Sticky Dead Microbes: rapid abiotic retention of microbial necromass in soil

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Abstract

Microbial necromass dominates soil organic matter. Recent research on necromass and soil carbon storage has focused on necromass production and stabilization mechanisms but not on the mechanisms of necromass retention. We present evidence from soil incubations with stable-isotope labelled necromass that abiotic adsorption may be more important than biotic immobilization for short-term necromass retention. We demonstrate that necromass adsorbs not only to mineral surfaces, but may also interact with other necromass. Furthermore, necromass cell chemistry alters necromass-necromass interaction, with more bacterial tracer retained when there is yeast necromass present. These findings suggest that the adsorption and abiotic interaction of microbial necromass and its functional properties, beyond chemical stability, deserve further investigation in the context of soil carbon sequestration.

Keywords

Soil organic matter; functional properties; stable isotope; grassland pasture; carbon sequestration; nitrogen

Main text

Soil organic matter (SOM) is a key indicator for healthy soil and sustainable agriculture (Paustian et al., 2016). Research into SOM stability has traditionally focused on the quality and quantity of plant inputs to soil (Lehmann and Kleber, 2015), however, recent research reveals that SOM is dominated by dead microbial products and residues (hereafter, 'necromass'; (Kallenbach et al., 2015; Liang et al., 2019, 2011)). The persistence of necromass in soil may be promoted via necromass uptake and immobilization into microbial biomass, but retention ultimately relies on microaggregate formation via adsorption to soil mineral surfaces. In actuality, both biotic (microbial immobilization) and abiotic retention (adsorption and molecular interaction) may co-occur but the relative importance of these short-term processes has not been assessed (Fig.1).

Necromass has been visualized on mineral surfaces (Kleber et al., 2011), supporting the paradigm that accumulation of stable SOM is dominated by organic-mineral adsorption, and is limited by mineral surface area (McNally et al., 2017). However, necromass is not detected as a smooth coverage on mineral surfaces, but clumpy (Dignac et al., 2017; Vogel et al., 2014), suggesting that SOM stabilization may also involve organic-organic interactions, or necromass adhering to other necromass and organic matter for example by ionic interactions, hydrogen bridges, van der Waals forces, and (partial) entrapment (Schweizer et al., 2018; Vogel et al., 2014) (Fig.1). Understanding the relative importance of these two abiotic adsorption processes will be critical for predicting upper limits of SOM persistence.

The chemistry of microbial necromass has been assumed unimportant as a regulator of SOM storage, because its chemical composition is more similar than diverse plant inputs (Liang et

al., 2017). Previous research on necromass chemistry and persistence has focused on its stability and emphasized chitin retention (Fernandez et al., 2016; Schreiner et al., 2014). However, cell chemistry can alter rates of cell-cell adhesion (Dufrêne, 2015) and organic-mineral adsorption rates (Meissner et al., 2015), particularly for N-rich necromass (Kopittke et al., 2017). Gram-positive bacterial envelopes have a thick cross-linked peptidoglycan layer outside the lipid membrane, whereas a Gram-negative cell envelope has an inner and outer lipid membrane enclosing a thinner peptidoglycan layer in the periplasm. Fungal cell walls are highly heterogeneous; yeast cell walls, for example, are composed of layered mannan, β-glucans and chitin outside a lipid membrane. Therefore, cell-membrane functional groups with a high N-content, such as peptidoglycan-rich Gram-positive bacteria, may be favored for organic-organic interaction, relative to Gram-negative bacteria or fungi. Given the potential for broad shifts in microbial community composition as a result of global change, land use change or even seasonality (Buckeridge et al., 2013; Ramirez et al., 2012), necromass cell chemistry may influence SOM stabilization at ecosystem scales.

We investigated the importance of biotic and abiotic necromass retention in grassland soil and the influence of necromass chemistry on this retention in short-term laboratory incubations. We hypothesized: H1. both biotic and abiotic necromass retention occur, and that biotic retention is more important; H2. more necromass is retained in soil with higher background concentrations of necromass (implying organic-organic adhesion); and H3. abiotic retention would be higher for Gram-positive bacterial membranes (implying cell chemistry is important). We tested these hypotheses with short-term (3-d) laboratory incubations of clay-loam agricultural pasture soil; the duration was chosen to limit biotic processing of rapid abiotic interactions. Detailed methods are available in supplementary

methods. Soil was assigned to live and sterile treatments with enhanced background necromass (~5.5 mg C and ~1.5 mg N g^{-1} soil fwt) from three soil microbial functional groups (Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*Micrococcus luteus*) and yeast (*Saccharomyces cerevisiae*)), and incubated with a no-addition control. We also added a small amount of isotopic tracer necromass consisting of $^{13}C^{15}N$ labeled (10 atm%) *E.coli* necromass (0.050 mg C and 0.016 mg N g^{-1} soil fwt) to all treatments; the dual label was used to aid assessment of biotic vs. abiotic retention. Headspace CO_2 and N_2O samples were collected throughout the 3-day incubation. At the end of the incubation, we used the chloroform-direct-extraction method to estimate microbial biomass immobilization of the tracer (measured in all, detected only in live soils), and the balance between immobilization and loss as CO_2 was used to calculate carbon use efficiency of the live microbes. The C, N, $\delta^{13}C$ and $\delta^{15}N$ of the post-extraction soils (i.e. removing any free or loosely-bound C or N) was used to assess total retention (assumed biotic + abiotic in the live soils and abiotic only in the sterile soils), and mixed models were used to assess the statistical significance (α=0.05) of our results.

Retention of C from the tracer necromass was lower in live vs. sterile soils (P=0.006), rejecting H1 and indicating that short-term retention of necromass C was dominated (>70%) by abiotic processes (Fig.2a). Our results, however, cannot confirm that biotic processes are unimportant for persistent SOM-C accrual. Our controlled environment incubation may have overestimated the importance of abiotic processes: in a more dynamic, natural system with active plant-microbial interactions, live microbial immobilization of necromass-C and plant and microbial uptake/immobilization of necromass-N is potentially critical to retention. Furthermore, our lab-grown, single-culture additions only approximate the

97 retention of chemically and taxonomically-complex native necromass. Nonetheless, our
98 results illustrate an important short-term effect of abiotic C retention in soil.

99 The lower retention of ^{13}C in live vs. sterile soils was not predicted and we suspected this
100 was either a side-effect of sterilization, or CO_2 loss from microbial activity. We discounted
101 the sterilization effect, because we did not see a parallel lower retention of necromass-^{15}N
102 in live compared to dead soils (Fig.2b). This, combined with no change in C:N ratio of tracer
103 retention in sterile soils and a drop in C:N ratio of retention in live soils ($P<0.001$, data not
104 shown) equates with live microbial processing of C, associated with immobilization/loss of
105 4-10% of the total C added, as extractable biomass or CO_2 (Fig.3a-b). Losses of N_2O from the
106 labeled necromass were comparable to CO_2 (<2%, Fig.3c) but we presume that N uptake to
107 microbial biomass (not measured) would be lower than C, reflecting the lower N demand for
108 growth in these N-rich pasture soils. C-starvation during incubation may have resulted in use
109 of necromass-C for maintenance, reflected in a low substrate use efficiency (CUE) (Fig.3d).

110 Higher C and N was retained in live and sterile soils with enhanced background necromass
111 compared to controls (~10-40 % higher, Fig. 2a-b), supporting the hypothesis (H2) that
112 necromass may be adhering to necromass. Organic-organic adhesion has been suggested
113 previously based on isotopic and visual evidence in laboratory and long-term field studies
114 (Schweizer et al., 2018). We did not observe higher C and N retention in soils with enhanced
115 peptidoglycan (*M. luteus*) necromass, rejecting H3, that abiotic retention would be higher
116 for Gram-positive bacterial membranes. However, we provide novel evidence that the
117 retention of C and N from the necromass tracer was higher in live and dead soils augmented
118 with *S. cerevisiae* necromass (C: $P<0.001$; N: $P=0.03$). This higher microbial C and N retention
119 in *S. cerevisiae*-amended soils does not appear to be a biotic process, because the CUE of

the tracer necromass in live soils did not differ between background necromass types (Fig. 3d) (despite all enhanced background necromass treatments being lower than the no-addition control, which was presumably a response to higher substrate concentration relative to the control (Geyer et al., 2019)). Instead, this enhanced retention of the tracer necromass in the presence of *S. cerevisiae* necromass may be indicative of faster or stronger interaction between the complex morphogenesis of the Gram-negative outer cell membrane and yeast cell walls, such as occur in live microbial communities (Dufrêne, 2015). Further, compound-specific research is required to understand if the properties of yeast necromass extend to other fungal necromass generally and to specific bacterial membrane and fungal cell wall compounds. Regardless, this result indicates that cell chemistry contributes to an adhesion mechanism that promotes necromass stability in soil.

We conclude that abiotic processes are important for short-term retention of necromass-C and N in soils and require greater emphasis in studies investigating SOM stability. Our results support research that indicates organic-organic interaction promotes retention of C and N and contributes novel evidence that this mechanism is regulated by cell chemistry. If this short-term abiotic retention occurs *in situ* and persists, then microbial community structure and possibly the fungal:bacterial ratio, may influence C and N stabilization through variations in community cell chemistry. Field additions of isotopically-labelled necromass from different taxa, in different soils, and followed by secondary ion mass spectrometry (nanoSIMS), would be a valuable start. These findings suggest that abiotic adsorption and interaction of microbial necromass and its functional properties beyond chemical stability (i.e. cell molecular property, aggregations, and morphology), deserve further investigation in the context of soil carbon sequestration.

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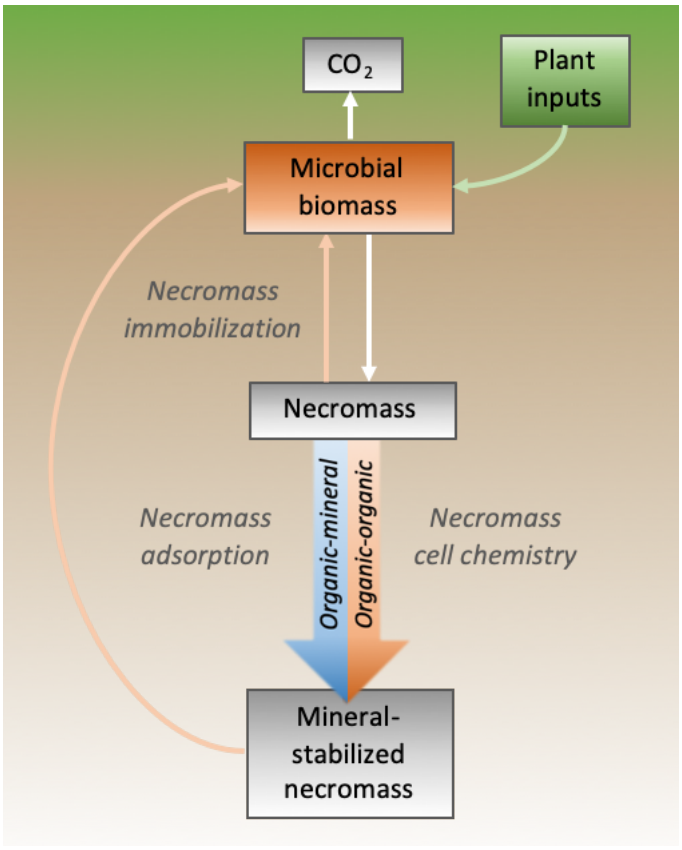
Figure captions

Figure 1. Schematic diagram of microbial necromass fate in mineral soils. In this diagram, necromass is presented as a substrate for microbes, similar to plant inputs (litter or exudates). Microbes can acquire unassociated or mineral-associated necromass for substrate. Immobilization includes recycling of necromass by microbes into new biomass, and eventually necromass, with potential for some loss as CO_2. Stabilization is assumed to be through adsorption to mineral surfaces ('organic-mineral'), especially on fine silts and clays. In this study we hypothesize that this process is not limited to mineral surface availability ('organic-mineral'), but that necromass also adheres to necromass ('organic-organic'), promoting retention, and that this organic-organic process may be influenced by necromass cell chemistry.

Figure 2. The retention of necromass ($^{13}C^{15}N$-*E.coli*) carbon and nitrogen in live and sterile soil with different background necromass. Data presented as the mean (± standard error, n=5) % retention of applied $^{13}C^{15}N$ necromass as **(a)** carbon (C) or **(b)** nitrogen (N) after 3 days incubation in live (microbial immobilization and adsorption) and sterile (adsorption

only) agricultural pasture soils. Tracer $^{13}C^{15}N$-labeled *E.coli* necromass was added (0.050 mg C and 0.016 mg N g^{-1} soil fwt) in combination with different background (i.e. unlabeled) necromass (control, *E.coli*, *M. luteus* or *S. cerevisiae* (~5.5 mg C and ~1.5 mg N g^{-1} soil fwt)). Sterile soils were either autoclaved or gamma-irradiated (results pooled) and thus presumably have a slightly higher level of background necromass C and N from the recently killed natural soil microbial community (~ 0.38 mg C and 0.030 mg N g^{-1} soil fwt). 'Retention' is calculated from the amount of label remaining in the water-extracted soil residue at the end of a short-term (3 d) incubation. Upper and lower-case letters within each plot indicate a significant difference between live and sterile treatments, and different letters indicate significant difference between necromass treatments (α=0.05).

Figure 3. Tracing the gaseous loss, biotic immobilization and carbon use efficiency of necromass tracer ($^{13}C^{15}N$-*E.coli*) in live soils with different background necromass. Data presented as the mean ($\pm$ standard error, n=5) % of applied $^{13}C^{15}N$ necromass in **(a)** CO_2, **(b)** microbial biomass ('MBC'), and **(c)** N_2O, and **(d)** the carbon use efficiency ('CUE') of necromass mineralization after 3 days incubation. Tracer $^{13}C^{15}N$-labeled *E.coli* necromass was added (0.050 mg C and 0.016 mg N g^{-1} soil fwt) in combination with different background (i.e. unlabeled) necromass (control, *E.coli*, *M. luteus* or *S. cerevisiae* (~5.5 mg C and ~1.5 mg N g^{-1} soil fwt)). Different letters within each plot indicate significant difference between necromass treatments (α=0.05).



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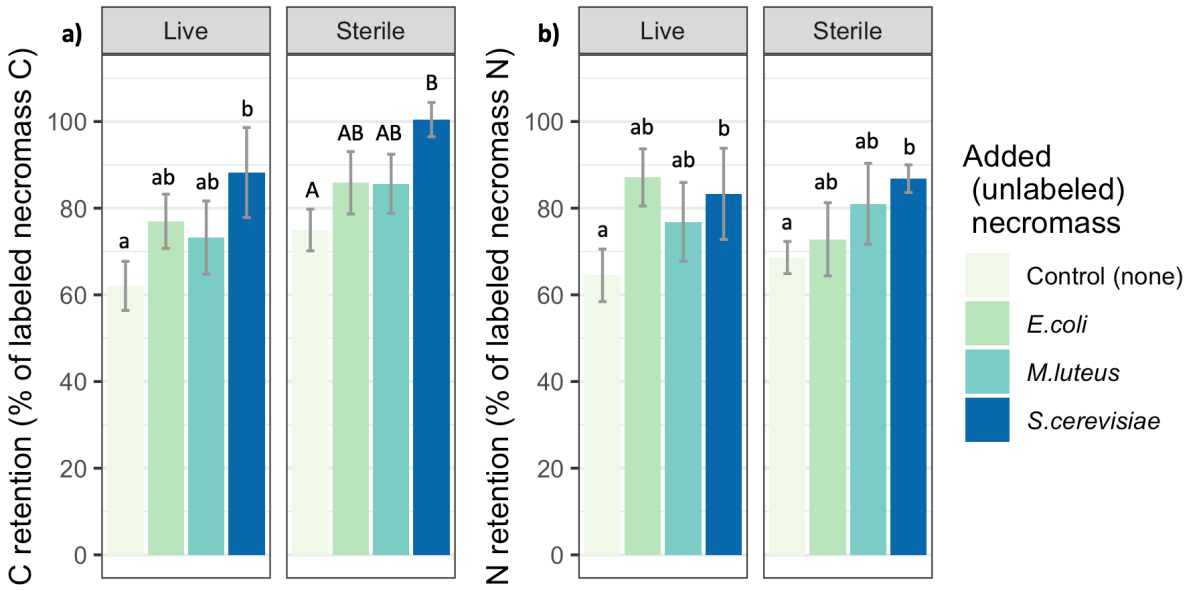

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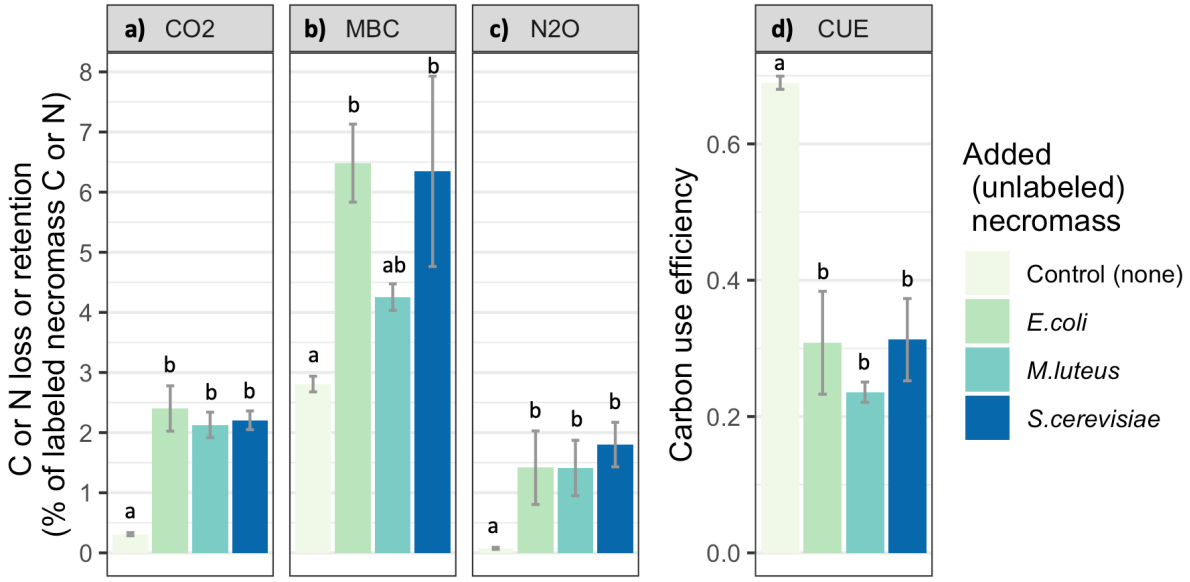

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275 **Figure 3. Tracing the gaseous loss, biotic immobilization and carbon use efficiency of**

276 **necromass tracer ($^{13}C^{15}N$-*E.coli*) in live soils with different background necromass.** Data

277 presented as the mean (± standard error, n=5) % of applied $^{13}C^{15}N$ necromass in **(a)** CO_2, **(b)**

278 microbial biomass ('MBC'), and **(c)** N_2O, and **(d)** the carbon use efficiency ('CUE') of

279 necromass mineralization after 3 days incubation. Tracer $^{13}C^{15}N$-labeled *E.coli* necromass

280 was added (0.050 mg C and 0.016 mg N/g) in combination with different background (i.e.

281 unlabeled) necromass (control, *E.coli*, *M. luteus* or *S. cerevisiae* (~5.5 mg C and ~1.5 mg

282 N/g)). Different letters within each plot indicate significant difference between necromass

283 treatments (α=0.05).

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Supplementary Methods (online only)

To test our hypotheses, we used short-term (3 day) live and sterile soil incubations of clay loam soil (order: Chromic Eutric Albic Stagnosol; pH: 6.8; texture: 53 % sand, 19 % silt, 28 % clay; bulk density: 0.96 g soil cm^{-3}; organic matter: 4.8 % C, 0.48 % N, 0.19 mg MBC (g dw soil)$^{-1}$) managed as an agricultural pasture (247 kg N ha^{-1} y^{-1} (slurry and manure), 2-3 mows y^{-1}, 0.21 livestock units ha^{-1} y^{-1} (cattle equivalent)), from northwest England (53°50'57"N, 2°47'01"W). The soil was collected from the top 10 cm of 1 m^2 plots separated by 20 m (n=5), sieved to 2 mm and adjusted to 60% water holding capacity, then subsampled to live and sterile treatments in 500 mL laboratory bottles fitted with septum-modified lids (γ-irradiated soils were sterilized in triple-layered polyethylene bags and added to sterile laboratory bottles at the start of the incubation). Soil sterilization was either via autoclaving (2x 120°C for 30 min) or γ-irradiation (>75kGy); we used two different methods to isolate effects of sterilization itself on C and N retention. Specifically, we were concerned that autoclaving more than γ-irradiation would release protected pools of C, confounding our results by providing new sites for C and N retention. We found no difference ($P<0.05$) between autoclaving and γ-irradiation, so we pool those data ('sterile') in our results (by averaging pairs to not inflate degrees of freedom; n=5). Necromass was made by growing cultures from streak plates in media (including unlabeled or labeled glucose (^{13}C) and ammonium chloride (^{15}N)), spinning down biomass to a pellet, rinsing, autoclaving twice, freeze-drying, subsampling the powder to aliquots and autoclaving again. The incubation included unlabeled necromass to enhance the background concentration of necromass in the soil: we assumed that soil necromass C is at least 50% of soil organic C (Liang et al., 2011) and doubled that amount by adding lab grown necromass (~5.5 mg C and ~1.5 mg N

308 g^{-1} soil fwt) from three functional group chemistries (Gram-negative and Gram-positive
309 bacteria (*Escherichia coli* (%C: 44.2, %N: 13.9, C/N: 2.7) and *Micrococcus luteus* (%C:
310 40.3, %N: 11.5, C/N: 3.0)) and a yeast (*Saccharomyces cerevisiae* (%C: 41.8, %N: 9.6, C/N:
311 3.7))), and maintained a no-addition control. To quantify retention, we added a very small
312 amount of isotopic tracer necromass consisting of $^{13}C/^{15}N$ labeled (10 atom %) *E. coli*
313 necromass (0.050 mg C and 0.016 mg N g^{-1} soil fwt) to all samples, included a natural
314 abundance control, and assessed retention of the tracer as a proxy for necromass retention.
315 To maintain sterile conditions, in a laminar flow hood we processed the jars (60 g fw soil
316 each) over the course of one day in groups of 6 jars (necromass-types and controls),
317 processed 3 times (live/sterile treatments), repeated for each soil replicate (n=5). Sterile
318 background necromass was added as a powder to addition treatments and all soils were
319 lightly shaken to distribute the powder through soil or to mimic this addition in the controls.
320 By jar, 3 mls of sterile labelled necromass solution or water was added, lids were
321 immediately and tightly closed, and initial headspace gas samples were collected through a
322 filtered syringe. Headspace gas samples were collected a total of 4 times throughout the 3-
323 day incubation, plus a final gas sample was collected for isotope analysis. At the end of the
324 incubation, we aseptically subsampled 20 g fw soil to chloroform-added and non-
325 chloroform-added extraction tubes, and used chloroform direct extraction (Fierer et al.,
326 2003), with 35 ml water as an extractant. Soil and extractant were shaken for 1 hr on a
327 rotary shaker, centrifuged, and filtered, then sparged to remove excess chloroform.
328 Extractable organic C (EOC) and extractable nitrogen (EN) of the sparged and blank-
329 corrected chloroform extracts and water extract controls were used to calculate microbial
330 biomass uptake of the label (measured in all but only detected in the live soils), on a
331 Shimadzu TOC-L CPN (Shimadzu UK Ltd., UK). To measure substrate uptake into biomass,

chloroform extracts were freeze-dried, ground and analyzed for $\delta^{13}C$ on a Costech CN streamed to a Picarro G-2131i CRDS. We analyzed CO_2 and N_2O concentrations in the headspace gas samples on a Perkin-Elmer Autosystem XL gas chromatograph (GC) fit with a methanizer and flame ionization detector (CO_2) and an electron capture detector (N_2O). The $\delta^{13}C$ and $\delta^{15}N$ signatures of the headspace gas samples were analyzed on Picarro G2131-*i* (CO_2) and Picarro G5131-*i* (N_2O) Isotope and Gas Concentration Analyzers (cavity ringdown spectrometers). We used CO_2, N_2O and MBC concentrations and their delta values in mixing models to calculate labelled necromass incorporation into live-treatment CO_2, N_2O, and MBC, and the carbon use efficiency of the live microbes. The C, N, $\delta^{13}C$ and $\delta^{15}N$ of the post-extraction soils (i.e. the extraction soil remaining on the filter) were used to assess total abiotic and biotic retention. Soils were oven-dried, ground and analyzed for $\delta^{13}C$ on a Costech CN Analyzer streamed to a Picarro G2131-*i* Isotope and Gas Concentration Analyzer, and for $\delta^{15}N$ on a Carlo Erba NA1500 Elemental Analyzer coupled to a Dennis Leigh Technologies IRMS. We used mixed models to assess the statistical significance (α=0.05) of our results with two fixed factors (live or sterilized, and added necromass type) and one random effect (plot) using the function lmer (package lme4), Anova (car) and glht (multcomp) in R (3.2.4).