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## Evidence of rapid non-targeted effects of cycloheximide on soil bacteria using $^{13}\text{C}$ -PLFA analysis

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### ABSTRACT

Stable isotope probing of phospholipid fatty acids (PLFA-SIP) is useful when studying bacterial contributions to soil processes and is an effective way to separate fungal and bacterial activity by linking  $^{13}\text{C}$  enrichment to specific PLFAs. Distinguishing bacterial contributions to soil processes often employs selective inhibitors; however, studies demonstrating their efficacy when using stable isotope probing of PLFAs are less common. Here we determined the effect of the fungal inhibitor cycloheximide (4.8 mg g<sup>-1</sup> dry soil) and the bacterial inhibitor bronopol (0.48 mg g<sup>-1</sup> dry soil) on microbial communities white spruce (*Picea glauca* (Moench) Voss) forest floor by measuring the uptake of  $^{13}\text{C}$ -enriched glucose (2 mg g<sup>-1</sup> dry soil) in microbial PLFAs. We targeted  $^{13}\text{C}$ -glucose uptake by the bacterial community conditioned to a stable soil environment of 23 °C for over 2 weeks rather than new bacteria generated from active colony growth caused by glucose addition. Nearly all bacterial PLFAs exhibited pronounced inhibition of  $^{13}\text{C}$  enrichment in the presence of bronopol. Limited inhibition of  $^{13}\text{C}$  enrichment in the presence of cycloheximide was observed as bacterial PLFA affected by cycloheximide had roughly one third less  $^{13}\text{C}$  enrichment than samples emended with  $^{13}\text{C}$ -glucose alone. Inhibitory effects only reduced  $^{13}\text{C}$  enrichment and did not affect total PLFA concentrations, implying that the inhibitors in the concentrations applied were impeding bacterial activity without causing cell death. Based on this work,

we conclude that bronopol is an effective inhibitor for bacteria. Additionally, non-targeted effects of cycloheximide on soil bacteria must be accounted for when it is used in soil incubations.

## KEYWORDS

PLFA;  $^{13}\text{C}$  enrichment; microbial inhibitors, bacteria, non-targeted effects

## INTRODUCTION

Stable isotope probing of phospholipid fatty acids (PLFA-SIP) has been described as a means of uncovering previously unknown biological functions of soil microorganisms (Yao et al. 2015). The PLFA-SIP approach is sensitive to changes in soil microbial communities (Watzinger 2015) and has been used to determine microbial contributions to soil biogeochemical processes (Boschker et al. 1998), plant-microbe interactions in the rhizosphere (Treonis et al. 2004) and microbial response to land management practices (Watzinger et al. 2014). Often, PLFA-SIP relies on simultaneously measuring the total concentration and the  $\delta^{13}\text{C}$  signature of individual microbial fatty acids. Further interpretations for eubacteria rely on PLFA biomarkers linked to general bacteria (Zelles 1997), gram distinction (O'Leary and Wilkinson 1988; Zelles 1997) and actinobacteria (Vestal and White 1989). The PLFA technique is now a fundamental method to characterise soil microbial communities but it continues to suffer from improper or overreaching interpretations based on inappropriate biomarker assignment (Frostegård et al. 2011). Therefore, more fundamental research is needed which utilises the strength of PLFA-SIP to confirm the links between individual PLFA and their microbial origins.

A primary use of PLFA biomarkers is to distinguish bacterial contributions to soil processes, with recent PLFA-SIP work (Rousk and Frey 2015) questioning long held assumptions regarding their roles in the decomposition of soil organic matter. Initially, Anderson and Domsch (1975) utilised selective inhibitors

to distinguish fungal and bacterial contributions to soil respiration. However, interpretation of results may be unreliable as inhibitors can affect both microbial groups (Rousk et al. 2009). The inhibitors bronopol, which can have static or toxic effects on cells (Shepherd et al. 1988) and cycloheximide, a fungicide, have been shown to exhibit fewer non-targeted effects than other commonly used microbial inhibitors (Rousk et al. 2009). Bronopol and cycloheximide have been used in tandem to determine bacterial and fungal contributions to nitrogen mineralization (Boyle et al. 2008), niche overlap in organic matter decomposition in soil (Rousk et al. 2008), and pH effects on substrate metabolism (Martin et al. 2012). While microbial inhibitors combined with PLFA-SIP has greatly increased our knowledge regarding the roles of bacteria and fungi on soil processes, the window in which microbial inhibitors first begin to affect the activity of microbial communities is less studied.

Using PLFA-SIP to target the physiological behaviour of a bacterial community within a stable soil environment (i.e.; microbial homeostasis) is challenging due to the rapid change initiated by the introduction of new substrates. During the lag phase of colony growth, bacteria in pure culture can exhibit major physiological adaptation by upregulating genes associated with lipopolysaccharide and fatty acid biosynthesis in 20 minutes after the introduction of a new substrate (Rolfe et al. 2011). Typically glucose amendments in moist soil conditions exhibit bacterial growth after 8 to 9 hours (Lovieno and Bååth 2008), yet SIP-PLFA probing using glycine has shown enrichment in eukaryotic PLFAs within 8 hours (Dungait et al. 2013). Hence, incubation times, preferably less than one hour, are required to capture the physiological behaviour of only the bacteria conditioned to the current soil environment.

In this study, we used  $\delta^{13}\text{C}$  values of PLFAs to understand how the presence of selective inhibitors affected the uptake of  $^{13}\text{C}$ -glucose into microbial PLFAs conditioned to a stable soil environment. We used total PLFA concentrations ( $\text{nmol g}^{-1}$  • dry soil) to observe any potential growth in the microbial biomass (White et al., 1979), or alternatively any cytotoxicity of inhibitors. We focused on how inhibitors

affected the  $\delta^{13}\text{C}$ -values and concentrations of PLFAs linked to bacteria as short-term incubations favour bacterial over fungal growth (Reischke et al. 2014). We also assessed the extent of non-targeted effects of cycloheximide (a fungal inhibitor) on bacteria.

## MATERIALS AND METHODS

### Pre-Incubation Soil Conditions

Organic forest floor (LFH) used in the incubation was collected from a white spruce (*Picea glauca* (Moench) Voss) stand found in Alberta's boreal forest with sampling and compositing described in Swallow and Quideau 2013. Field capacity moisture content was determined on a bulk air-dried forest floor according to Parent and Caron (1993). Prior to the start of the experiment, the forest floor material was brought to field capacity and incubated at 23 °C for over 2 weeks to avoid interference from any rewetting effects on the microbial community. Field capacity moisture content was monitored and maintained during the preconditioning period by daily weighing and application of lost moisture. The forest floor pH was 4.6 determined with a 1:4 dilution with 0.01  $\text{CaCl}_2$  (Karla and Maynard 1991).

### Inhibitor Application and Incubation

Samples for the incubation consisted of 15 g of moist bulk forest floor transferred into 130 ml glass flat-bottomed culture tubes ( $n=7$ ). Prior to substrate addition, samples were left overnight at 23 °C (approximately 16 hours) to allow for evaporation and water loss was determined by reweighing the samples the following day. The recorded moisture loss was used as the volume of water to establish identical concentrations of  $^{13}\text{C}$ -glucose and inhibitors in each sample. While each sample lost roughly a similar amount of moisture, this method ensured that the concentrations of  $^{13}\text{C}$ -glucose and inhibitors in the soil water were similar in all samples.

### $^{13}\text{C}$ -Glucose and Inhibitors

$^{13}\text{C}$ -glucose (Sigma Aldrich, 99% D-Glucose-1- $^{13}\text{C}$ ) was applied to all samples at a rate of  $2 \text{ mg g}^{-1}$  dry soil for adequate PLFA enrichment. The bacterial inhibitor bronopol, and the fungal inhibitor, cycloheximide, were applied together with  $^{13}\text{C}$ -glucose at rates reported to inhibit cellular activity without causing cell death for target organisms and to limit inhibitory effects on non-targeted organisms (Blagodatskaya and Anderson 1998; Rousk et al. 2009;). Bacterial inhibited samples received  $0.48 \text{ mg g}^{-1}$  dry soil of bronopol and fungal inhibited samples received  $4.8 \text{ mg g}^{-1}$  dry soil of cycloheximide.

### Incubation and Sampling

Samples were incubated for 45 minutes to ensure  $^{13}\text{C}$ -glucose use was limited to the bacterial community conditioned to the soil environment prior to the onset of colony growth. After 45 minutes, 5 g of sample was frozen, and then freeze dried. Roughly 0.30 g of freeze dried sample was extracted for PLFAs using a modified Bligh and Dyer (1959) extraction (Hannam et al. 2006) where PLFAs were extracted in a methanol:chloroform solvent, purified on a SPE column (Agilent Technologies, Santa Clara, USA), and converted to fatty acid methyl-esters prior to being separated using a GC-FID (Agilent 6890 Series capillary gas chromatograph) equipped with a 25 m Ultra 2 (5%-phenyl)-methylpolysiloxane column. The Sherlock Microbial ID system software package (MIDI, Inc., Newark, DE) was used for PLFA identification. Quantification of individual PLFAs relied on response values of the internal standard of 19:0 added to each sample at the onset of the extraction and expressed in  $\text{nmol g}^{-1}$  dry soil.

Individual PLFAs were described based on the composition of the aliphatic chain as  $\text{X:Y}\omega\text{Z}$ , where X indicates the number of carbon atoms, Y indicates the number of unsaturated bonds, and  $\omega\text{Z}$  identifies the location of the first unsaturation from the aliphatic end. When present, the suffix 'c' indicates the cis geometric isomer. Branching occurring in saturated fatty acids is indicated by distance from the aliphatic end where ISO indicates branching at the second carbon and ANTE indicates branching from the third carbon. Finally, cyclopropane fatty acids are indicated by CYC, while the presence of methyl branching at

the 10 carbon from the carboxyl end of the fatty acid is indicated by ME. Distinction between bacterial and eukaryotic sourced lipids was based on the summary of PLFA origins found in Ruess and Chamberlain (2010). In brief, biomarker PLFA linked to bacteria are limited to PLFA less than 20 carbon atoms in length and include all saturated PLFAs, 10-Methyl branched PLFAs, iso/antieso methyl branched PLFAs, cyclopropyl PLFAs and monounsaturated PLFAs. Biomarker PLFA for soil fungi and other soil eukaryotes are limited to PLFA which are polyunsaturated, and 18 carbon in length or longer..

### Isotopic Analysis

Compound specific  $^{13}\text{C}$  analysis of the PLFAs was conducted using a 6890N Agilent GC equipped with an HP-Ultra 2 column (50 m length, 0.2 mm i.d., 0.33 mm film thickness), and interfaced to a Finnigan Deltaplus Advantage Isotopic Ratio Mass Spectrometer (IRMS; ThermoFinnigan, Bremen, Germany) *via* a GC Combustion III unit. Results were expressed in the  $\delta$ -notation, part per thousand variations from the standard Pee Dee Belemnite, and were corrected for the methyl group added during transesterification.

### Data Analysis

Statistical analysis was conducted using R (R Development Core Team 2017) within the R-studio (Rstudio team, 2017) graphical environment. The Imperm package (Wheeler and Torchiano 2016) was used to conduct permutational ANOVA (aovp) followed by Tukey's adjustment for multiple comparisons (TUKEY HSD) at  $\alpha$  of 0.05. Data transformations were not necessary, as data did not show heteroscedasticity. Graphical figures were created using ggplot2 (Wickham 2009).

## RESULTS

### Saturated and Branched Saturated PLFAs

As expected, the highest  $\delta^{13}\text{C}$  values (‰) occurred with glucose in the absence of inhibitors (Figure 1A). All saturated PLFAs showed similar decreases in  $\delta^{13}\text{C}$  values following addition of the inhibitors. The presence of cycloheximide reduced  $\delta^{13}\text{C}$  values in relation to the glucose control, but nearly all saturated PLFAs in cycloheximide samples still showed positive  $\delta^{13}\text{C}$  values. Bronopol on the other hand, severely restricted the uptake of  $^{13}\text{C}$  from the enriched glucose, with most saturated PLFAs having  $\delta^{13}\text{C}$  values less than -20 ‰ (Figure 1a). For saturated PLFA, cycloheximide  $\delta^{13}\text{C}$  values were on average 29% (3.8% standard error) lower than the glucose control  $\delta^{13}\text{C}$  values when compared against  $\delta^{13}\text{C}$  values in bronopol. Many branched saturated PLFAs followed a similar pattern as the saturated PLFAs (Figure 1b). Branched saturated PLFA with significantly different  $\delta^{13}\text{C}$  values between glucose control and cycloheximide treatments (Figure 1b), had on average 25% (1.4% standard error) lower  $\delta^{13}\text{C}$  values in cycloheximide samples when compared against  $\delta^{13}\text{C}$  values in bronopol. However, in the case of a17:0 and 10Me18:0,  $\delta^{13}\text{C}$  values were lower than those for other branched saturated PLFAs, and they were not impacted by the presence of cycloheximide. On the other hand, cyc17:0 had similar  $\delta^{13}\text{C}$  values among all three treatments. Additionally,  $\delta^{13}\text{C}$  values in cyc17:0 were generally very low (-28 ‰). Overall, PLFA concentrations were not affected by the presence of either bronopol or cycloheximide (Fig 2a/b).

### Unsaturated PLFAs

All monounsaturated PLFAs followed a similar response pattern to saturated PLFAs, with many having positive  $\delta^{13}\text{C}$  values when incubated only with enriched glucose (Fig 1c). The presence of inhibitors also affected them similarly to saturated PLFAs, namely with a strong inhibition of  $^{13}\text{C}$  uptake in the presence of bronopol and a limited effect in the presence of cycloheximide. Monounsaturated PLFA with significantly different  $\delta^{13}\text{C}$  values between glucose control and cycloheximide treatments (Figure 1c),

had on average 38% (8.1% standard error) lower  $\delta^{13}\text{C}$  values in cycloheximide samples when compared against  $\delta^{13}\text{C}$  values in bronopol. In contrast, polyunsaturated PLFAs, 18:2 $\omega$ 6c and 20:4 $\omega$ 6c, exhibited no difference in  $\delta^{13}\text{C}$  values with or without the presence of any inhibitor (Fig 1c) with low  $\delta^{13}\text{C}$  values of -26.3 and -29.1 ‰, respectively. Concentrations of unsaturated PLFAs were similar in the presence or absence of inhibitors (Fig 2c).

## Discussion and Conclusion

In samples with glucose but no inhibitors, the enrichment of  $^{13}\text{C}$  in saturated, branched saturated and monounsaturated PLFAs indicates that glucose was used by the bacterial community. This enrichment, coupled with the fact that PLFA concentrations did not change with the addition of inhibitors, verified that our study targeted the pre-existing bacterial community as intended. When applied at higher concentrations (10 mg g<sup>-1</sup> dry soil and 2 mg g<sup>-1</sup> dry soil) bronopol appears to act as a biocide resulting in lower bacterial abundances based on 16S rRNA gene copies even 6 weeks after its addition (Pan et al. 2019). In our study, any evidence of cell death caused by inhibitors would coincide with a decrease in PLFA concentrations and a lack of  $^{13}\text{C}$  enrichment, as PLFAs are traditionally associated with living soil microbial biomass (Janzen et al. 1994). Rangl et al. (2017) demonstrated *in vitro* that activated phospholipases can completely consume the outer membrane of gram-negative bacteria in minutes upon cell death. However, it should be noted that PLFA turnover might be affected by the presence of inhibitors as the inhibitors could also potentially interfere with the phospholipases responsible for lipid degradation (Frostegård et al. 2011). Overall, the similar PLFA concentration seen with the addition of glucose in the presence and absence of inhibitors indicate that the inhibitors were not at levels high enough to cause significant cell death but were high enough to inhibit the  $^{13}\text{C}$  incorporation from glucose into microbial lipids.

The limited effects of cycloheximide on bacterial PLFAs corroborate recent (Chen et al. 2014) and more traditional (Anderson and Domsch 1973) views that when applied at the correct concentrations, cycloheximide exhibits limited non-targeted effects on the bacterial community in soil. However, for nearly all bacterial PLFAs, cycloheximide coincided with lower  $\delta^{13}\text{C}$  values than the inhibitor free samples, indicating that cycloheximide did impact bacteria to a limited extent and more specifically, reduced  $\delta^{13}\text{C}$  values by 28-30% when compared to glucose samples without cycloheximide. Recent work by Pan et al. (2019) corroborates this finding as their work found that non-targeted effects of cycloheximide on soil bacteria are generally low in short-term incubations but become more pronounced over 6 weeks. Therefore, the confounding effects of cycloheximide on bacteria must be taken into account when it is used as a fungal inhibitor in soil incubations. The confounding non-targeted effects of cycloheximide on bacteria will become even more pronounced when differences between fungal and bacterial contributions are small.

Lack of enrichment in polyunsaturated PLFAs (18:2 $\omega$ 6c and 20:4) indicated that the short incubation time selected ensured that the bacterial community was primarily responsible for glucose use with non-detectable use by soil fungi and other eukaryotes. Additionally, cyclopropyl fatty acids (cyc17:0) exhibited no enrichment in the absence of inhibitor, which coincides with the traditionally held view that this PLFA is synthesized during times of metabolic stress (Bossio and Scow 1998; Zhang and Rock 2016).

10Me18:0 is a common biomarker for actinobacteria (Brennan 1988), and it responded to bronopol in a similar manner as other bacterial PLFAs. However, unlike other bacterial PLFAs, 10Me18:0 had generally lower levels of enrichment and appeared to not be affected by cycloheximide as the absence or presence of cycloheximide with glucose resulted in similar enrichment levels. The pattern observed in this study has also been observed in recent work by Bore et al. (2017) who found that  $^{13}\text{C}$  enrichment in

actinobacteria PLFA biomarkers derived from labelled glucose was substantially less than other bacterial PLFAs. Our results suggest that actinobacteria, in addition to sharing similar functional roles as fungi in soil organic matter decomposition (Helfrich et al. 2015), can rapidly access simple sugars in soil, albeit at rates less than other bacteria.

Bronopol appears to be an adequate inhibitor for studies requiring the suppression of bacterial activity without causing cell death in the short-term. Though cycloheximide causes non-targeted effects on soil bacteria by potentially reducing  $\delta^{13}\text{C}$  values by roughly one third, the extent of the non-targeted effects can be accounted for indicating that it is still an effective inhibitor for soil fungi. Overall, selective inhibitors coupled with PLFA-SIP are an effective tool to uncover the contribution of fungi and bacteria to the degradation of substrates and subsequently to investigate larger questions related to carbon biogeochemistry in soil.

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### Figure Captions

Figure 1. Mean  $\delta^{13}\text{C}$  values (‰) with standard error of a) saturated, b) branched saturated, and c) unsaturated PLFAs in response to glucose [grey circles]; glucose and bronopol [black circles]; and glucose and cycloheximide [white circles]. Lower case letters indicate statistical ( $P < 0.05$ ) differences among treatments for each specified PLFA.

Figure 2. Mean concentrations ( $\text{nmol g}^{-1}$  dry soil) with standard error of a) saturated b) branched chain and c) unsaturated PLFAs in response to glucose [grey], glucose and bronopol [black], glucose and cycloheximide [white]. Lower case letters indicate statistical ( $P < 0.05$ ) differences among treatments for each specified PLFA.

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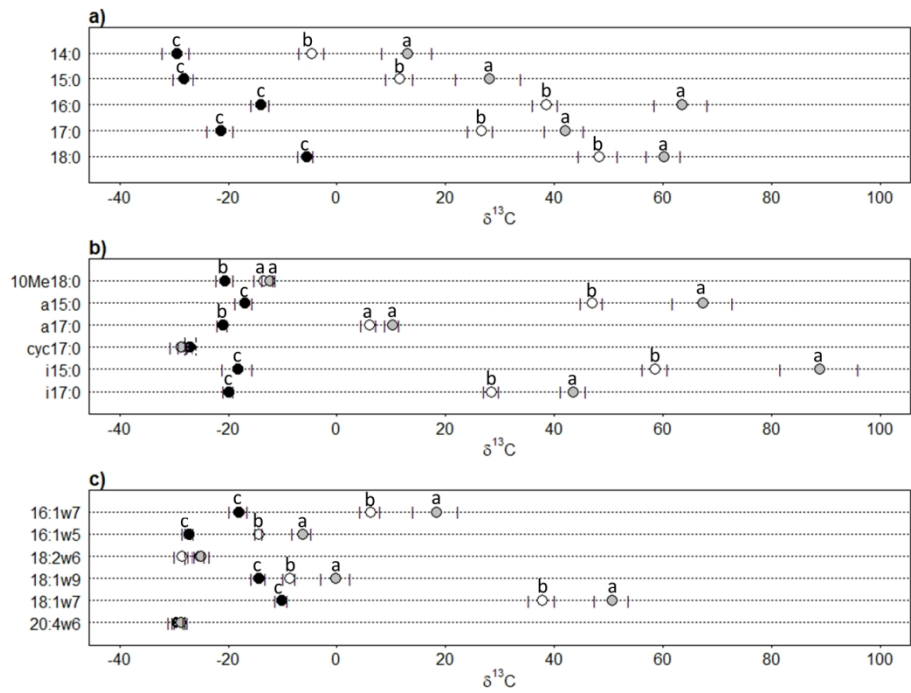


Figure 1. Mean  $\delta^{13}\text{C}$  values (‰) with standard error of a) saturated, b) branched saturated, and c) unsaturated PLFAs in response to glucose [grey circles]; glucose and bronopol [black circles]; and glucose and cycloheximide [white circles]. Lower case letters indicate statistical ( $P < 0.05$ ) differences among treatments for each specified PLFA.

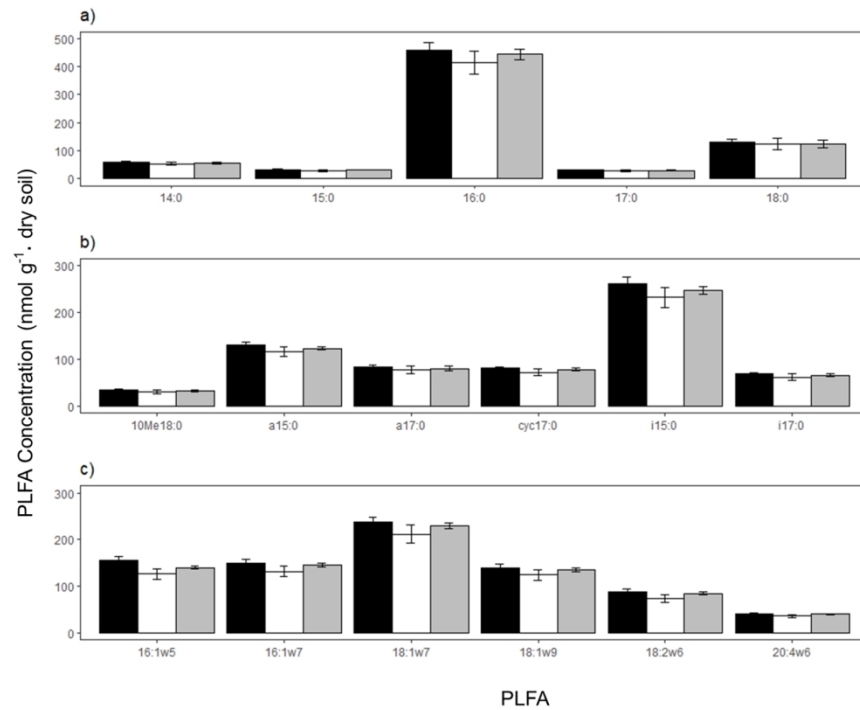


Figure 2. Mean concentrations (nmol g<sup>-1</sup>•dry soil) with standard error of a) saturated b) branched chain and c) unsaturated PLFAs in response to glucose [grey], glucose and bronopol [black], glucose and cycloheximide [white]. Lower case letters indicate statistical (P<0.05) differences among treatments for each specified PLFA.