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# Separating cellular metabolism from exoenzyme activity in soil organic matter decomposition

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ABSTRACT

Soil organic matter (SOM) decomposes both inside and outside of cells. Cellular metabolism and extracellular depolymerization normally operate simultaneously in soil but are difficult to separate in practice. To learn more about the extracellular component of SOM decomposition, we sterilized a semiarid annual grassland soil to inhibit cellular metabolism, and then assayed cell viability, exoenzyme activities, and pathways of carbon dioxide ($CO_2$) emission. Chloroform ($CHCl_3$) fumigation was intended to disrupt cellular activities while leaving biochemical processes intact. Gamma ($\gamma$) irradiation and autoclaving were intended to disrupt both cellular and extracellular biochemical processes while leaving abiotic processes intact. We measured the potential activities of eight enzymes (six hydrolytic, two oxidative) and $CO_2$ emission induced by seven substrates (glucose, three amino acids, three tricarboxylic acid [TCA] cycle intermediates). We found that all three sterilization techniques clearly disrupted cellular metabolism. Chloroform and irradiation decreased cultivable cell counts by 2–3 orders of magnitude, inhibited $CO_2$ emission pathways associated with glucose and amino acids, and decreased the hydrolytic activities of α-glucosidase and xylosidase by 72–82%. The other hydrolytic enzymes (β-glucosidase, cellobiohydrolase, NAGase, phosphatase) were less sensitive to both $CHCl_3$ and irradiation. All hydrolytic activities that we assayed were inhibited by autoclaving, indicating that biochemical reactions and other extracellular processes drive hydrolytic SOM decomposition. Oxidative activities, on the other hand, did not stop after autoclaving or even combusting at 500 °C. This supports other studies which have found that mineral catalysts partly drive oxidative SOM decomposition. Unexpectedly, $CO_2$ emission from TCA intermediates decreased by only 26–47% after sterilization suggesting that the required dehydrogenase enzymes for decarboxylation are still active when cells are dead but relatively intact. Because $CHCl_3$ had slightly smaller effects on exoenzyme activities compared to irradiation, and because it may be continuously applied, limiting the potential for recolonization and regrowth (unlike irradiation), we suggest it is an adequate and more accessible method for separating the activity of exoenzymes from cellular metabolism under realistic soil conditions.

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## 1. Introduction

Microbes decompose soil organic matter (SOM) by both cellular metabolism and extracellular depolymerization. To breakdown plant detritus, both mechanisms are required; but there are many compounds in soil that may not require exoenzyme processing, such as root exudates and even the constituents of "stabilized SOM" (Guenet et al., 2012; Stockmann et al., 2013). We still know surprisingly little about the relative contribution of these mechanisms for SOM decomposition because they are difficult to separate in practice. Which substrates depend most on cellular metabolism for their degradation (Gralnick and Newman, 2007)? Which enzymes continue to function after cells die (Burns, 1982; Wallenstein and Weintraub, 2008)? And which pathways of carbon dioxide ($CO_2$) emission persist in sterile soil (Peterson, 1962; Ramsay and Bawden, 1983; Lensi et al., 1991; Maire et al., 2013)?

Ecosystem carbon models based solely on biology (e.g., microbial biomass; Moorhead et al., 2013) may be ignoring important biochemical mechanisms (exoenzymes) and even purely abiotic chemical reactions (Hall and Silver, 2013) involved in OM breakdown. These mechanisms are expected to be most important when microbes are less active or abundant due to limiting factors such as drought (i.e., slow diffusion), temperature (i.e., extreme hot and cold), and organic matter removal (e.g., erosion and tree

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harvesting) (Dungait et al., 2012; Schimel and Schaeffer, 2012; Ruamps et al., 2013). But even when there are few microbes alive to produce exoenzymes, dead microbes may leave a legacy on SOM decomposition through the continued action of their enzymes. To study this action, we need to eliminate cellular activities without eliminating extracellular activities.

Separating cellular from extracellular activities in soil is difficult, because you must either disable cellular metabolism without affecting exoenzymes or vice versa. In order to better understand extracellular SOM decomposition, we therefore need an effective sterilization technique that does not substantially alter soil structure, chemistry, or enzyme activities. Heat, for example, is effective sterilization; both moist (i.e., autoclaving) and dry heat kill microbes by denaturing cellular proteins, damaging DNA, disrupting cell wall, and liquefying cell membranes. But heat also alters soil chemistry and structure by oxidizing organic matter (Skipper and Westermann, 1973; Powlson and Jenkinson, 1976; Anderson and Magdoff, 2005), destroying exopolysaccharides (EPS) (Roberson et al., 1995; Or et al., 2007; Berns et al., 2008; Holden, 2011), and denaturing extracellular proteins (Shih and Souza, 1978; Van Stokkum et al., 1995). Toxic chemicals, such as mercuric chloride ($HgCl_2$) and sodium azide ($NaN_3$), inhibit many microbial activities, but they do not necessarily sterilize (Skipper and Westermann, 1973; Trevors, 1996), which is the only way to ensure that cellular metabolism is minimized. Fumigants such as ethylene and propylene oxide react with organic molecules and can increase soil pH (Clark, 1950; Allison, 1951; Wolf et al., 1989), which could indirectly alter enzyme activity by influencing ionic binding and stabilization (Sarkar et al., 1989; Allison, 2006). Therefore, none of these sterilization approaches are well suited to separate cellular from exoenzyme activities.

Two possible sterilization techniques that might fit the role are chloroform ($CHCl_3$) fumigation and gamma ($\gamma$) irradiation. Chloroform is chemically inert but is hydrophobic and so disrupts cell membranes by disorganizing the lipid bilayer, causing cells to lyse and to release their cell contents into the soil. Chloroform's ability to lyse cells is why it is used to estimate soil microbial biomass C and N (Jenkinson and Powlson, 1976; Brookes et al., 1985). Some enzymes are unaffected by $CHCl_3$ (Zelles et al., 1997), but others are (Klose and Tabatabai, 2002). In theory, those enzymes least damaged by cell lysis should be those best suited for the extracellular environment. The fumigation itself typically lasts 1–3 days (Jenkinson and Powlson, 1976; Foster, 1988; Dickens and Anderson, 1999). If given enough time, $CHCl_3$ diffuses into soil micropores and kills most—but not all—soil bacteria and fungi (Ingham and Horton, 1987; Alphei and Scheu, 1993; Toyota et al., 1996). Soils may also be incubated under $CHCl_3$ to prevent microbial regrowth.

Gamma irradiation is used widely in industry to sterilize medical equipment and food. It has also been used to sterilize soil (e.g., McLaren, 1969; Powlson and Jenkinson, 1976; Allison, 2006). Gamma is an ionizing radiation that damages DNA and, like $CHCl_3$, punctures the cell membrane causing lysis. Gamma irradiation is an effective sterilizing agent in soil (Jackson et al., 1967; Brown, 1981; Wolf et al., 1989), especially at doses above 20 kGy (Clark and Coleman, 1970; Lensi et al., 1991; McNamara et al., 2003). Even at high doses, gamma irradiation does not disturb soil structure and chemistry as much as autoclaving (Ramsay and Bawden, 1983; Alphei and Scheu, 1993; Denisova et al., 2005). However, we did expect that physical damage to proteins caused by ionizing radiation would disrupt enzyme activity more strongly than $CHCl_3$ (Brown, 1981; Vasileva-Tonkova and Chomoneva, 2004; Allison, 2006; Constantinovici et al., 2009), thus giving us a way to separate biotic, biochemical, and abiotic pathways of SOM decomposition without destroying soil structure.

To attempt to separate cellular metabolism from exoenzyme and abiotic activities in SOM decomposition, we subjected soil to three sterilization techniques and then assayed: (1) cell viability by culturing and live-dead staining with microscopy; (2) depolymerization by measuring the potential activities of eight enzymes; and (3) pathways of $CO_2$ emission by measuring respiration induced by seven substrates. We expected that gamma irradiation and autoclaving would disrupt both cellular and extracellular activities (Tiwari et al., 1988; Carter et al., 2007), whereas $CHCl_3$ fumigation would primarily disrupt cellular activities.

With respect to depolymerization, we expected assays of hydrolytic enzymes to be more sensitive to sterilization than assays of oxidative enzymes. Oxidative activities can be catalyzed by minerals (Brown, 1981; Hall and Silver, 2013) and, thus, we expected the oxidative assay to have a stronger abiotic component. Therefore, sterilization should have little effect if enzyme activities are extracellular and independent of microbial biomass (Moorhead et al., 2013). With respect to pathways of $CO_2$ emission, we wanted to see which types of substrates would be converted to $CO_2$ in sterile soil, much like a community-level physiological profiling (Garland and Mills, 1991) for the extracellular soil matrix itself. We expected all the added substrates to be processed only through cellular metabolism, and therefore to produce little or no $CO_2$ in sterile soil. We added glucose because it requires glycolysis before releasing $CO_2$ in the tricarboxylic acid (TCA) cycle. We also added TCA intermediates (pyruvate, citrate, α-ketoglutarate) and amino acids that undergo decarboxylation (L-glutamic acid, L-serine, L-phenylalanine).

## 2. Methods

### 2.1. Site description

Surface soils (0–10 cm) were collected in February 2013 from the University of California Sedgwick Reserve near Santa Ynez, CA (370 m ASL, 34.7120 °N, 120.0388 °W). The site experiences a Mediterranean-Type climate with hot dry summers and cooler wet winters. The mean annual precipitation and temperature are 380 mm and 17 °C, respectively. Roughly 90% of annual precipitation falls between November and April. The soil is mapped as a thermic *Pachic Argixeroll* with silty clay loam texture, pH 6.0, 2.2% C, and 0.21% N. Vegetation is dominated by nonnative Mediterranean annual grasses; primarily *Bromus diandrus*, *Bromus hordaceous*, and *Avena fatua*.

### 2.2. Soil collection and processing

We collected three 5 cm diameter soil cores—each roughly 20 m apart. The soil cores were sealed in plastic bags and transported to the laboratory at University of California Santa Barbara. Soils were stored at room temperature (20–22 °C) and air dried for 1 week. The air-dried soils (0.054 g $H_2O$ $g^{-1}$ soil) were then sieved (4 mm) and homogenized to make one composite sample. From this composite sample, three analytical replicates per sterilization treatment were used for the exoenzyme assays, and four analytical replicates were used for substrate-induced respiration (SIR) assays.

We weighed the air-dried soil subsamples (20 g) into autoclaved 50-ml glass beakers. The soil depth in the beaker was roughly 2.5 cm. Soil moisture was adjusted to 35% of water-holding capacity (WHC) by adding 3.5 ml of deionized water with a pipette (WHC = 0.69 g $H_2O$ $g^{-1}$ soil; 35% WHC = 0.24 g $H_2O$ $g^{-1}$ soil). All soils were then pre-incubated in the dark at 100% humidity at room temperature (20–22 °C) for 10 days to allow time for the effects of the wetting itself (i.e., Birch Effect) to subside. Soils in the 'alive' treatment were stored in the dark at 100% humidity for two weeks