Isothermal microcalorimetry provides new insight into terrestrial carbon cycling

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ABSTRACT

Energy is continuously transformed in environmental systems through the metabolic activities of living organisms, but little is known about the relationship between the two. In this study, we tested the hypothesis that microbial energetics are controlled by microbial community composition in terrestrial ecosystems. We determined the functional diversity profiles of the soil biota (i.e. multiple substrate-induced respiration and microbial energetics) in soils from an arable ecosystem with contrasting long-term management regimes (54 y). These two functional profiling methods were then related to the soils' microbial community composition. Using isothermal microcalorimetry, we show that direct measures of energetics provide a functional link between energy flows and the composition of belowground microbial communities at a high taxonomic level (Mantel R = 0.4602, P = 0.006). In contrast, this link was not apparent when carbon dioxide (CO_2) was used as an aggregate measure of microbial metabolism (Mantel R = 0.2291, P = 0.11). Our work advocates that the microbial energetics approach provides complementary information to soil respiration for investigating the involvement of microbial communities in belowground carbon dynamics. Empirical data of our proposed microbial energetics approach can feed into carbon-climate based ecosystem feedback modeling with the suggested conceptual ecological model as a base.

INTRODUCTION

Life above- and belowground has evolved complex and diverse communities and a key issue in ecology is to explore the functional significance of community composition. Despite the central role of soil microorganisms in the Earth's biogeochemical cycles, the importance of microbial diversity in ecosystem functioning is still debated[1]. The regulation of our climate and the carbon cycle is an important ecosystem service and function. Soil organic matter is the largest carbon pool in terrestrial ecosystems and soils are therefore major players in the global carbon cycle[2]. Organic matter contains energy-rich bonds and is the primary energy source for the abundant and diverse soil biological communities. Through metabolic activities, heterotrophic microorganisms utilize energy stored in organic matter and exchange it within the biosphere and with the atmosphere.

According to the second law of thermodynamics, high order energy (exergy) dissipates as low order energy from a system over time and this process is irreversible. From an energy point of view, soil ecosystems can be characterized as open systems of non-equilibrium thermodynamics with the decomposition of soil organic matter to carbon dioxide (CO_2) as a dissipative process that increases entropy[3,4]. Microbial metabolism is divided into two categories: catabolic reactions that release energy and anabolic reactions that demand energy. An example of catabolic reactions in soils is the breakdown of organic material into smaller compounds which releases energy necessary for anabolic biosynthetic reactions. Energy not required for anabolic processes is dissipated as heat and CO_2 is released from the soil system into the atmosphere. However, we do not grasp in detail how life belowground abides by the second law of thermodynamics[5,6].

Isothermal microcalorimetry provides information on heat flows of all processes with very high precision[7]. It is of particular interest for studying microbial involvement in soil carbon dynamics as it quantifies all microbial metabolic processes (i.e. the net outcome of catabolic and anabolic processes) not only accounted for by CO_2 measurements. As such, it is an alternative, yet complementary, approach to CO_2 production for exploring microbial activity and carbon dynamics in soil systems. Further information on the use, advantages and challenges of isothermal microcalorimetry in soil and environmental sciences can be found in comprehensive reviews[7-9]. The calorespirometric ratio (ratio of heat-to-CO_2-C) has been used to evaluate metabolism and metabolic efficiency in soil systems[10,11], and this ratio appears to vary among soil systems with different land uses[11,12]. Recently, Harris and co-workers[13] proposed a dimensionless index of microbial thermodynamic efficiency determined using isothermal microcalorimetry. The index is based on the ratio of energy output in relation to energy input. Small values of this index indicate that microbial energetics are efficient; in other words that the biota has the ability to minimize energy dissipation from a system whilst maintaining metabolism. Although it is known that soil organisms require both energy and carbon to drive belowground processes, little is known about how energy flows are linked to the carbon cycle and if there is a relation between microbial energetics and microbial community composition in the soil. A better understanding of the relationship between the two is likely to help evaluate the efficiency of carbon allocation in soil ecosystems and the consequences of the different efficiencies.

Soil organisms have developed diverse life strategies to assimilate carbon and energy for maintenance, growth and reproduction[14], and they can rapidly adapt to changes in external environmental conditions[15] through alternative biochemical pathways[16]. Although the

allochtonous *r*- versus zymogenous K-selection concept[17] has been criticized as being an oversimplified view of the processes of natural selection in ecology[18], it is still consistent with modern interpretation of community type and soil microbial functioning[14]. In general, allochtonous *r*-strategists are adapted to rapidly acquiring resources when abundant and maximizing their growth rate. These organisms generally release a larger fraction of organic material to the atmosphere as CO_2. In comparison, zymogenous K-strategists have developed a suite of extracellular enzymes[19-21] to break down complex organic material and they are therefore adapted to competing and surviving when resources are limited. In ecosystems dominated by K-strategists, it is assumed that more of the organic material is sequestered in soils through carbon allocation to microbial cell maintenance and synthesis of extracellular components such as enzymes, polysaccharides, metabolites, proteins etc.[22]. Consequently, different soil microbial communities are likely to call upon different biochemical pathways resulting in different carbon and energy flows through the communities and ecosystems. Under this scenario there may be divergences between CO_2 production and energy utilization among microbial communities with different makeups in the short-term and potentially long-term consequences for the carbon cycle in soil.

Here, we tested the hypothesis that the composition of microbial communities in soils and their functioning controls energy flows as soil organisms have developed diverse biochemical pathways and life strategies. The general assumption is that measurements of microbial energetics provide a more subtle description of microbial processes related to the carbon cycle than do measurements of microbial CO_2 production. Soils from an arable ecosystem which differ only in their contrasting long-term organic matter inputs were chosen[23] to avoid the confounding effects of major soil properties such as soil texture or pH (SI Table 1). For illustration purposes,

we also proposed a conceptual ecological model of microbial energetics in terrestrial ecosystems in which the different energy flows are explicitly described (Scheme 1).

EXPERIMENTAL SECTION

We established a laboratory experiment in which we added a range of carbon substrates to soils from an arable ecosystem in order to test the hypothesis. Seven substrates (see SI Table 1 for details on all substrates used) or Milli-Q water as control were added separately to either non-sterile or gamma-irradiated sterile soil samples. The release of heat or CO_2 after substrate and water additions to gamma-irradiated soil was also included in order to account for abiotic processes (abiotic CO_2 evolution[24] or substrate interactions with soil matrix[25]). We then measured substrate-induced CO_2 production[26] and energy flow profiles and determined the strength of the relationship between these profiles and microbial community composition[27].

Site and Sample Collection. Soils were sampled from the Ultuna Long-Term Soil Organic Matter Experiment (Uppsala, Sweden; 60°N, 17°E)[23]. The experiment was started in 1956 on a postglacial clay loam classified as an Eutric Cambisol. In this experiment, soils (2 x 2 m blocks) have been treated with mineral nitrogen fertilizers (80 kg N $ha^{-1}y^{-1}$; applied annually as either $Ca(NO_3)_2$ or $(NH_4)_2SO_4$) or organic amendments (biennial addition at 8 Mg ash-free organic matter $ha^{-1}y^{-1}$). The treatments are replicated in four blocks, but one of the four blocks does not have randomly distributed treatments and was therefore omitted from the present study. At the end of May 2010, four treatments were selected, *viz.* (i) Green Manure, (ii) Straw+calcium nitrate, (iii) Farmyard Manure and (iv) Peat+calcium nitrate (approx. 6 months after the last application of organic manure). Eight sub-samples from 0-7 cm depth were taken from each replicate block, sieved < 2mm, composited and mixed per replicate block and stored frozen until

spring 2012. Soils were then adjusted to 45% of their water holding capacity (WHC) and pre-incubated for two weeks at 25°C to allow any disturbance due to sieving to subside.

Substrate-Induced Respiration. The use of multiple substrate-induced respiration (MicroResp™ approach[26]) is often used to evaluate the functional diversity status of the soil biota and to investigate carbon dynamics in soils. The correct use of this approach requires that sufficient substrate is provided to saturate the microbial respiratory metabolism. For this study, seven substrates and recommended carbon concentrations[26] were selected: γ-amino butyric acid, D-glucose, citric acid and α-ketoglutaric acid were prepared so that 30 mg of C substrate per mL soil water were supplied to each well; substrates that did not readily dissolve in water (i.e. *N*-acetyl glucosamine, L-alanine and α-cyclodextrin) were supplied at a concentration of 7.5 mg C mL^{-1} soil water. These substrates are commonly used in functional diversity profiling and they have shown to discriminate between different soil microbial communities.[26,28] For each soil treatment, soil samples (300 µL total volume per well, approx. 0.5 g dry soil) were added to a 96-well microtiter deep well plates and then 30 µL of each substrate was dispensed to each deep well (four replicate wells per substrate plus four Milli-Q water controls). The substrate addition brought the water content to 65% of WHC and soils were incubated at 25° for eight hours. After 2 hours, the gel detector plates were mounted onto the microtiter plate system and substrate induced respiration was measured between 2-8 h. The gel detector plates were then read in a plate reader (Multiskan RC, Labsystem Finland). A calibration curve of absorbance (x) versus headspace equilibrium CO_2 concentration (y) was measured independently and absorbance data from microtiter deep well plates were fitted to a power decay model ($R^2 = 0.976$) as follows: $y = 0.0499x^{-2.702}$.

143 **Microbial Energetics.** For each soil treatment, eight aliquots of soil (5 g) were placed into 20
144 mL glass reaction vessels and each vessel was sealed with an admix ampoule set up consisting of
145 two 1 ml syringes (SI Fig. 1). Each admix ampoule contained either one of the seven substrates
146 mentioned above or Milli-Q water as control (SI Table 1). The samples where then introduced
147 into a TAM Air isothermal micro-calorimeter (TA Instruments Sollentuna, Sweden) with the
148 thermostat set to 25°C. The calorimeter was then sealed and the samples were allowed to
149 equilibrate for 3 hours. After equilibration, the plungers of the two syringes were slowly pressed
150 down to add the C substrates and Mill-Q water control drop wise (60 µL per gram of soil
151 corresponding to the same volume as for substrate-induced respiration described above) and heat
152 flows were determined over 8 hours after substrate addition.

153 **Assessment of Abiotic Processes.** For each soil treatment, (i) one set of soil samples (300 µL
154 total volume per well) were added to a 96-well microtiter deep well plates to assess CO_2
155 evolution due to abiotic processes[24], and (ii) eight aliquots of each soil treatment (5 g soil) were
156 weighed into 20 mL glass reaction vessels to evaluate substrate interactions with soil physical
157 properties. The plates and reaction vessels were covered with aluminum foil and samples were
158 then gamma-irradiated to sterilize them (CODAN Steritex APS, Espergaerde, Denmark) at a
159 minimum of 25 kGy. Samples were then kept in a laminar flow cabinet for 36 hours to avoid
160 contamination. To ensure complete sterilization, gamma-irradiation was repeated and samples
161 were then allowed to settle for four weeks. Seven C substrates (see above for substrate selection
162 and concentrations) and Milli-Q water control were filter sterilized with a DMSO Safe
163 Acrodisc® Syringe Filter (0.2 µm Nylon Membrane, 25 mm). For substrate-induced respiration,
164 30 µL of each filter sterilized substrate or Milli-Q water controls had been dispensed to each
165 deep well and samples have been treated as described above. For microbial energetics, the admix

166 ampoules (SI Fig. 1) were thoroughly cleaned with ethanol and rinsed repeatedly with filter
167 sterilized Milli-Q water prior addition of one of the C substrates or MilliQ water as control. The
168 samples were then introduced into a TAM Air isothermal microcalorimeter and heat flows were
169 determined as described above.

170 **Microbial Community Profiles.** Phospholipid fatty acid (PLFA) profiling was used to
171 assess the composition of the microbial communities using the method of Frostegård et al.[27].
172 This analysis was used to determine which of the two functional diversity profiling methods, i.e.
173 substrate-induced CO_2 respiration or microbial energetics, was best related to microbial
174 community composition. Phospholipids were extracted from approximately 7-g fresh soil using
175 chloroform, methanol and citrate buffer to the ratio of 1:2:0.8 (v/v/v), fractionated by solid phase
176 extraction, depolymerized and then derivatized by mild alkaline methanolysis. The resultant fatty
177 acid methyl esters were analyzed by gas chromatography (Agilent/HP model 5890N, Santa
178 Clara, California, USA). Mono-unsaturated and cyclopropyl fatty acids were taken as gram-
179 negative bacteria (G-) biomarkers[29], iso- and anteiso-fatty acids as grampositive bacteria (G+)
180 biomarkers[30], C18:2(9,12) as a fungal biomarker[27] and carboxylic acids with a methyl function
181 on the carbon chain as biomarkers for actinobacteria[31].

182 **Statistical Analysis.** All statistical analyses were performed in R version 2.15.1[32] using the
183 'Vegan: Community Ecology Package'[33]. The resultant data was analyzed by one-way analysis
184 of variance (ANOVA) and homogeneous groups of means established using Duncan's multiple
185 range test. Levene's test was used to evaluate variance homogeneity and, where necessary, data
186 were log-transformed prior further statistical analysis. PLFA and functional diversity profiling of
187 the soils were examined with principal component analysis (PCA) using normalized covariance
188 of %mol of PLFA data, substrate-induced respiration or substrate-induced heat flow data,

189 respectively. Significant differences between soil treatments along ordination axes were analyzed
190 by post-hoc one-way ANOVA followed by Bartlett's test and Tukey multiple pair test
191 comparison on PC scores. The association between the substrate-induced respiration, heat release
192 and PLFA data was determined by comparing the dissimilarity matrices of each of the datasets
193 using the Mantel test based on the Pearson product-moment correlation coefficient (999
194 permutations). Pearson correlation analysis was used to evaluate linear regression between PLFA
195 biomarkers of fungal-bacterial ratio data (X-axis) and respiration as well as microbial energetics
196 data (Y-axis).

197 **Model description and parameterization.** A conceptual ecological model of microbial
198 energetics (catabolic and anabolic processes) in terrestrial soil ecosystems under aerobic, dark
199 conditions was devised and it is presented in Scheme 1. As such, reactions requiring light (e.g.
200 autotrophy) are not included in the model which only considers oxygen as a terminal electron
201 acceptor because nitrate and sulfate reduction are negligible in aerobic systems.

202 Water amended control soils show significant specific heat flows ($Q_{Control}$) with respect to
203 basal metabolism[13]. It is therefore essential to correct the heat output of each substrate-amended
204 soil in order to obtain heat produced from substrate addition only ($Q_{Substrate}$):

205 $$Q_{Substrate} = Q_{Total} - Q_{Control} \quad (1)$$

206 where Q_{Total} (mJ g^{-1}soil h^{-1}) and $Q_{Control}$ (mJ g^{-1} soil h^{-1}) are the heat flow of each substrate-
207 amended and water amended control soil, respectively.

208 Heat dissipated from abiotic processes was also removed in order to obtain heat flows due to
209 metabolic activity of microbial substrate decomposition only ($Q_{Metabolism}$, mJ g^{-1} soil h^{-1}).

Assuming that the abiotic processes that occur in sterile soils and in non-sterile soils generate equal heat flows, $Q_{\text{Metabolism}}$ can then be obtained by subtracting the heat flow of substrate-amended sterile soils (Q_{Abiotic}):

$$Q_{\text{Metabolism}} = Q_{\text{Substrate}} - Q_{\text{Abiotic}} \quad (2)$$

When there are no abiotic processes then heat produced from substrate addition only ($Q_{\text{Substrate}}$) is equal to heat flow due to soil biological activity ($Q_{\text{Metabolism}}$).

The heat signal $Q_{\text{Metabolism}}$ is heat dissipated from the soil system and it corresponds to the net outcome of catabolic (energy releasing) and anabolic (energy demanding) processes. It is the sum of energy conversions associated with (i) complete biological oxidation of the added substrate to CO_2 (Q_{CO_2}); and (ii) the sum of incomplete decomposition and anabolic soil processes ($Q_{\text{Net soil}}$). Incomplete decomposition processes result in intermediate products (intermediary catabolism with CO_2 not being the decomposition end product; Scheme 1 red arrow in $Q_{\text{Net soil}}$) and anabolic soil processes include microbial growth and maintenance, production of secondary metabolites, synthesis of extracellular enzymes, extracellular polysaccharides and so forth (biosynthetic anabolism; Scheme 1 green arrows in $Q_{\text{Net soil}}$).

The maximum theoretical available energy that becomes dissipated as heat during metabolism is associated with the complete oxidation of the added substrate carbon to CO_2. In this case, no energy is conserved within the system. The heat dissipated during the complete oxidation to CO_2 (Q_{CO_2}) is derived from the following equation assuming that heat production from possible priming effects of native soil organic matter is negligible in comparison with decomposition of the added substrate:

$$Q_{CO_2} = \Delta H_c^o \text{ (kJ mol}^{-1}) \times \frac{[n(CO_2)_{Substrate} - n(CO_2)_{Control}]}{N_C} \quad (3)$$

where ΔH_c^o (kJ mol^{-1}) is the standard molar enthalpy of combustion of the added substrates (SI Table 1); $n(CO_2)_{substrate}$ and $n(CO_2)_{control}$ is the amount of CO_2 mineralized (mol) in the substrate-amended and water-amended control soils, respectively, and N_C is the number of carbon atoms in the substrate.

All intermediary catabolic processes release less heat than the heat associated with the complete oxidation to CO_2 (Q_{CO_2}). The net outcome between intermediary processes and biosynthetic anabolic reactions ($Q_{Net\ soil}$) can be calculated by the difference between heat dissipated from overall metabolic activity ($Q_{Metabolism}$) and Q_{CO_2}):

$$Q_{Net\ soil} = Q_{Metabolism} - Q_{CO_2} \quad (4)$$

The carbon involved in transformations associated with the net outcome of $Q_{Net\ soil}$ remains in the soil system, but CO_2 is lost to the atmosphere.

RESULTS AND DISCUSSIONS

Assessment of Abiotic Processes. The addition of carboxylic acids to the sterile soils induced significant heat signals with the shape of the curve resembling that of non-sterile soils but of lower magnitude (SI Fig. 2a and b). In contrast to heat production, abiotic CO_2 production was negligible (*cf.* Fig. 1 and SI Fig. 2; SI Table 2). The other substrates and water amended control soils did not result in any measurable abiotic CO_2 production (SI Table 2) or heat flow apart from an initial (less than 30 minutes) small wetting enthalpy peak when adding the substrates to the sterile soils (SI Fig. 2c). The absence of any significant heat signal in water amended sterile

control soils beyond 30 minutes indicates that possible enzymes or metabolites released from microbial cells into soil solution during gamma-sterilization had no discernible effect on energy flows. In contrast with the sterile samples, adding the substrates and water to the non-sterile samples resulted in a significant substrate or water-induced heat release (SI Fig. 2d).

The origin of the abiotic heat signals upon carboxylic acid addition (SI Fig. 2a and b) is not known, but neutralization reactions and ligand binding of weak acids onto organic material are known to cause substantial exothermic reactions[25,34]. In non-sterile soil it is, however, uncertain if abiotic and biotic reactions have similar strengths or if one of them is a stronger sink for breakdown of carboxylic acids. Sensitivity analysis was therefore required to validate if our assumption of equal abiotic heat flows in sterile and non-sterile soils was violated (see below in the following section). Because the first initial immediate reaction was no longer apparent after two hours (SI Fig. 2a and b), we opted for the use of the 2-8 hour incubation period to evaluate the relationship between microbial community composition and functional diversity profiles.

Relationship between microbial community and functional diversity profiles. The principal component analysis (PCA) of the microbial energetics data ($Q_{\text{Metabolism}}$) revealed a clear separation among soil treatments ($P < 0.01$, Fig. 2a), but only green manure and straw+calcium nitrate amended soils were separated along PC1 in the respiration data ($P = 0.034$; Fig. 2b). Furthermore, microbial community composition was also significantly different among soil treatments with actinobacteria (10Me-C18:0)[31], Gram-negative bacteria/fungal (C18:1ω9c)[35,36] and fungal biomarkers (C18:2ω6,9)[27] being the main variables responsible for the separation of the different soil management regimes ($P < 0.001$, Fig. 2c). Pairwise comparison of dissimilarity matrices between overall microbial metabolic heat profiles and microbial community profiles revealed a significant similarity between the two data sets (Mantel $R = 0.4602$, $P = 0.006$, *cf.* Fig.

2a and c), but no such similarity was detected between respiration and community profiles (Mantel R = 0.2291, P = 0.11; *cf.* Fig. 2b and c). These data clearly show that the composition of the microbial community was related to the metabolic processes that occurred in the samples and that this relationship was not apparent when CO_2 evolution was used as an aggregate measure of microbial metabolism. Microbial metabolism in soils consists of a plethora of processes including reactions that do not produce CO_2 as an end-product.[11] Isothermal microcalorimetry quantifies all metabolic processes and therefore accounts for the different processes that occur within different microbial communities, regardless of the different life strategies of soil organisms[14]. This is not always the case with respiration measurements.

Moreover, the mantel test for dissimilarity matrices indicated that overall microbial heat flow ($Q_{\text{Metabolism}}$) and CO_2 data provided different information, i.e. that there are divergences between the two. This was independent of whether the analysis was based on pairwise comparison between metabolic heat profiles and respiration profiles of all seven substrates (Mantel R = 0.2173, P = 0.112; *cf.* Fig. 2a and b) or when the two carboxylic acids, which generated significant heat flows in sterile soils, were excluded from the analysis (Mantel R = 0.03488, P = 0.426). The overall microbial heat flows ($Q_{\text{Metabolism}}$) were based on the assumption that the abiotic processes that occur in sterile soils generate equal heat flows in non-sterile soils. This is a challenging assumption to validate however. Sensitivity analysis was done on microbial heat flows assuming (i) $Q_{\text{Substrate}} = Q_{\text{Metabolism}}$, i.e. there was no abiotic heat release upon carbon substrate addition or (ii) that abiotic heat release was 50% of that determined in sterile soils. The analysis resulted in the same conclusion, namely that there were divergences between heat profiles and CO_2 data ((i) Mantel R = 0.2887, P = 0.059; *cf.* Fig. 2b and SI Fig. 3a; (ii) Mantel R = 0.2266, P = 0.114; *cf.* Fig. 2b and SI Fig. 3b). Thus, potential violations of this assumption are

297 unlikely to affect overall conclusion drawn from this experiment. In contrast, Currie[37] found that
298 heat flows and CO_2 were closely related when combining energy balance with a model that was
299 parameterized through bomb calorimetric analysis, i.e. measurements of stored energy in organic
300 material. However, the two studies are not directly comparable, as different approaches were
301 used. Nevertheless, they warrant further investigation into the relation between energy and
302 carbon cycling in terrestrial ecosystems.

303 Long-term organic inputs of peat+calcium nitrate resulted in the greatest fungal-to-bacterial
304 ratio among the different long-term management regimes (Table 1). The other management
305 regimes had lower ratios and were ranked in the order green manure > farmyard manure >
306 straw+calcium nitrate amended soils (Table 1). Soils amended with peat+calcium nitrate
307 dissipated the least heat ($Q_{Substrate}$ and $Q_{Metabolism}$) (Fig. 1a and b), and the net outcome of heat
308 dissipated between anabolic and intermediary catabolic reactions ($Q_{Net\ soil}$) was lowest in
309 peat+calcium nitrate or green manure amended soils (Fig. 1a and c). Conversely, green manure
310 amended soils showed the highest CO_2 production among the four soil systems (Fig. 1a and d).
311 Such differences in respiration and heat flows strongly suggest that carbon and energy allocation
312 varied among the four soil management systems. All soils received the same amount of energy
313 (Σ energy input carbon substrates: 1.29 kJ g^{-1} soil; SI Table 1). The fact that less heat was
314 dissipated in green manure and peat+calcium nitrate systems may be merely due to overall lower
315 metabolic activities. However, lower calorespirometric ratios, i.e. heat output $Q_{Metabolism}$ per unit
316 CO_2 (Table 1) indicate that the green manure and peat+calcium nitrate systems, with higher
317 relative abundances of fungi, may have a more efficient microbial metabolism. Consequently,
318 more energy is retained within soil systems that contain higher proportions of fungi. Specifically,
319 Pearson correlation analysis based on all field replicates (n = 12) revealed a negative linear

relationship between the fungal-bacterial ratio and the sum of all energy heat flows (Fig. 3a; $P < 0.001$), but there was no relationship between the fungal-bacterial ratio and overall respiratory activity (Fig. 3b; $P = 0.66$). Normalizing each substrate by the sum of overall heat release response revealed a negative relationship between the fungal-bacterial ratio and substrate-induced heat release of *N*-acetyl glucosamine additions ($Q_{Metabolism}$: X = -5.17, $r^2 = 0.73$ or $Q_{Net\ soil}$: X = -6.90, $r^2 = 0.83$). Fungal cell walls contain chitin which is a long-chain polymer of *N*-acetyl glucosamine[38]. Hence, *N*-acetyl glucosamine was used in anabolic processes and therefore less heat was dissipated into the atmosphere from soil systems that contain relatively more fungi than bacteria. Overall, our results are in line with a long-standing paradigm in microbial ecology that microbial communities dominated by fungi are more efficient in carbon assimilation[39] and nutrient resource retention[40] than bacterial-dominated communities.

Implications for carbon cycling in terrestrial ecosystems. Our findings demonstrate that the composition of microbial communities in soil and their functioning are related to energy flows. These findings provide an indication that microbial communities may not be functionally redundant with respect to carbon cycling as hitherto thought. If this were to be confirmed, we would therefore have to re-evaluate the concept of functional redundancy in soil microbial ecology. In the present study, microbial energetics were related to microbial communities at a high taxonomic level and described under optimal, saturated microbial metabolism. Although PLFA profiles only provide a description of microbial community composition at a high taxonomic level, recent research syntheses[41,42] accentuate that this level may matter for ecosystem function. In the future it will be necessary to evaluate (i) if the taxonomic level of diversity matters, i.e. different taxonomic levels of diversity for example at the species level may result in a different relationship with energy flows, (ii) if microbial energetics are similar under

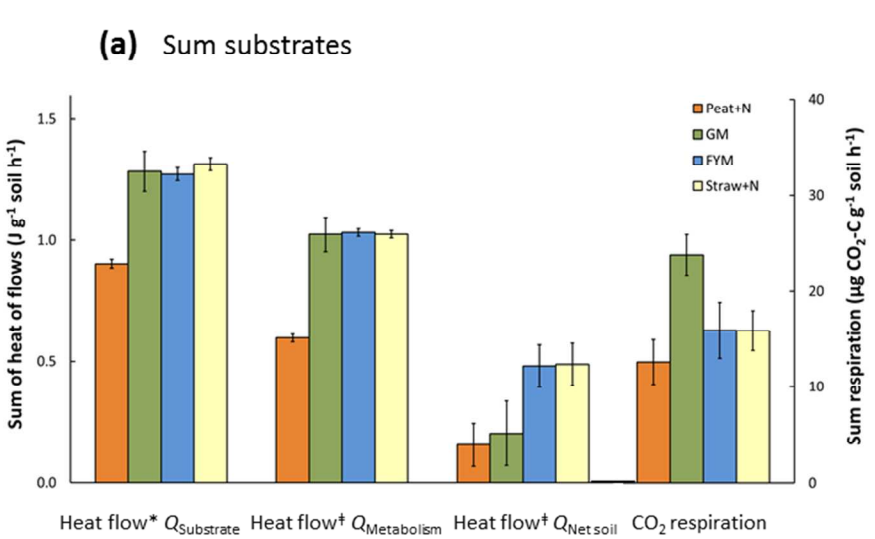
343 ecologically relevant substrate levels, i.e. poorer carbon conditions and (iii) if microbial
344 communities with different energy flows respond differently to external forces such as flooding,
345 heat or cold stress and so forth.

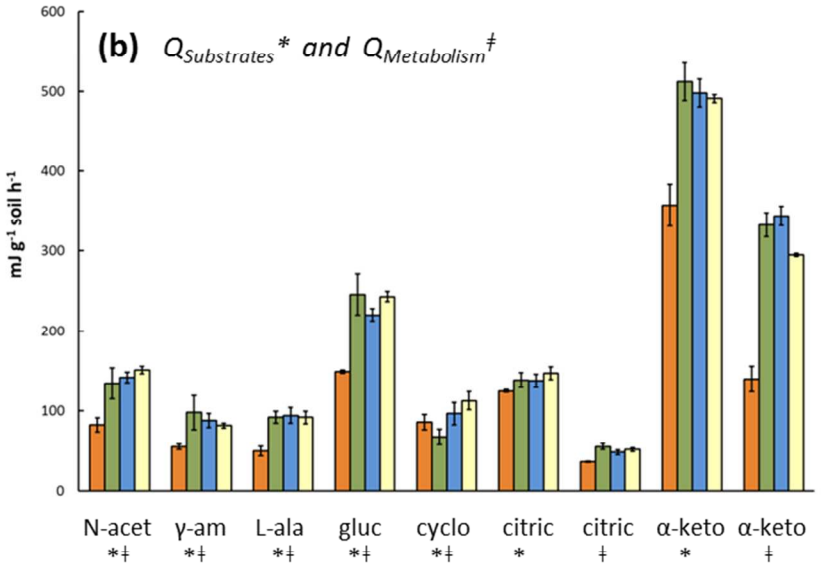
346 Our results have significant implications for carbon cycling in terrestrial ecosystems and
347 support the emerging view of carbon sequestration. The classical view that carbon sequestration
348 belowground is mainly due to the molecular property of residing organic matter is increasingly
349 considered obsolete. It is replaced by a conceptual model which describes carbon stabilization as
350 an ecosystem property[43] with soil microorganisms as important facilitators[41]. Data from the
351 present study furthermore confirm that soil systems that contain relatively more fungi may have
352 the ability to sequester more carbon belowground in comparison with systems with relatively
353 more bacteria. Allison and co-workers[44] have suggested that changes in microbial metabolism,
354 resulting in a decrease in the fraction of assimilated carbon allocated to growth, can explain the
355 apparent acclimation to warming that is often observed for soil respiration. Subtle changes in
356 metabolism, not apparent when aggregate measures such as soil respiration are used as an
357 indicator of community activity, may thus potentially have significant consequences for
358 ecosystem-scale function. Such metabolic changes may therefore have to be accounted for to
359 fully understand terrestrial climate change feedback mechanisms. It is therefore imperative to
360 develop our knowledge of soil microbial community functioning using a microbial energetics
361 approach, if we are to construct a complete understanding of carbon dynamics in soils. The work
362 presented here provides empirical data that can feed into emerging microbial-enzyme carbon-
363 climate based feedback models[44,45], and the proposed ecological model of microbial energetics in
364 soil ecosystems can be used as a start.

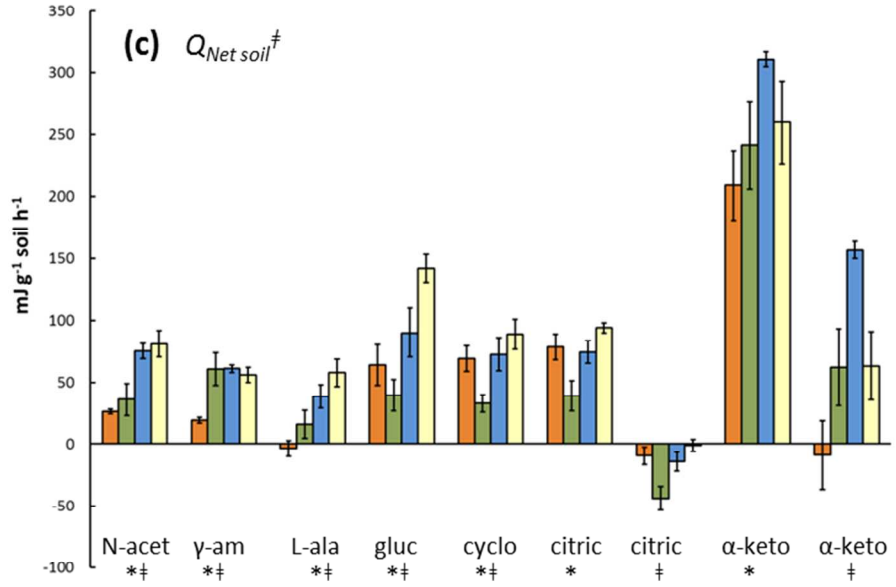
In the last century, theoretical ecological frameworks of ecosystem bioenergetics have been proposed[46,47] and energy budget of organic forest floors[48] were established. Currie[37] evaluated the relation between carbon and energy and our proposed work on microbial energetics in terrestrial soil ecosystems further develops the area of ecosystem bioenergetics. However, the ecological model is still in its infancy within soil science and there is a clear scope for further development. Soils are structurally heterogeneous and external environmental conditions do not have a uniform effect throughout the soils, resulting in a large diversity of micro-habitats. Future studies could examine microbial energetics under various environmental conditions. Here, soils could be exposed to photoperiods, flooded conditions or oxygen-free atmosphere to estimate the importance of e.g. autotroph[49], methanogen[50], sulfate- or nitrate[51] reducing microorganisms on microbial energetics. In a broader perspective, the microbial energetics approach has the potential to provide further information when employing ecological theory into microbial ecology to better understand microbial systems. In particular, it provides new insights into the relation between biodiversity and land use extensification[52], ecosystem development[53,54] as well as key ecosystem functioning such as carbon sequestration[43] and nutrient retention[52]. By taking an energetic view of soil microbial metabolism, we may improve our understanding of the significance of microbial biodiversity on ecosystem function and thus improve prediction of microbial feedback mechanisms and ecosystem responses to climate change.

FIGURES

Figure 1. Substrate-induced heat flows and respiration. (a) Overall responses of four soils from the Ultuna Long-Term Field Experiment. Mean values represent sum of responses to all seven substrates. (b-d) Responses of each carbon substrate separately (N-acet = N-acetyl glucosamine; γ-am = γ-amino butyric acid; L-ala = L-alanine; gluc = D-glucose; cyclo = α-cyclodextrin; citric = citric acid and α-keto = α-ketoglutaric acid): (b) Heat flows $Q_{Substrate}$ and $Q_{Metabolism}$, (c) $Q_{Net\ soil}$ and (d) respiration. Heat flows and respiration were determined by isothermal microcalorimetry or MicroResp, respectively; for explanation of heat flow abbreviations see Scheme 1. The error bars indicate standard deviation (n=3). Peat+N = peat+ $Ca(NO_3)_2$; GM = Green manure; FYM = farmyard manure; Straw+N = straw+$Ca(NO_3)_2$. $Q_{Substrate} = Q_{Metabolism}$ when there are no abiotic substrate interactions with physical properties and these substrates are suffixed with*□. Substrates suffixed with * are $Q_{Substrate}$ and substrates suffixed with □ are $Q_{Metabolism}$. In the latter, $Q_{Substrate}$ were corrected for heat outputs derived from sterile soils (Table S2, equation (2)) on the assumption that abiotic substrates interactions with soil matrix are occurring in the same order of magnitude in non-sterile and sterile soils.


(a) Sum substrates
Peat+N
GM
FYM
Straw+N
Sum of heat of flows (J g-1 soil h-1)
Sum respiration (µg CO2-C g-1 soil h-1)
Heat flow* QSubstrate
Heat flow‡ QMetabolism
Heat flow‡ QNet soil
CO2 respiration


(b) QSubstrates* and QMetabolism‡
mJ g-1 soil h-1
N-acet *‡
γ-am *‡
L-ala *‡
gluc *‡
cyclo *‡
citric *
citric ‡
α-keto *
α-keto ‡


(c) QNet soil‡
mJ g-1 soil h-1
N-acet *‡
γ-am *‡
L-ala *‡
gluc *‡
cyclo *‡
citric *
citric ‡
α-keto *
α-keto ‡

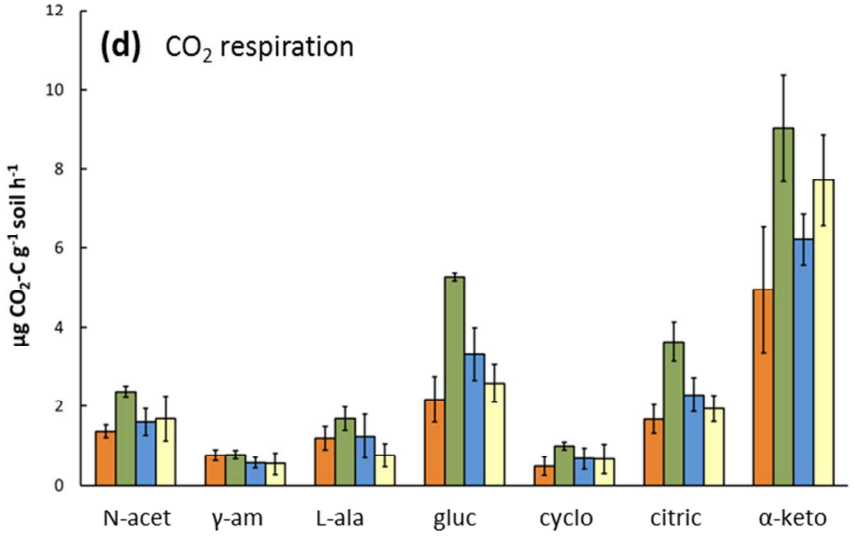

(d) CO2 respiration
µg CO2-C g-1 soil h-1
N-acet
γ-am
L-ala
gluc
cyclo
citric
α-keto

Figure 2. Functional diversity profiling and composition of soil microbial communities. Principal component analysis representing the effect of contrasting long-term organic matter inputs on (i) the functional diversity profiling of the soil biota based on utilization of 7 different substrates via (a) overall microbial metabolic activity ($Q_{Metabolism}$); and (b) CO_2 respiration and (ii) (c) the composition of the soil microbial communities by PLFA. Values in parentheses on axis labels denote % variation accounted for by the respective components, and 95% confidence ellipses are provided for each soil treatment. Peat+N = peat+ $Ca(NO_3)_2$; GM = Green manure; FYM = farmyard manure; Straw+N = straw+$Ca(NO_3)_2$.

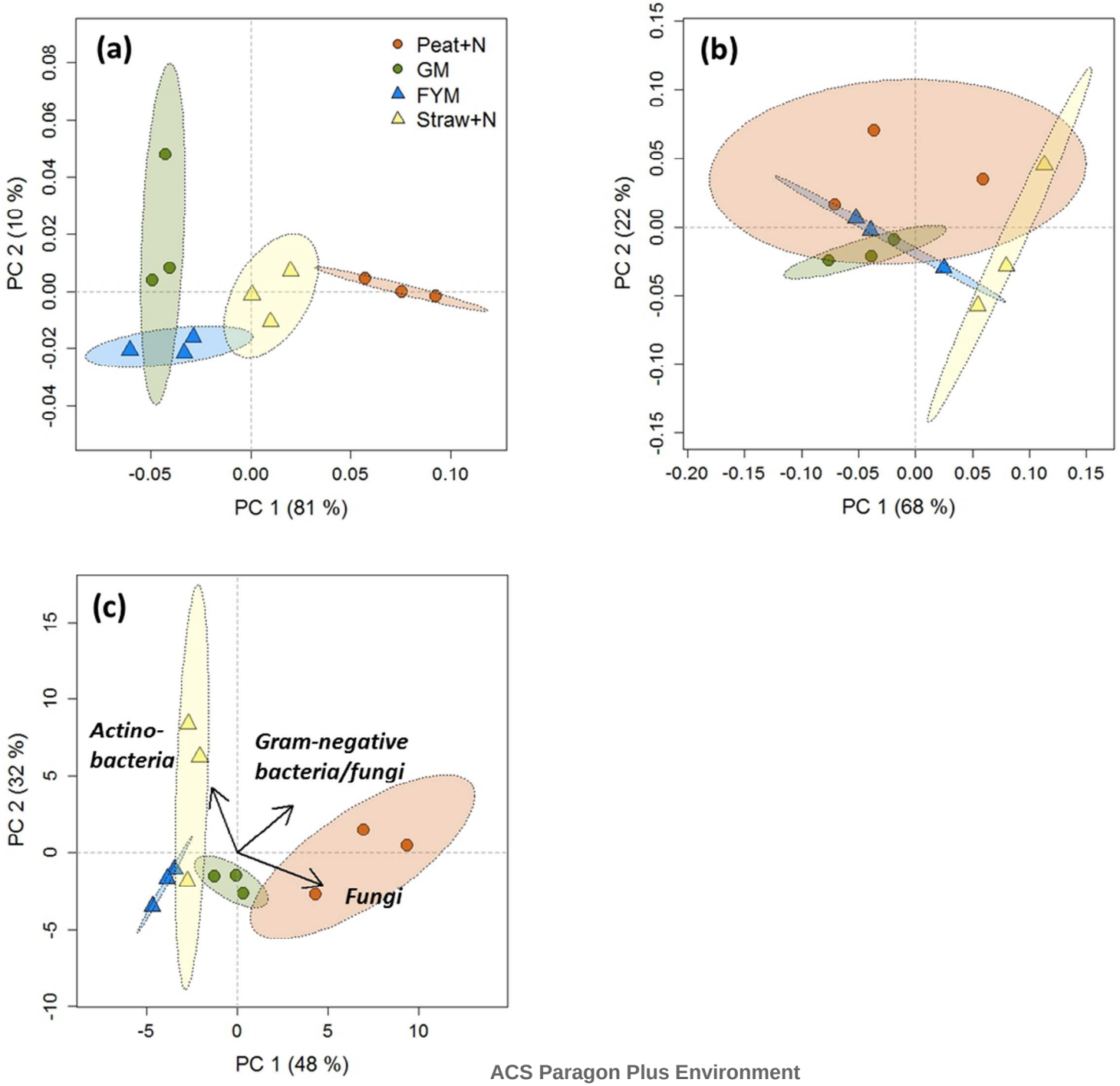
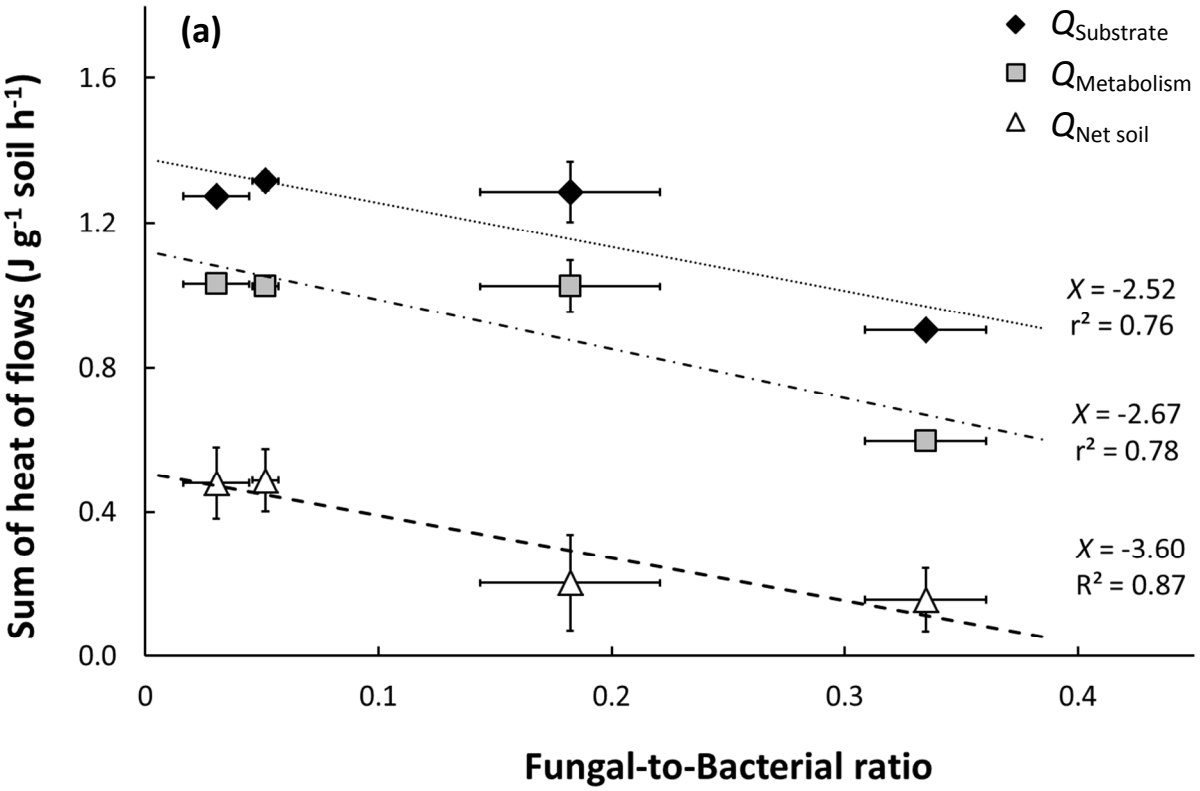
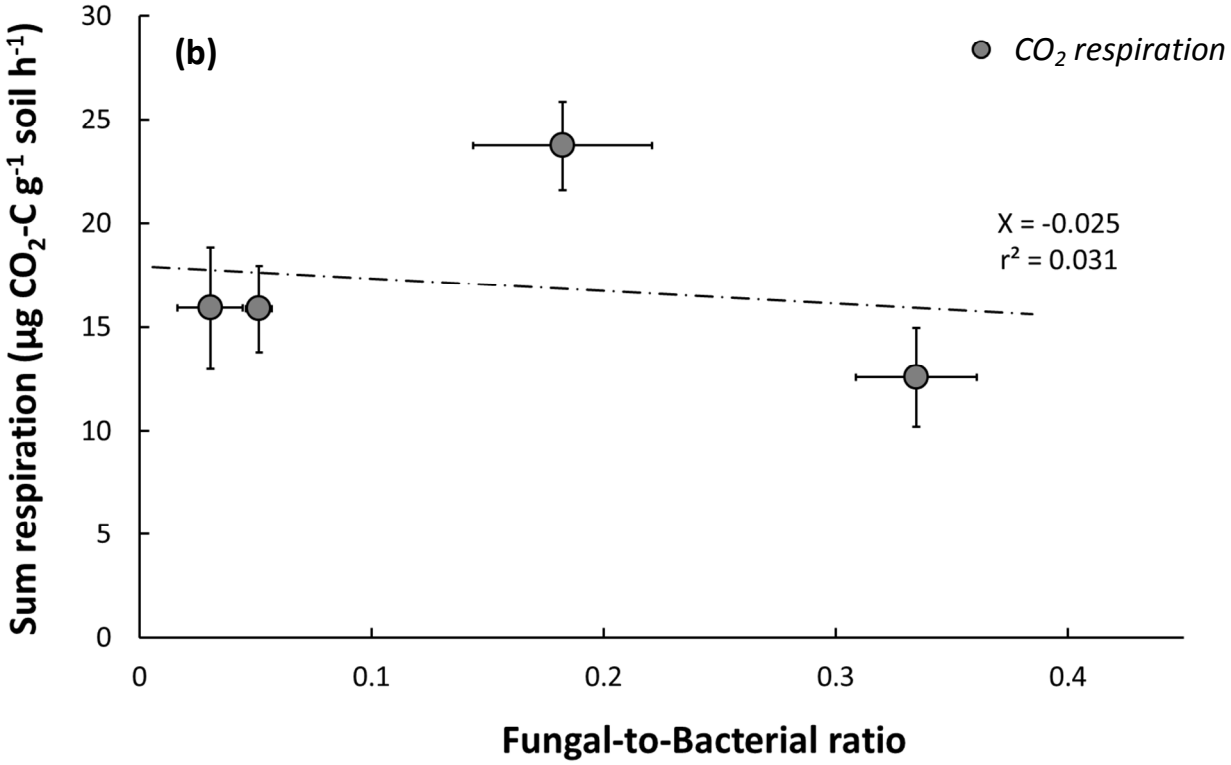

(a)
Peat+N
GM
FYM
Straw+N
PC 2 (10 %)
PC 1 (81 %)
(b)
PC 2 (22 %)
PC 1 (68 %)
(c)
Actino-
bacteria
Gram-negative
bacteria/fungi
Fungi
PC 2 (32 %)
PC 1 (48 %)

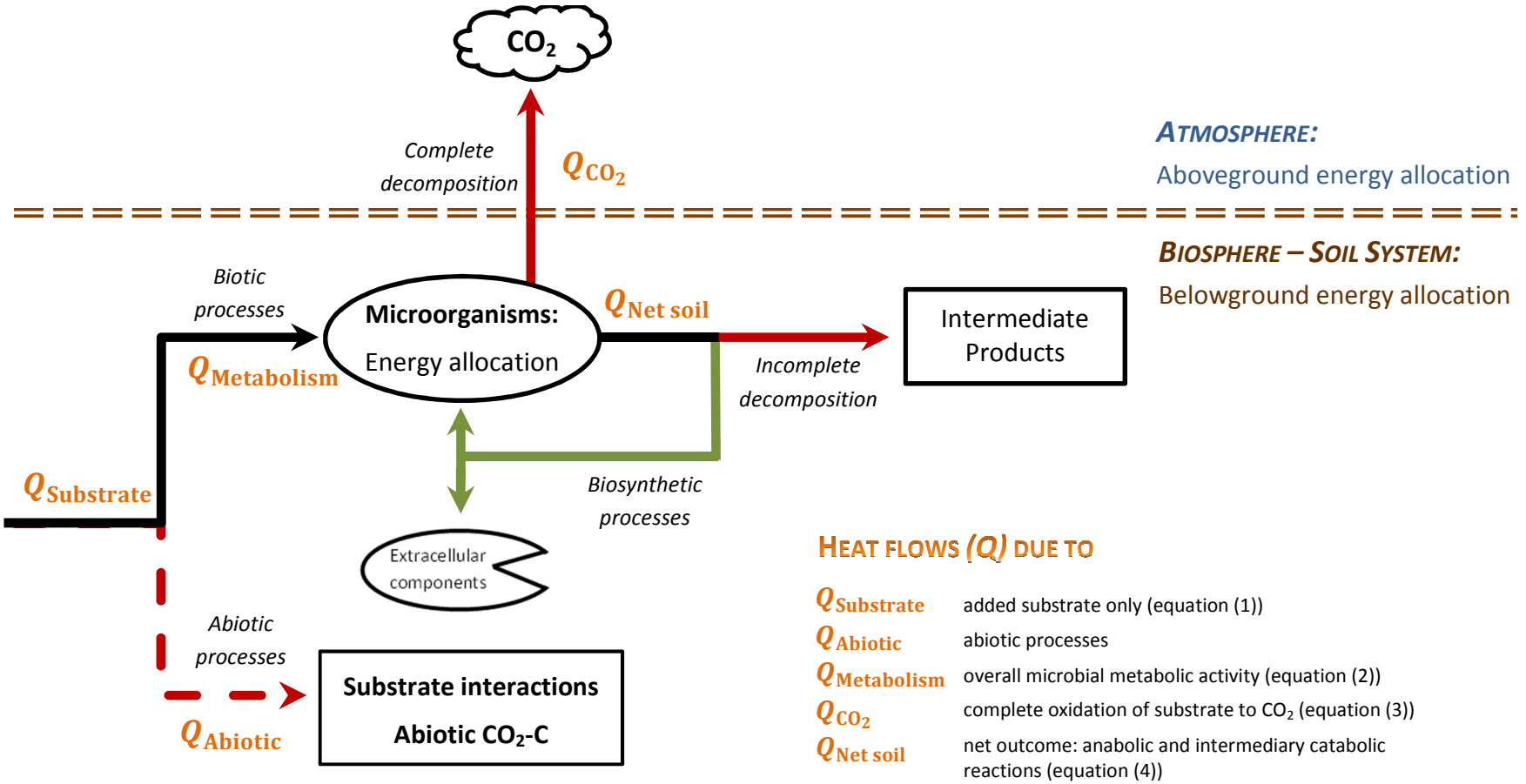
Figure 3. Pearson correlation analysis. Linear correlation analysis between fungal-to-bacterial ratio (x-axis) and (a) heat flows and (b) CO_2 respiration (n = 12).


(a)
$Q_{Substrate}$
$Q_{Metabolism}$
$Q_{Net\ soil}$
X = -2.52
r² = 0.76
X = -2.67
r² = 0.78
X = -3.60
R² = 0.87
Sum of heat of flows (J g-1 soil h-1)
Fungal-to-Bacterial ratio
0.0
0.4
0.8
1.2
1.6
0
0.1
0.2
0.3
0.4


(b)
CO_2 respiration
X = -0.025
r² = 0.031
Sum respiration (µg CO_2-C g-1 soil h-1)
Fungal-to-Bacterial ratio
0
5
10
15
20
25
30
0
0.1
0.2
0.3
0.4

SCHEMES

Scheme 1. Conceptual model of microbial energetics of metabolism in aerobic soils. Red and green arrows represent catabolic and anabolic processes, respectively. Heat flows ($Q_{\text{Subscript}}$) are represented in orange. Solid lines indicate dominant processes whereas dashed lines represent minor processes.



HEAT FLOWS (*Q*) DUE TO

$Q_{Substrate}$	added substrate only (equation (1))
$Q_{Abiotic}$	abiotic processes
$Q_{Metabolism}$	overall microbial metabolic activity (equation (2))
Q_{CO_2}	complete oxidation of substrate to CO_2 (equation (3))
$Q_{Net\ soil}$	net outcome: anabolic and intermediary catabolic reactions (equation (4))

TABLES.

Table 1. Basic characteristics including fungal-to-bacterial ratios (F:B ratio) and calorespirometric ratio (heat output $Q_{Metabolism}$ per unit CO_2; mJ μg^{-1} CO_2-C) of soils used in study. Mean values (n = 3); common letters show homogenous means using Duncan's multiple range test at 1% significance level.

Treatment	C (%)	N (%)	C-to-N ratio	Microbial biomass (μg C g^{-1} soil)	pH (H_2O)	F:B ratio*	calorespirometric ratio mJ μg^{-1} CO_2-C
Green Manure	1.7 A	0.19 A	9.7 A	205 A	5.9 A	0.18 A	44 A
Straw+$Ca(NO_3)_2$	2.0 B	0.17 B	10.7 B	254 B	6.4 B	0.03 B	65 B
Farmyard Manure	2.3 C	0.23 C	10.1 C	298 C	6.4 B	0.05 B	66 B
Peat+$Ca(NO_3)_2$	3.9 D	0.22 C	17.6 D	186 A	5.8 A	0.33 C	49 A

*Fungal-to-bacterial ratio (F:B ratio) was based on the abundance of the fungal PLFA biomarker 18:2 (9, 12)[27] and the sum of 8 bacterial PLFA biomarkers.

ASSOCIATED CONTENT

Supporting Information. Additional information noted in the text is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CO_2, carbon dioxide; Q_{Total}, heat flows of substrate-amended soils; $Q_{Substrate}$, heat flows from added substrate; $Q_{Control}$, heat flows from water amended soils/basal metabolism, $Q_{Metabolism}$, heat flows from overall microbial metabolic acitivity; $Q_{Abiotic}$), heat flows in sterile soils/abiotic processes; $Q_{Net\ soil}$, net outcome: heat flows of anabolic and intermediary catabolic reactions; (Q_{CO_2}), heat dissipated during complete biological oxidation of the added substrate; H_c^o, standard molar enthalpy; $n(CO_2)_{substrate}$ and $n(CO_2)_{control}$, CO_2 mineralized (mol) in substrate-amended and water-amended control soils, respectively; N_C, the number of carbon atoms in substrate added.

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