

Can we measure microbial production in forest soils using labeled organics?

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Abstract

I tested an aquatic method for measuring microbial production in forest soils by adding a labeled organic substrate (^{14}C -leucine) and measuring the $^{14}\text{CO}_2$ respired. Other studies have used this method in the soil and found that the final percent of added label that gets respired is constant regardless of the amount added. They also find that the bulk of the respiration occurs within the first 1-2 hours and that all of the labeled substrate is taken up. This is evidence that amino acid additions are not causing microbes to grow but instead activates them to immediately respire whatever they are given. We found similar results, concluding that this method is not useful to measure the natural rate of amino acid respiration or microbial production in soil.

Introduction

Microbes are essential components of all ecosystems. They are also abundant with 10^6 microbes mL^{-1} in aquatic systems and 10^9 microbes mL^{-1} in forest and grassland systems. Ecologists want to measure their activity in natural systems because microbes control the recycling of nutrients in ecosystems. They do this by breaking down dead organic matter into mineral forms that plants can use.

One way to study microbes is to measure their production. In a laboratory, this can be done by culturing bacteria and counting them under a microscope. In aquatic systems this can be done by adding a small (1-10nM) amount of ^{14}C -labeled amino acid to a water sample and incubating it in an airtight flask for 1-3 hours. Scientists have measured both the evolution of $^{14}\text{CO}_2$ and ^{14}C incorporated into microbial protein using this technique. The $^{14}\text{CO}_2$ is captured on paper that has been soaked in the organic base, phenethylamine. The ^{14}C incorporated can be measured by filtering the microbes out of the water.

This method has also been used in soils by Jones et al (2005) and Vinolas et al 2001. Jones added five (0.1, 1, 10, 1000, 10,000 μM) concentrations of ^{14}C -Glycine to grassland soil samples. These levels were chosen based on the natural glycine levels in soil of 2-4 μM . They found that 15% of the glycine was mineralized to $^{14}\text{CO}_2$ within 2 hours, and mineralization stopped at 30% after 24 hours for all concentrations.

Using the 30% respiration they found in 24 hours and the total standing stock of amino acids in the soil (38 μM), Jones calculated the mineralization rate of 11 μM per day. Jones also found that all of the glycine not respired was removed from solution. Jones' results show rapid turnover of amino acids in soil, however the constant percentage of the added ^{14}C -glycine respired with different concentrations is evidence that this method may be inducing rapid respiration by the large pool of inactive bacteria in the soil.

The goal of this project study was to determine whether or not this method is adequate to directly measure the natural rate of microbial production in forest soils. I did this by dissecting the method as it has been used in grassland (Jones et al. 2005) and aquatic systems (Hobbie and Crawford 1969) and changing it where necessary to work on forest soils. The biggest change we made to the previous method used in soils (Jones et al 2005) was decreasing the concentration of amino acid added by several orders of magnitude. Rather than adding micro-molar (μM) concentrations we added the amino

acid, leucine at three (nM) concentrations. The hypothesis was that if the method is a good predictor of measuring microbial production, we will see less $^{14}\text{CO}_2$ evolved at the low concentrations because of isotope dilution.

Along with adding leucine alone, I performed several tests of the method including bacterial and fungal inhibitions, glucose addition, and homogenization of the soil. Adding un-labeled glucose along with ^{14}C -leucine was another way to test whether leucine additions were inducing respiration or growth. Kirchman et al. (1990) found that adding glucose in aquatic experiments stimulates microbial uptake of amino acids. Based on these results, I expected that if microbes were taking up ^{14}C -leucine for growth, adding glucose would increase bacterial growth and decrease the amount of ^{14}C -leucine that was respired. This is because leucine should be used by microbes to make proteins rather than respired for energy. Glucose could eliminate the need for energy.

A large source of error in soil studies is the method of handling samples. It is common practice to dry, homogenize, and grind soil samples before using them in studies. It is possible that through these practices, scientists are activating bacteria or mechanically extracting more amino acid from soils than is actually available to microbes. I tested this practice by running a time series similar to the control, except I ground the soil samples first. The goal was to determine whether or not grinding had a significant effect on amino acid respiration. I expected that it would either release amino acids that were held in soil aggregates or activate dormant microbes to respire more of the labeled substrate.

Methods

Soil collection

I collected soil from oak-dominated fertilized and non-fertilized (control) plots at the Falmouth Wastewater Treatment Forest. The OC site was an undisturbed oak stand while the OF site had been sprayed with inorganic-N rich effluent for 15 years, stopping treatment which ended in 2005.

I filled two 1-gallon bags of organic-layer soil from each forest and brought them back to the lab where they were opened and refrigerated. I also took a 10x10 cm sample from the oak control (OC) plot. This sample was used to find the soil's water-holding capacity. To do this, I saturated the soil completely then measured how much of the added water drained out naturally. I did this in order to determine how much water to add to each sample.

Addition of ^{14}C Leucine

The ^{14}C -leucine addition was from the aquatic method originally used by Hobbie and Crawford (1969) and Jones et al. (2005) in soil. I added 3 grams of soil (approximately 15 mL) to a 25 mL Erlenmeyer flask. ^{14}C -Leucine solution and DI water were added to make a total volume of 2.25mL. This volume was calculated as 50% of the organic layer field capacity (Lipson et al. 1999). The three ^{14}C -leucine concentrations were 400, 58 and 5.8 nM.

Immediately after ^{14}C -leucine addition, I closed the flasks with an airtight serum stopper. A plastic cup holding a 2 inch piece of Whatman no.1 chromatographic paper was suspended into the flask. The paper was folded like an accordion in order to fit into the cup. At the end of the incubation, I injected 0.2 mL of phenethylamine onto the chromatographic paper using a needle to pierce the serum stopper. I then left the sample on a shaker table for 1 hour. The original method (Hobbie and Crawford 1969) also

injected 0.2 mL of 2N H₂SO₄ into their aquatic samples to release dissolved CO₂, however we found that this was not necessary for a soil experiment. We began by adding the soil-equivalent of this (10mL of H₂SO₄). However repeating the experiments without it did not show significantly different results and we feared that adding this much volume to the airtight flask might be forcing gas out.

The chromatographic paper was then placed in a scintillation vial with 5 mL of ScintiVerse scintillation cocktail. Samples were read on the scintillation counter to determine the disintegrations per minute of ¹⁴C. From this we converted to moles of ¹⁴CO₂ evolved during the incubation.

Samples for the time series were taken 1, 4, 16, 24, and 48 hours after ¹⁴C-leucine addition. I also conducted a 6-hour short term series, taking samples every 30 minutes for the first 3 hours and every hour for the second three hours.

Bacterial and Fungal Inhibitions

Inhibitions were done using the method of Baath et al. (2003). To inhibit bacteria, 6 mg of Streptomycin Sulfate (Sigma Aldrich) was mixed with each ¹⁴C-leucine solution before adding it to the soil. Fungi were inhibited with 24 mg of Cycloheximide (Acros Organics) per sample. Inhibitions were run on the OC and OF soil samples with 58 and 5.8 nM ¹⁴C-leucine concentrations. The experiment ran for 48 hours, since this was the point at which the rate of uptake was similar in the original time series.

Glucose Addition

I added 1 mL of 0.5 uM glucose to each sample (OC, OF) for a total of 50 umoles per sample, and ran a full five point time series for the bottom two concentrations (58 and 5.8 nM).

Disrupting aggregates

I homogenized a subset of samples (OC, OF, 58nM, 5.8nM) and ground them with a mortar and pestle before adding ¹⁴C-leucine and running a time series. These samples were treated the same as the original time series.

Results and Discussion

The 48-hour time series from both OC and OF plots showed a distinctive pattern of rapid amino acid respiration. Both plots show between 4 and 6% of the added ¹⁴C evolved as CO₂ within the first 6 hours for all three concentrations (Figures 1 and 2). After 24 hours a total of 8-10% was respired for both forest types and the rate of ¹⁴CO₂ production had slowed significantly. The shape of this time series curve is similar to that of Jones et al. (2005), though the final percent respired is one third lower in this study. Jones et al. used the same method; however they added glycine rather than leucine which may explain the large difference in final percent respired. Crawford et al (1974) found that leucine tends to be respired less than glycine (14 versus 28%). Our highest concentration (400nM) is within the range that Jones et al. (2005) added, so it is fair to say that there is overlap between the two studies.

Results for the short term, 6-hour incubation are varied however they reveal that the majority of amino acid respiration actually happens within the first half hour of incubation (Fig. 3). This is consistent with the results of Jones et al (2007) who found that the majority of respiration occurred in the first 30-60 minutes.

We found that all of the ¹⁴C-leucine was either taken up by the soil and microbes or respired for both OC and OF plots (Fig. 4a-b). Jones et al. (2005) found similar results

for grassland soils, suggesting that it is common for soil microbial communities to absorb all of the amino acid available to them.

Bacterial and fungal inhibitors did not stop respiration of leucine alone or together at any concentration, and actually induced respiration in the lowest concentration. The samples with cycloheximide alone had especially high leucine respiration, between 50 and 60% (Fig. 5). One reason for this could be that cycloheximide was used as a substrate and induced a large portion of the previously inactive microbial community allowing them to respire a high percentage of the added ^{14}C leucine. Another explanation is that it induced microbial growth. The fact that growth inhibitors did not have a negative effect is either evidence that amino acid addition does not cause growth and that microbes are ready to take up any carbon substrate and respire it immediately.

Results for glucose addition were not significantly different from those without glucose. Though the final percent respiration was slightly lower (6% rather than 10%) than without glucose, the majority of $^{14}\text{CO}_2$ respiration still happened within the first 6 hours for all concentrations and forest sites (Fig. 6). Kirchman et al. (1990) followed a similar procedure and found that microbes in the ocean incorporate a higher percentage of added amino acids into their biomass when glucose is present than without glucose, suggesting that they were energy-limited as well as carbon-limited. We expected that if this method is effective in soil, the percent of added amino acid that is respired would decrease to make up for an increase in assimilation. The mineralization of amino acids by microbes in soil, rather than direct incorporation into proteins, is evidence that microbial growth is energy or carbon-limited. The lack of response to glucose addition in this experiment, however, is evidence that soil microbes are respiring amino acids for reasons other than carbon-limitation. It may be an over simplification to call soil microbes limited by any nutrient in fact since, as David White (1995) has described, microbes in soil can remain inactive until nutrients appear.

Disrupting soil aggregates has a large positive effect on amino acid respiration in the OC soil but not in OF soil. The positive effect could be caused by releasing bacteria that were previously locked up in aggregates and unable to reach the added leucine. There was less of a positive effect in the OF soil because there were fewer aggregations in that soil to break up. From observation, the soil texture was very different in the OF site than the OC site.

Conclusions

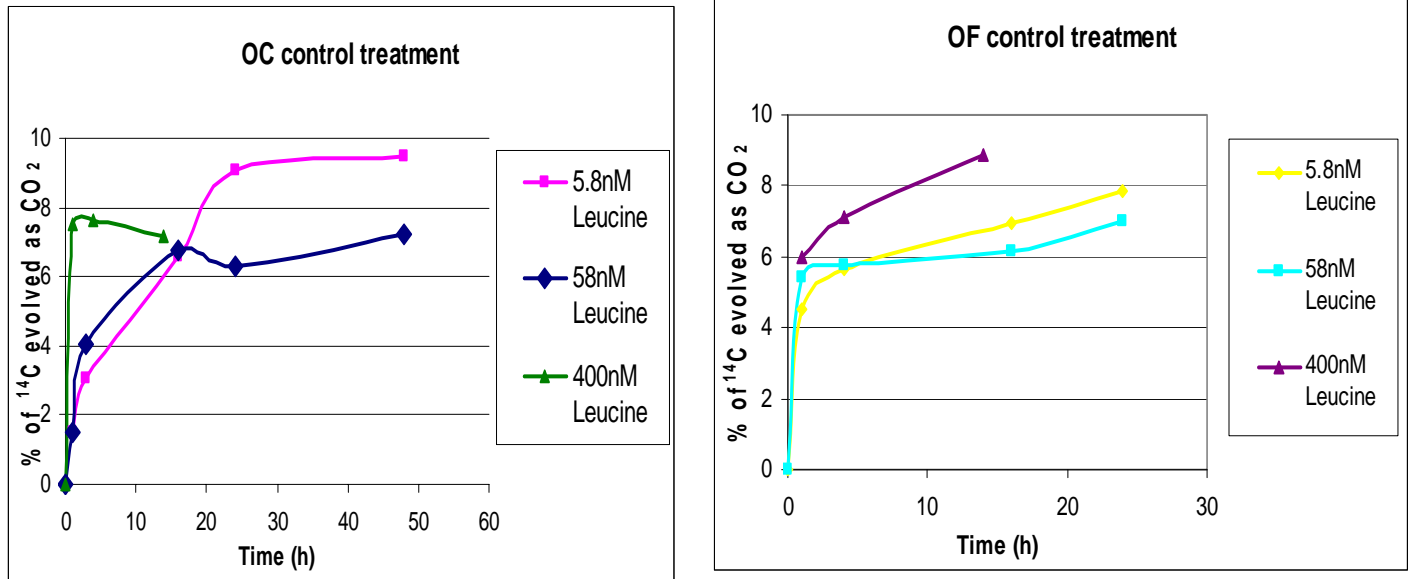
The difference between aquatic and terrestrial systems for this method is that there are a tremendous number of inactive bacteria in the soil. This is why amino acid addition causes substrate-induced respiration. It is impossible to measure the natural rate of respiration by adding labeled substrate because any addition will only induce microbes to that level. The results of this study also suggest that although all of the added amino acid is taken up as soon as it becomes available, it does probably not stimulate microbial growth, at least in the time frame of this experiment. While it is likely that the natural rate of amino acid turnover is quite high, it is impossible to measure it with this method in soil.

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Figures



Figures 1 and 2. Percentage of total added ^{14}C -leucine addition that was respired as $^{14}\text{CO}_2$ in the OC and OF plots.

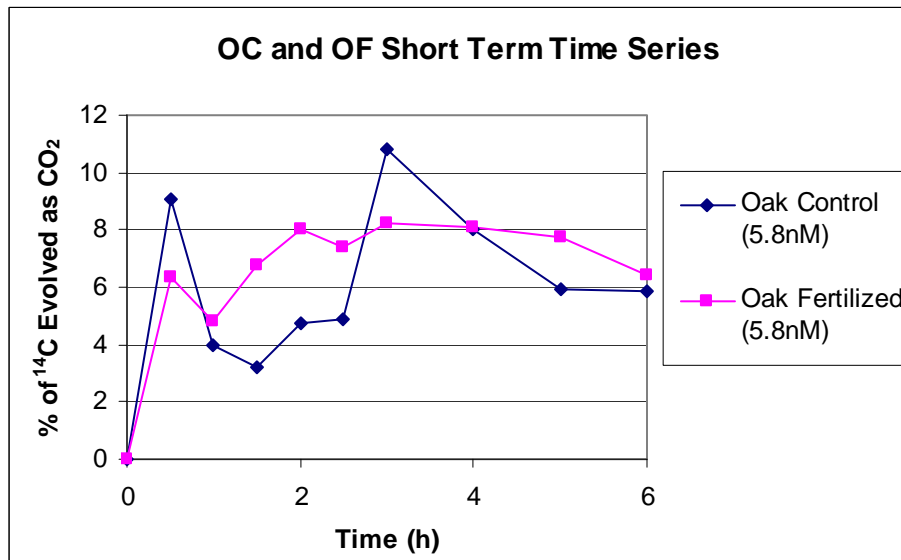


Figure 3. A short term time series for OC and OF plots with 5.8 and 58 nM ^{14}C -leucine addition. It is clear from this time series that the majority of amino acid respiration occurs

within the first half hour after addition.

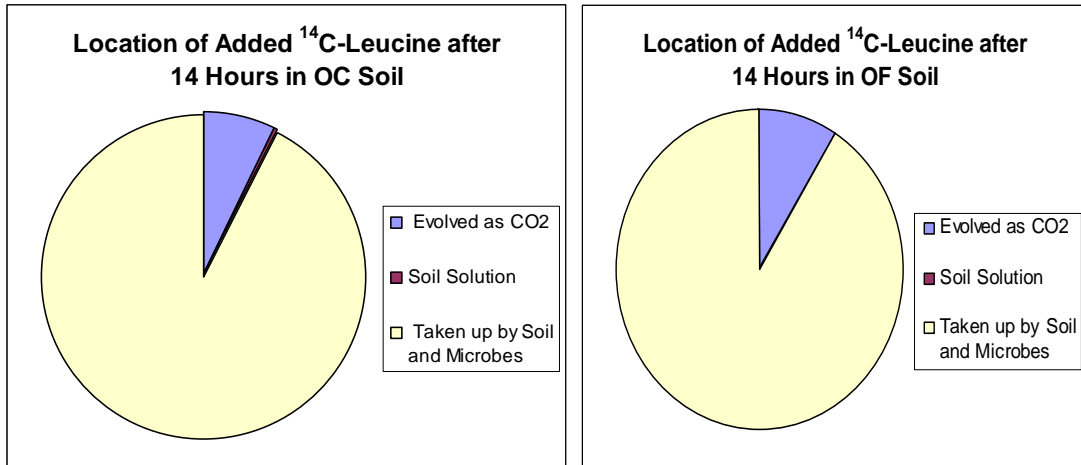


Figure 4a-b. ¹⁴C budget for OC and OF plots. The three possible locations for labeled leucine were 1) in CO₂, 2) in soil solution and 3) in soil microbial biomass. The CO₂ and soil solution were measured and the soil and microbial biomass was estimated by mass balance.

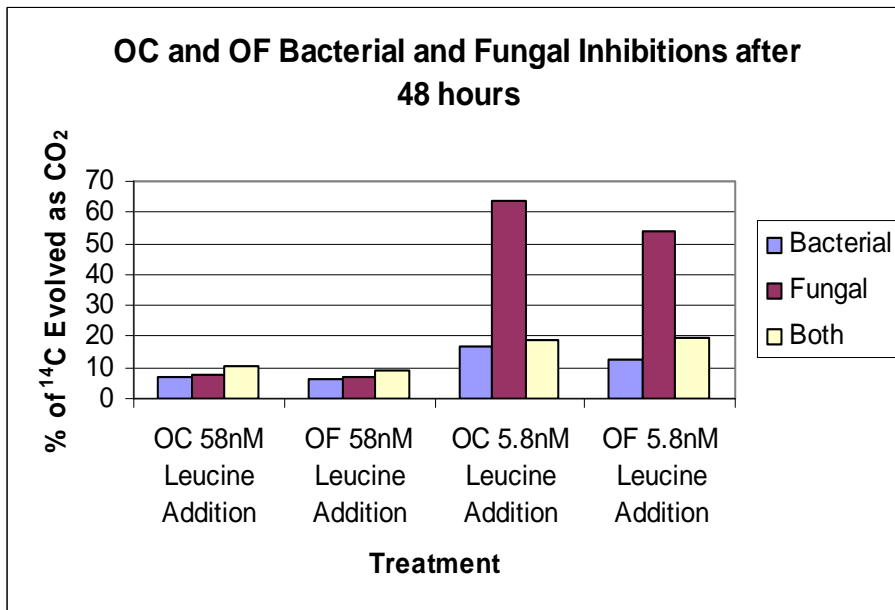


Figure 5. Results for bacterial and fungal growth inhibitor addition. The fungal inhibitor was 26 mg of Cycloheximide per sample and the bacterial inhibitor was 6 grams of

streptomycin per sample. These were all incubated for 48 hours.

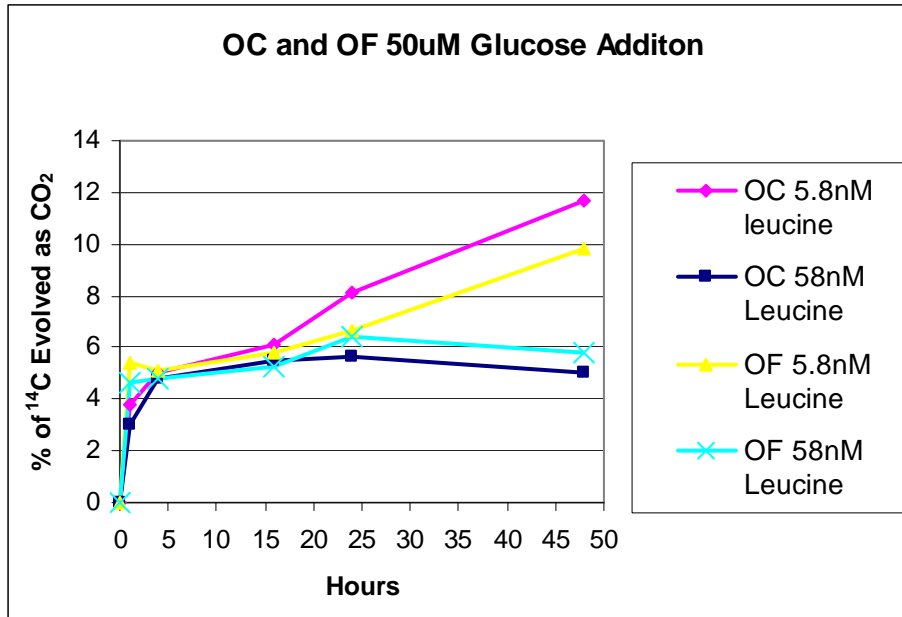


Figure 6. This shows the results for a time series with glucose addition. One mL of 0.05 M glucose (50 umoles total) was added to each sample along with 1 mL of either medium (58 nM) or low (5.8 nM) ¹⁴C-leucine. From these results, it does not appear that the microbes strongly preferred glucose over leucine.

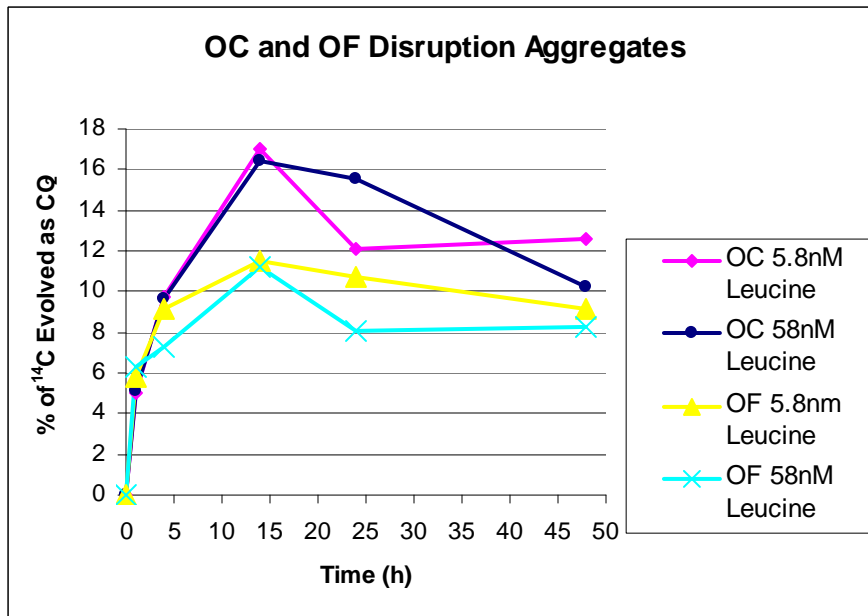


Figure 7. Disrupting soil aggregates was done with a mortar and pestle shortly before ¹⁴C-leucine addition. The results for this time series suggest that doing this increases the initial active microbial population either by growth or releasing the inhibitions of previously inactive microbes.

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