.

To study microbial responses to freezing, position-specific and uniformly ^{13}C labelled glucose (C-1, C-2, C-4, C-6 and U-G) was applied to soil samples and incubated at +5, -5 and -20 °C for 3 days. Total CO_2 release from the soil and position-specific ^{13}C patterns in PLFAs were determined. Due to expected membrane structural adjustments in response to freezing, we

hypothesized that the glucose C-1 position would be highly oxidized via PPP and microbial community structure will shift to compensate for loss of more sensitive populations.

2.2.2. Material and Methods

2.2.2.1. Sampling site

The soils were collected from an agriculturally-used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 m a.s.l.) in August 2014. The average annual temperature and precipitation are 7 °C and 874 mm, respectively. The soil properties were pH (H_2O) – 6.48, cation exchange capacity (CEC) – 13 $cmol_c$ Kg^{-1}, total organic carbon (TOC) – 1.77%, and total nitrogen (TN) – 0.19 %. The soils were collected from 0 – 10 cm depth, sieved through a 2 mm mesh, and stored for 1 day at +5 °C, except for subsamples (n = 3) used to determine dry weight.

2.2.2.2. Experimental design

The soils were incubated in airtight screw-cap glass microcosms with a base layer of quartz sand. About 80 g of soils were weighed into metal sampling rings fitted with ceramic plates at the base and placed into the screw-cap glasses. To equalize densities, an equal pressure was applied to all soils. The soils were re-wetted to field capacity at +5 °C for 2 days. Thereafter, the microcosms were preconditioned at +5, -5 and -20 °C for 1 day. These temperatures were chosen to simulate the 1) mean annual temperature of the sampling site which served as control, 2) mean winter temperature, and 3) lowest temperatures in some winters at this site and common deep freezer storage temperatures, respectively. About 28 mg of four position-specific ^{13}C-labeled isotopomers of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4 and ^{13}C-6), uniformly labelled (U-^{13}C) and non-labelled glucose to serve as natural backgrounds were each dissolved in 60 ml of ultra-pure water at room temperature to give a 2.55 mM stock solution. Each glucose isotopomer, uniformly labelled and non-labelled glucose were replicated four times with two sampling dates and three temperature levels resulting in 144 individual microcosms. With aid of four fine tip pipettes, 5 ml of each tracer solution including non-labeled glucose were dropwise applied to cover entire soil surface in individual microcosms 1 day after preconditioning at each temperature. Small glass caps filled with 5 ml of 1 M NaOH were placed on the soil surface in the microcosms to trap the released CO_2.

To depress freezing point at -20 °C, 20% (w/v) of NaCl was dissolved in NaOH traps. The microcosms were sealed and incubated at the respective temperatures for 1 and 3 days.

2.2.2.3. Sampling and sample preparation

NaOH traps were exchanged after 10 hours, 1, 2 and 3 days and soils were destructively sampled after 1 and 3 days. The samples were immediately mixed and transferred to plastic bags. The plastic bags were then squeezed and rolled to drive out the air in order to minimize respiration during storage. All the soil samples were stored at -20 °C for a maximum of 3 weeks and subsequently subjected to PLFA-extraction as described below.

2.2.2.4. Analytical methods

Respired CO_2 and $\delta^{13}C$ Analysis

0.4 ml of each CO_2 trap was diluted with ultrapure water in the ratio 1:10. CO_2 content was then measured with a non-dispersive infrared (NDIR) gas analyser (TOC 5050, Shimadzu Corporation, Kyoto, Japan). $SrCO_3$ was precipitated from the remainder of the CO_2 traps using $SrCl_2$. The precipitates were separated by centrifuging at 2000 xg for 10 minutes and washing in between with Millipore water to pH 7. After drying the precipitates at 60 ˚C, bulk $\delta^{13}C$ values were determined by weighing 1-2 g of each sample into a tin capsule and measuring with a Flash 2000 Elemental Analyser coupled by a ConFlo III interface to a Delta V advantage IRMS (all Thermo Fisher, Bremen, Germany). ^{13}C content in CO_2 was calculated according to a mixing model (Gearing et al., 1991), where the C content of the background in Eq.1 was substituted according to Eq.2.

$$[C]_{CO_2} \cdot r_{CO_2} = [C]_{BG} \cdot r_{BG} + [C]_{appG} \cdot r_{rappG} \quad (1)$$

$$[C]_{CO_2} = [C]_{BG} + [C]_{appG} \quad (2)$$

Where $[C]_{CO_2/BG/appG}$ is C content of the sample/Background/applied Glucose (mg C g^{-1}soil); $r_{CO_2/BG/appG}$ is ^{13}C atom %-excess of labelled sample/Background/applied Glucose (at %)

PLFA extraction and analysis

PLFA extraction and purification

PLFAs were extracted and analysed according to a method by Frostegård et al. (1991), modified and described in detail by Gunina et al. (2014). Briefly, 25 µL of the first internal standard (phosphatidylcholine-dinonadecanoic acid – IS1) was added to 6 g of each soil sample, which were semi-thawed for about 40 minutes, crushed and mixed. PLFAs were extracted with 18 mL followed by 6 mL of extraction buffer consisting of a mixture of 0.15 M citric acid, methanol and chloroform in the ratio 0.8:2:1 (v:v:v), respectively. The extract was subjected to liquid-liquid extraction using chloroform. Phospholipids were separated from neutral and glycolipids by solid phase extraction in columns packed with activated silica gel. The phospholipids were then hydrolysed in 0.5 M NaOH and derivatized to fatty acid methyl esters (FAME) using boron trifluoride. The FAMEs were extracted by liquid-liquid extraction method using hexane. Fifteen microliter of the second internal standard (tridecanoate methyl ester – IS2) and 185 µL of toluene were added to the extracted FAMEs and transferred to GC vials for analysis.

PLFA quantification and $\delta^{13}C$ analysis

FAME quantification and $\delta^{13}C$ analysis were determined on a GC-C-IRMS consisting of an Autosampler unit (*AS 200*) and combustion unit (*Combustion Interface III*) interfaced to a *Delta plus* isotope ratio mass spectrometer (all units from Thermo Fisher Scientific, Bremen, Germany). One microliter samples were injected with one minute splitless time into a liner at 280 ˚C. Peak separation was accomplished with two capillary columns (DB1-MS, 15 m, 250 µm ID, 0.25 µm film thickness and DB-5 MS, 30 m 250 µm ID, 0.25 µm film thickness) and Helium (99.9996% pure) as carrier gas with a flow of 1.7 mL/min.

FAME peaks were integrated and the $\delta^{13}C$ values (‰) calculated with ISODAT 2.0. Drift correction was performed by linear regression of CO_2 (99,995% pure) peaks repeatedly injected as reference gas between sample peaks. Unknown $\delta^{13}C$ values contributed by derivatizing agents and concentration-dependent isotopic fractionation were corrected (Glaser and Amelung, 2002; Schmitt et al., 2003). FAME amounts were calculated by relating their areas to the area of IS2 followed by quantification from calibration curve by linear

regression using five increasing concentrations of external standards consisting of 27 fatty acids (see supplementary Table 1). Extracted PLFAs were corrected based on recovery of IS1. ^{13}C enrichment in PLFAs was determined according to Equations (1) and (2) above.

2.2.2.5. Pathway discrimination index

Two main pathways of glucose transformation are the PPP and glycolysis. To discriminate between these two pathways based on ^{13}C incorporated into PLFAs from glucose positions, we designed a pathway discrimination index (PDI) defined as:

$$PDI = (C6 - C1)/C6 \quad (3)$$

where C1 and C6 are ^{13}C amounts (%) incorporated from glucose C-1 and C-6 positions into microbial PLFAs

When the PPP activity is maximal, glucose C-1 and C-4 are completely respired. As a result, incorporation of C-1 into PLFAs equals 0, giving a PDI value of 1. Alternatively, when glycolysis is maximal and PPP is zero, only the glucose C-4 position is completely respired. This leads to equal incorporation of C-1 and C-6 into PLFAs, resulting in a PDI value of 0. When the two pathways are at equilibrium, the PDI value equals 0.5.

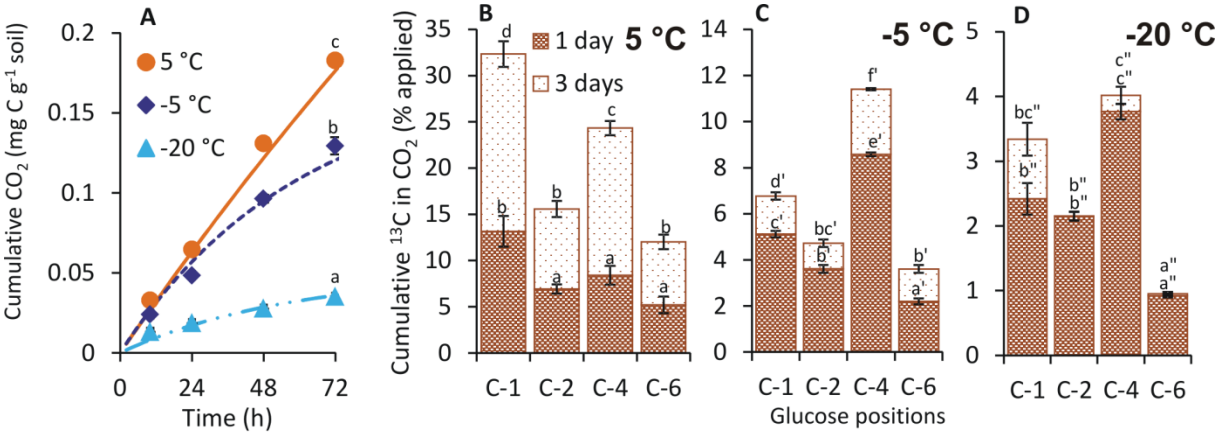
2.2.2.6. Statistical analysis

A Nalimov outlier test was performed on CO_2 with a 95% significance level. ^{13}C incorporated into CO_2 and PLFAs were tested for significant differences between positions, incubation duration and temperatures with a factorial analysis of variance (ANOVA). When normality and homogeneity of variance assumptions were not met, the data was validated by a nonparametric Kruskal Wallis ANOVA. Tukey's Honest Significance Difference (Tukey HSD) post-Hoc test was used to determine significant differences with a 95% confidence level. Factor analysis of PLFA concentrations was conducted to group the microorganisms. Microbial grouping was achieved if PLFAs loaded on the same factor higher than 0.5 and if literature data on pure cultures revealed a common origin of the PLFAs loading on the same factor (Zelles et al., 1995; Zelles, 1999). Statistica (version 12.0, Statsoft GmbH, Hamburg, Germany) was used for all statistical analyses.

2.2.3. Results

2.2.3.1. CO_2 release and ^{13}C recovery

Cumulative CO_2 released and position-specific ^{13}C patterns were highly sensitive to temperature. Compared to +5 °C, cumulative CO_2 released was 2- and 5-fold lower at -5 and -20 °C after 3 days, respectively (Fig. 1A). Similarly, ^{13}C in CO_2 from uniformly labelled glucose decreased 3- and 9-fold at -5 and -20 °C compared to +5 °C, respectively (supplementary Figure 1). Position-specific ^{13}C patterns in CO_2 revealed high oxidation of the C-1 position at +5 °C (Fig. 1B). This pattern switched at subzero temperatures, with the C-4 position exhibiting a nearly 2-fold higher oxidation than the C-1 position at -5 °C (Fig. 1C). Interestingly, this metabolic shift was moderated at -20 °C, with the C-1 and C-4 positions exhibiting relatively equal oxidation rates (Fig.1D). Moreover, the proportion of ^{13}C respired between day 1 and 3 decreased with decreasing temperature.



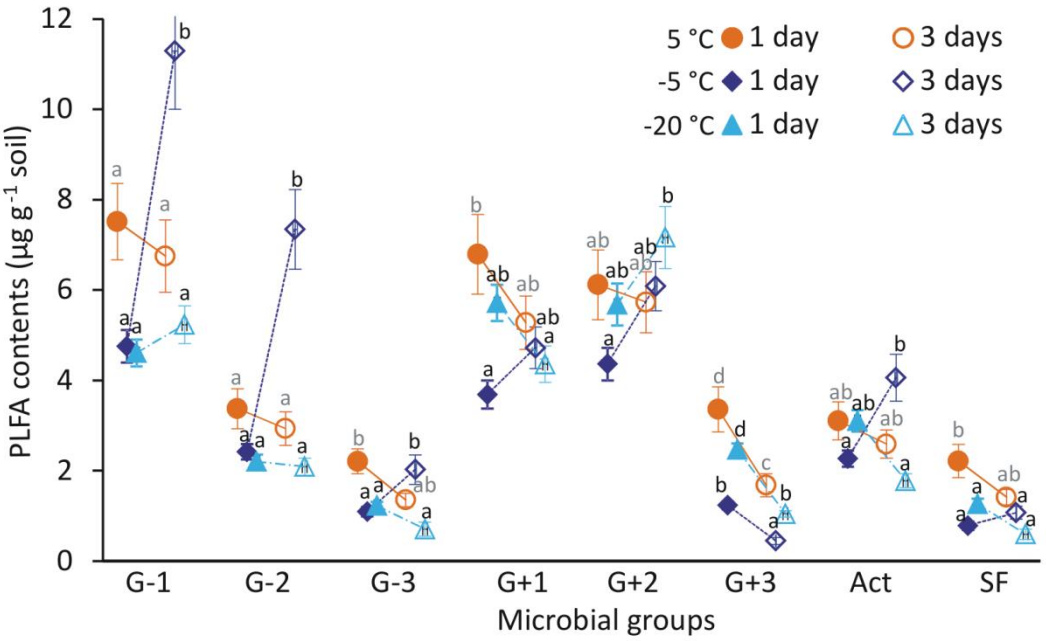
S2 Figure 1: *cumulative CO_2 (mean±SEM) (**A**) and ^{13}C (mean±SEM) recovered in CO_2 respired from position-specific labelled ^{13}C glucose at +5 (**B**), -5 (**C**) and -20 °C (**D**). Significant effect of temperature on cumulated CO_2 at day 3 and significant differences ($p<0.05$) between incubation duration and individual glucose positions respired at each temperature are indicated by letters (+5 (a), -5 (a') and -20 °C (a")) above the error bars. C-1, C-2, C-4 and C-6 show the position of ^{13}C in glucose molecule. Note different Y scales for the 3 temperatures.*

2.2.3.2. PLFA

PLFA contents

Grouping of PLFAs by combining factor loadings of the concentrations and known PLFA finger-prints from the literature on pure cultures (Zelles et al., 1995; Zelles, 1999) resulted in

8 microbial groups (Supplementary Table 2). Generally, the biomarkers of bacteria (G-1, G+1 and G+2) dominated the fingerprint irrespective of temperature (Fig. 2). A switch of temperature from +5 °C to -5 °C, significantly decreased PLFA concentration of G-3, G+1, G+2, G+3 and SF 1 day after glucose addition (Fig. 2).

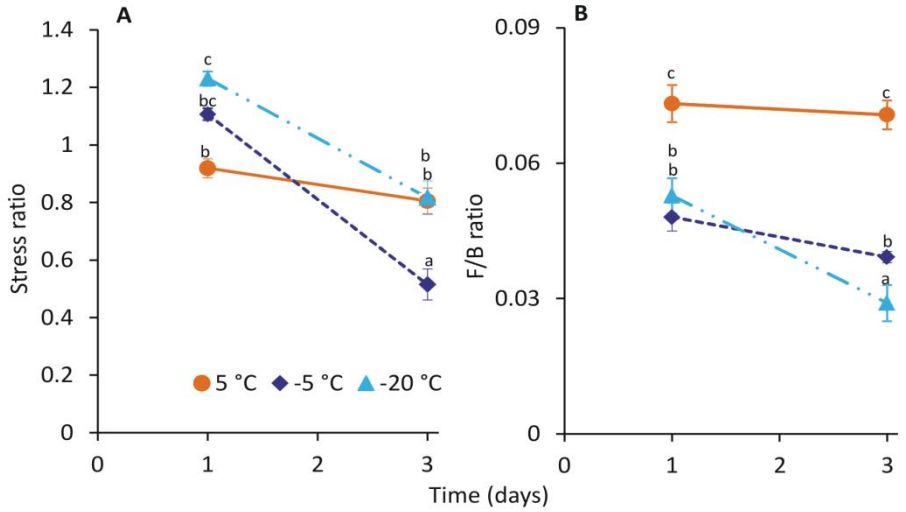


S2 Figure 2: *PLFA contents (mean ± SEM) of microbial groups at +5, -5 and -20 °C, grouped according to a factor analysis (Table 1). The letters above the error bars indicate significant differences (p<0.05) between incubation duration and temperature within microbial groups' fatty acid content.*

Three days later at -5 °C, PLFA concentration of gram-negative bacteria (G-1 and G-2) increased strongly, exceeding their concentration at +5 °C. The PLFA concentration of gram-negative 3 (G-3) and actinomycetes (Act) doubled after 3 days at -5 °C attaining the same level as at +5 °C. In overall, PLFA concentration of G+3 decreased significantly between day 1 and 3 at each temperature (Fig. 2).

Stress and fungal/bacterial ratio

The stress ratio was determined by dividing the sum of PLFAs Cy17:0 and Cy19:0 by the sum of PLFAs 16:1ω7 and 18:1ω7 (Fig. 3A). Stress ratios were significantly higher at -20 °C compared to +5 °C on the first day. However, the stress ratios decreased 1.5- and 2-fold at -20 and -5 °C after 3 days, respectively.

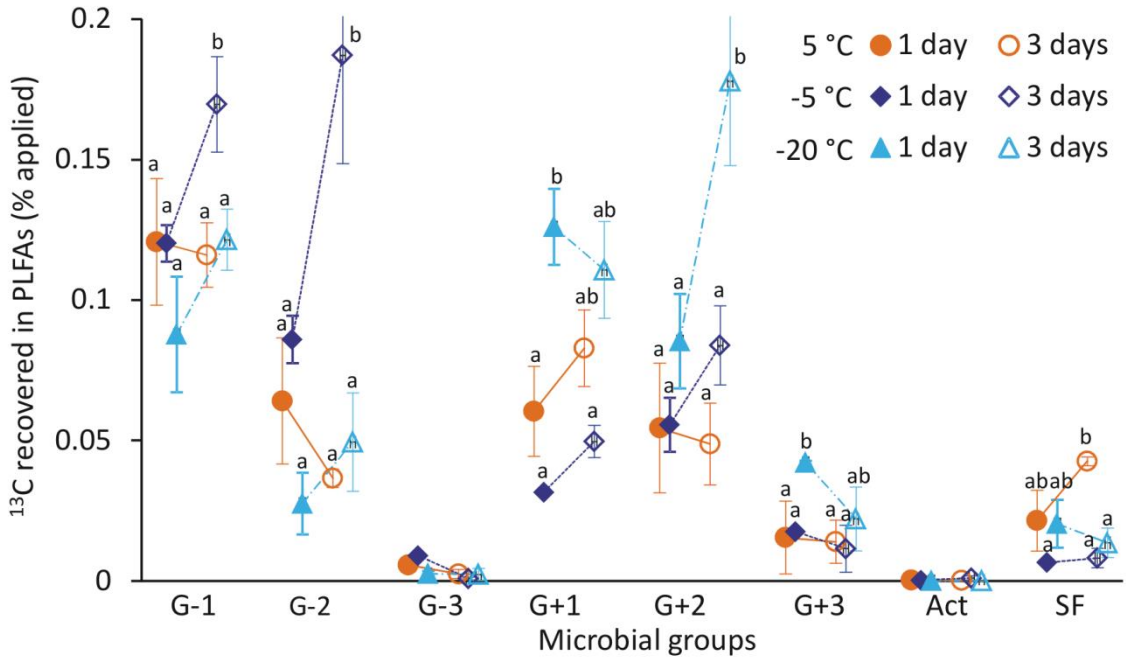


S2 Figure 3: *stress ratio (mean ± SEM) of G-1 bacteria (**A**) and fungal/bacterial ratio (mean ± SEM) (**B**). Letters above the error bars indicate significant differences (P<0.05) between temperature and incubation duration.*

The fungal/bacterial PLFA (F/B) ratio was 1.5-fold lower at subzero temperatures than +5 °C on the first day (Fig. 3B). The ratio decreased 2-fold between day 1 and 3 at -20 °C. This F/B ratio, which demonstrates a shift in microbial community structure over the 3 day period resulting from a relative decrease in fungi compared to bacteria, was strongest at -20 °C.

^{13}C incorporation into PLFA

G-1 bacteria were the most active in glucose utilization at +5 °C (Fig. 4). G-1 incorporated 0.12% of applied ^{13}C from uniformly labelled glucose into their PLFAs, while each of the other microbial groups incorporated less than 0.06% at +5 °C. The ^{13}C incorporated into the PLFAs of G-1 and G-2 increased by factors of 1.5 and 2 after 3 days at -5 °C, respectively (Fig. 4).

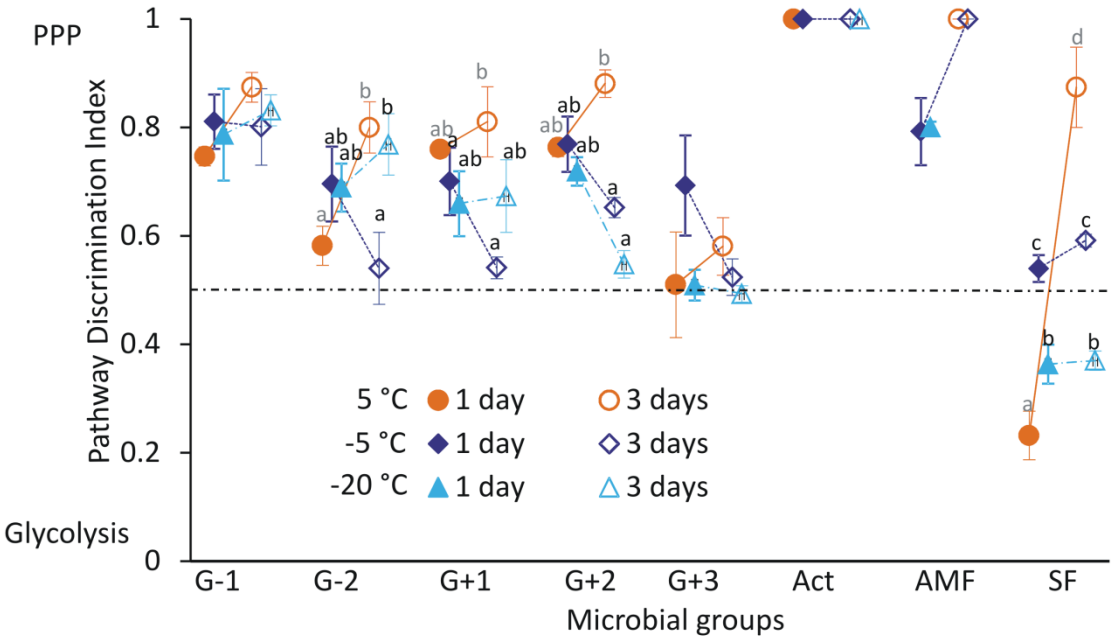


S2 Figure 4: *^{13}C (mean ± SEM) recovered in microbial PLFAs from uniformly labelled glucose at +5, -5 and -20 °C. The letters above the error bars indicate significant differences (p<0.05) between the temperatures and incubation duration of individual microbial groups.*

On the contrary, gram-positive bacteria dominated glucose utilization at -20°C, with G+1 and G+3 incorporating twice as much ^{13}C into their PLFAs at -20 °C than at higher temperatures (-5 and +5 °C) on the first day. G+2 doubled ^{13}C incorporation into their PLFAs 3 days after glucose application at -20 °C. Subzero temperatures decreased significantly ^{13}C incorporation into PLFAs of SF at day 3 compared to +5 °C (Fig. 4). G-3 and Act incorporated less than 0.009% ^{13}C into their PLFA irrespective of temperature and time despite a 2-fold increase in fatty acid content between day 1 and 3 at -5 °C.

2.2.3.3. Pathway Discrimination index

The pathway discrimination index (PDI) revealed an influence of temperature on the pathway of glucose transformation for the majority the microbial groups. This excludes G-3 (at +5 and -20 °C) and Act (at each temperature), whose ^{13}C amounts in PLFAs from glucose positions were undetectable or extremely low and did not allow calculation of the PDI. At +5 °C, pentose phosphate pathway (PPP) activity increased significantly between day 1 and 3 in G-2 and SF (Fig. 5).



S2 Figure 5: *Pathway Discrimination Index (PDI), reflecting the ratio of the pentose phosphate pathway to glycolysis of individual microbial group at +5, -5 and -20 °C, respectively. The letters above the error bars indicate significant effect (p<0.05) of temperature and incubation duration within individual microbial group.* ^{13}C *incorporation from glucose positions into PLFAs of each microbial group are presented in Supplementary Table 4.*

A switch to -5 °C significantly decreased PPP activity 3 days after glucose addition in G-2, G+1, G+2 and SF when compared to +5 °C at the same time. At -20 °C, G+2 was the only microbial group that significantly decreased PPP activity at day 3 when compared to +5 °C at the same time. SF sustained low (below 0.5) PPP activity at -20 °C irrespective of the duration (Fig. 5). G-1 is the only microbial group that did not alter the pathways with temperature fluctuations.

2.2.4. Discussion

2.2.4.1. Microbial respiration

CO_2 decreased 2- and 5-fold at -5 and -20 °C compared to +5 °C, respectively (Fig. 1A). This reduction agrees with previous studies reporting temperature sensitivities of CO_2 production in frozen soils (Panikov et al., 2006). Position-specific ^{13}C recovery in CO_2 showed high oxidation of the glucose C-1 position (Fig.1B) revealing high PPP activity at +5 °C, which is

consistent with other studies (Dijkstra et al., 2011c). The pattern switched to a high oxidation of the glucose C-4 position at subzero temperatures, reflecting a switch to glycolysis (Dijkstra et al., 2011c; Apostel et al., 2015). This response sums up the total activity of the microbial community in soil. To understand the cause of this shift, we assessed microbial metabolic pathway patterns of individual microbial groups at various temperatures based on the PDI.

2.2.4.2. Specific pathways of glucose utilization by individual microbial groups

The PDI revealed high PPP activity in individual microbial groups' at +5 °C, confirming the metabolic fluxes observed in CO_2. Among the microbial groups that incorporated detectable ^{13}C into their PLFAs at -5 °C, PPP activity significantly declined in G-2, G+1, G+2 and SF at day 3 when compared to +5 °C (Fig. 5). This reduction in PPP activity over time at -5 °C led to an increase in glycolysis, with equilibrium between the two pathways being achieved within 3 days. This explains the high oxidation of the C-4 position observed in CO_2 fluxes at -5 °C (Fig. 1C). The response was driven by an increased demand for intracellular antifreeze metabolites (Bore et al., 2017a). At -20 °C, G+2 was the only microbial group that switched pathways in favour of glycolysis at day 3 when compared to +5 °C (Fig. 5). This switch was attributed to an increased demand for both intra- and extracellular antifreeze compounds (Bore et al., 2017a). The response may have contributed to a doubling of ^{13}C incorporation into PLFAs of G+2 between day 1 and 3 at -20 °C (Fig. 4). SF sustained the high glycolysis level at -20°C (Fig. 5), which goes along with low ^{13}C incorporation into their PLFAs at this temperature (Fig. 4). The switch in metabolic pathways reflects a change in the physiological state of microorganisms in response to freezing (Wouters et al., 2000). Nevertheless, utilization of the same pathways by G-1 irrespective of temperature (Fig. 5) suggests that not all microbes respond to freezing in the same way. Under environmental stress, gram-negative bacteria are unable to properly synthesize fatty acids 16:1ω7 and 18:1ω7, resulting in production of the by-products Cy17:0 and Cy19:0, respectively (Jahnke and Nichols, 1986). Stress level of G-1 was significantly higher at -20 °C than +5 °C on the first day (Fig. 3A). The stress ratio decreased ≈2-fold within 3 days at subzero temperature, suggesting physiological alterations which resulted in a better adaptation to the new conditions. However, not only

metabolic adaptations in the short-term, but also microbial community structure adaptations in the medium-term, are likely to be induced by temperature shifts.

2.2.4.3. Microbial community structure in frozen soils

Temperature, especially below 0 °C, is one of the primary regulators of microbial activity in soil, as it influences intracellular kinetics of metabolic processes, soil moisture content and substrate availability via diffusion in the extracellular environment. Thus, microbial community structure may shift to compensate for the loss of populations more sensitive to freezing. Previous studies reported dominance of fungal communities in frozen alpine and forest soils (Schadt et al., 2003; Haei et al., 2011). On the contrary, F/B ratios in our study was 1.5-fold lower at subzero temperatures than at +5 °C, demonstrating a decrease in fungal populations (Fig. 3B). These results are consistent with other studies on frozen agricultural (Koponen et al., 2006) and permafrost soils (Mykytczuk et al., 2013; Tuorto et al., 2014), which are frequently dominated by bacteria. Among the bacterial communities, PLFA contents of G-1, G+1 and G+2 were highest, suggesting that these groups were dominant at low temperatures. Nonetheless, PLFA contents do not reflect the microbial activity, as many groups maintain metabolic activity while down-regulating cell-proliferation or cells can be lysed, leading to preservation of 'non-living' PLFA under frozen conditions. Thus, PLFA content provides a snapshot of the combined effects of both active and inactive microorganisms. Moreover, this proxy is hindered by the fact that microorganisms adapt their phospholipids to new temperatures to maintain the appropriate fluidity for optimal substrate uptake (Nedwell, 1999), resulting in potentially altered PLFA signatures with temperature shifts. To circumvent this challenge, we compared these phospholipid abundance results with ^{13}C incorporation into PLFA, which reflects the activity of microbial groups utilizing glucose.

2.2.4.4. Microbial activity at subzero temperatures

Glucose utilization was dominated by bacteria, as demonstrated by the high levels of ^{13}C incorporation into their PLFAs. However, the activity of individual bacterial groups differed with temperature. ^{13}C incorporated into PLFAs of G-1 was 2 times higher than those of G-2, G+1 and G+2 at +5 °C (Fig. 4). When the temperature was reduced to -5 °C, ^{13}C incorporation

into PLFAs of G-1 and G-2 increased by factors of 1.5 and 2, respectively, within 3 days. Gram-negative bacteria exhibit high levels of competition for easily available substrates such as glucose (Treonis et al., 2004; Bölscher et al., 2017). This group-specific behaviour was reversed at -20 °C, where gram-positive bacteria out-competed gram-negative bacteria and approximately doubled their incorporation. The ability for microbes to sustain their activity at subzero temperatures, especially uptake of substances through membranes, was related to alterations in their phospholipid composition (Drotz et al., 2010). In response to low temperatures, microorganisms adjust their protein expression, antifreeze metabolite content and membrane lipid composition, with the latter aspect geared toward maintaining the appropriate membrane fluidity (Russell et al., 1995; Berry and Foegeding, 1997; Russell, 2002). Maintenance of fluidity is crucial in sustaining membrane functions, such as the activity of membrane bound enzymes and solute transport (Russell et al., 1995; Russell, 2002). However, adaptations of membrane lipid composition overlays community structure changes. Thus, adaptations in PLFA contents must be thoroughly discussed with regard to both processes.

2.2.4.5. Structural adaptation mechanisms at subzero temperatures

Unsaturation of fatty acid chains is the most common change that occurs with decreasing temperatures (Beales, 2004). Desaturation, which increases fluidity of the membrane by creating disturbances in the packing of fatty acid chains in the bilayer, is achieved via rapid responses of desaturases in the membrane (Russell et al., 1995; Beales, 2004), i.e. the intact phospholipids are modified without removal of fatty acids from the backbone or incorporation of new C atoms. In a recent study, the proportion of phospholipids containing recently-synthesized, ^{13}C-containing compounds, and average degree of unsaturation of the corresponding acyl chains, was found to be higher at -4 °C than at temperatures above 0 °C (Drotz et al., 2010). Their findings are consistent with our results, which show the PLFAs (16:1ω7, 16:1ω5, 18:1ω7 and 18:1ω9) characterizing gram-negative bacteria are mono-unsaturated and dominant at -5 °C. The dominance of gram-negative PLFAs at -5 °C was attributed to desaturation within existing fatty acid chains (Nichols and Russell, 1996; Russell, 2002). Moreover, strong ^{13}C incorporation into PLFAs of G-1 and G-2 between day 1 and 3 at -5 °C reflects *de novo* synthesis of the unsaturated fatty acids from the applied glucose to

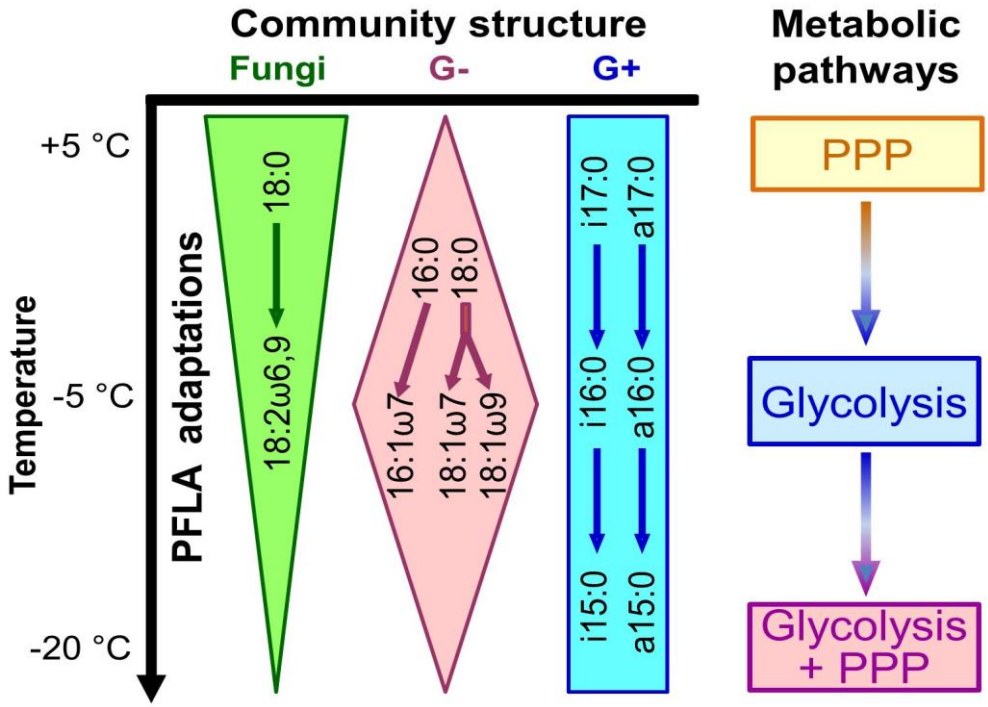
increase their proportion. Therefore, not only did alterations of existing membrane chains occur, but a community shift towards gram-negative bacteria also occurred at -5 °C.

^{13}C incorporation into Act PLFAs was undetectable irrespective of temperature and duration, confirming that this group was less competitive for the readily available glucose (Bölscher et al., 2017). Nevertheless, Act PLFA contents doubled between day 1 and 3 at -5 °C, attaining similar levels as at +5 °C. Such an increase without ^{13}C incorporation can be attributed to recycling of microbial necromass (Dippold and Kuzyakov, 2016) from vulnerable microbes. The characteristic PLFAs of Act (10Me16:0 and 10Me18:0), show that Act PLFAs are long saturated chains with mid-chain branching. Synthesis of branched fatty acids might have favoured survival and growth of this group at -5 °C. Such changes in composition of membrane lipids can change the transition temperature over a range of 100 °C, allowing microbial activity to occur even at extremely low temperatures (Price and Sowers, 2004; Panikov et al., 2006). Accordingly, syntheses of mono-unsaturated and branched fatty acids were crucial for survival and growth of these microbial groups at -5 °C.

Other modifications that improve membrane fluidity with decreasing temperature include increasing the proportion of short-chain branched fatty acid (Russell et al., 1995). Reducing fatty acid chain length reduces carbon-carbon interactions between neighbouring chains, while branching disrupts the packing of fatty acid chains in the bilayer (Russell et al., 1995; Russell, 2002). In our study, the proportion of ^{13}C incorporation into PLFAs of gram-positive groups (G+1 and G+2) relative to other groups was highest at -20 °C. Additionally, ^{13}C incorporation into the PLFAs of these microbial groups was higher at -20 °C than at -5 and +5 °C. The PLFAs characterizing G+1 are a15:0 and a16:0 and G+2 are i15:0 and i16:0. These PLFAs contain short-chain saturated and branched acyl groups. Modification of these fatty acids requires *de novo* synthesis of the whole phospholipid molecule by cytoplasmic enzymes linked to growth (Russell, 2002). Therefore, the increased ^{13}C incorporation into PLFAs of gram-positives at -20 °C reflects their high levels of *de novo* synthesis, demonstrating their increased competitiveness at freezing temperatures. The shift to gram-positives goes along with the synthesis of these short-chain saturated and branched fatty acids. The proportion of a15:0 and i15:0 was double those of a16:0 and i16:0 in both G+1 and G+2 (see Supplementary Table 3), confirming preferential synthesis of short-chain

branched PLFAs at this temperature. This is consistent with a study showing an increased synthesis of 15:0 at the expense of 17:0 fatty acids with decreasing temperature in pure bacterial cultures (Puttmann et al., 1993).

G+2 doubled the incorporation of ^{13}C into their PLFAs between day 1 and 3 at -20 °C. On the contrary, ^{13}C incorporation into PLFAs of G+1 did not differ with time. This implies that -20 °C was more favourable to G+2 with *iso*-branching (i15:0 and i16:0) PLFAs than G+1 with *anteiso*-branching (a15:0 and a16:0). This contradicts other findings, which suggested an increase in the ratio of *anteiso*- to *iso*-fatty acids with decreasing temperatures in pure bacterial cultures (Suutari and Laakso, 1994). However, it should be noted that none of these studies were performed at subzero temperatures, where the pure kinetic effect of temperature alteration is combined with many other stress factors such as lack of water. A study conducted to determine the effect of freeze-thaw cycles (between -17 and +4 °C) on microbial communities in agricultural soil did not find any changes in the ratio of *anteiso*- to *iso*- branched PLFAs (Koponen et al., 2006). However, this study was based solely on PLFA contents and thus could not make any conclusions on microbial activity or formation of new fatty acids – a shortcoming we overcame by using labelled ^{13}C glucose. Therefore, the increases in ^{13}C incorporation at -20 °C in this microbial group was a culmination of a community shift to gram-positive bacteria, synthesis of saturated short-chain branched fatty acids and switch of metabolic pathway to glycolysis.



S2 Figure 6: *Changes of community structure, PLFA adaptations to decreasing temperatures and switch of metabolic pathways with decreasing temperature. The shapes reflect changes in PLFA contents with temperature. The acronyms G- and G+ mean gram-negative and gram-positive bacteria, respectively.*

2.2.5. Conclusions

Combining position-specific labelling and compound-specific ^{13}C analysis revealed new mechanisms of soil microbial responses to freezing as summarized in figure 6. 1) Switch of metabolism: Glucose was metabolized via PPP at +5 °C, while the metabolic pathway shifted to glycolysis at subzero temperatures. 2) Membrane adjustments: Adaptation to freezing, includes, but not limited to, altered phospholipid composition in order to maintain membrane fluidity at subzero temperatures. Syntheses of unsaturated fatty acids dominated microbial activity at -5 °C. On the contrary, short branched *iso*- and *anteiso*-fatty acids dominated composition of phospholipids at -20 °C. The fatty acid unsaturation at -5 °C and dominance of *iso and anteiso*- fatty acids at -20 °C reflects membrane adaptations of microbial groups to freezing. 3) Adaptation of microbial community structure: A 1.5-fold decrease in F/B ratio at subzero temperatures, compared to +5 °C, indicates a shift in microbial community structure towards bacteria. Gram-negatives were active in glucose utilization at -5 °C, while gram-positives became highly competitive for glucose at -20 °C. This discriminate glucose utilization by microbial groups further supports the shift in community structure. Based on these results, we recommend that: 1) storage periods of soil samples for PLFA analysis at -20 °C should be minimized due to ongoing microbial activity, and 2) when sampling the soils, the temperature should be put into consideration because it influences microbial composition. These findings will be useful not only in understanding C dynamics in high latitude and altitude ecosystems with seasonally or permanently frozen soils and controlling pathogenic and spoilage organisms in food industry, but will also be useful in bioprospecting psychrophiles for biotechnological and medical applications.

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Supplementary information

Supplementary Table 1: Fatty acids in the external standards

FA-type	Name	Common name	Abbreviation	Retention time (s)*
Saturated	Tetradecanoic acid	Myristic acid	14:0	1382
	Pentadecanoic acid	-	15:0	1665
	Hexadecanoic acid	Palmitic acid	16:0	2026
	Heptadecanoic acid	Margaric acid	17:0	2463
	Octadecanioc acid	Stearic acid	18:0	3211
	Eicosanoic acid	Arachidic acid	20:0	4348
Branched chain	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	1311
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	1294
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	1575
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	1551
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	1910
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	1880
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	2329
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	2292
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	2381
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	3698
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	2202
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	3328
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	1351
	cis-11-Hexadecenoic acid	-	16:1w5c	1974
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	1936
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	2986
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	2849
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	4228
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2w6,9	2772
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	2814
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	3885

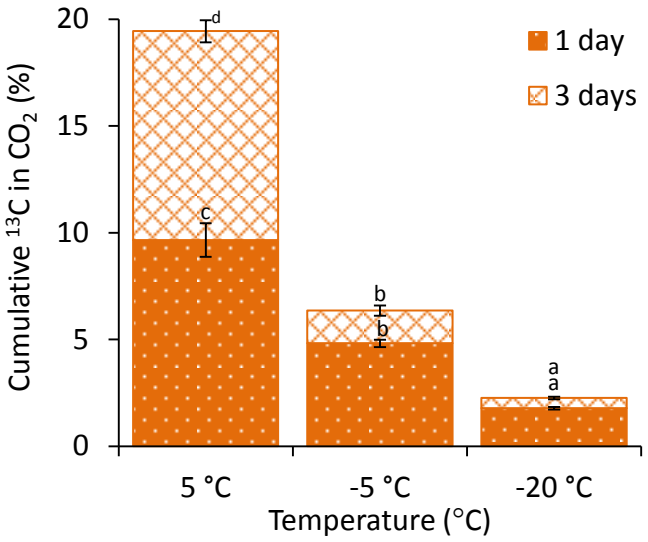
*for 45 m ± 0.5 m column lengths, deviations of ± 15 s possible

Supplementary Table 2: Microbial groups based on fatty acid grouping after factor loadings from factor analysis and comparing with pure culture results (Zelles, 1997; Zelles, 1999)

Group	Abbreviation	Fatty Acids
Gram-negative 1	G-1	16:1w7, 18:1w7
Gram-negative 2	G-2	18:1w9c
Gram-negative 3	G-3	16:1w5c
Gram-positive 1	G+1	a15:0, a16:0
Gram-positive 2	G+2	i15:0, i16:0
Gram-positive 3	G+3	i14:0, i17:0
Actinomycetes	Act	10Me16:0, 10Me18:0
Saprophytic fungi	SF	18:2w6,9

Supplementary Table 3: PLFA contents (mean ± SEM) characterizing gram-positive bacteria 3 days after glucose application

	a15:0	i15:0	a16:0	i16:0	a17:0	i17:0
5 °C	4.90 (0.70)	5.25 (1.08)	0.74 (0.13)	2.11 (0.38)	1.32 (0.18)	1.04 (0.24)
-5 °C	6.04 (1.19)	7.32 (1.54)	0.08 (0.02)	1.73 (0.36)	0.64 (0.10)	0.34 (0.07)
-20 °C	5.87 (1.28)	7.12 (1.57)	0	2.35 (0.60)	0.63 (0.21)	0.44 (0.11)



Supplementary Figure 1: Cumulative ^{13}C (mean ± SEM) in CO_2 from uniformly labeled glucose. Letters indicate significant differences ($p < 0.05$) between incubation duration and temperatures.

Supplementary Table 4: Relative ^{13}C incorporation (mean ± SEM) expressed as % of applied ^{13}C from glucose positions into PLFAs of individual microbial groups. SEM of each value are presented in parenthesis below them

			G-1	G-2	G+1	G+2	G+3	Act	AMF	SF
5 °C	Day 1	C-1	0.03963	0.029612	0.024671	0.0215648	0.027152	0	0.002804	0.073523
			(0.01017)	(0.009099)	(0.004822)	(0.00889)	(0.00267)	0	(0.00053)	(0.02198)
		C-2	0.1032154	0.0526306	0.0299295	0.0233069	0.002895	0	0.002011	0.023963
			(0.01939)	(0.01996)	(0.01054)	(0.00815)	(0.00144)	0	(0.00101)	(0.00269)
		C-4	0.010241	0.0003019	0.0003496	0	0.001848	0	0	0
			(0.00476)	(0.00030)	(0.00024)	0	(0.00081)	0	0	0
		C-6	0.1564154	0.0708045	0.1026369	0.0910179	0.055379	0.00058	2.42E-05	0.09569
			(0.04205)	(0.02111)	(0.02854)	(0.01944)	(0.0089)	(0.00003)	0	(0.02333)
	Day 3	C-1	0.0221158	0.017048	0.0289076	0.018188	0.009277	0	0	0.011493
			(0.00495)	(0.00451)	(0.00531)	(0.00314)	(0.0006)	0	0	(0.0066)
		C-2	0.1105499	0.0268288	0.0535224	0.0441504	0.006947	0.000422	0	0.031028
			(0.01935)	(0.00970)	(0.01120)	(0.00401)	(0.0001)	(0.00004)	0	(0.00221)
		C-4	0.0208154	0.0021271	0.0021098	0.0012213	0.001249	0	0	0.000516
			(0.00816)	(0.00123)	(0.00122)	(0.00057)	(0.0004)	0	0	(0.0003)
		C-6	0.1757278	0.0851874	0.1524923	0.1525959	0.022116	0	0.004724	0.091194
			(0.02835)	(0.01424)	(0.03616)	(0.02157)	(0.04079)	0	(0.0014)	(0.0138)
-5 °C	Day 1	C-1	0.0343804	0.0501404	0.0191511	0.0325897	0.011235	0	0.003217	0.007073
			(0.00289)	(0.00366)	(0.00150)	(0.00203)	(0.0006)	0	(0.0001)	(0.0009)
		C-2	0.1551461	0.0557893	0.0557802	0.0540002	0.015436	0.001114	0.01048	0.006712
			(0.00241)	(0.020055)	(0.00903)	(0.01852)	(0.0066)	(0.0011)	(0.0013)	(0.0023)
		C-4	0.0293253	0.0047043	0.0007806	0	0	0	0.000165	0
			(0.00811)	(0.00176)	(0.00033)	0	0	0	(0.0001)	0
		C-6	0.1816076	0.164718	0.0639304	0.1411401	0.036564	0.00436	0.015492	0.01536
			(0.02881)	(0.02673)	(0.01064)	(0.01984)	(0.0059)	(0.0005)	(0.0030)	(0.0028)
	Day 3	C-1	0.0381155	0.1609993	0.0390367	0.0608336	0.004215	0	0	0.011338
			(0.01028)	(0.04448)	(0.01546)	(0.01714)	(0.0007)	0	0	(0.00284)
		C-2	0.1992477	0.1889635	0.0671432	0.0862262	0.003931	0.000784	0.002304	0.013543
			(0.04287)	(0.08132)	(0.02407)	(0.01643)	(0.0015)	(0.0007)	(0.0014)	(0.00538)
		C-4	0.0336655	0.0127985	0	0.0011861	0.000192	0.000493	0	0
			(0.01053)	(0.00640)	0	(0.00079)	(0.0001)	(0.0004)	0	0
		C-6	0.1915064	0.3499272	0.0850382	0.1748718	0.008849	0.000675	0.00221	0.027737
			(0.05725)	(0.13133)	(0.03866)	(0.06701)	(0.0030)	(0.00036)	(0.0013)	(0.00219)
-20 °C	Day 1	C-1	0.0291238	0.017606	0.0934464	0.0769036	0.037201	0	0.001	0.028555
			(0.00949)	(0.00254)	(0.014051)	(0.01039)	(0.00504)	0	(0.0006)	(0.0013)
		C-2	0.1147434	0.0275495	0.1450665	0.1034451	0.054027	0	0.002456	0.039049
			(0.01945)	(0.00970)	(0.01464)	(0.02769)	(0.0118)	0	(0.00231)	(0.01236)
		C-4	0.0097809	0.0003092	0.008145	0.0030947	0.006667	0	0	0.000564
			(0.00363)	(0.00030)	(0.00199)	(0.00129)	(0.0027)	0	0	(0.00056)
		C-6	0.1365183	0.0566319	0.2743941	0.2735598	0.075768	0	0.005008	0.044832
			(0.01397)	(0.01841)	(0.09510)	(0.09956)	(0.0426)	0	(0.0020)	(0.0258)
	Dey 3	C-1	0.0341803	0.0243826	0.0709245	0.1240645	0.019715	0	0	0.018677
			(0.00267)	(0.00169)	(0.00860)	(0.02701)	(0.0038)	0	0	(0.0007)

C-2	0.2120319	0.0556422	0.1358213	0.1600894	0.032831	0	0.003162	0.028944
	(0.03137)	(0.01986)	(0.01072)	(0.03813)	(0.0069)	0	(0.0006)	(0.0167)
C-4	0.0409735	0.0050637	0.0056703	0.0089586	0.002722	0	3.14E-05	0.000624
	(0.0083)	(0.00207)	(0.00082)	(0.00325)	(0.00055)	0	0	(0.0003)
C-6	0.2028313	0.1054089	0.2170883	0.2741377	0.038873	0.001271	0	0.029645
	(0.03049)	(0.01780)	(0.03960)	(0.01942)	(0.0039)	(0.00082)	0	(0.0131)

2.3. Study 3: Soil microorganisms can overcome respiration inhibition by coupling intra- and extracellular metabolism: ^{13}C metabolic tracing reveals the mechanisms

Ezekiel K. Bore[1], Carolin Apostel[1,2], Sara Halicki[1], Yakov Kuzyakov[1,2], Michaela A. Dippold[1]

[1] Agricultural Soil Science, Georg-August-University Goettingen, Germany

[2] Soil Science of Temperate Ecosystems, Georg-August-University Goettingen, Germany

Conflict of interest

The authors declare no conflict of interest.

Corresponding Author:

Ezekiel K. Bore

Department of Agricultural Soil Science

Georg-August University of Goettingen

Buesgenweg 2

37077 Goettingen

email: ezekielbore7@gmail.com

Tel.: 0551-3922106

Fax.: 0551-3933310

Abstract

CO_2 release from soil is commonly used to estimate toxicity of various substances on microorganisms. However, the mechanisms underlying persistent CO_2 release from soil exposed to toxicants inhibiting microbial respiration, e.g. sodium azide or heavy metals (Cd, Hg, Cu), remains unclear. To unravel these mechanisms, NaN_3-amended soil, was incubated with position-specifically ^{13}C-labeled glucose and ^{13}C was quantifed in CO_2, bulk soil, microbial biomass and phospholipid fatty acids (PLFA). High ^{13}C recovery from C-1 in CO_2 indicates that glucose was predominantly metabolized via the pentose phosphate pathway irrespective of inhibition. Although NaN_3 prevented ^{13}C incorporation into PLFA and decreased total CO_2 release, ^{13}C in CO_2 increased by 12% compared to control soils due to an increased use of glucose for energy production. The allocation of glucose-derived carbon towards extracellular compounds, demonstrated by a 5-fold higher ^{13}C recovery in bulk soil than in microbial biomass, suggests the synthesis of redox active substances for extracellular disposal of electrons to bypass inhibited electron transport chains within the cells. PLFA content doubled within 10 days of inhibition, demonstrating recovery of the microbial community. This growth was largly based on recycling of cost-intensive biomass compounds, e.g., alkyl chains, from microbial necromass. The bypass of intracellular toxicity by extracellular electron transport permits the fast recovery of the microbial community. Such efficient strategies to overcome exposure to respiration-inhibiting toxicants may be exclusive to habitats containing redox-sensitive substances. Therefore, the toxic effects of respiration inhibitors on microorganisms are much less intensive in soils than in pure cultures.

Keywords: Soil functions, Respiration inhibition, Extracellular electron transport, Sodium azide, Metabolic tracing

2.3.1. Introduction

Soil microbes are the primary drivers of soil organic matter (SOM) cycling. However, microbial activity can be altered by human activities such as fertilization, use of pesticides or soil contamination by heavy metals from mines, industrial, agricultural and technological application. The rate and amount of CO_2 evolved from soil are used to evaluate the effects of toxicants (e.g. heavy metals and other pollutants) on heterotrophic microorganism activity in SOM decomposition (Babich and Stotzky, 1985; ISO16072, 2002). Persistent CO_2 release from soils exposed to toxicants (Voroney and Paul, 1984; Trevors, 1996) or contaminated with heavy metals (Bond et al., 1976; Ausmus et al., 1978; Fliessbach et al., 1994) remains unclear. Understanding the mechanisms responsible for CO_2 emission under such disturbances is important for predicting the response of nutrient and C cycles to future anthropogenic environmental changes (Schimel, 2013). Knowledge of metabolic pathways through which organic substances are oxidized to CO_2 is crucial in unraveling these mechanisms, making metabolic tracing an invaluable tool for identifying alterations in microbial transformation pathways of organic substances under these unfavorable conditions (Scandellari et al., 2009; Dijkstra et al., 2011b; Dippold et al., 2014).

Microorganisms break down complex plant materials such as cellulose to produce easily available water-soluble substances such as glucose, the most abundant monomer in soil, from which about 60-70% is incorporated into cellular compounds while 30-40% is oxidized for energy (Fischer et al., 2007; Gunina and Kuzyakov, 2015). Sufficient amounts of glucose in soil solution can activate microbial metabolism and induce growth (Blagodatskaya and Kuzyakov, 2013; Mau et al., 2015) hence accelerating SOM decomposition. In sterile soil, approximately 98% of glucose remains K_2SO_4-extractable within 24 hours (Bremer and van Kessel, 1990). Glucose lacks physical or chemical interactions with soil due to an absence of charged functional groups or hydrophobic parts (Fischer et al., 2010; Apostel et al., 2015), making it a potent candidate for tracing soil metabolic processes. Furthermore, the use of position-specifically labeled glucose allows the fate of individual molecular positions to be determined (Scandellari et al., 2009; Dijkstra et al., 2011b; Apostel et al., 2015) permitting detailed reconstruction of microbial metabolic pathways and de-novo formed products (Dippold and Kuzyakov, 2013). This provides the toolbox required to elucidate the source of

CO_2 emission under respiration-inhibited conditions and thus to identify the mechanisms to overcome intoxication in contaminated environments.

In this study, microbial respiration was inhibited via NaN_3 addition, a potent electron transport chain inhibitor at cytochrome oxidase and catalase (Keilin, 1936), resulting in cell asphyxiation (Winter et al., 2012). Paradoxically, azide addition increases CO_2 efflux from soil (Rozycki and Bartha, 1981; Trevors, 1996). This cannot be attributed to errors in CO_2 determination caused by formation of volatile hydrazoic (HN_3) acid (Rozycki and Bartha, 1981; Trevors, 1996), as chloroform fumigation also results in persistent CO_2 emission (Voroney and Paul, 1984; Blankinship et al., 2014). The CO_2 emitted from soil following microbial metabolic inhibition was previously ascribed to active oxidative extracellular enzymes (EXOMET) already present in soil or released from dead cells (Maire et al., 2013). However, since this study solely traced catabolism, the ultimate source of the emitted CO_2 could not be definitively concluded.

This study aimed to establish the origin and underlying mechanisms of persistent CO_2 release from soils exposed to model toxicants inhibiting respiratory chains. Analysis of position-specific ^{13}C patterns in CO_2, soil, microbial biomass and PLFA was performed to elucidate mechanisms underlying inhibition-induced CO_2 release. Measuring the production of de-novo formed microbial compounds was used as criteria to confirm or reject existence of intracellular metabolism following inhibition. Production of phospholipid-derived fatty acids (PLFAs), components of microbial cell membranes only formed by intact and proliferating cells, is frequently utilized to confirm the presence of active intracellular metabolism. We hypothesized that a lack of ^{13}C incorporation from labeled glucose into microbial biomass and PLFA after inhibition would confirm the proposed EXOMET theory.

2.3.2. Materials and methods

2.3.2.1. Sampling site

The soil was sampled from agriculturally used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 a.s.l.) with a mean annual temperature of 7 °C, and a mean annual precipitation of 874 mm. The soil had a pH (KCl) of 4.88, a pH (H_2O) of 6.49, a TOC and TN content of 1.77% and 0.19%, respectively. CEC was 13 $cmol_C\,kg^{-1}$. The soil was collected from 0-10 cm, air dried, sieved to 2 mm, and stored at 5 °C until use.

2.3.2.2. Experimental design

Incubations were conducted in screw-cap glass microcosms with a base layer of quartz sand. 80 g of dry soil was transferred to soil sample rings and installed on ceramic plates above the quartz sand. Half of the rings received NaN_3 to inhibit microbial activity while the second set was not treated with NaN_3 (Control). The 10 mL of 2 mM NaN_3 solution added to each ring was subdivided into two portions: 1) 2 mL was added directly onto the soil surface and, 2) 8 mL to the sand (to be taken up through the ceramic plates) while control soils received 10 mL of water. The added volume of water rewetted the soil to field capacity. All microcosms were preconditioned for 24 hours at 5 °C. This temperature was used to eliminate interference of the CO_2 determination by volatile HN_3 formed from the conversion of NaN_3 in soil. Four position-specific ^{13}C-labeled isotopomeres of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4 and ^{13}C-6), uniformly ^{13}C-labeled (U-^{13}C) and non-labeled glucose (natural abundance background) were applied to the soil in separate microcosms with 4 replicates each and 3 sampling dates resulting in 144 individual microcosms. 5 mL of 2.55 mM glucose solution was applied onto the soil surface in each microcosm. Glucose solution for the treated soil contained 1 mM NaN_3 to maintain continued inhibition. Cups filled with 5 ml of 1 M NaOH were placed into each microcosm to trap CO_2. Microcosms were sealed and incubated at 5 °C in the dark.

2.3.2.3. Sampling and sample preparation

NaOH in the vials was sampled and replaced after 10 hours, 1, 2, 3, 6 and 10 days. Soils were sampled after 1, 3, and 10 days. Each sample was divided into two fractions: 30 g of each sample was immediately subjected to chloroform-fumigation extraction as described below, while the remainder was stored at -20 °C for PLFA analysis.

2.3.2.4. Analytical methods

Amount and $\delta^{13}C$ value of CO_2

0.4 ml of each CO_2 trap was diluted 1:10 with ultrapure water and CO_2 content was determined by a non-dispersive infrared (NDIR) gas analyzer (TOC 5050, Shimadzu Corporation, Kyoto, Japan). The remaining volume was precipitated with 5 ml of 0.5 M $SrCl_2$ solution. Precipitates of $SrCO_3$ were separated by four-fold centrifuging at 2000 xg for 10 min and washing in between with millipore water to remove NaOH until pH 7 was reached. Dried $SrCO_3$ samples (1-2 mg) were loaded into tin capsules and $\delta^{13}C$ value was measured with

Flash 2000 Elemental analyzer coupled by a ConFlo III interface to a Delta V advantage Isotope Ratio Mass Spectrometer IRMS (all units from Thermo Fisher Scientific, Bremen, Germany). ^{13}C respired from the applied glucose was calculated according to a mixing model (Eq.1 and 2), where the C content of the background ($[C]_{BG}$) in Eq.1 was determined by Eq.2. (Gearing, 1991)

$$[C]_{CO_2} \cdot r_{CO_2} = [C]_{BG} \cdot r_{BG} + [C]_{appG} \cdot r_{appG} \quad (1)$$

$$[C]_{CO_2} = [C]_{BG} + [C]_{appG} \quad (2)$$

where:

$[C]_{CO_2/BG/appG}$ C content of the sample/background/applied Glucose (mg C g^{-1}soil)

$r_{CO_2/BG/appG}$ ^{13}C atom %-excess of labeled sample/background/applied glucose (at %)

Bulk soil C and ^{13}C content measurement

Aliquots of samples were freeze dried, ground in ball mill and 13-15 mg were weighted into tin capsules. Carbon stable isotope measurement were performed with EuroVektor elemental analyzers (HEKAtech GmbH, Wegberg, Germany) coupled by a ConFlo III interface to a Delta Plus XP IRMS (both units from Thermo Fisher Scientific, Bremen, Germany). Incorporation of ^{13}C from applied glucose into soil was calculated according to equation 1 and 2.

Microbial biomass ^{13}C determination

Microbial biomass C and $\delta^{13}C$ values were determined by chloroform fumigation extraction. Soil samples were divided into two subsets of 12 g each. One subset was extracted directly and the other subset was first fumigated with chloroform for 3 days in a desiccator to lyse microbial cells. Organic C was extracted with 36 ml of 0.05 M K_2SO_4 on an orbital shaker for 1.5 h. Samples were centrifuged for 10 min at 2000 rpm and the supernatant was filtered and frozen at -20 °C until C content analysis on a TOC/TIC analyzer (Multi C/N 2100 AnalytikJena, Jena, Germany). Thereafter, the extracts were freeze dried and about 25 mg (fumigated) and 40 mg (unfumigated) were used for $\delta^{13}C$ values determination via EA-IRMS. Incorporation of ^{13}C into fumigated and unfumigated samples was calculated using

equations 1 and 2. Microbial biomass C was calculated by subtracting unfumigated from fumigated C and dividing the product by a correction factor of 0.45 (Wu et al., 1990).

PLFA extraction and analysis

PLFA extraction and purification

PLFAs were analysed according to a modified method by Frostegård et al. (1991) with each step described in detail in Gunina et al. (2014). Briefly, 25 µg of an internal recovery standard (phosphatidylcholine-dinonadecanoic acid) was added to 6 g frozen soil. PLFAs were repeatedly extracted (first with 18 ml, then with 6 ml) with a 2:1:0.8 mixture of methanol, chloroform, and 0.15 M citric acid adjusted to pH 4. A two phase mixture was generated from the combined extracts by addition of chloroform and citric acid. After shaking the lower chloroform phase was removed by liquid-liquid extraction. Neutral and glycolipids were separated from phospholipids on a solid phase extraction column packed with activated silica gel by elution with chloroform, acetone and methanol, respectively. Phospholipids were hydrolyzed in 0.5 M NaOH in methanol for 10 min on 100 °C and derivatized to fatty acid methyl esters (FAME) by heating with 10% boron trifluoride in methanol at 80 °C for 15 min. 15 µg of the second external standard (tridecanoate methyl ester) together with 185 µl toluene were added to the sample and then transferred into 1.5 ml GC vials.

Analysis of $\delta^{13}C$ values on GC-C-IRMS

1 µl of PLFA samples were injected with a one minute splitless time into a liner at 280 ˚C of a Trace GC coupled via a GC-C III interface to a Delta plus IRMS (all units from Thermo Fisher Scientific, Bremen, Germany). Peak separation was accomplished with two capillary columns (DB1-MS, 15 m, 250 µm ID, 0.25 µm film thickness and DB-5 MS, 30 m 250 µm ID, 0.25 µm film thickness) with helium (He 99.99% pure) as carrier gas at a flow rate of 1.7 ml per min.

FAME peaks were integrated and the $\delta^{13}C$ values (‰) calculated via ISODAT 2.0. Drift correction was performed via repeated injection of the reference gas (CO_2 99.995% pure) during measurement by linear regressions between the gas peaks surrounding the sample peaks. Correction functions according to Glaser and Amelung (2002) were used to account for the unknown $\delta^{13}C$ value of the derivatization agents and concentration-dependent isotopic fractionation during the measurement (Schmitt et al., 2003). PLFA-C was quantified by 1) relating each FAME area to the area of IS 2, 2) calculating calibration curves by linear

regression of external standards consisting of 27 fatty acids at five increasing concentrations (see supplementary Table 1) and 3) correcting for the recovery of the initially added phospholipid standard.

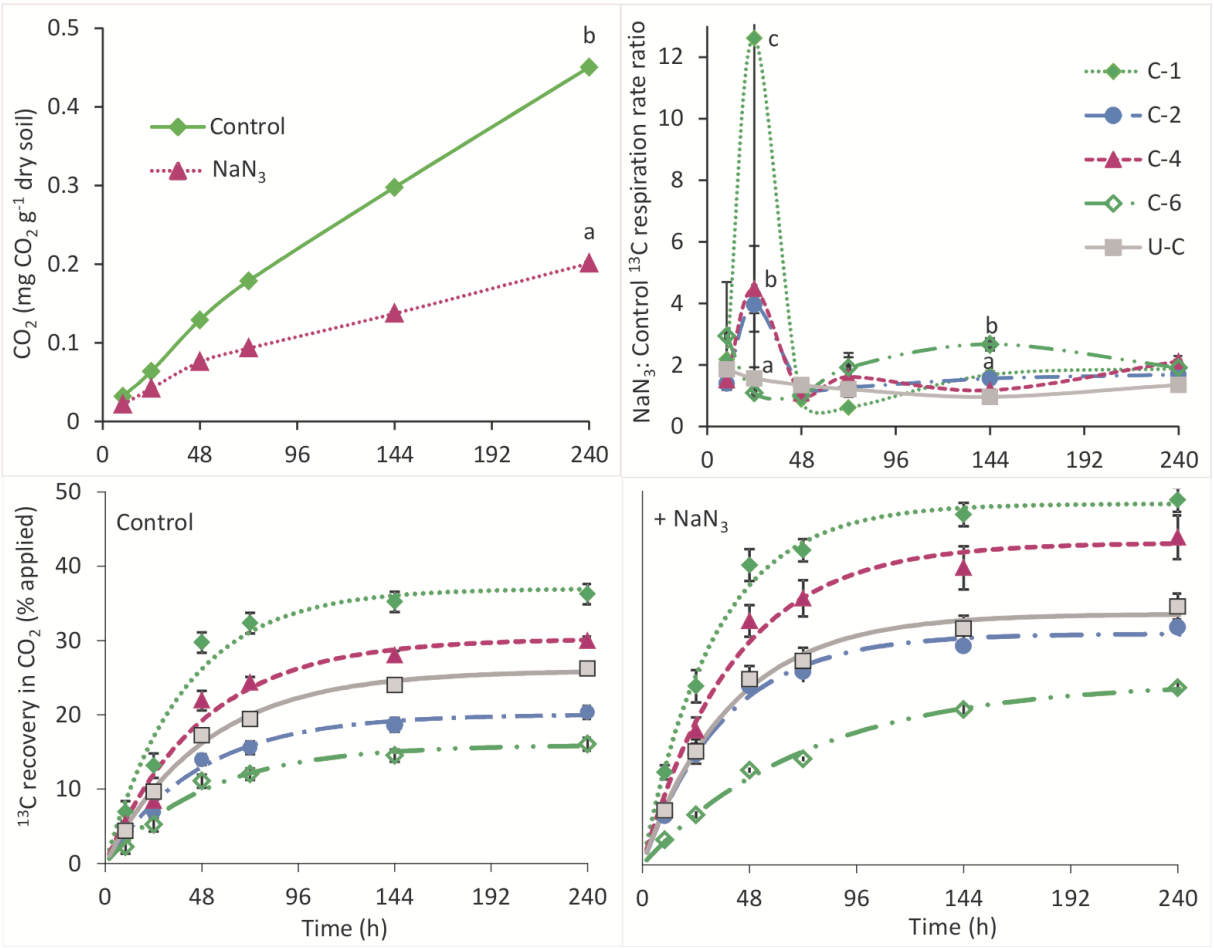
2.3.2.5. Statistical analysis

A Nalimov outlier test was performed for the respiration data with significance levels of 95% in case of four replicates. Microbial grouping was done by factor analysis of relative PLFA amounts (Apostel et al., 2013; Gunina et al., 2014). PLFA were grouped to one microbial group if they loaded on the same factor higher than 0.5 and if literature data on pure cultures proved their common origin (Zelles et al., 1995; Zelles, 1999). Incorporation of ^{13}C into bulk soil, microbial biomass, PLFAs and into CO_2 was tested for significant differences between the positions, incubation time and effect of NaN_3 addition via factorial analysis of variance (ANOVA). If assumptions of normal distribution and homogeneous variances were not met, then outcomes were validated by a nonparametric Kruskal Wallis ANOVA. Significant differences were determined with Tukey Honest Significance Difference (Tukey HSD) post-hoc test at a confidence level of 99.95%. Statistical tests were performed with Statistica (version 12.0, Statsoft GmbH, Hamburg, Germany).

2.3.3. Results

2.3.3.1. CO_2 efflux and ^{13}C recovery

Ten days after glucose application, total CO_2 respired from azide-treated soil was 2-fold lower compared to control (Fig. 1, top left). Two stages of glucose mineralization independent of inhibition were observed: 1) high ^{13}C recovery in CO_2 within the first 3 days and 2) low ^{13}C recoveries thereafter (Fig. 1, bottom).

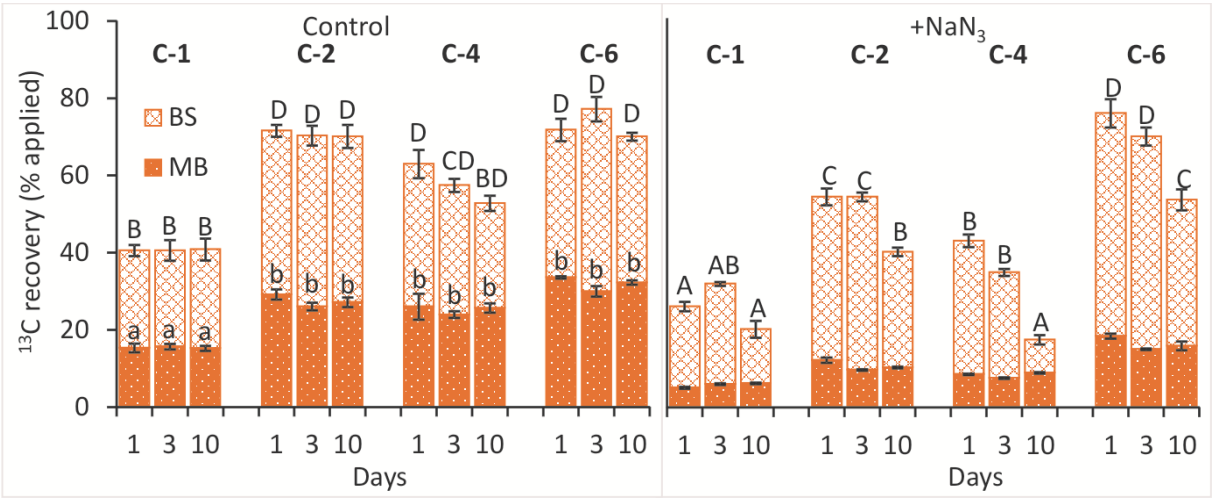


S3 Figure 1: *Cumulative CO_2 (mean± SE) from ^{13}C uniformly labeled glucose respired during 10 days in control and NaN_3 treated soils (top left), +NaN_3:Control ^{13}C (mean ± SE) respiration rate ratio from individual glucose position (top right) and cumulative ^{13}C (mean ± SE) recovered in CO_2 released from position-specific ^{13}C labeled glucose applied to soil with control (bottom left) and azide-exposed (bottom right) . ^{13}C curves were fitted with nonlinear least-square regressions according to an exponential equation ($cum^{13}C(t) = {}^{13}C_{max}*(1-e^{-kt})$), where; cum ^{13}C (t) is the cumulative ^{13}C amount depending on time, $^{13}C_{max}$ is the parametrically determined maximum of ^{13}C, k is the mineralization rate and t is time (parameter estimates in Supplementary Table 4). Steven' runs test for the fitted ^{13}C curves revealed no deviation from linearity (Supplementary Table 5). Significant differences between fitted curves are displayed in Supplementary Table 6.*

There were clear differences in ^{13}C recovery from the individual glucose positions in CO_2, especially in the first phase. Position-specific ^{13}C recovery in CO_2 followed a classical pattern characteristic of high pentose-phosphate pathway activity, with C-1 > C-4 > C-2 > C-6, irrespective of inhibition. On average, ^{13}C recovery in CO_2 was 12% higher in inhibited soil compared to control (Fig. 1, bottom), with the largest difference occurring within the first day (Fig. 1, top right).

2.3.3.2. Glucose ^{13}C incorporation into bulk soil and microbial biomass

Azide addition resulted in alteration of the ^{13}C recovery patterns. At day 1, ^{13}C recoveries from C-2 and C-4 in inhibited bulk soil were 17% and 20% lower compared to control (Fig. 2). ^{13}C recovery in bulk soil with inhibition did not differ between day 1 and 3 but decreased by over 12% at day 10 for each position, suggesting a shift in C transformations between day 3 and 10. In contrast, ^{13}C recovery in control soil did not differ between the days for each glucose position. Moreover, the ^{13}C recovery in bulk soil was 5 times higher than in microbial biomass, 3 days after inhibition (Fig. 2), whereas in control only twice as much ^{13}C was in bulk soil than in microbial biomass after 3 days.



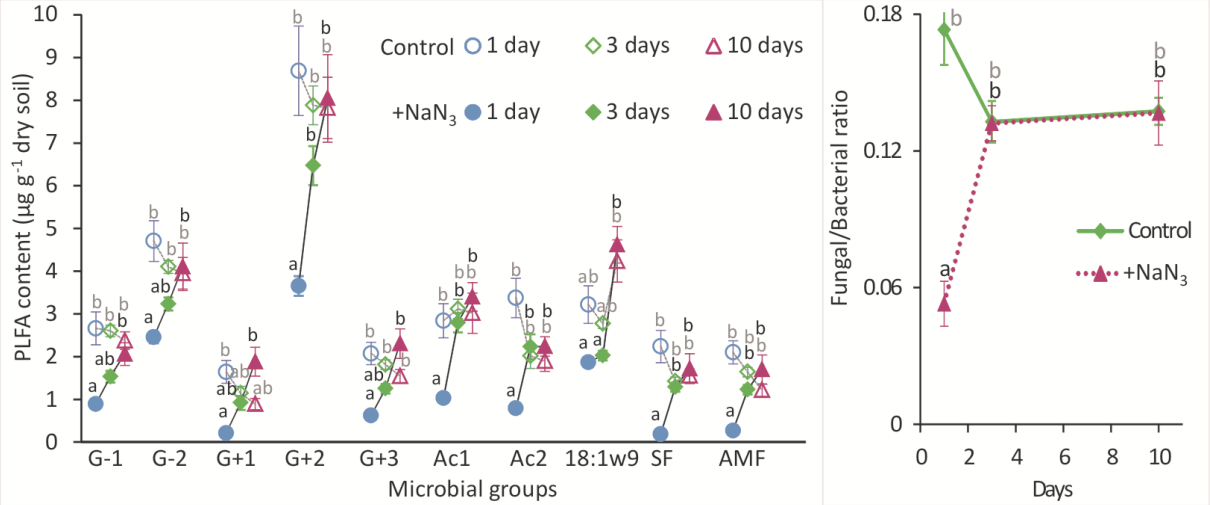
S3 Figure 2: *^{13}C recovery (mean ± SE) from various glucose positions in bulk soil and extractable microbial biomass, 1, 3 and 10 days after application in control (left) and azide-exposed soils (right). Significant effects of NaN_3 addition, days and individual glucose positions, as according to Tukey Honest Significance Difference (Tukey HSD) post-hoc test, in bulk soil (BS) are indicated by upper case letters above the error bars, while extractable microbial biomass (MB) by lower case letters.*

Position-specific patterns of ^{13}C incorporation into microbial biomass were similar irrespective of respiration inhibition by NaN_3. However, ^{13}C recovery was 3-fold lower in microbial biomass after inhibition compared to control (Fig 2).

2.3.3.3. Microbial community composition

Grouping of PLFAs resulted in 10 functional microbial groups (supplementary Table 2). In general, the biomarkers of gram positive 2 (G+2) were dominant followed by those of gram negative 2 (G-2) irrespective of inhibition (Fig. 3, left). At day 1, the amounts of fatty acids extracted were over 2-fold lower in inhibited soils compared to control (Fig. 3, left). Ten days

after inhibition, fatty acid contents doubled to levels similar to control and pattern of fatty acid did not differ between treatments suggesting complete recovery of the microbial community in inhibited soil.

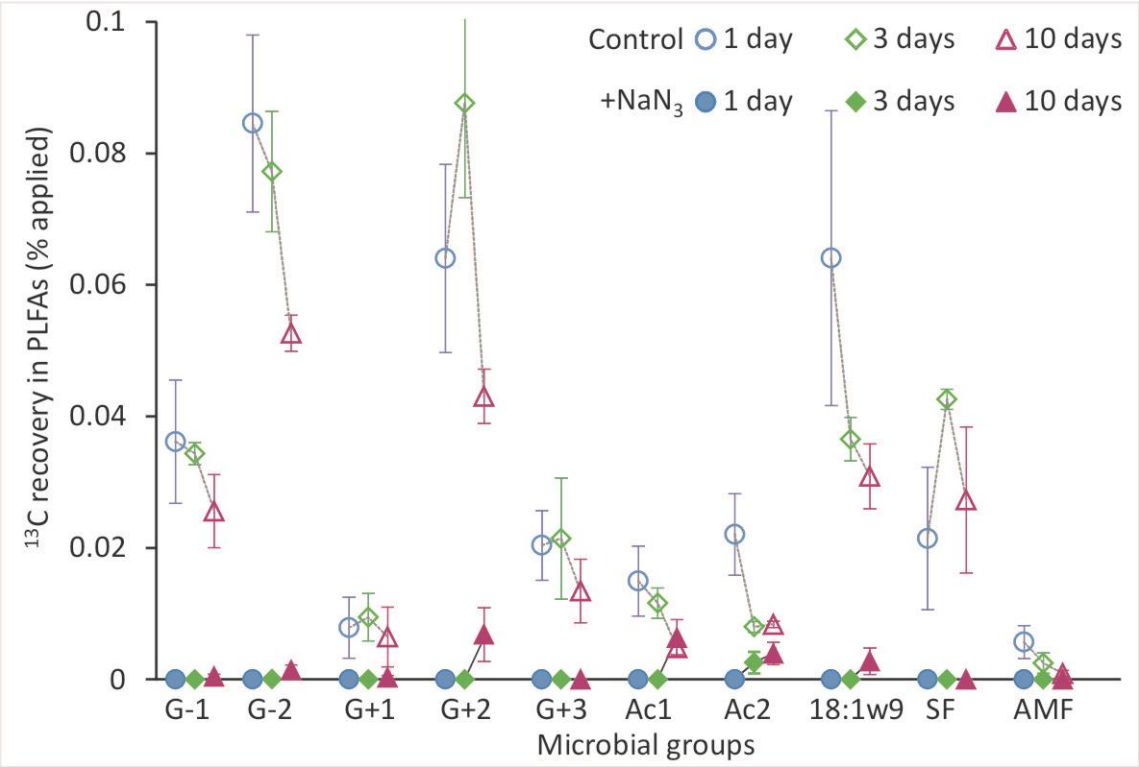


S3 Figure 3: *Absolute PLFA contents (mean ± SE) of microbial groups (µg g^{-1} dry soil), grouped according to a factor anaysis (factor loading see Supplementary Table 2) in control (open markers) and azide-exposed (solid markers) (left) and fungal/bacterial ratios (mean ± SE) (right). The letters indicate significant differences ($p<0.05$) between incubation time (within microbial groups' fatty acid content) and the effect of NaN_3 addition. Meaning of microbial group acronyms are: G-1 = Gram negative 1, G-2 = Gram negative 2, G+1 = Gram postive 1, G+2 = Gram positive 2, G+3 = Gram positive 3, Ac1 = Actinomycets 1, Ac2 = Actinomycetes 2, 18:1w9 = Fatty acid not associated with any microbial group, SF = Saprophytic Fungi and AMF = Arbuscular Micorrhizal Fungi.*

The fungal/bacterial (Fu/Ba) ratio did not differ between the days in control soils (Fig. 3, right). In azide-treated soil, however, Fu/Ba ratio increased 2-fold between day 1 and 3, attaining similar levels at day 3 in both treatments and remained constant till day 10 (Fig. 3, right).

2.3.3.4. Glucose ^{13}C uptake by microbial groups

In control soil, ^{13}C recovery was highest in PLFAs of G-2 and G+2, with each group containing approximately 0.09% of the applied glucose ^{13}C in their membrane fatty acids. The other groups only incorporated between 0.01- 0.04% of applied ^{13}C. Incorporation of ^{13}C into the PLFA of each microbial group did not differ between the days in both treatments (Fig. 4).



S3 Figure 4: *^{13}C recocery (mean ± SE) from applied uniformly labeled ^{13}C glucose in PLFAs of microbial groups in control (open markers) and azide-exposed (solid markers). Meaning of microbial group acronyms are: G-1 = Gram negative 1, G-2 = Gram negative 2, G+1 = Gram postive 1, G+2 = Gram positive 2, G+3 = Gram positive 3, Ac1 = Actinomycets 1, Ac2 = Actinomycetes 2, 18:1w9 = Fatty acid not associated with any microbial group, SF = Saprophytic Fungi and AMF = Arbuscular Micorrhizal Fungi.*

In respiration inhibited soil, however, there was no ^{13}C incorporation into microbial PLFAs within the first 3 days (Fig. 4). After 10 days, less than 0.007% of applied ^{13}C was recovered in PLFAs of G+2, Actinomycetes 1 and 2 (Ac1 and Ac2) (Fig. 4). The other microbial groups did not incorporate measurable ^{13}C into PLFAs 10 days after azide addition despite of an increase in their fatty acid content (Fig. 3, left), revealing dependence on other C sources for their growth.

2.3.4. Discussion

2.3.4.1. Total CO_2 efflux and glucose mineralization

CO_2 is persistently released from soil exposed to respiration inhibiting toxicants (e.g. NaN_3) or heavy metals inhibting respiration (e.g. cadmium (Cd), mercury (Hg) and copper (Cu)) (Ausmus et al., 1978; Rozycki and Bartha, 1981; Fliessbach et al., 1994; Trevors, 1996).

Application of ^{13}C glucose to NaN_3-inhibited and control soils resulted in high ^{13}C recovery in CO_2 within the first 3 days (Fig.1, bottom), consistent with intense glucose mineralization. Lower ^{13}C recoveries thereafter reflects mineralization of glucose-derived metabolites after exhaustion of glucose (Blagodatskaya et al., 2011). The ^{13}C recovery pattern in CO_2 was similar (C-1>C-4>C-2>C-6) under both conditions. The high ^{13}C recovery from glucose C-1 position in CO_2 reveals that glucose was predominantly catabolized via the pentose phosphate pathway (Caspi et al., 2008; Dijkstra et al., 2011c; Apostel et al., 2015).

Average ^{13}C recovery in CO_2 was 12% higher from inhibited soil compared to control. In contrast, total CO_2 from inhibited soil was half emitted from control (Fig. 1, top left). High CO_2 release with low ^{13}C content from control points towards a priming effect, i.e. a glucose-induced acceleration of SOM decomposition (Kuzyakov, 2010; Blagodatskaya and Kuzyakov, 2013). Increased ^{13}C recovery and reduced total CO_2 after NaN_3 inhibion could not be explained by a shift towards fermentative metabolism resulting from respiration inhibition, as $^{13}CO_2$ from fermentation would be C-4 dominated instead of C-1. High CO_2 emission from soils with minimized microbial activity can be attributed to release of active oxidative extracellular enzymes (EXOMET) from dead organisms (Maire et al., 2013). To prove or reject the relevance of EXOMET compared to cellular metabolism, investigation of intracellularly formed metabolites (i.e. de-novo formed microbial biomass) was conducted.

2.3.4.2. ^{13}C incorporation into microbial biomass and bulk soil

Glucose is utilized by microorganisms for biosynthesis of various cellular building blocks and as an energy source (Gunina et al., 2014). Recovery of glucose-derived ^{13}C in microbial biomass is indicative of active intracellular biosynthetic processes. ^{13}C recovery in microbial biomass from C-6, C-4, and C-2 was higher than C-1 in control soil (Fig. 2, left), with a similar pattern arising in control bulk soil, confirming that microbial products, and not untransformed glucose, are the predominant source of extracellular ^{13}C. The position-specific ^{13}C recovery pattern in bulk soil and microbial biomass complemented the metabolic fluxes observed in CO_2 and confirm that glucose was predominantly metabolized via pentose phosphate pathway. The dominance of this pathway at 5 °C under moderate C supply reflects the classical metabolic C allocation observed in previous studies (Dijkstra et al., 2011c). Temperature decrease shifts metabolic activity from glycolysis and NADH production to pentose phosphate pathway and NADPH production in microbial cultures (Wittmann et

al., 2007) to meet the pentose and NADPH demands for biosynthesis (Fuhrer and Sauer, 2009). High ^{13}C recovery from positions C-6, C-4 and C-2 implies that, after loss of the C-1, the remaining part of the molecule was allocated to biosynthesis (Gunina et al., 2014). Levels of ^{13}C in microbial biomass did not change between the days in control soils, suggesting that glucose was consumed within the first day and was incorporated into C pools with slower turnover.

In inhibited soil, glucose ^{13}C recovery from C-6 and C-2 in bulk soil and microbial biomass was higher than of C-1 and C-4 (Fig. 2, right), suggesting intracellular transformation to pyruvate via the pentose phosphate pathway (Dijkstra et al., 2015) followed by oxidization for energy production. Increased glucose oxidation after pyruvate formation also accounts for the altered pattern of ^{13}C recovery observed in bulk soil, with even stronger decrease in C-4 and C-2 incorporation at day 1 compared to control soil (Dijkstra et al., 2015). Intracellular transformation of glucose after NaN_3 inhibition opposes the concept of extracellular oxidative metabolism (Maire et al., 2013), as surviving microbes must be responsible for the observed transformation. The ≈3-fold lower ^{13}C recovery in microbial biomass and higher recovery in CO_2 following inhibition compared to control conditions (Fig. 2 and Fig. 1, bottom) implies that the surviving microbes utilized substantial amount of glucose for energy production. This shift towards energy production may represent an adaptation mechanism by tolerant bacterial strains to overcome inhibition, as microorganisms that survive heavy metal intoxication also divert a large amount of energy from added substrate towards energy-intensive physiological detoxification mechanisms (Gordon et al., 1993). Resistance to toxicants shifts microbial community structure to compensate for the loss of more sensitive populations (Giller et al., 1998). Furthermore, resistance of some Trichoderma strains to NaN_3 was reported by Kelley and Rodriguez-Kabana (1981). Therefore, we conducted PLFA analysis to identify those microbial groups that survive inhibition.

2.3.4.3. ^{13}C incorporation into PLFAs

^{13}C was incorporated into PLFAs of each microbial group in control soils with G-2 and G+2 incorporating 2 times more than other microbial groups (Fig. 4). At room temperatures, growth rate of Gram negative bacteria is dependent upon the concentration of easily available substrate (Treonis et al., 2004). Therefore, they dominate the rhizosphere, where

low molecular weight organic substances are present in high concentrations (Gunina et al., 2014; Apostel et al., 2015). In contrast, Gram positive bacteria are typically abundant in bulk soil and incorporate C from old SOM (Kramer and Gleixner, 2006). Therefore, ^{13}C recovery within the same range in PLFA of G-2 and G+2 in control soils implies that gram positive bacteria profited from lower competitiveness of gram negatives at low temperature (5 °C). The lack of difference in ^{13}C incorporation into PLFAs between the days was similar to results in microbial biomass, confirming complete glucose incorporation within the first day and slow transformation of ^{13}C in microbial products thereafter.

Ten days after inhibition, incorporation of glucose-derived ^{13}C into fatty acids was only observed in G+2 and actinomycetes (Fig. 4) i.e. groups known to use old soil organic matter or microbial necromass (McCarthy and Williams, 1992; Kramer and Gleixner, 2006). Therefore, their ^{13}C incorporated into PLFA is likely to occur by recycling of glucose-derived excretion products and not intact glucose. Such recycling of extracellular glucose-derived metabolites explains the decrease in ^{13}C recovery by over 12% in bulk soil 10 days after inhibition. There was no detectable glucose ^{13}C incorporation in any PLFA 3 days after inhibition (Fig. 4), despite of more than 9% recovery in microbial biomass, suggesting either: 1) Absence of growth and membrane repair or 2.) Glucose C was not allocated to fatty acid biosynthesis. To determine the most likely scenario, we examined the fatty acid content of microbial biomass to determine the growth status of the microbial populations.

2.3.4.4. Effects of NaN_3 on microbial community structure

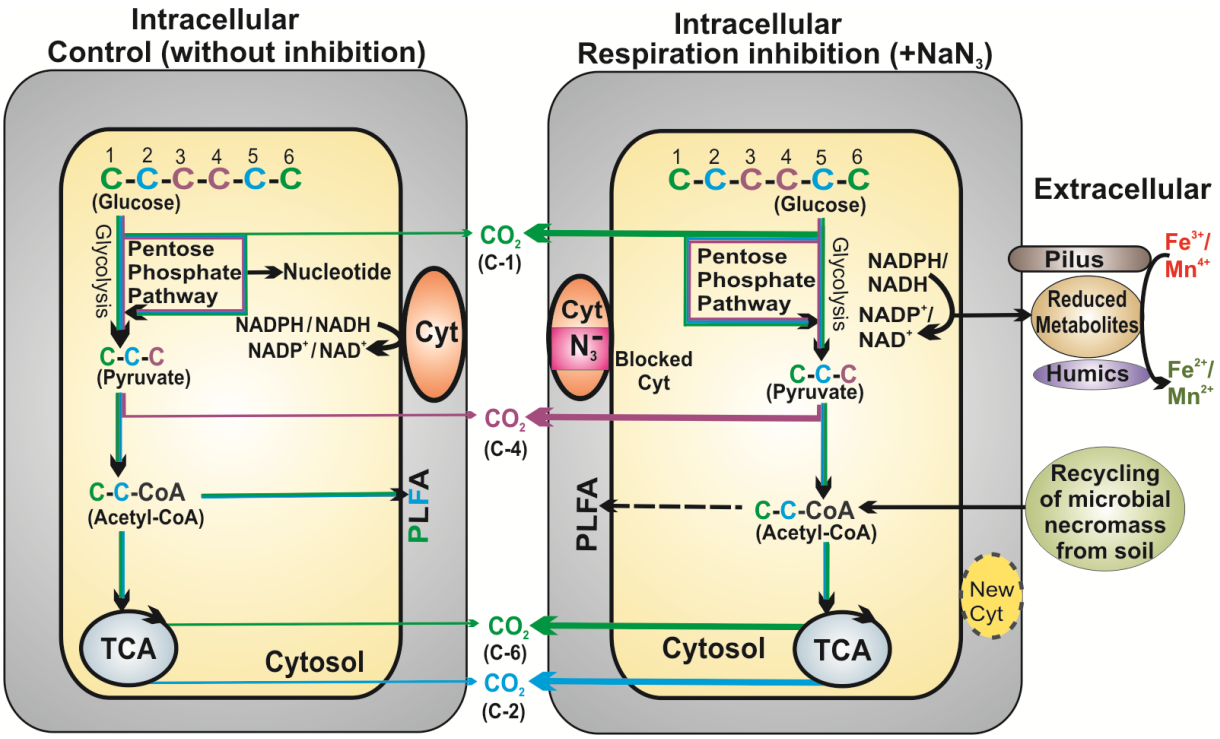
Comparing fungal/bacterial ratio in inhibited and control soils at day 1 demonstrated a greater short-term susceptibility of fungi to azide inhibition (Fig. 3, right). However, a 2-fold increase of the fungi/bacteria ratio to a level equal to control soils within 3 days shows fast recovery of fungal biomass.

The fatty acid contents associated with each microbial group were more than 2-fold lower after inhibition compared to control soil at day 1, likely resulting from cell asphyxiation (Winter et al., 2012). However, the 2-fold increase in fatty acid content associated with each microbial group in inhibited soils between day 1 and 10 implies that there was growth of the affected microbial groups. This contradicts previous assumptions that microorganisms stressed by exposure to toxicants divert energy from growth to maintenance (Killham, 1985).

Furthermore, the increasing fatty acid contents raise the question how microorganisms grow without incorporating glucose into their PLFA. Living microorganisms can utilize microbial necromass from soil (Dippold and Kuzyakov, 2016) (Fig. 5), whose pool was likely substantial resulting from the death of a high percentage of microorganisms after inhibition. The energy for transforming or recycling of such compounds was provided by an increase in glucose oxidation, which was proven by the 12% increased ^{13}C recovery in CO_2 and the ≈3-fold lower ^{13}C recovery in microbial biomass after inhibition compared to control. Preferential recycling of the cost-intensive alkyl chains of PLFA and other biomass compounds occur under such conditions. This recycling is accompanied by *de novo* formation of other cheaper biomass compounds. Therefore, ecosystems are self-regulating systems that evolve mechanisms of self-repair and their biological populations are adapted to resist and recover from environmental fluctuations (EFSA, 2016). Diminishing effects of inhibition over time lead to potential recovery of the microbial groups (EFSA, 2016) as indicated by increase in PLFA content.

2.3.4.5. Adaptation mechanisms to respiration inhibition

Azide inhibits electron transfer in non-phosphorylating submitochondrial particles at cytochrome oxidase and catalase (Keilin, 1936) resulting in cell asphyxiation (Winter et al., 2012). Continued respiration after inhibition, with intensive intracellular transformation via pentose phosphate pathway raises the question: How do microorganisms manage intracellular respiration without electron acceptors (NAD^+ or $NADP^+$), which cannot be regenerated after azide inhibition? A common response of bacteria to electron-acceptor limitation is to produce electrically conductive pilus-like appendages called bacterial nanowires (Reguera et al., 2005; Gorby et al., 2006), which anchor between the periplasmic and outer membranes and allow transfer of electrons from the cell to minerals containing Fe^{3+} and Mn^{4+} in the extracellular environment (Reguera et al., 2005; Gorby et al., 2006) (Fig. 5). Other demonstrated mechanisms to overcome inhibition include 1) Electron shuttling between the cell and extracellular minerals via humic substances in solution (Lovley et al., 1996; Bi et al., 2013; Piepenbrock et al., 2014) and solid state (Roden et al., 2010) or 2) Excretion of reduced metabolites by microorganisms, including quinones (Newman and Kolter, 2000) and phenolic compounds (Vempati et al., 1995; Pentrakova et al., 2013), that transfer electrons to the extracellular environment (Fig. 5).



S3 Figure 5: *Microbial glucose transformation pathways in control (left) and mechanisms adapted to overcome inhibition (right). Colored arrows correspond to glucose C positions and indicates their fate. The thickness of the colored arrows towards CO_2 is proportional to amount. Black straight and broken arrows indicate metabolites formed or recycled. Black curvy arrows indicate redox processes.*

Processes outlined above demonstrate that electron transport chain inhibition does not stop intracellular microbial metabolism in soil. Without compound-specific ^{13}C measurement of the extracellular metabolite pool, it is not possible to point out which of these processes for extracellular electron disposal dominated after NaN_3 addition. However, extracellular electron transfer by excretion of reduced metabolites explains the high ^{13}C recovery in the bulk soil compared to microbial biomass during the first 3 days following inhibition. The effectiveness of NaN_3 as a bacteriostat in liquid samples such as antibodies, milk (Winter et al., 2012) or water samples does not contradict our findings, because such samples lack of humic substances or minerals (Fe^{3+} and Mn^{4+}) permitting extracellular electron transfer via the above mentioned mechanisms.

Previously, persistent CO_2 release from soil with eliminated or inhibited respiration was attributed to abiotic processes (Rozycki and Bartha, 1981; Trevors, 1996) and extracellular oxidative metabolism (Maire et al., 2013). Our data clearly demonstrate that inhibition of electron transport chain cannot stop intracellular glucose metabolism: microorganisms circumvent respiration inhibition via temporary extracellular electron transfer, giving

organisms a chance to reconstruct new electron transport chains and resume normal (aerobic) respiration. Thus, even under respiration inhibition no indication for extracellular glucose metabolism could be perceived. The ability of microorganisms to overcome limitations of intracellular metabolism by utilizing SOM resources is important for understanding the origin of CO_2 emitted from soils with respiration-inhibiting toxicants (Bond et al., 1976; Ausmus et al., 1978; Trevors, 1996). Heavy metals such as Cd, Hg and Cu, which are widespread in the environment due to their industrial, agricultural, medical and technological application, inhibit respiration (Belyaeva et al., 2012). However, their presence in organic or clayey soils does not stop respiration (Bond et al., 1976; Ausmus et al., 1978; Fliessbach et al., 1994), a phenomenon which could similarly be explained by extracellular electron transfer because such soils are rich in humic substances and minerals (Fe^{3+} and Mn^{4+}) functioning as extracellular electron acceptors. Microorganisms' tolerance and resistance to copper based fungicides could also be attributed to extracellular electron transfer. Therefore, the mechanisms proven from metabolic perspective in this study contribute to a better understanding of microbial resilience and resistance to direct respiration inhibiting toxicants (e.g. azides, cyanides), pesticides (e.g. phosphides and phosphines) or heavy metals (e.g. Cu and Hg) and subsequent ecological recovery after perturbation. Such mechanisms are limited to portion of microorganisms activated by glucose (Monard et al., 2008) and toxicants that inhibit respiratory chains directly.

2.3.5. Conclusions

Combining position-specific ^{13}C labelling with compound specific ^{13}C-PLFA analysis proved to be a valuable tool to understand how microorganisms overcome respiration inhibition. Glucose was metabolized by soil microorganisms via pentose phosphate pathway irrespective of respiration inhibition. NaN_3 reduced total CO_2 efflux 2-fold but increased ^{13}C recovery in released CO_2 by 12% compared to control soils. The low ^{13}C recovery in microbial biomass, increased pyruvate oxidation and increased proportion of glucose-derived metabolites in extracellular microbial products following NaN_3 application provide evidence for increased glucose use for energy production and synthesis of extracellular electron transport compounds to bypass inhibition. Resources for growth were recycled from the large pool of microbial necromass resulting from toxicant addition. Consequently, to overcome intracellular inhibition of the electron transport chain, microorganisms most likely

coupled intracellular metabolism with extracellular redox processes. This is possible only in soil and similar environments rich in electron acceptors. Construction of new electron transport chains and resumption of aerobic respiration as well as recovery of microbial groups occurred within 10 days at 5 °C. We assume this bypass of respiration inhibition will be much faster at high temperatures.

These results suggest that the persistent CO_2 efflux after azide addition to soil is as a result of intracellular oxidation of SOM followed by extracellular electron disposal. This mechanism is also likely to account for microbial tolerance to e.g. heavy metals and other toxicants directly altering microbial respiration in soils but requires confirmation by extending this position-specific labelling approach on soils contaminated with a broad spectrum of toxicants. Finally, this metabolic tracing approach provides an understanding of the impacts of chemicals such as azides, cyanides and heavy metals on soil C cycling following contamination and enables development of unique insights concerning soil-specific microbial mechanisms to overcome respiration inhibition.

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Supplementary information

Table 1. Fatty acid in the external standards

FA-type	Name	Common name	Abbreviation	Retention time (s)*
Saturated	Tetradecanoic acid	Myristic acid	14:0	1282
	Pentadecanoic acid	-	15:0	1653
	Hexadecanoic acid	Palmitic acid	16:0	2013
	Heptadecanoic acid	Margaric acid	17:0	2448
	Octadecanioc acid	Stearic acid	18:0	3196
	Eicosanoic acid	Arachidic acid	20:0	3935
Branched chain	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	1299
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	1282
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	1563
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	1539
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	1900
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	1870
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	2316
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	2279
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	2365
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	3437
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	2190
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	3496
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	1338
	cis-11-Hexadecenoic acid	-	16:1w5c	1662
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	1926
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	2970
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	2839
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	3871
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2w6,9	2759
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	2807
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	3684

*for 45 m ± 0.5 m column lengths, deviations of ± 10 s possible

Table 2. Fatty acid grouping following factor loadings from factor anaysis and pure culture results (Zelles, 1997; Zelles, 1999)

Group	Abbreviation	Fatty acids
Gram negative 1	G-1	16:1w7
Gram negative 2	G-2	18:1w7
Gram positive 1	G+1	i14:0
Gram positive 2	G+2	i15:0, a15:0
Gram positive 3	G+3	i16:0
Actinomycetes 1	Ac 1	10Me16:0,a17:0
Actinomycetes 2	Ac 2	i17:0,10Me 18:0
18:1w9	18:1W9	18:1W9
Saprophytic fungi	SF	18:2w6,9
AM fungi	AMF	16:1w5

Table 3. Total microbial biomass C and extracted PLFA (mean ± SE) during incubation (nd-no data).

	∑MBC (µg C g^{-1} dry soil)			∑PLFA (µg C g^{-1} dry soil)		
	1	3	10	1	3	10
Control	251.36	199.31	231.51	58.26	47.87	46.22
	(6.47)	(13.90)	(7.89)	(6.53)	(4.24)	(3.84)
+NaN_3	286.74	240.85	259.58	23.61	37.46	52.91
	(14.71)	(15.17)	(3.01)	(2.56)	(5.69)	(13.06)

Table 4. Parameter estimates for the fitted ^{13}C curves

		Control				+NaN3			
Position		Estimate	Standard	t-value	p-value	Estimate	Standard	t-value	p-value
C-1	^{13}C(max)	36.98275	1.363439	27.1246	0	48.29878	1.142347	42.28032	0
	k	0.025597	0.002859	8.95439	8.65E-09	0.030986	0.00239	12.96721	8.85E-12
C-2	^{13}C(max)	20.05699	0.662117	30.29222	0	30.90886	0.702	44.0297	0
	k	0.02166	0.002035	10.64384	0	0.02728	0.001922	14.19772	0
C-4	^{13}C(max)	30.26421	1.059578	28.56252	0	43.09849	1.755119	26.26516	0
	k	0.02094	0.002061	10.15853	0	0.02449	0.002776	8.82383	0
C-6	^{13}C(max)	15.95253	0.757332	21.06412	0	24.46448	0.622012	39.3312	0
	k	0.01983	0.002595	7.64155	0	0.0134	0.000813	16.47672	0

Table 5. Steven's runs test for linearity: Regression analysis parameters for fitted ^{13}C curves (nd- no data)

Treatment	Position	R^2	$P_{slope\neq0}$	$P_{runs\ test}$	$P_{equlity\ of\ slopes}$	$P_{equality\ of\ intercepts}$
Control	C-1	0.5353	0.0001	0.3000	0.3215	0.009379
	C-2	0.5980	< 0.0001	0.3000		
	C-4	0.8295	< 0.0001	0.3000		
	C-6	0.7541	< 0.0001	0.3000		
+NaN3	C-1	0.7061	< 0.0001	0.3000	0.02117	nd
	C-2	0.8232	< 0.0001	0.3000		
	C-4	0.5070	0.0003	0.4000		
	C-6	0.9029	< 0.0001	0.4000		

Table 6. Significant differences ($p<0.05$) between the fitted curves for individual treatment are indicated by superscript letters on $^{13}C(max)$ values.

Treatment	Positions			
	C-1	C-2	C-4	C-6
Control	36.98275^{d}	20.05699^{b}	30.26421^{c}	15.95253^{a}
$+NaN_3$	48.29878^{d}	30.90886^{b}	43.09849^{c}	24.46448^{a}

2.4. Study 4: Sorption of Alanine changes microbial metabolism in addition to availability

Carolin Apostel[1,2]*, Michaela A. Dippold[2], Ezekiel Bore[2], Yakov Kuzyakov[1,2]

[1] Department of Soil Science of Temperate Ecosystems, Büsgenweg 2, Georg-August-University Göttingen, 37077 Göttingen, Germany

[2] Department of Agricultural Soil Science, Büsgenweg 2, Georg-August-University Göttingen, 37077 Göttingen, Germany

* Corresponding author E-mail address: carolinapostel@yahoo.com

Abstract

Sorption is one of the main processes stabilizing organic matter in soil against microbial mineralization. We hypothesize that besides reduced accessibility for microorganisms and enzymes, changes in microbial metabolism additionally intensify this organic matter stabilization effect of sorption.

Position-specifically ^{14}C labeled Alanine was applied to soil as solution or sorbed on sterilized soil to investigate the mechanisms underlying this metabolism related stabilization effect. Sorption decreased initial mineralization of Alanine by ~80% and doubled the duration until the mineralization maxima ($^{14}CO_2$ peak). Almost all Alanine was taken up by microorganisms independent on sorption, and C-1 was completely (> 99%) decarboxylated during glycolysis after one day. Sorption could not prevent microbial utilization of Alanine, but increased the carbon use efficiency (CUE) of sorbed Alanine for 60% compared to Alanine in solution and increased C incorporation in microbial biomass up to four times. The position-specific pattern of ^{14}C in soil and in microbial biomass showed that oxidation of C-2 from sorbed Alanine was strongly lowered compared to free Alanine. Both higher CUE and delayed C-2 mineralization were achieved by a higher C flux towards efficient anabolism, or/and to slower cycling cell components.

Limitation of accessibility for microorganisms alone does not explain the stabilizing effect of sorption on organic substances like amino acids and the observed changed position specific pattern. Even though all sorbed Alanine was taken up by microorganisms within 3 days, C partitioning towards anabolism, slower microbial turnover and increased CUE increased C retention from sorbed compounds in soil even after microbial uptake. Position-specific labeling clearly showed that LMWOS are stabilized by sorption not as intact molecules, but after microbial metabolization – as released metabolites or microbial biomass. We conclude that the indirect effects of sorption, namely 1) more C partitioned to anabolism, 2) slower decomposition, 3) higher incorporation into microbial biomass and 4) increased carbon use efficiency promote C retention in soil and may be even more important than the direct effect, namely inaccessibility. The finding that stabilization did not significantly impede microbial utilization, but sorption greatly increased carbon use efficiency has major implications for conceptual and numerical representation of organic matter stabilization and losses in soils.

Keywords: Amino acid sorption, Metabolic tracing, Alanine transformation, Position-specific labeling, Radioisotope applications, Soil microorganisms, Soil organic matter, Carbon sequestration

2.4.1. Introduction

Sorption of organic molecules to soil particles is a key mechanism of soil organic matter (SOM) stabilization (Guggenberger and Kaiser, 2003): Although those mineral-organic associations only consist to 0.2 – 20% of carbon, 70 - 100% of SOM is stabilized within those complexes (Christensen, 2001; Lützow et al., 2006; Schmidt et al., 2011; Sollins et al., 2007, 1996) and turnover of mineral-associated C is on average four times slower than those of non-associated OM (Baisden et al., 2002; Kögel-Knabner et al., 2008). The mechanisms behind the stabilization effects of sorption, however, are not yet fully understood (Kleber et al., 2015). The majority of studies analyzing the effects of sorption on SOM retention were carried out in simplified systems, either: a) in suspension, b) with the addition of pure minerals or c) by inoculating with individual bacterial strains or a combination thereof (Barré et al., 2014). Even with the application of simplified systems, results on SOM retention varied from no effect on biodegradation to a total stop of biodegradation (Barré et al., 2014). It is therefore necessary to measure not only the effect of sorption on SOM mineralization, but also to identify the mechanisms behind this effect. The most common explanation is that sorption of SOM decreases availability to microorganisms and enzymes (Vieublé Gonod et al., 2006), which are the most important drivers of C dynamics in soil (Kögel-Knabner, 2002). Indeed, sorption strength negatively correlates with microbial metabolization of amino acids (Jones and Hodge, 1999) , but this effect varies strongly with soil type, minerology and experimental approach (Barré et al., 2014). On the other hand, microorganisms facilitate sorption. To interact with mineral surfaces, OM needs to be water-soluble and therefore requires chargeable functional groups (Kleber et al., 2015). Plant-derived primary OM from litter or root exudates often does not possess these traits before being microbially metabolized to low molecular weight organic substances (Oades, 1989). The application of position-specifically labeled Alanine also showed that stronger sorption to mineral particles not only decreased microbial C uptake, but also shifted metabolic pathways towards a higher flux through the anabolism (Dippold et al., 2014). If substrates are used to a higher portion in anabolic pathways, the carbon use efficiency (CUE) increases (Dijkstra et al., 2011; Giorgio and Cole, 1998), while CO_2 production decreases. To understand how sorption stabilizes OM, it is therefore necessary not only to quantify changes of C-fluxes from SOM to CO_2 or microbial biomass, but also to identify changes in microbial metabolism induced by sorption. To achieve this, free and sorbed tracers that are position-specifically ^{14}C

labeled need to be applied. In contrast to uniformly labeled tracers, position-specific labeling allows the reconstruction of metabolic pathways. The allocation of C from individual molecule positions towards CO_2, i.e. mineralization, can be compared with the known network of metabolic pathways and the fate of individual molecule positions therein. From such a comparison, metabolic pathways can be identified based on the position-specific fingerprint in individual C pools like CO_2.

Such experiments require position-specific metabolic tracers, which enter the basic metabolism of glycolysis, pentose-phosphate pathway and citric acid cycle at a central, branching point. Pyruvate is one of these tracers entering the metabolism at the interface from glycolysis and pentose-phosphate pathway to citric acid cycle (Caspi et al., 2014). Its aminated form – Alanine – can easily be transferred by transamination to pyruvate and thus is an equivalent metabolic tracer. Furthermore, Alanine, is a neutrally charged amino acid, with a negatively chargeable carboxylic position C-1 (-COOH), a positively chargeable amino-bound position C-2 ($-CHNH_2$) and a methylic position C-3 ($-CH_3$). Therefore, Alanine can be sorbed to soil particles by 1) cation exchange, 2) anion exchange or 3) ligand exchange. Alanine is also the most abundant amino acid in dissolved organic matter (Fischer et al., 2007). In previous studies using position-specifically labeled Alanine, the microbial metabolization in soil could successfully be reconstructed (Apostel et al., 2013). The effect of sorption to various pure minerals in aquatic suspension on the Alanine metabolization could also be assessed by position-specifically labeled tracers (Dippold et al., 2014). Therefore, position-specifically labeled Alanine is a suitable tracer to disentangle effects and mechanisms of sorption on organic C stabilization at mineral surfaces in soil and validate existing studies on pure minerals.

We hypothesize that sorption affects microbial utilization and stabilization of Alanine C in two ways. The direct effect: Uptake of Alanine sorbed to mineral particles by microorganisms will be slower and decreased compared to free Alanine, due to stabilization by mineral surfaces and lower accessibility. The indirect effect: sorbed Alanine will be microbially metabolized to a larger extent by anabolic pathways because slow desorption and slow continuous uptake by microorganisms lead to more efficient C use compared to fast C utilization of Alanine from solution.

2.4.2. Materials and Methods

2.4.2.1. Experimental design

The experiment consisted of two treatments – sorbed Alanine and free Alanine – in which tracers were added to soil from the same site (description see section 2.2). To produce soil with sorbed tracer, solutions of Alanine labeled with ^{14}C on each of the three positions (C-1 = -COOH, C-2 = -$CHNH_2$, C-3 = -CH_3) were added to soil sterilized by γ-radiation. The soil with sorbed Alanine was added to non-sterilized soil at the beginning of the incubation. In the free Alanine treatment, position-specifically labeled Alanine solutions were applied. During the 10 days of incubation, incorporation of ^{14}C from Alanine into CO_2, extractable organic carbon, microbial biomass and soil was analyzed. This enabled to compare the dynamics of Alanine in solution and sorbed Alanine during the 10 days of the experiment and thus, to test hypothesis 1. As position-specifically labeled tracers were applied, the metabolic pathways utilized after the uptake could also be reconstructed, allowing to test hypothesis 2.

2.4.2.2. Preparation of sorbed tracer soil

The soil used throughout this experiment was taken from the Ap horizon of an agriculturally used loamy Luvisol (pH_{KCl} 4.88, pH_{H2O} 6.49, TOC 17.7 g · kg^{-1},TN of 1.9 g · kg^{-1}, CEC 13 cmolc · kg^{-1}) in northern Bavaria (49°54’ northern latitude; 11°08’ eastern longitude, 500 a.s.l.), sieved to 2 mm and dried. To prepare soil with sorbed Alanine, a subset of soil was sterilized by γ-radiation (10 h at 53 kGy) at Synergy Health (Radeberg, Germany). To remove cytosolic products of the microbial cells lysed by the radiation that would compete with the tracer for exchange places on the soil particles, we pre-extracted the sterilized soil with 1 M K_2SO_4 for 1 h on a horizontal shaker. Microbial extracts were removed by filtering on 300 °C pre-heated glass fiber filters. Then, uniformly and position-specifically labeled tracer solutions (^{14}C-1 Ala, ^{14}C-2 Ala, ^{14}C-3 Ala and U ^{14}C Ala) with an activity of ~50 Bq · g $soil^{-1}$ were added to the soil. All substances were purchased from American Radiolabeled Chemicals Inc (St. Louis, USA) with an activity of 3,7 MBq · ml^{-1}. The soil-tracer-suspensions were shaken on a horizontal shaker for 2 h to enable the Alanine to sorb to the soil particles. The remaining solutions, containing the non-sorbed alanine, were removed from the soil by filtering. Additional non-sorbed Alanine was removed by repeated post-extraction with 100 ml Millipore water. After filtering, the soils with sorbed Alanine were freeze-dried and their ^{14}C

activity was quantified: Approximately 50% of the added ^{14}C remained sorbed to the soil. As the recovery from all C positions was the same, we conclude that a) Alanine sorbed to the soil as intact molecule and b) no microbial decomposition took place before the start of the experiment. Their ^{14}C activities were determined, and solutions with similar ^{14}C activity and Alanine contents were prepared for the treatment, where free Alanine was added in solution to soil.

2.4.2.3. Experimental setup

The incubations were conducted in screw-cap microcosms with a layer of quartz sand at the bottom. In the sorption treatments, each sample consisted of ~10 g of freeze-dried soil containing sorbed ^{14}C-alanine and ~80 g of dry, non-labeled, non-sterilized soil. In the free Alanine treatments, each sample consisted of ~90 g of dry, non-labeled, non-sterilized soil. The soils were filled into soil sample rings installed on ceramic plates. To equalize bulk densities in all samples, a defined pressure was applied. Soils with the sorbed Alanine were rewetted by dripping 10 ml of Millipore water onto the soil surface. Another 10 ml were added to the sand, to be taken up into the soil through the ceramic plate up to field capacity. In the free treatment, the ^{14}C-Alanine solutions with equal activity to the respective sorbed samples were added to the soil for rewetting instead of pure Millipore water. For each of the three sampling times, four replicates of each of the four labeled substances (^{14}C-1 Ala, ^{14}C-2 Ala, ^{14}C-3 Ala and U-^{14}C Ala) were prepared, as were three replicates of non-labeled backgrounds. Due to the sorption-desorption equilibrium, it is possible that a certain portion of the Alanine tracer that is added in solution will sorb to the soil particles after addition. The sorption pre-experiment suggests that this portion is lower than 10%. Vice versa, despite intensive washing of the sterilized soil with the adsorbed alanine, we cannot fully exclude that a very small portion of the sorbed tracer desorb abiotically in the first phase of incubation.

2.4.2.4. Analytical methods

$^{14}CO_2$-measurements

Cups with NaOH were placed inside the screw-cap glasses to trap CO_2. NaOH was replaced at 15, 30, 45 and 60 min and 2, 3, 4 and 6 h. After that, the NaOH cups were replaced daily. NaOH with trapped CO_2 was mixed 1:5 with scintillation cocktail (Rotiszint® eco plus, Poth,

Karlsruhe, Germany) and stored in the dark for 24 h to exclude chemoluminescence. ^{14}C in CO_2 was determined by scintillation counting on a multipurpose scintillation counter (LS 6500, Beckmann Coulter, Krefeld, Germany).

Bulk soil measurements

^{14}C remaining in soil after one, three and ten days was oxidized by combustion on an Oxidizer (Ox500, Zinsser Analytic, Frankfurt, Germany) and the resulting $^{14}CO_2$ was trapped in Oxysolve C400 (Zinsser Analytic, Frankfurt, Germany) and measured on a liquid scintillation counter (Packard-Tricarb low level, Perkin Elmer, Waltham, MA, USA). From the three C positions and the two treatments, different proportions of the applied ^{14}C were recovered in soil and CO_2 combined. It is possible that a very small part of the $^{14}CO_2$ was lost during the replacement of the NaOH traps. For this reason and to compensate for uncertainties in the amount of added tracer, we did the not refer the tracer recoveries of day 1, 3 and 10 to the added amount but to the recovered tracer amount, which is the sum of the cumulatively evolved $^{14}CO_2$ and the bulk soil ^{14}C at the respective time point.

Chloroform fumigation extraction

Incorporation of ^{14}C into microbial biomass was determined by chloroform fumigation extraction (Wu et al., 1994). Two subsets of 15 g of soil were extracted with 45 ml of 0.05 M K_2SO_4. The first subset was fumigated with chloroform for 3 days prior to extraction. The K_2SO_4 extracts of fumigated and non-fumigated soil were mixed 1:5 with scintillation cocktail (Rotiszint® eco plus, Poth, Karlsruhe, Germany) and stored in the dark for 24 h to exclude chemoluminescence, before being measured on a scintillation counter (LS 6500, Beckmann Coulter, Krefeld, Germany). The ^{14}C contents of the non-fumigated samples were defined as dissolved organic ^{14}C ($DO^{14}C$). The ^{14}C content of the fumigated samples minus $DO^{14}C$ corrected by a factor of 0.45 for less extractable cell components was defined as ^{14}C in microbial biomass.

2.4.2.5. Carbon use efficiency

Carbon use efficiency (CUE) describes how much of the assimilated C is used by the microbial community for growth (Giorgio and Cole, 1998). CUE can be determined by comparing catabolic to anabolic C fluxes (Dijkstra et al., 2011a, 2015) or catabolic to anabolic energy

fluxes (Herrmann et al., 2014; Herrmann and Bölscher, 2015). In tracer experiments, CUE can be approximated according to Spohn and Chodak (2015) as:

$$CUE = \frac{C_B}{C_U} = \frac{C_B}{CO_2 + C_B} \quad \textbf{(1)}$$

Where C_B is tracer C incorporated in microbial biomass C and C_U is tracer C taken up by microorganisms. Plants and therefore root respiration was excluded. Accordingly, all CO_2 was released by microbial respiration, and C_U was defined as the sum of $^{14}CO_2$ and microbial biomass ^{14}C.

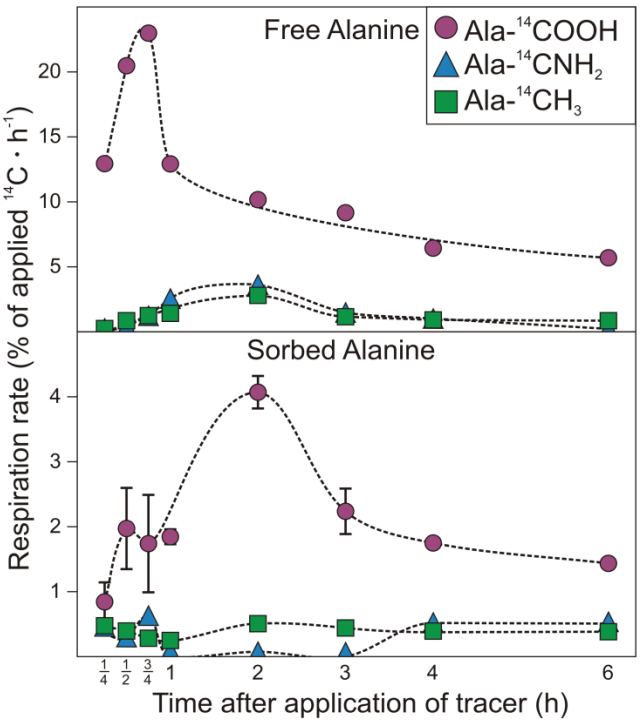
2.4.2.6. Statistical analysis

For the replicates, a Nalimov outlier test with significance levels of 95% (when four replicates were available) or 99% (when three replicates were available) was performed. Incorporation of ^{14}C from the three positions of free and sorbed Alanine into CO_2, microbial biomass, DOC and soil was tested with a two-way analysis of variance (ANOVA). Significant differences between positions and treatments on each day were determined with the Tukey Honest Significance Difference (Tukey HSD) post-hoc test with $p < 0.05$. Differences between incorporation of individual positions on successive days were tested with a Student's t-test. All statistical tests were done with RStudio version 0.98.1103.

2.4.3. Results

2.4.3.1. Initial respiration rates

The maximum respiration rates for all three Alanine positions (C-1 = -COOH, C-2 = -$CHNH_2$, C-3 = -CH_3) were 4 to 5 times higher from free Alanine than from sorbed Alanine (max. respiration rates: $C\text{-}1_{free}$ ~ 22, $C\text{-}1_{sorb}$ ~ 4, $C\text{-}2_{free}$ ~ 4.5, $C\text{-}2_{sorb}$ < 1, $C\text{-}3_{free}$ ~ 4, $C\text{-}3_{sorb}$ < 1 % · h^{-1}) (Fig. 1). The mineralization peak of ^{14}C-1 from free Alanine occurred within the first 45 min after applying the tracer solutions. In contrast, mineralization of ^{14}C-1 from sorbed Alanine displayed two peaks: the first, lower peak also within the first 45 min, the second within 2 h after application. The two peaks indicate that mineralized, sorbed Alanine was derived from two pools.

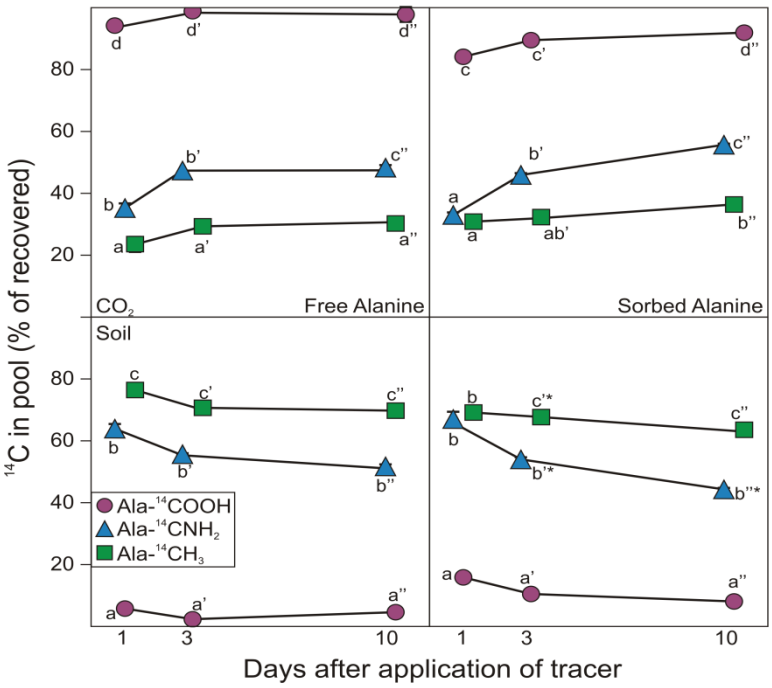


S4 Figure 1: *Respiration rate in % of applied tracer per hour of free (top) and sorbed (bottom) Alanine C-1 (Ala-COOH), C-2 (Ala-CNH_2) and C-3 (Ala-CH_3) up to 6 h after application*

In general, the position-specific pattern for both free and sorbed Alanine in CO_2 was: C-1 > C-2 > C-3. This reflects a microbial use by similar metabolic pathways, but the utilization of the sorbed Alanine was delayed.

2.4.3.2. Alanine mineralization to CO_2 and stabilization in soil

The higher initial CO_2 release from all positions of free Alanine observed in the first 6 h was almost equal after one day (Fig. 2). Only cumulated $^{14}CO_2$ from sorbed ^{14}C-1 was 10% lower than from free ^{14}C-1, and cumulated mineralization from positions C-2 and C-3 was equal in both treatments. After 3 days, the position-specific pattern of Alanine-^{14}C in cumulated CO_2 was the same for sorbed and free C: ~ 100% of ^{14}C-1, ~ 45% of ^{14}C-2 and ~ 30% of ^{14}C-3 were mineralized. Accordingly, Alanine-derived ^{14}C remaining in soil after three days had an identical position-specific pattern, regardless of whether Alanine had been added free or sorbed.



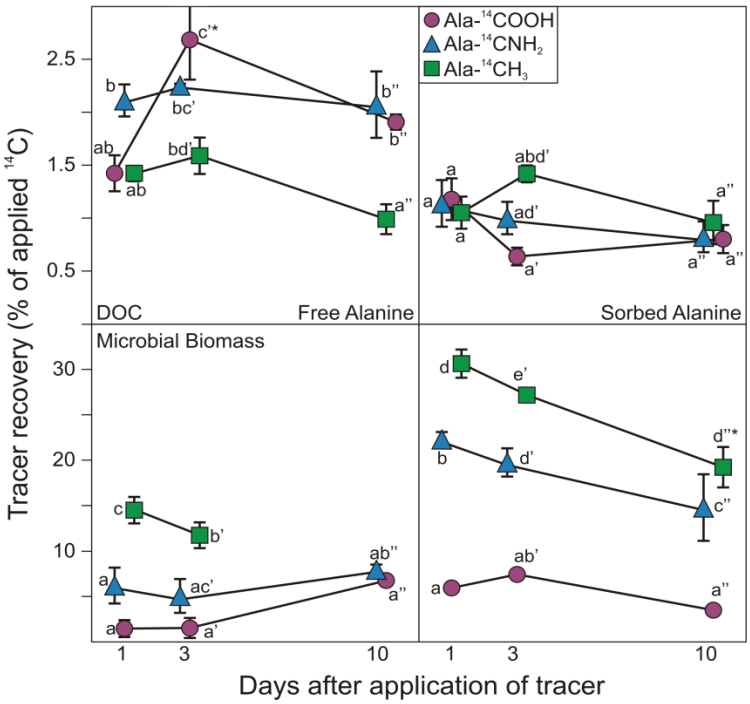
S4 Figure 2: *Tracer ^{14}C from free (left) and sorbed (right) Alanine C-1 (Ala-COOH), C-2 (Ala-CNH$_2$) and C-3 (Ala-CH$_3$) recovered in CO_2 (top) and bulk soil (bottom). Amounts are given in % of recovered ^{14}C, which is the sum of cumulative $^{14}CO_2$ and bulk soil ^{14}C. Letters (a, a', a'') indicate significant differences between positions in one pool on one day (p > 0.05); * indicates that the incorporation of a certain position is significantly different to the incorporation of the previous sampling day (p > 0.05)*

The general pattern of sorbed and free Alanine-^{14}C positions incorporated in soil was complimentary to the pattern in CO_2: C-1 < C-2 < C-3. However, already on the first day after tracer application, less C-2 than C-3 from free Alanine was remaining in soil ($p < 0.05$). From sorbed Alanine, C-2 and C-3 remained in soil equally on day 1, and only after day 3 C-2 was recovered less than C-3 ($p < 0.05$). Although sorption did not prevent the uptake by microorganisms, sorption altered Alanine transformation pathways at least over the first days.

2.4.3.3. Tracer Alanine ^{14}C in dissolved organic matter and microbial biomass

Only 2 – 1% of the applied free and 1% of sorbed Alanine ^{14}C remained in the DOC pool after 10 days (Fig. 3). The position-specific pattern of ^{14}C from free Alanine in DOC after 3 days corresponded to the pattern in CO_2 (highest incorporation of C-1, followed by C-2 and C-3), suggesting similar sources of both, CO_2 and DOC. In contrast, the three positions from sorbed Alanine were equal in DOC through day 10, indicating non-metabolized Alanine. Both

the activity and the position-specific pattern of ^{14}C from sorbed Alanine in DOC remained constant over 10 days.

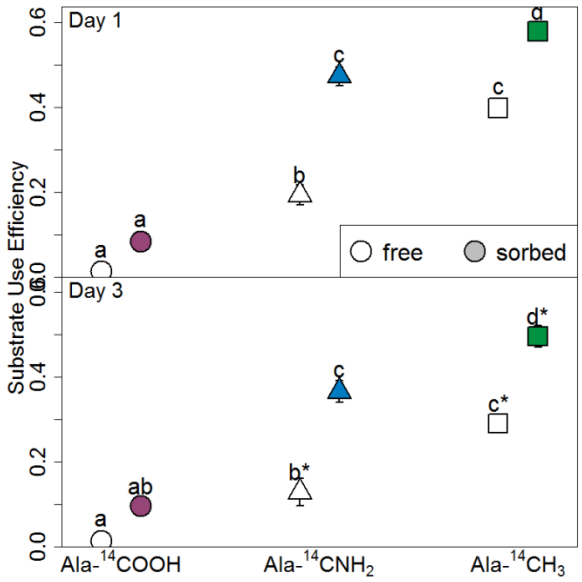


S4 Figure 3: *Tracer ^{14}C from free (left) and sorbed (right) Alanine C-1 (Ala-COOH), C-2 (Ala-CNH_2) and C-3 (Ala-CH_3) recovered in DOC (top) and microbial biomass (bottom). Letters (a, a', a'') indicate significant differences between positions in one pool on one day ($p > 0.05$); * indicates that the incorporation of a certain position is significantly different to the incorporation of the previous sampling day ($p > 0.05$). Incorporation of free C-3 on day 10 could not be reliably measured*

Incorporation of ^{14}C from all positions of sorbed Alanine into microbial biomass was higher than from the same positions from free Alanine ($p < 0.05$) (except C-1 on day 10). Thus, sorption increased the C amount retained in microbial biomass. The preference of sorbed over free C was different for all three Alanine positions: C-2 from sorbed Alanine was incorporated nearly four times as much on days 1 and 3; on day 10 it was still twice as much as C-2 from free Alanine ($p < 0.05$). C-3 and C1 from sorbed Alanine in microbial biomass was also more than twice as high as from free Alanine on days 1 and 3. Sorbed Alanine C-1 was also incorporated 2-3 times more than from free Alanine on days 1 and 3. The fact that C from all three positions of sorbed Alanine were incorporated more than their free counterparts, but each to a different extent, suggests that free and sorbed Alanine are used in different metabolic pathways.

2.4.3.4. Carbon use efficiency

As C-1 from free and sorbed Alanine was mineralized to CO_2 almost completely, its CUE was the same in both treatments (Fig. 4). CUE for C-2 and C-3, however, was about twice as high for sorbed than for free Alanine on day 1 and 3 ($p < 0.05$), indicating increased anabolic use of sorbed versus free C.



S4 Figure 4: *Carbon use efficiency of free and sorbed Alanine 1 (top) and 3 (bottom) days after application*

2.4.4. Discussion

2.4.4.1. Sorption delays Alanine uptake and mineralization to CO_2

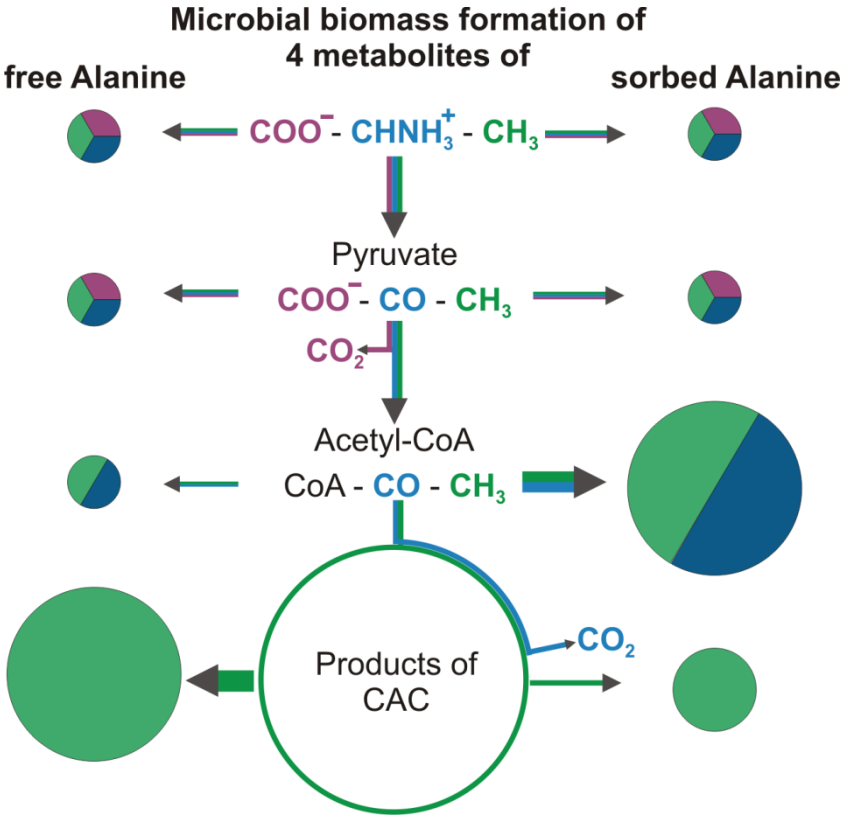
During the initial 6 h, respiration rates from all positions (C-1 = -COOH, C-2 = -$CHNH_2$, C-3 = -CH_3) of sorbed Alanine were ~75% lower than from the corresponding positions of free alanine (Fig. 1). Moreover, the time until respiration rates peaked from the sorbed Alanine was doubled compared to free Alanine. This decreased and delayed CO_2 release corresponds to decreased accessibility of Alanine due to sorption. In contrast, half-lives of free Alanine in DOC of less than 10 min have been observed (Jones et al., 2009). Sorbed, position-specifically labeled Acetate was taken up within minutes (Fischer and Kuzyakov, 2010), however, Alanine has a higher sorption potential due to its three possible sorption mechanisms. Small peaks in the respiration rates of C-1 and C-2 of sorbed Alanine in the first

hour of the experiment correspond to the peaks of the same positions of free Alanine. This indicates that some of the sorbed Alanine desorbs very quickly after rewetting. Those molecules were sorbed by weak sorption mechanisms and were vulnerable to fast exchange reactions, e.g. with cations or other charged organic anions in the soil solution (Saidy et al., 2012). Dippold et al. (2014) compared the metabolization of position-specifically labeled Alanine sorbed to clay particles, iron oxides and activated charcoal: Alanine sorbed to clay minerals reached a maximum mineralization of 15-17% $\cdot$ h^{-1} for C-1 and was therefore only slightly more stabilized than the free Alanine in our experiment (maximum respiration rate: ~20% $\cdot$ h^{-1} for C-1). Respiration rates of Alanine sorbed to soil (maximum respiration rate ~4% $\cdot$ h^{-1} for C-1) lie between the maximum mineralization rates of Alanine sorbed to clay minerals and iron oxides (Dippold et al., 2014). As soils are heterogeneous mixtures of various minerals and organic matter, naturally more than one sorption mechanisms can be present. In contrast to the initial mineralization rates, the cumulative mineralization of Alanine C-1 within 10 days was the same for free and sorbed Alanine – almost 100% of added C. This means that none of the initial Alanine remained irreversibly sorbed to the soil (Fischer et al., 2010): everything was taken up by microorganisms and decarboxylated during glycolysis. Decreased accessibility (Dungait et al., 2012) due to sorption did not promote Alanine C stabilization. It is well known that sorption and desorption in the soil can occur very quickly and that a sorption-desorption equilibrium will be established very fast. Therefore, a part of the tracer added in solution will sorb to the soil particles before being taken up by microorganisms, while some of the Alanine from the sorption treatment will be released into the solution prior to microbial uptake. With our experimental approach, it is not possible to finally estimate the portion of alanine that was exchanged by the abiotic processes of the sorption-desorption equilibrium. However, the difference in dynamics of Alanine mineralization to CO_2 between the two treatments can only be explained if microbial uptake is faster than the abiotic sorption-desorption kinetics (Fischer et al., 2010) – which would lead to an equal behaviour of ^{14}C in both treatments.

2.4.4.2. Sorption changes microbial metabolic pathways

While cumulative mineralization to CO_2 of free and sorbed Alanine was similar after 10 days, the position-specific ^{14}C patterns in soil and microbial biomass indicate a shift in metabolic pathways induced by sorption (illustrated in Fig. 5). Within the basic metabolization of

Alanine in microbial cells, first the carboxylic C (C-1 position) is respired during glycolysis. The C-2 position is the second to be respired. This occurs almost instantly if C is used in the citric acid cycle (Caspi et al., 2014). If biomass produced from Acetyl-CoA is further metabolized, C-2 will also be lost before C-3. This process, however, takes several days and occurs in many anabolic pathways during recycling of anabolic products e.g. fatty acids (Apostel et al., 2013). More C-2 than C-3 of free Alanine is lost from soil already after one day (Fig. 2). In contrast, C-2 and C-3 of sorbed Alanine remain in soil in equal proportions until day 3. Therefore, C from sorbed Alanine was either used less for energy production in the citric acid cycle, or it was fed into slower cycling microbial pools. In both cases, the amount of C from sorbed Alanine that remains stabilized in soil is higher than from free Alanine. Cell residues from dead microorganisms contribute greatly to SOM (Miltner et al., 2012). Accordingly, if more C is partitioned to production of high molecular weight biomass from sorbed low molecular weight C sources, more C remains stabilized after cell death (Apostel et al., 2015).



S4 Figure 5: *Metabolic pathways of Alanine: expected C-fluxes (arrows) and position-specific pattern (pie charts) in microbial biomass from free and sorbed Alanine. Size of arrows / charts represents amount of C in the flux / pool, colors represent position-specific pattern (purple = COOH, blue = $CHNH^{+}_{3}$, green = CH_3)*

The trends observed in bulk soil are even stronger in microbial biomass (Fig. 3): C from all positions of sorbed Alanine is incorporated more into microbial biomass than their free counterparts. Especially the incorporation of C-2 from sorbed versus free Alanine into microbial biomass is higher than in the bulk soil. This corresponds to a decrease of C fluxes through the citric acid cycle (Caspi et al., 2014) and/or incorporation into slower cycling microbial biomass pools. Microorganisms increase investment in microbial biomass formation pathways when C accessibility decreases (Dippold et al., 2014) by two potential mechanisms: a) uptake of sorbed C sources increases demand for anabolic C investment (e.g. biofilm formation on surfaces, …) (Dashman and Stotzky, 1982) and/or b) microbial specialists with a more efficient metabolism are taking up C from sorbed, but not from free Alanine. Which of these explanations is valid can be investigated in the future either 1) by tracing extracellular polysaccharide formation by compound-specific isotope analysis of microbial monosaccharides or 2) by tracing metabolic pathways of individual microbial groups based on compound-specific isotope analysis of microbial biomarkers.

2.4.4.3. Sorption increases carbon use efficiency

As the incorporation of the carboxylic group was close to 0 in both treatments, no difference between CUE of free and sorbed C-1 could be detected. For both C-2 and C-3, however, the CUE of sorbed Alanine was between 20 and 60% higher than that of free Alanine (Fig. 4). Several reasons why sorbed Alanine was processed more efficiently than free Alanine are possible: (Blagodatskaya et al., 2014) attributed high CUEs in soil to the formation of storage compounds, not actual microbial growth. Such a shift in C flux towards biosynthesis pathways – not necessarily solely for storage compounds but perhaps also for extracellular polysaccharides or other biofilm compounds (Gorbushina, 2007) – would also explain the delayed loss of C-2 both in bulk soil and microbial biomass (Fig. 2, Fig. 3). (Dijkstra et al., 2015), however, used metabolic flux modelling of position-specifically labeled Glucose and identified balanced growth with production of various metabolites, rather than only production of storage compounds, as the major process leading to high CUE.

Another explanation for the increased CUE of sorbed Alanine might be a preferential uptake and metabolization by microbial specialists, which possess a more efficient metabolism. Measuring incorporation of ^{14}C in microbial biomarkers and extracellular polysaccharides would reveal whether the higher CUE from sorbed Alanine occurs due to a metabolic shift

towards anabolism within the whole microbial community or due to a stronger impact of microbial specialists' metabolism.

2.4.5. Conclusions

Sorption is one of the main factors stabilizing organic matter in soil and it is tacitly accepted that the spatial accessibility for microbial uptake and enzymatic decomposition is the solely mechanism responsible for stability of sorbed compounds. By using position-specifically labeled Alanine, the dominant amino acid in soil, we showed a new mechanism of organic matter stabilization by sorption: a shift in the microbial metabolism of compounds initially sorbed on minerals.

Due to reduced accessibility of Alanine sorbed to soil and its slow release into the solution , the initial CO_2 release is decreased by ~80% and the time until the mineralization peak is doubled compared to free alanine. After one day, however, the carboxylic C-1 is mineralized almost completely, in the sorbed and free treatment. This means that sorbed Alanine is taken up by microorganisms intact and transformed according to the basic metabolism of C_3-molecules (glycolysis). However, up to four times more C from sorbed than free Alanine was retained in microbial biomass. Although sorption did not stabilize the primary amino acid, more retention in microbial biomass facilitates the stabilization of secondary microbial products.

Although the direct effect of sorption – the inaccessibility - was of short-term importance for this amino acid, sorption of Alanine did increased the CUE by 20-60% and the C incorporation in microbial biomass up to four times. The position-specific pattern also revealed that sorption increased C-2 retention in soil and microbial biomass either due to a) a shift from C utilization in the catabolic citric acid cycle to anabolic production of biomass or/and b) a shift towards production of slower cycling cell components. Both mechanisms increase the metabolic stabilization of sorbed Alanine C, especially because close proximity to mineral phases facilitates sorption.of the microbial transformation products, which might be more likely stabilized as they are in average of higher molecular weight than the initially added Alanine.

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2.5. Study 5: Persistence of sugar-derived C in soil is controlled by the initial use of the C positions in the microbial metabolism

Ezekiel K. Bore[1], Yakov Kuzyakov[1,2,3], and Michaela A. Dippold[1]

[1] Department of Agricultural Soil Science, University of Goettingen, 37077 Goettingen, Germany

[2] Department of Soil Science of Temperate Ecosystems, University of Goettingen, 37077 Goettingen, Germany

[3] Institute of Environmental Sciences, Kazan Federal University, 420049 Kazan, Russia

Corresponding Author:

Ezekiel K. Bore

Department of Agricultural Soil Science

University of Goettingen

Buesgenweg 2

37077 Goettingen

email: ezekielbore7@gmail.com

Tel.: 0551-3922106

Fax.: 0551-3933310

Abstract

Transformation of sugars by microorganisms is a key process influencing carbon (C) stabilization in soil. To reveal the mechanisms responsible for the persistence of labile compounds in soil, the fate of position-specific and uniformly ^{13}C labeled glucose and ribose were studied under field conditions for 800 days. ^{13}C was quantified in bulk soil, microbial biomass and extractable organic carbon (EOC).

Incorporation of glucose C-4 and C-1 were lower than C-2 and C-6 in bulk soil and microbial biomass 3 days after tracer application. Ribose utilization showed lower incorporation of C-1 than C-5 in bulk soil and microbial biomass on day 3. This reveals that glycolysis and the pentose phosphate pathway (PPP) were simultaneously involved in the transformation of sugars. However, the mean residence time (MRT) of uniformly labeled ^{13}C ribose in bulk soil and microbial biomass were 3 and 2 times higher than glucose, respectively. This suggests that ribose and glucose were incorporated into different cellular components. Continuous decrease of glucose ^{13}C in soil and microbial biomass over time revealed that recycling dominated glucose transformation in soil. The differences in MRT of individual glucose C positons indicate intensive metabolization and incorporation of modified compounds. Unexpectedly, the MRT of glucose C-1 and C-4 was higher than C-2 and C-6 in bulk soil and microbial biomass. This could reflect portions of intact glucose molecules that were directly incorporated into cellular components such as amino sugars, which are known to have high persistence in nature. On contrary, ribose ^{13}C in soil stabilized after 250 days, implying stabilization in non-living soil organic matter (SOM). As expected, the MRT of ribose C-1 was 2- and 3-fold lower in bulk soil and microbial biomass than C-5 throughout the experiment, respectively, confirming that recycling of ribose occur to a minor extend, as position-specific fingerprint was preserved. One direct use of ribose is the formation of the ribonucleotide backbone of nucleic acids. After cell death, the nucleic acids can interact strongly with mineral surfaces in the soil, becoming unavailable for microbial reuse and thus stabilized without transformation in soils. This reflects the differences in biochemical utilization of ribose and glucose, and subsequent differences in stabilization mechanisms in soil.

Persistence of glucose C in soil was dominated by recycling while stabilization in non-living SOM accounted for persistence of ribose. This information is not only useful in broadening the understanding of the persistence of labile compounds in soil, but also underscores the role microbial processes in SOM stabilization.

Key words: Microorganisms, Recycling, SOM stabilization mechanisms, metabolic tracing, labile compounds

2.5.1. Introduction

Carbon (C) atoms of labile compounds like sugars have been shown to persist longer in soil than those of chemical compounds of high recalcitrance such as lignin (Gleixner et al., 2002; Kiem and Kogel-Knabner, 2003; Amelung et al., 2008; Schmidt et al., 2011). This contradicted the notion linking turnover of organic substances in soil to their molecular structure. Recycling has been shown to be a major process accounting for high mean residence time (MRT) of many SOM fractions such as sugars (Gleixner et al., 2002; Basler et al., 2015a). However, it is not clear whether recycling entails intact re-utilization or intensive metabolization and incorporation of modified compounds. To shed more light on this process, we combined compound-specific isotope analysis and position-specific labeling of sugars to track the fate of the entire molecule and individual molecular positions under field conditions for 800 days.

Sugars are the most abundant organic substances in the biosphere because they constitute structural components of most living biomass (Koegel-Knabner, 2002). Plant cell walls for example, contain cellulose as a major structural component. Thus, cellulose is the most abundant biopolymer, and its subsequent hydrolysis by exoenzymes lead to release of glucose into soil solution. Glucose can also be synthesized by soil microorganisms in addition to being the main constituent of rhizodeposits (Kuzyakov, 2010). Hence, glucose concentration in soil solution is higher than other low molecular weight organic substances (LMWOS) (Fischer et al., 2007). Glucose is not only utilized by microorganisms as building block for various cellular components, but also as an energy source (Gunina and Kuzyakov, 2015). Ribose on the other hand is mainly plant derived and becomes available in soil through hydrolysis of hemicellulose (Cheshire et al., 1971; Koegel-Knabner, 2002). Ribose can directly be utilized in the formation of ribonucleotide backbone of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). After cell death, part of microbial necromass and residues are transformed into non-living soil organic matter (SOM). Additionally, sugars play an important role in aggregate formation. Polysaccharides act as glue, binding both mineral and organic particles, resulting in microaggregate formation (Six et al., 1999). Therefore, understanding the fate of sugars, will improve estimation of C cycling in soil and consequent stabilization in SOM.

In a recent study, turnover time of glucose C in cytosol was found to be approximately 150 days (Gunina et al., 2017). This turnover time reflects the mean of all glucose C atoms

assimilated to highly heterogeneous cell components of varying chemical structure and molecular weight dissolved in the cytosol (Malik et al., 2015). Estimating the turnover time of individual sugar C atoms still lacks, despite such studies being able to distinguish whether the turnover occurs mainly as intact glucose molecule or glucose is immediately transformed to a broad variety of cytosolic compounds. Each sugar C atom is allocated via the basic metabolic pathways to different cell components. These components will subsequently have deviating half-life times in soil once the necromass is transformed as part of the SOM. Therefore, tracking each C atom can improve estimation of the microbial residue contribution to SOM.

Studies tracking the fate of individual sugar C atoms by position-specific labeling have been used to characterize and model microbial metabolic pathways under ordinary (Scandellari et al., 2009; Dijkstra et al., 2011b; Apostel et al., 2015) and disturbed conditions (Bore et al., 2017a; b). However, to date no systematic studies tracking turnover of individual sugar C atoms in soil have been conducted. Using position-specific and uniformly ^{13}C-labeled glucose and ribose, this study examined turnover times of sugar-derived C in bulk soil, microbial biomass and extractable organic carbon (EOC) with an aim of improving the understanding of the contribution of microbial residue to SOM and consequent C stabilization mechanisms. We hypothesized that 1) turnover time of glucose C-6 and ribose C-5 will be higher than the other positions because these positions (glucose C-6 and ribose C-5) are mainly oxidized in tricarboxylic acid cycle (TCA) unlike other positions that are oxidized via pentose phosphate pathway (PPP) and glycolysis before TCA, and 2) pathway-specific, positional fingerprints in bulk soil, microbial biomass and EOC will be lost with increasing time after tracer application due to microbial residue recycling and simultaneous existence of decomposing and constructing pathway, which leads to mixing of C positions.

2.5.2. Materials and methods

2.5.2.1. Field experiment

Experimental site

The experiment was conducted on agriculturally used loamy Luvisol in Hohenpoelz, Germany (49°54' northern latitude; 11°08' eastern longitude, 500 m a.s.l.) in August 2010, for over 2 years. The site has a mean annual temperature and precipitation of +7 °C and 874 mm

respectively. The soil chemical properties are; a pH (H_2O) of 6.49, a total organic carbon (TOC) and total nitrogen (TN) content of 1.77% and 0.19%, respectively, and a cation exchange capacity (CEC) of 13 $cmol_C\ kg^{-1}$. The last crop on the field before the onset of the experiment was *Triticale*.

2.5.2.2. Experimental design

A 12 × 12 m field was divided into four quadrants, each representing a replicate. Columns, each with diameter of 10 cm and height of 13 cm were installed in the quadrants to a depth of 10 cm. Four position-specifically ^{13}C-labeled isotopomers of glucose (^{13}C-1, ^{13}C-2, ^{13}C-4, and ^{13}C-6), two position-specifically ^{13}C-labeled isotopomers of ribose (^{13}C-1, ^{13}C-5), uniformly ^{13}C-labeled (U-^{13}C) and unlabeled glucose and ribose were applied to the soils in separate columns in the quadrants, organized in randomized block design. 10 ml of 0.09 mM C monosaccharide solutions (glucose and ribose) were applied to the soil at five points in the columns via syringes to allow uniform distribution. This C concentration was chosen to simulate the natural sugar pool in soil solution (Fischer et al., 2007). To prevent leaching of the added monosaccharides due to rainfall, a roof was installed over the plot for the first 10 days. Since the columns had no vegetation growth, monosaccharide uptake was mainly microbial. Soils in the columns were destructively sampled and weighed after 3, 10, 50, 250, 430 and 800 days. Soil moisture content of subsamples from each column was determined by drying for 24 hours at 105 °C to constant weight. 30 g of each sample sieved to <2 mm, was stored at +5 °C until chloroform fumigation-extraction, as described below.

2.5.2.3. Analytical methods

Bulk soil C content and $\delta^{13}C$ signature

Aliquots of freeze dried soil were ground in a ball mill and about 5 mg were weighed into tin capsules. ^{13}C isotope measurements were performed with elemental analyzer (Eurovector, Milan, Italy) coupled by a ConFlo III interface to a Delta V advantage IRMS (both units from Thermo Fisher Scientific, Bremen, Germany). ^{13}C recovered from applied monosaccharides was calculated according to a mixing model equation 1 and 2 (Gearing et al., 1991), where the C content of the background ($[C]_{BG}$) in Eq.1 was determined by Eq.2.

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C\text{-}BG} + [C]_{appS} \cdot r_{appM} \quad (1)$$

$[C]_{soil} = [C]_{BG} + [C]_{appM}$ (2)

where:

$[C]_{CO_2/BG/appM}$ is C content of the sample/background/applied monosaccharide (mg C g^{-1}soil)

$r_{CO_2/BG/appM}$ is ^{13}C atom %-excess of labeled sample/background/applied monosaccharide (at %)

Quantification of C and ^{13}C in microbial biomass and dissolved organic matter

C content of microbial biomass was determined by chloroform fumigation-extraction. Two subsamples weighing 15 g were taken from each soil sample. One set of subsamples was extracted directly, while the other was first fumigated with chloroform for 5 days in a desiccator to lyse microbial cells. 45 ml of 0.05 M K_2SO_4 was used to extract organic C on an orbital shaker for 1.5 h. Samples were centrifuged for 10 min at 2000 rpm, the supernatant filtered and the C concentration determined by total organic/inorganic C (TOC/TIC) analyzer (Multi C/N 2100 AnalytikJena, Jena, Germany). The extracts were then frozen and freeze-dried thereafter. About 25 mg (fumigated) and 35 mg (unfumigated) of freeze-dried extracts were used for $\delta^{13}C$ determination via EA-IRMS (same instrument coupling used for bulk soil $\delta^{13}C$ determination). Incorporation of glucose ^{13}C into fumigated and unfumigated samples was calculated according to Eq. 1 and 2. Microbial biomass C and ^{13}C were calculated by subtracting unfumigated from fumigated values and dividing by an extractability correction factor of 0.45 (Wu et al., 1990). C concentration and ^{13}C recovery in unfumigated samples are extractable organic carbon (EOC) and its ^{13}C content, respectively.

2.5.2.4. Statistical analysis and calculations

^{13}C incorporation into bulk soil, microbial biomass, and EOC were tested for significant differences between the positions and duration with a factorial analysis of variance (ANOVA). If assumptions of normality and homogeneity of variances within groups were not met, the data was square root transformed or outcomes were validated by a nonparametric Kruskal Wallis ANOVA. Significant differences were determined with Tukey Honest Significance Difference (Tukey HSD) post-hoc test at 95% confidence level. Statistical tests were performed with Statistica (version 12.0, Statsoft GmbH, Hamburg, Germany).

Recovery of monosaccharide-derived ^{13}C (expressed as % of total applied ^{13}C and present as $^{13}C_{rec}$) and ^{13}C enrichment of bulk soil, microbial biomass or EOC (expressed as ‰ of total C in the pool and presented as $^{13}C_{enr}$) were calculated according to Eq. 3 and 4, respectively.

$$^{13}C_{rec}\ (\%) = (^{13}C_{l}/^{13}C_{app}) \times 100 \quad (3)$$

$$^{13}C_{enr}\ (‰) = (^{13}C_{l}/C_{pool}) \times 1000 \quad (4)$$

where $^{13}C_{l}$ is monosaccharide-derived ^{13}C incorporated into bulk soil, microbial biomass or EOC as calculated by Eq. 1 and 2, $^{13}C_{app}$ is the amount of applied monosaccharide ^{13}C, and C_{pool} is the total amount of C in bulk soil and microbial biomass.

To describe decomposition rate of ^{13}C, a single first-order kinetic Eq. 5 was applied to the enrichment of ^{13}C in bulk soil and microbial biomass (Kuzyakov, 2011).

$$^{13}C_{enr(t)} = {}^{13}C_{enr\ (0)} \,.\, \exp^{-kt} \quad (5)$$

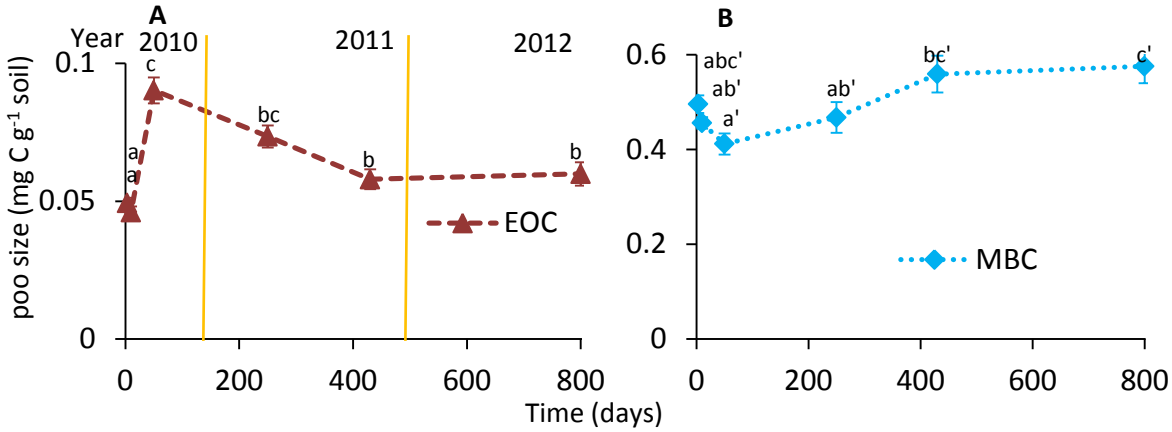
where $^{13}C_{enr\ (t)}$ is the ^{13}C enrichment (‰) of bulk soil and microbial biomass at any time *t* as determined in Eq. 4, $^{13}C_{enr\ (0)}$ is the ^{13}C enrichment (‰) of bulk soil and microbial biomass at time *0, k* is the rate constant (‰ d^{-1}), and *t* is time (days).

The mean residence time (MRT) of C in each pool was calculated as 1/*k*, where *k* values were obtained in Eq. 5.

2.5.3. Results

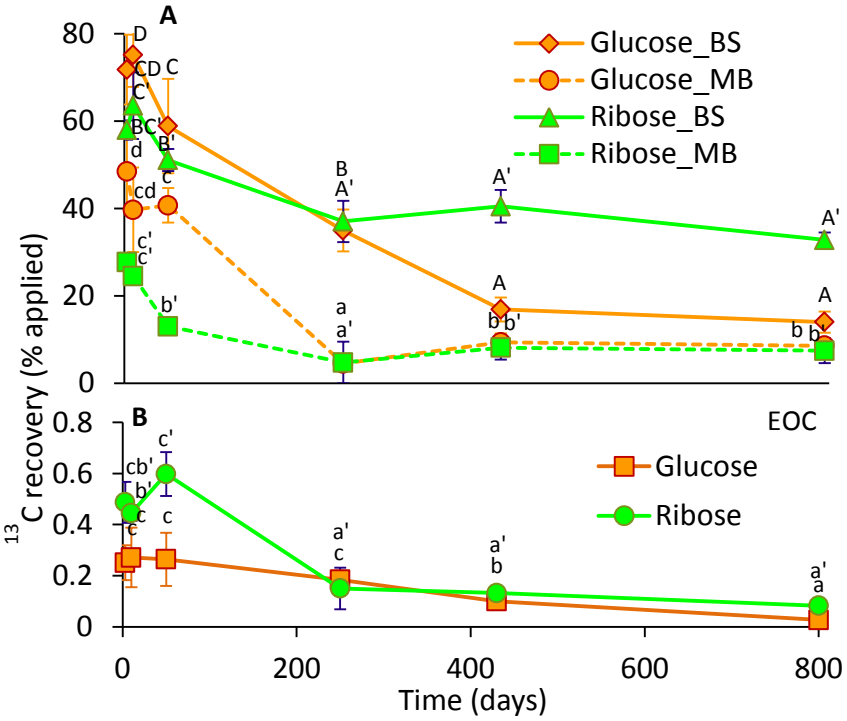
2.5.3.1. Pool sizes and ^{13}C recovery of uniformly labelled monosaccharides

On average, extractable microbial biomass C accounted for 3.3% of the soil C stock and EOC was 0.32% (Fig. 1A and B). Extractable microbial biomass C was significantly lower at day 50 than after day 430. On contrary, EOC was significantly higher at day 50 (fall) than all the other days.



S5 Figure 1: *Total C contents (mean ± SEM) in EOC (**A**) and microbial biomass (**B**). Letters above the error bars indicate significant effects (p<0.05) of time on pool sizes (EOC = a, and extractable microbial biomass (MB) = a').*

^{13}C in bulk soil from both monosaccharides strongly decreased from day 3 to 250 where there was convergence (Fig. 2A). While ribose ^{13}C remained constant afterwards, glucose ^{13}C decreased continuously till day 430 before stabilizing. Similarly, glucose- and ribose-derived ^{13}C in microbial biomass strongly decreased, converging at day 250 and remained stable afterwards. After 250 days, a 5-fold higher ribose ^{13}C in bulk soil than in microbial biomass was sustained over time (Fig. 2A). On contrary, a 2-fold higher glucose ^{13}C in bulk soil than microbial biomass was sustained after 430 days. In EOC, glucose and ribose-derived ^{13}C were less than 0.5%. Nonetheless, ribose ^{13}C in EOC was 2-fold higher than glucose but it decreased strongly; converging with glucose ^{13}C at day 250 and both remained constant afterwards (Fig. 2B).

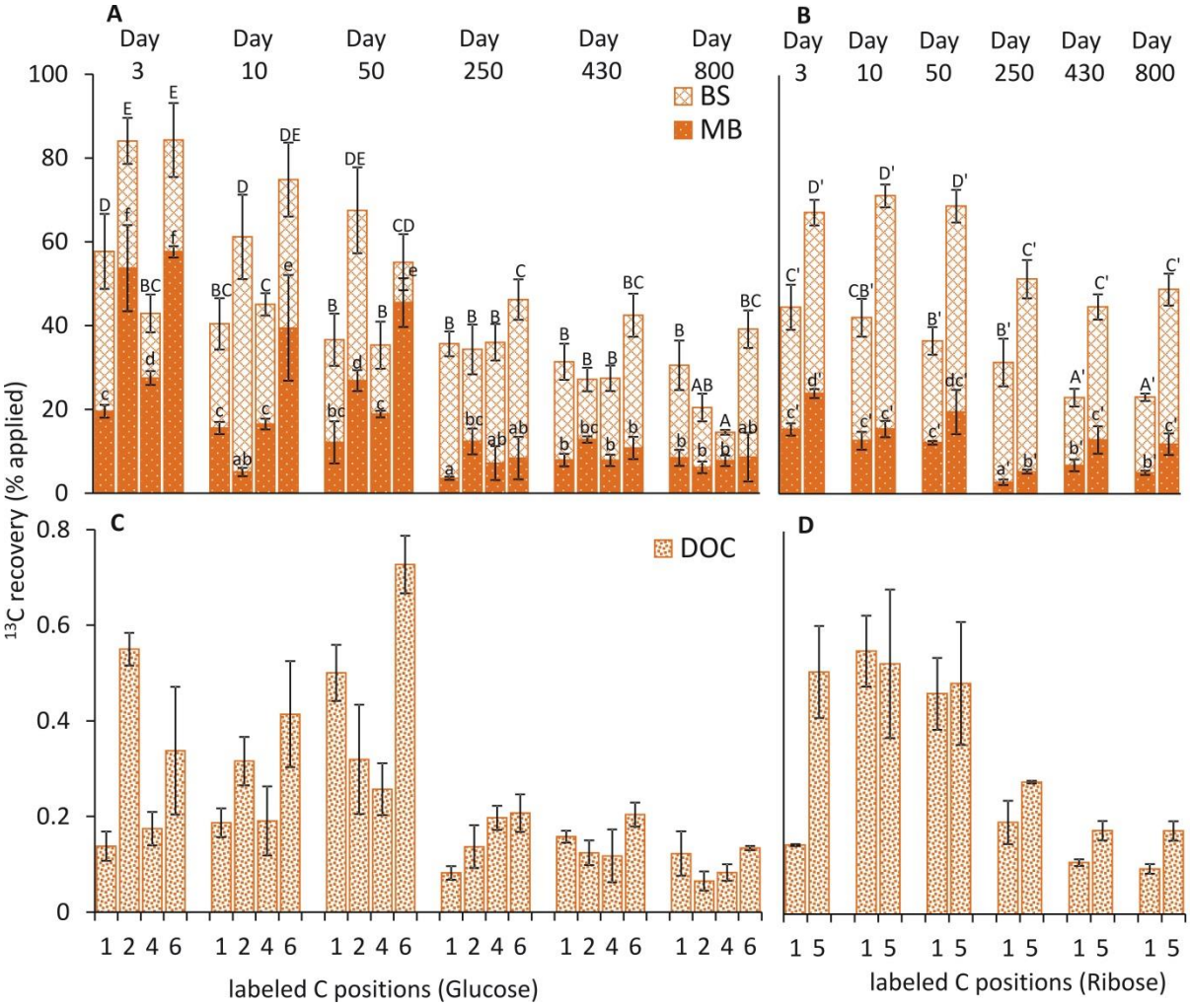


S5 Figure 2: *^{13}C incorporation (mean ±SEM) of uniformly labelled monosaccharides into bulk soil and microbial biomass (**A**) and into EOC (**B**). Letters above the error bars indicate significant effects (p<0.05) of time on ^{13}C recovery (ribose ^{13}C in bulk soil (BS) = A', ribose ^{13}C in extractable microbial biomass (MB) = a', glucose ^{13}C in BS = A and glucose ^{13}C in MB = a).*

2.5.3.2. Position-specific ^{13}C incorporation into soil, microbial biomass and EOC

The position-specific pattern of ^{13}C incorporated into soil differed with time. Glucose C-1 and C-4 were significantly lower than C-2 and C-6 in soil from day 3 to 50 (Fig. 3A). Thereafter, this pattern progressively got lost, with individual glucose C position approaching convergence on day 430 in soil. However, strong decrease of C-4 was later observed on day 800. Glucose C-1 that was stabilized in soil at day 10 remained until day 800. For ribose, C-1 was significantly lower than C-5 for the entire period in soil (Fig. 3B). Ribose C-1 attained stabilization in soil on day 430 while C-5 was on day 250. In microbial biomass, ^{13}C incorporation of glucose positions was a reflection of the pattern in soil on day 3 and 50. Glucose C-2 in microbial biomass was significantly lower than the other positions on day 10. Lower glucose C-1 than C-2 in microbial biomass was still evident on day 250. Thereafter, all positions were equally recovered, leading to the loss of the pathway-specific fingerprint. Like ^{13}C incorporation in soil, ribose C-1 was lower than C-5 in microbial biomass on day 3 and day 250 onwards. C-5 decreased strongly on day 10, leading to the loss of pathway-specific

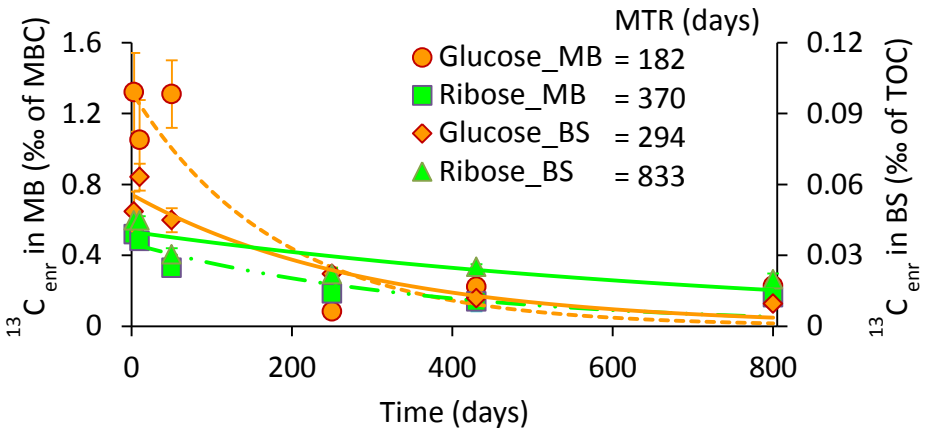
fingerprint in microbial biomass, which lasted until day 50. Of all the days, ribose C-1 and C-5 was lowest on day 250 in microbial biomass. In EOC, big variations in recovery of the sugar positions the first 50 days could not allow significant differences to be determined. Nonetheless, lower glucose C-1 and C-4 than C-2 and C-6 could be observed on day 3 and 10 (Fig. 3C). The pattern switched to lower C-2 and C-4 than C-1 and C-6 on day 50. Notable also on day 50, glucose C-1 and C-6 in EOC was twice as much as on day 3 and 10. On day 250 onwards, glucose positions were mixed up in EOC and pathway-specific fingerprints disappeared. For ribose, C-1 was over 2-fold lower than C-5 in EOC on day 3 (Fig. 3D). Thereafter, this pattern disappeared and recovery of C-1 and C-5 were indistinguishable. Additionally, ribose C-1 in EOC increased strongly between day 3 and 10 and remained constant till day 50. However, ribose C-1 and C-5 in EOC decreased strongly between day 50 and 250, attaining a steady state afterwards.



S5 Figure 3: *^{13}C recovery (mean ± SEM) of position-specific labelled glucose (in bulk soil (BS) and extractable microbial biomass (MB) (**A**), and in EOC (**C**)) and ribose (in BS and MB (**B**), and in EOC (**D**)). Letters above the error bars indicate significant differences ($p<0.05$) between ^{13}C recovered from monosaccharide positions and time in BS (glucose = A, ribose = A'), MB (glucose = a, ribose = a').*

2.5.3.3. ^{13}C mean residence times of uniformly labeled monosaccharide

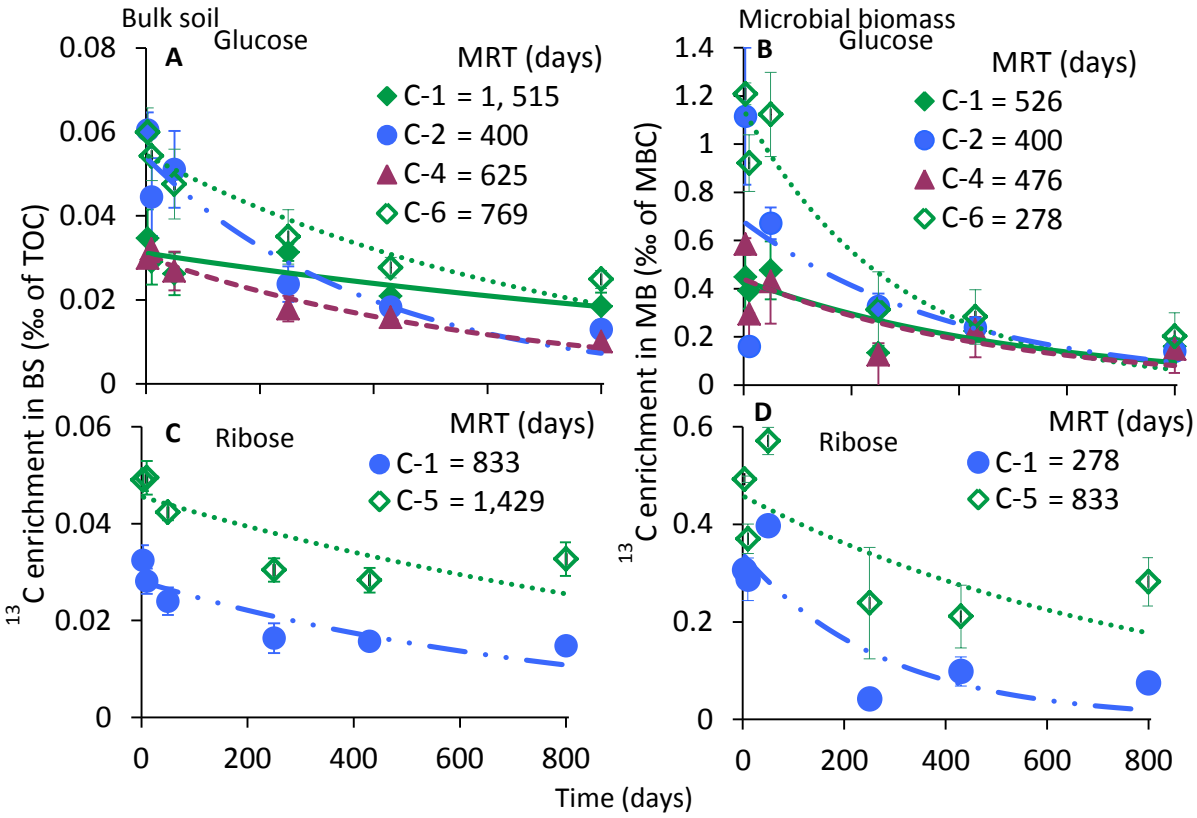
Based on decrease in ^{13}C enrichments with time of uniformly labeled monosaccharides, MRT of glucose C in bulk soil and microbial biomass were estimated as 294 and 182 days, respectively (Fig. 4). The MRT of ribose C in bulk soil and microbial biomass were as 833 and 370, respectively (Fig. 4). Thus, the MRT of glucose C in bulk soil was thrice lower than ribose C. The MRT of glucose C in bulk soil was also double its value in microbial biomass.



S5 Figure 4: *^{13}C enrichment (mean ± SEM) in BS and in MB by uniformly labeled monosaccharides. The curves were fitted with nonlinear least-square regressions according to exponential Eq. 5 and parameter estimates are presented in supplementary Table 1. MRT is an abbreviation for mean residence time.*

2.5.3.4. ^{13}C mean residence times of individual monosaccharide C positions

Glucose C-2 and C-6 corresponds to ribose C-1 and C-5, respectively, if glucose is transformed via PPP. However, they behave strongly different in bulk soil and microbial biomass. MRT of glucose C-1 was highest in bulk soil and microbial biomass (Fig. 5A and B). On contrary, glucose C-2 had the lowest MRT in soil while C-6 was lowest in microbial biomass. For ribose, the MRT of C-5 was higher than C-1 in bulk soil and microbial biomass (Fig. 5C and D).



S5 Figure 5: *^{13}C enrichment (mean ± SEM) in BS and MB by position-specific labeled glucose (**A** and **B**) and ribose (**C** and **D**). The curves were fitted with nonlinear least-square regressions according to exponential Eq. 5 and parameter estimates are presented in supplementary Table 2, 3, 4, and 5.*

2.5.4. Discussion

2.5.4.1. Microbial sugar utilization

Sugars are not only utilized by microorganisms as building block for various cellular components, but are also preferred energy source (Gunina and Kuzyakov, 2015). Less than 0.5% of the applied glucose or ribose ^{13}C could be recovered in EOC 3 days after application (Fig. 2B). Such low ^{13}C recovery is attributed to rapid sugar uptake by microorganisms, which occur within a few seconds to minutes (Jones and Murphy, 2007). After uptake, certain portions of the sugar-derived C are 1) mineralized to CO_2, 2) incorporated into anabolic products, and 3) released into the soil as microbial products (Cheshire, 1979; Gunina and Kuzyakov, 2015). On 3 days, ^{13}C recovered in soil from both ribose and glucose were 58% and 72% of applied ^{13}C, respectively (Fig. 2A). This reveals that larger proportion of ribose was oxidized to CO_2 than glucose. The mean residence time (MRT) of glucose in microbial cells has been shown to be <30 minutes, implying that mineralization is a very rapid process

(Gunina and Kuzyakov, 2015). Therefore, the amount of ^{13}C in bulk soil on day 3 mainly comprises sugar-derived metabolites in microbial biomass and extracellular environment. To get a picture regarding distribution of sugar-derived C in soil, we quantified ^{13}C in MB.

Half and two thirds of ribose and glucose ^{13}C in soil were contained microbial biomass on day 3, respectively. This reveals large proportion of glucose C is incorporated into anabolic products than ribose. This difference reflects transfer of C to different compound classes by specific pathways (Apostel et al., 2015). This is supported by the recovery of ribose ^{13}C in EOC, which was 2 times higher than glucose even up to day 50 (Fig. 2B). High recovery of ribose ^{13}C in EOC could reflect microbial utilization of pentoses, which are a preferentially used for the formation of extracellular compounds e.g. exopolysaccharides (EPS) and might have been exported faster than glucose C. However, elucidation of transformation pathways of these sugars is not possible with uniformly labeled sugars. Therefore, we used position-specific ^{13}C-labeling to track the fate of individual C in the sugars in order to reconstruct their transformation pathways and predict the resulting metabolic product.

2.5.4.2. Reconstruction of metabolic pathways

Characterization and modeling of microbial metabolic pathways based on position-specific labeled substances have gained momentum recently (Scandellari et al., 2009; Dijkstra et al., 2011b; Apostel et al., 2017). Tracking the fate of individual molecular positions of substances, permit reconstruction of metabolic pathways and prediction of resulting metabolic products (Apostel et al., 2013; Dippold and Kuzyakov, 2013). Three days after glucose application, ^{13}C recovery in bulk soil revealed that glucose C-4 was highly oxidized (Fig. 3A). This high oxidation of C-4 position implies that glycolysis was the major pathway of glucose transformation (Dijkstra et al., 2011c; Apostel et al., 2015). Although incorporation of glucose C-1 into bulk soil was significantly higher than C-4 on day 3, it was 1.5 times lower than C-6. This reveals that, in addition to glycolysis, portions of glucose molecules were transformed via pentose phosphate pathway (Dijkstra et al., 2011c; Apostel et al., 2015). Functioning of the pentose phosphate pathway is further reflected by lower incorporation of ribose C-1 than C-5 in soil on day 3 (Fig. 3B). Ribose is transformed via pentose phosphate pathway in the same step as glucose after oxidation of C-1 position (Caspi et al., 2008). Functioning of both pathways in parallel reflects demand for metabolic precursors from both pathways. Similar to soil, recovery of glucose C-1 and C-4 were significantly lower than C-2

and C-6 in microbial biomass on the day 3. Ribose also showed similar pattern of ^{13}C incorporation between bulk soil and microbial biomass on day 3. The similarity of ^{13}C recovery pattern between bulk soil and microbial biomass reflects the contribution of microbial products to SOM (Miltner et al., 2012). However, to link sugar C with possible microbial metabolites, knowledge of sugar C turnover rates is a crucial guide. Therefore, we estimated MRT of sugar-derived ^{13}C based on uniform and position-specific labeling.

2.5.4.3. Turnover of sugar-derived C

Microbial sugar transformation results in partitioning of C positions into different compound classes with different turnover rates. The mean residence time of glucose-derived ^{13}C (uniformly labeled) in microbial biomass was 182 days. This value is in the same range as those reported in a recent study (Gunina et al., 2017). The extractable compounds consists of metabolite fraction of the microbial biomass with low molecular weight organic substances and high molecular weight cellular biosynthetic components, with latter forming a major proportion of the extracts (Simpson et al., 2007; Ma et al., 2012; Malik et al., 2016). This reflects heterogeneity of microbial biomass components, hence, the C turnover represents an average turnover of the various cellular components (Malik et al., 2013; Gunina et al., 2017). Turnover of cellular components lead to the release of microbial products into the soil. For example, complementary trends were observed between microbial biomass C and EOC (Fig.1A and B), suggesting that the degradation of microbial biomass C is a major source of EOC. The decrease in microbial biomass C, which resulted in an increase in EOC up to day 50, could be attributed to release of extracellular compounds e.g. EPS to protect the cells against decreasing temperatures in fall. An increase in EPS concentrations was found to correlate positively with EOC at low temperature in marine samples (Krembs et al., 2002). In spring (day 250), an increase in microbial biomass C led to a decrease in EOC, reflecting intense microbial metabolism due to favorable temperature conditions. Thus, the relationship between microbial biomass C and EOC reflects a strong link between intracellular and extracellular processes, which have season variations as main controlling factor. Since microbial biomass releases C periodically into EOC pool, we determined the MRT of microbial biomass C based on ^{13}C pulse labeling data.

The MRT of glucose-derived ^{13}C in microbial biomass was 1.6 times lower than in soil, revealing a higher turnover of extractable microbial biomass compounds than microbial residues in soils (Gunina and Kuzyakov, 2015). Similarly, the MRT of ribose-derived ^{13}C in microbial biomass was 2 times lower than bulk soil, confirming higher turnover rates of extractable microbial biomass components than necromass portion in SOM. Furthermore, MRT of ribose-derived ^{13}C in bulk soil and microbial biomass were 3 and 2 times higher than glucose, respectively. Therefore, it is apparent that the C from these two substances partitions into different compound classes, ribose-derived products in living and in non-living SOM having low turnover rates. To reveal these compound classes, mean residence time of individual C positions of each substance was determined.

The MRT of individual C positions of the sugars differed from each other, suggesting fragmentation and incorporation of sugar molecules into biosynthetic molecules with different turnover time. As predicted, turnover time of ribose C-5 was higher than C-1 irrespective of the pool. Preferential incorporation of C-5 into biosynthetic molecules is higher than C-1 because its degradation only occurs if it transferred to TCA. On contrary, ribose C-1 has a high probability of oxidation via pyruvate dehydrogenase and TCA.

A complete opposite pattern is observed when glucose molecule is considered. Glucose C-1 and C-4, with high chance of oxidation via PPP and pyruvate dehydrogenase, respectively, had higher MRT than C-2 and C-6 irrespective of the pool. The discrepancy between transformation of glucose and ribose C positions can be attributed to their intensity of recycling in the cells. Whereas glucose can be intensely fragmented via glycolysis and PPP and reconstructed via gluconeogenesis (Apostel et al., 2015), ribose is internally recycled to a minor extend (Gunina and Kuzyakov, 2015). High MRT of C-1 and C-4 could be representative of glucose fragments that are initially assimilated into stable biosynthetic molecules. Estimations show that it takes about 30 minutes for glucose C atoms to spread over each metabolic pathway in the cell (Gunina and Kuzyakov, 2015), with C-1 and C-4 being preferentially mineralized (Fig. 3A). This means that part of the remaining portions of C-1 and C-4 could represent intact glucose molecules that were directly incorporated into biosynthetic molecules such amino sugars, which are known to have high persistence in nature (Gunina et al., 2017). Other portions of remaining C-1 and C-4 are likely to have been incorporated in cellular pools with short turnover times, which subsequently have a fast decomposition rate if they enter necromass pool. However, the turnover of different

biosynthetic molecules in the cell is not the same. Therefore, a detailed assessment of microbial metabolic processes will give a crucial lead in uncovering the environmental persistence of C atoms derived from labile compounds.

2.5.4.4. Dynamics of sugar-derived C atoms in soil

Mechanisms controlling the long-term kinetics of labile compounds in soil such as sugars still remain elusive. Interaction of sugars with soil matrix is inconsequential to their fate (Bremer and van Kessel, 1990). This means that sugar transformation in soil is mainly controlled by microorganisms. The glucose-derived ^{13}C in bulk soil was about 70% of the applied ^{13}C, with two thirds of this amount contained in microbial biomass on day 3. On day 800, 14% of applied glucose ^{13}C was recovered in the soil, with half of it being in microbial biomass (Fig. 2A). This is consistent with results showing 15-20% of glucose ^{14}C remained in soil 3 years after application (Cheshire, 1979). The continuous decrease of ^{13}C in soil can be attributed to sustained intracellular and extracellular recycling and mineralization of the components. Sustained pathway-specific, positional fingerprint in soil and microbial biomass (Fig. 3A) from days 3 to 50 was a reflection of slow metabolic process due to decreasing temperatures in fall. After day 250, glucose C positions converged in microbial biomass and pathway-specific, positional fingerprint was no longer visible (Fig. 5B). Existence of decomposing and constructing pathways in parallel within microbial metabolism, which is intensive is spring, especially after winter, leads to a mixing of C positions after 250 days (Scandellari et al., 2009). Furthermore, MRT of glucose ^{13}C in microbial biomass was consistent with turnover of bacterial biomass in soil (Moore et al., 2005). This could mean that bacteria dominated glucose transformation as already observed in other studies working with these soils (Gunina et al., 2014; Apostel et al., 2015; Gunina et al., 2017). After death of bacteria biomass, continuous recycling of their necromass by living microbes could also result in mixing of the glucose C positions and loss of pathway-specific fingerprint. Therefore, the mechanism controlling the remaining glucose ^{13}C in soil after 250 days can be attributed to recycling within microbial cells and microbial products in extracellular environment.

The MRT of individual glucose C position was different from each other irrespective of the pool (Fig. 5A and B). Moreover, none of the glucose C position MRT could be related to turnover time of entire molecule based on uniform labeling. The differences in turnover time of glucose C positions reveal intensive metabolization and incorporation of modified

compounds. A recent study revealed that the microbial biomass components consist of fast turnover components such as amino acids, sugars, organic acids and alcohols, which is a minor portion of the extracts (Malik et al., 2016). Majority of the components were high molecular weight polymeric compounds of lipid, protein and polysaccharide origin (Hart et al., 2013; Malik et al., 2016). A universal substrate like glucose, which spreads in each metabolic pathway in cells and can thus be found in each of these compounds as fragments or individual atoms (Apostel et al., 2015). Recycling within the living biomass can lead to continuous metabolization of the incorporated ^{13}C in these compounds. Besides, microbial products containing the label can be released into the soil (Cheshire, 1979). Additionally, microbial necromass containing the label is released into the soil after cell death. These compounds can be recycled back to the living biomass or stabilized soil as non-living SOM (Miltner et al., 2009; Miltner et al., 2012; Dippold and Kuzyakov, 2016). Proteinaceous compounds such as amino acids and constituents of cell such as amino sugars, which may contain some label have been found to be stabilized in soil via sorption on mineral soil fractions or by entrapment into more recalcitrant organic material (Lützow et al., 2006; Miltner et al., 2012). However, desorption and release of entrapped components due to environmental perturbation can allow recycling into living microbial biomass (Miltner et al., 2012). Thus, persistence of glucose C in soil can be attributed mainly to recycling, with stabilization playing a minor role. To ascertain that recycling indeed accounts for persistence of labile compounds in soil, ribose transformation was assessed as well.

Eight hundred days after sugar application, 33% and 14% of applied ribose and glucose ^{13}C were recovered in soil, respectively. This difference indicates that stabilization mechanisms of ribose C could be different from glucose. One possible direct biochemical ribose C utilization is the formation of ribonucleotide backbone of DNA and RNA (Caspi et al., 2008; Keseler et al., 2009). Persistence of ^{13}C in nucleic acids has previously been observed in other studies (Griffiths et al., 2006; Malik et al., 2015). This could be attributed to stabilization of nucleic acids in SOM and microbial C recycling. In nutrient-limited environments, microorganisms often employ the salvage pathway of nucleotide synthesis, which involves recycling nucleotide in SOM rather than the *de novo* synthesis (Nyhan, 2005). However, if recycling played a major role, we would expect at least slow depletion of ^{13}C in SOM over time. On the contrary, ^{13}C in soil remained fairly constant after day 250, strongly indicating stabilization in non-living SOM. Furthermore, MRT of ribose ^{13}C in bulk soil and microbial

biomass were 3 and 2 times higher than glucose, reinforcing the differences in biochemical utilization and stabilization mechanisms in SOM. It is possible that after microbial death, nucleic acids strongly interacts with mineral and organic fractions of the soils, inhibiting their availability for microbial reuse (Lützow et al., 2006). Additionally, ribose can be utilized by microorganisms for formation of EPS, a major component of biofilms secreted by microbes under unfavorable conditions such as low temperatures or nutrient limitation. Sorption of EPS on soil surfaces increases potential of ribose stabilization in non-living SOM. These mechanisms could account for 5-fold higher ribose ^{13}C in bulk soil than in microbial biomass after 250 days (Fig. 2A). Whereas persistence of glucose C is largely attributed to recycling, stabilization in non-living SOM accounts for persistence of ribose C in SOM. This is consistent with suggestion that plant-derived sugars (mostly pentoses like ribose) are governed by stabilization processes in soil (Basler et al., 2015b). However, we emphasize that microbial modification plays a critical role in the stabilization process. Therefore, persistence of substance classes is depended on biochemical utilization of the substance.

2.5.5. Conclusions

Sugars were transformed in soil via glycolysis and PPP, with both processes occurring in parallel. Recycling dominated the persistence of glucose C in soil, leading to continuous decrease of ^{13}C in soil and microbial biomass over time. The convergence of glucose C positions in microbial biomass after 250 days led to loss of pathway-specific positional fingerprint, consistent with our interpretation of continued recycling and use of the ^{13}C in various anabolic pathways. This indicates that position-specific labeling may loses its metabolic tracing potential with continuous transformation. The differences in MTR of individual glucose-derived C positions reveal intensive metabolization and incorporation of modified compounds during microbial recycling and demonstrate that the initial use of a molecule influences its persistence in the long-term. Glucose C-1 and C-4 with high probability of oxidation had higher MRT than C-2 and C-6, contradicting our first hypothesis. This revealed that after oxidation of C-1 and C-4, the remaining portion was directly incorporated into cellular components with low turnover such as amino sugars.

On contrary, persistence of ribose C in soil was attributed to stabilization in non-living SOM. Ribose can directly be utilized in the formation ribonucleotide backbone of nucleic acids or

secreted as EPS. Strong interaction of nucleic acids with mineral fractions in the soil after cell death, can account for constant ribose ^{13}C after 250 days. Additionally, secretion of EPS during formation of biofilms on soil surfaces, have potential to be subsequently stabilized. Consistent with our hypothesis, MRT of ribose C-1 was lower than C-5 confirming that ribose was recycled to a minor extend in the living biomass. Thus, stabilization mechanisms of the substance classes in soil should be considered individually, without generalizing. This information not only broadens the understanding of persistence of labile compounds in soil, but also underscores the role of microbial processes in SOM stabilization.

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Supplementary information

Table 1: Parameter estimates for the fitted curves of uniformly labeled glucose and ribose.

		Estimate	Standard - error	t-value - df = 22	p-level
Glucose_BS	a	0.055749	0.003432	16.24245	9.79E-14
	k	0.003415	0.000649	5.259586	2.82E-05
Glucose_MB	a	1.32308	0.117111	11.29766	1.25E-10
	k	0.005524	0.001655	3.337099	0.002987
Ribose_BS	a	0.039981	0.002086	19.16938	3.33E-15
	k	0.001203	0.000233	5.163148	3.55E-05
Ribose_MB	a	0.466191	0.035782	13.02868	8.06E-12
	k	0.002723	0.000639	4.259045	0.00032

Table 2: Parameter estimates for position-specific labeled glucose in bulk soil

Positions		Estimate	Standard - error	t-value - df = 22	p-level
C1	a	0.031148	0.002519	12.36529	0
	k	0.00066	0.000282	2.34237	0.028617
C2	a	0.053955	0.003853	14.00406	0.000000
	k	0.002505	0.000549	4.56170	0.000153
C4	a	0.030269	0.001540	19.66071	0.000000
	k	0.001583	0.000269	5.88587	0.000006
C6	a	0.054541	0.003318	16.43691	0.000000
	k	0.001325	0.000287	4.61734	0.000134

Table 3: Parameter estimates for position-specific labeled glucose in microbial biomass

Positions		Estimate	Standard - error	t-value - df = 22	p-level
C1	a	0.434345	0.049428	8.787486	0.000000
	k	0.001921	0.000694	2.766044	0.011270
C2	a	0.678214	0.115159	5.889372	0.000006
	k	0.002472	0.001289	1.917039	0.068311
C4	a	0.439472	0.040319	10.89986	0.000000
	k	0.002117	0.000607	3.49005	0.002073
C6	a	1.141707	0.085098	13.41631	0.000000
	k	0.003598	0.000834	4.31629	0.000279

Table 4: Parameter estimates for position-specific labeled ribose in bulk soil

Position		Estimate	Standard - error	t-value - df = 22	p-level
C-1	a	0.028074	0.001661	16.89796	0.000000
	k	0.001194	0.000263	4.53628	0.000163
C-5	a	0.045595	0.002123	21.47959	0.000000
	k	0.000726	0.000167	4.34192	0.000262

Table 5: Parameter estimates for position-specific labeled ribose in microbial biomass

Positions		Estimate	Standard - error	t-value - df = 22	p-level
C-1	a	0.338917	0.028513	11.88631	0.000000
	k	0.003591	0.000937	3.83106	0.000910
C-5	a	0.458261	0.041597	11.01671	0.000000
	k	0.001188	0.000403	2.94994	0.007404

Other contributions

Apostel, C., Herschbach, J., Bore, E. K., Spielvogel, S., Kuzyakov, Y., Dippold, M., 2017. Food for microorganisms: Position-specific ^{13}C labeling and ^{13}C-PLFA analysis reveals preferences for sorbed or necromass C. *Geoderma* 312, 86-94.

Declaration

Declaration by the doctoral candidate
at the Georg-August-Universität Göttingen

Name: Bore, Ezekiel
(Surname, First Name)

Address: Büsgenweg 2, 37077 Goettingen, Germany
(Country/Postcode/Place)

I intend to produce a dissertation on the topic of: “Unfavorable environmental conditions: Consequences on microbial metabolism and C stabilization in soil” at Georg-August-Universität Göttingen.

In this, I shall be supervised by Prof. Dr. Michaela Dippold.

I submit the following declaration:

1. The opportunity for the existing doctoral project was not made commercially available to me. Especially, I have not engaged any organization that seeks thesis advisers against a fee for the preparation of dissertations or performs my obligations with respect to examination components entirely or partly.
2. I have until now and shall in future accept the assistance of third parties only in a scope that is scientifically justifiable and compliant with the legal statutes of the examinations. I shall specifically complete all parts of the dissertation myself; I have neither, nor will I, accept unauthorized outside assistance either free of charge or subject to a fee.

   Furthermore, I am aware of the fact that untruthfulness with respect to the above declaration repeals the admission to complete the doctoral studies and/or subsequently entitle termination of the doctoral process or withdrawal of the title attained.

   Goettingen, November 14, 2017

   (Ezekiel K. Bore)