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Various aspects of soil microbial ecology as revealed by phospholipid fatty acid (PLFA) analysis

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Ph.D. Thesis

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Annotation

The PLFA profiling method was adopted and used to determine changes in microbial community structure and abundance along natural and human-induced environmental gradients. The presented studies were based on field sampling campaigns combined with targeted laboratory experiments. According to the aims of particular studies, microbial PLFA fingerprinting was combined with the auxiliary below- and aboveground ecosystem characteristics to identify the drivers of microbial responses to environmental changes or with ^{13}C-labelling and metagenomics to obtain more complex information about running processes and involved microorganisms.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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Petr Kotas

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List of papers and manuscripts with author's contribution

The thesis is based on the following papers:

I. **Kotas, P.**, Choma, M., Šantrůčková, H., Lepš, J., Tříska, J., Kaštovská, E., 2016. Linking above- and belowground responses to 16 years of fertilization, mowing, and removal of the dominant species in a temperate grassland. Ecosystems 20, 354-367. (IF = 4.19)

Petr Kotas performed the PLFA analysis, analyzed all data and wrote the paper.

II. **Kotas, P.**, Šantrůčková, H., Elster, J., and Kaštovská, E. Soil microbial biomass, activity and community composition along altitudinal gradients in the High Arctic (Billefjorden, Svalbard), Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-184, Biogeosciences (in review). (IF = 3.85)

Petr Kotas designed the study, conducted sampling, collected and analyzed all data, and wrote the manuscript.

III. Šantrůčková, H., **Kotas, P.**, Bárta, J., Urich, T., Čapek, P., Palmtag, J., Eloy Alves, R. J., Biasi, Ch., Diáková, K., Gentsch, N., Gittel, A., Guggenberger, G., Hugelius, G., Laschinsky, N., Martikainen, P. J., Mikutta, R., Schleper, Ch., Schnecker, J., Schwab, C., Shibistova, O., Wild, B., Richter, A. Significance of dark CO_2 fixation in arctic soils, accepted in Soil Biology & Biochemistry. (IF = 4.86)

Petr Kotas contributed to the incubation experiments, performed the compound specific isotopic analysis of PLFAs and contributed to writing and revisions of the manuscript.

IV. Čapek, P.**, Kotas, P.**, Manzoni, S., Šantrůčková, H., 2016. Drivers of phosphorus limitation across soil microbial communities. Functional Ecology. 30, 1705-1713. (IF = 5.63)

Petr Kotas performed the PLFA analysis and contributed to interpretation of the data.

Content

Abbreviations

AM – arbuscular mycorrhiza
C – carbon
CLPP – community level physiological profiling
DNA – deoxyribonucleic acid
EL – PLFA – ester-linked phospholipid fatty acid
FA – fatty acid
FAME – fatty acid methyl ester
GC-MS – gas chromatography - mass spectrometry
MCS – microbial community structure
MUFA – monounsaturated fatty acid
N – nitrogen
NEL-PLFA – non ester-linked phospholipid fatty acid
NL – neutral lipid
NLFA – neutral lipid fatty acid
OHFA – hydroxy-substituted fatty acid
P – phosphorus
PCA – principal component analysis
PCR – polymerase chain reaction
PHB – poly-β-hydroxybutyrate
PL – phospholipid
PLEL – phospholipid ether lipids
PLFA – phospholipid fatty acid
PUFA – polyunsaturated fatty acid
RNA – ribonucleic acid
SATFA – saturated fatty acid
SOM – soil organic matter
TSFAME – total soil fatty acid methyl ester
UNSATFA – unsaturated fatty acid

1. General introduction

The General introduction section was from the original Ph.D. Thesis removed due to copyrights and publishing purposes. The original text is available in printed form in the Academic library of the University of South Bohemia in České Budějovice. The content of the General introduction section was in full text submitted for publication as review article named "Use of phospholipid biomarkers in soil microbial ecology" to Pedobiologia Journal.

2. Aims and objectives

The foregoing literature review demonstrated that the PLFA profiling is an increasingly important approach to acquire information about soil microbial community structure (MCS), microbial response to various environmental changes, and utilization of ^{13}C-labelled carbon substrates. The general aim of this thesis was to adopt the PLFA profiling method and fully employ its potential to gain ecologically relevant information about soil microbial communities and their functioning. Each of the presented studies focused on different aspect of soil microbial ecology. The **studies I** and **II** focused on determination of key environmental factors which affect microbial community structure and functioning in two different ecosystems: the temperate species-rich mesotrophic meadow and the High Arctic tundra. The objective of **study III** was to quantify heterotrophic CO_2 fixation by soil microbial community and determine its incorporation into microbial biomass in permafrost affected soils. Finally, the **study IV** aimed to elucidate whether microbial community structure and functioning is a general driver of soil microbial community demand for phosphorus. The specific objectives were as follows:

Objectives of study I – To determine the main and combined effects of fertilization, mowing, and dominant plant species removal on soil microbial and physico-chemical properties, and to link the belowground response with aboveground plant characteristics and document the adaptation of the whole grassland to a long-term management. We hypothesized that the treatments will affect the soil microbial properties through their effect on SOM content, nutrient availability and soil acidity, and that fertilization and mowing will affect these soil properties in opposite way. We further expected that the combined application of fertilization and mowing would balance the effect of individual treatments.

Objectives of study II – To describe the spatial heterogeneity of abiotic and microbial properties in the soils along three altitudinal transects in the High Arctic (Billefjorden, Central Svalbard) and identify key environmental factors driving the altitudinal variability of the soil microbial community structure and activity in this Arctic ecosystem. We hypothesized that microbial biomass, the fungal proportion in the microbial community, and microbial activity will be positively related to SOM content, which will decrease along with increasing elevation.

Objectives of study III – To perform a $^{13}CO_2$-labelling experiment and determine the ^{13}C enrichment of soil microbial PLFAs in different layers of permafrost-affected soils to provide direct evidence of microbial heterotrophic CO_2 fixation and subsequent C utilization. We hypothesized that the rate of heterotrophic CO_2 fixation increases with increasing soil depth due to lowering the C availability, and that the composition of microbial communities utilizing inorganic C differs between the different soil layers.

Objectives of study IV – To determine the microbial PLFA composition in the worldwide soils with variable microbial community demand for phosphorus and use the microbial community structure together with other soil characteristics as explanatory variables to identify the main drivers of soil microbial community critical C-to-P ratio ($C:P_{CR}$). We expected that the $C:P_{CR}$ vary among soils and generally increases in P-poor soils as a result of microbial community adaptation to P limitation.

3. Results and conclusions

The unifying element of studies presented in this thesis was the determination of microbial community structure and functioning using the analyses of phospholipid fatty acid (PLFA) biomarkers in combination with microbial activity measurements. In all conducted studies, the microbial characteristics were related to auxiliary abiotic and biotic environmental variables and nutrient sources to portrait broader picture about the functioning of investigated soil systems. Besides the fact that PLFAs provided information about microbial community structure and abundance, each of these studies employed the PLFA fingerprints in a different way according to specific objectives of particular studies. The aim of this chapter is to summarize main findings and emphasize the various aspects of PLFA interpretation in the broader ecosystem context.

In the **studies I** and **II**, the PLFAs represented primary data of the conducted research. The **study I** investigated impacts of long-term management regime on above- and belowground compartments in the wet temperate grassland subjected for 16 years to fertilization, mowing, and dominant plant species removal. The biotic response to treatments was significant aboveground, where the plant productivity, plant biomass elemental stoichiometry and community composition significantly reflected fertilization and mowing. In contrast, the majority of soil microbial characteristics including microbial biomass, microbial C:N:P stoichiometry, enzyme activities and abundances of main microbial groups (detected by PLFAs specific for Gram-negative and Gram-positive bacteria, actinobacteria, and fungi) remained unaffected despite significant changes in soil physico-chemical properties and pronounced aboveground response. However, the evaluation of the whole PLFA pattern by multivariate statistics revealed significant shifts in microbial PLFA composition in fertilized plots connected mainly with lower soil pH. Given the narrow overall pH range in the experimental plots (4.18-3.85), our results indicate that the PLFA profiling allows highly sensitive detection of microbial response to minor changes in soil acidity. Furthermore, we observed close links between microbial and plant community responses to the treatments, indicated by the significant correlation between sample scores of main principal components extracted from PCA based on microbial PLFA profiles and plant community composition.

In summary, the PLFA profiling enabled very sensitive indication of management effects on microbial community composition. The combination of microbial PLFA fingerprints with information about plant community composition revealed mutual adaptation of plant and microbial communities to particular grassland management.

In the **study II,** the natural gradients of soil geochemical and microbial properties along three altitudinal transects in the High Arctic ecosystem were investigated. In order to meet objectives of the study, representative soil samples from four different altitudes (25, 275, 525 and 765 m a.s.l.) were gathered along each transect. These climosequences ranged from tundra vegetation to sites characterized by bare soil and biological soil crusts. Each sample was characterized by an array of geochemical and microbial parameters, including PLFA fingerprints. Abundances of main microbial groups based on specific PLFA markers were compared to determine important shifts in MCS. Besides significant horizontal variability in proportions of notional bacterial groups (Gram-positive and Gram-negative bacteria actinobacteria), the systematic altitudinal shift in fungi-to-bacteria ratios within microbial communities along all three gradients was observed. In contrast to our expectations, microbial communities with highest fungal proportions occurred at the most elevated sites, while microbial communities from lower altitudes were dominated by bacteria. The multivariate statistics retained the Mg^{2+} availability as the main factor driving the horizontal heterogeneity of soil MCS while changes in soil pH, SOM content and soil moisture were mainly responsible for the elevational shifts in MCS. In general, most of the changes in soil properties and MCS were directly or indirectly connected with decreasing coverage and occurrence of vascular plants along with increasing elevation. Compared to the **study I** was the PLFA profiling used in the **study II** to describe microbial biomass and community structure along natural gradients of environmental conditions on a regional spatial scale. The results help to describe current state and estimate the future development of the High Arctic ecosystems in Svalbard.

Unlike the two previous rather descriptive studies, the **studies III** and **IV** were focused on phenomena with wider ecological implications. **Study III** investigated the significance of CO_2 fixation by heterotrophic microorganisms in arctic soils. Even though the occurrence of this process in soils is generally accepted (Miltner et al., 2004; Šantrůčková et al., 2005), its importance for soil

C sequestration was unknown. Therefore, the study was conducted with the aim to elucidate the role of dark CO_2 fixation in arctic soils from a range of tundra ecosystems around the northern circumpolar region. The abundance and taxonomic affiliation of various carboxylase genes was determined by shotgun metagenomics and $^{13}CO_2$ pulse labelling was used to quantify the rate of inorganic C incorporation into microbial biomass. The ^{13}C enrichment of microbial PLFAs provided the direct evidence of heterotrophic CO_2 fixation and subsequent C utilization by soil microorganisms. Moreover, unique patterns in the ^{13}C enrichment of specific PLFA markers (PLFA-SIP) within organic, mineral and cryoturbated horizons were observed which indicates different contributions of fungi and bacteria to overall CO_2 assimilation in these soil layers. In the context of the thesis, the **study III** demonstrated that multiphasic approach combining determination of presence and magnitude of a given process (^{13}C-labelling and PLFA-SIP analysis) with the method yielding less direct, but more specific information about the soil potential for a certain function (diversity and taxonomic affiliation of carboxylase gene) enables to depict more complex picture about microbial processes in soil.

In the **study IV**, the PLFA fingerprints were used as predictors of half saturation constant (K_M) of a modified Monod equation, which was employed to describe the effect of P availability on microbial community growth rate. Previous empirical studies have shown that the ratio between available C and N at which the microbial growth starts to be N limited (C:N critical ratio) varies between ≈20 and 200 (e.g. Zechmeister-Boltenstern et al., 2015). The **study IV** was the first attempt to assess potential variability and drivers of relative microbial community demand for P (i.e. critical C:P ratio - $C:P_{CR}$) in the soil. Our results have shown that the microbial $C:P_{CR}$ ranged between 26.5 and 465 in 18 soil samples from various ecosystems. The high variability of $C:P_{CR}$ was mainly related to differences in K_M parameter. The forward selection of the best K_M predictors retained, among all available auxiliary data, the sample scores from PCA on microbial PLFA composition. Our results thus indicate that the microbial $C:P_{CR}$ is largely determined by the particular MCS of the soils. However, the MCS is shaped by the array of edaphic factors like pH, temperature, moisture, substrate and nutrient availability, and their combinations which indicates complex environmental control over the microbial community $C:P_{CR}$. In summary, the **study IV** represents the rather unusual use of microbial PLFA fingerprints. In contrast to a majority of studies,

where the soil PLFA profiles were used to detect microbial response to certain environmental change (e.g. **study I** and **II**), the **study IV** utilized the PLFA patterns as variables explaining the more general characteristic of microbial communities, the $C:P_{CR}$.

In conclusion, this thesis presents results from four studies that employed the PLFA profiling as an approach to acquire information about microbial abundance (**I - III**), microbial community structure (**I - IV**), and activity (**III**). The PLFAs were used to compare soil microbial communities on various spatial scales – on local (**I**), regional (**II**) and larger scales (**III**, **IV**). The results from conducted studies confirmed that the PLFA profiling has the large potential to reveal differences in MCS. The sensitivity of this method was demonstrated mainly in the **study I**, where even subtle response to fertilization treatment was detected while other microbial characteristics (with exception of C and N mineralization rates) did not respond to pronounced changes in soil and vegetation characteristics. In the **studies I** and **II,** the PLFA data were combined with numerous of auxiliary environmental variables, which allowed us to reveal coupling between microbial PLFA patterns and plant community composition (**I**) and to identify abiotic and biotic factors shaping the MCS (**I, II**). Such integrated approach allowed for the more complex understanding of ecosystem functioning and response to changes. In this context, I would like to stress the need of using multivariate statistical tools (PCA, redundancy analyses, variation partitioning, forward selection of explanatory variables) when using PLFA data either separately or in combination with other factors and environmental characteristics. The comparison of PLFA profiles among various soils highlights the main disadvantage of the PLFA-based approach. Despite its undisputable power to recognize shifts in MCS, the interpretative value of such information is limited due to the low taxonomic resolution (see section 1.3). The use of extended extraction method that yields significantly higher number of PLFAs (see section 1.1) than the simple extraction method used in conducted studies could partially compensate this deficit and enhance the sensitivity of detection of shifts in the MCS. However, the potential increase in the interpretative power in case these studies would be relatively small compared to the extra processing time and financial expenses needed. The **study III** proved the large potential of the combination of PLFA-SIP analysis and metagenomics. Such multiphasic approach can be especially valuable for the deeper understanding

of particular C sequestration processes. The use of certain FAs ratios as indicators of metabolic or physiological stress was not applicable in our studies. Despite significant differences in soil characteristics, such as soil pH (**studies I**, **II**), aeration, and C availability (**study III**), which were previously reported to trigger the stress response, no significant trends in the commonly used stress indicators were observed in our studies. Even when there were minor changes in indicator ratios observed, these could be caused by shift in microbial community composition and not induced by stress (see section 1.5). Our findings thus underpin the evident limitations of using PLFA-based stress indicators in studies which aim to investigate microbial communities in soils along natural environmental gradients.

4. Future prospects

I´m currently working on a study focused on the combined effect of fertilization and soil water level on the partitioning of C assimilated during photosynthesis in the plant-microbe-soil system (Kotas et al., in preparation; see section 1.6). We employed here the $^{13}CO_2$-labelling of wetland plant species (*Carex acuta*) grown in controlled mesocosms and tracked the assimilated C in different compartments of the experimental systems including microbial PLFAs. Our results indicate that the treatments interacted in their effect on C partitioning in the system including rhizodeposition intensity and subsequent utilization of root-released C by fungal and bacterial biomass.

In the future, I would like to continue in the research of interactions between plants and soil environment. These interactions are mediated by the regular input of exudates and other rhizodeposits into the surrounding of plant roots, which strongly affects diversity and activity of soil microorganisms and results in the rhizosphere priming effect, the process largely affecting C and N cycling in the soil (Kuzyakov, 2010). The microbially-driven processes in the rhizosphere are thus directly linked with root exudation. However, it is poorly understood how closely root exudation corresponds with the plant metabolome and how it is related to plant traits and changing nutrient availability. Therefore, my main prospect for future work is to focus on finding the links between the soil nutrient availability, plant functional traits, especially the root exudation intensity and composition, and microbial activity. To fulfil these goals, I plan to exploit my extensive experience with various chromatographic methods and mass spectrometric techniques as well as with stable isotope methods and immerse myself into the field of metabolomics.

5. References

Abraham, W.R., Hesse, C., Pelz, O., 1998. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Applied and Environmental Microbiology 64:4202–4209.

Ahlgren, G., Gustafsson, I.B., Boberg, M., 1992. Fatty-acid content and chemical composition of fresh-water microalgae. Journal of Phycology 28:37–50.

Amaral, J.A. and Knowles, R., 1995. Growth of methanotrophs in oxygen and methane counter gradients. FEMS Microbiology Letters 126:215–220.

Amelung, W., Brodowski, S., Sandhage-Hofmann, A., Bol, R., 2008. Combining biomarker with stable isotope analyses for assessing the transformation and turnover of soil organic matter. *In:* Sparks, D. (Ed.), Advances in Agronomy, vol. 100. Academic Press, Burlington, pp. 155–250.

Anthony, C., 1982. The biochemistry of methylotrophs. Academic Press Ltd., London.

Arao, T., Okano, S., Nishio, T., 2001. Comparison of bacterial and fungal biomass determined by phospholipid fatty acid and direct microscopical analysis in 4 types of upland soils. Soil Microorganisms 55:29–36.

Bååth, E., 2003. The Use of neutral lipid fatty acids to Indicate the physiological conditions of soil fungi. Microbial Ecology 45:373–383.

Bååth, E. and Anderson, T-H., 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFAbased techniques. Soil Biology & Biochemistry 35:955–63.

Bååth, E., Frostegård, Å., Pennanen, T., Fritze, H., 1995. Microbial community structure and pH response in relation to soil organic matter quality in wood-ash fertilized, clear-cut or burned coniferous forest soils. Soil Biology & Biochemistry 2:229–240

Bailey, V.L., Peacock, A.D., Smith, J.L., Bolton, H. Jr., 2002. Relationships between soil microbial biomass determined by chloroform fumigation–extraction, substrate-induced respiration, and phospholipidfatty acid analysis. Soil Biology & Biochemistry 34:1385–1389.

Balasooriya, W.K., Denef, K., Huygens, D., Boeckx, P., 2014. Translocation and turnover of rhizodeposit carbon within soil microbial. Plant and Soil 376:61–73.

Balkwill, D.L., Leach, F.R., Wilson, J.T., McNabb, J.F. and White, D.C., 1988. Equivalence of microbial measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. Microbial Ecology 16:73–84.

Bardgett, R. and van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature 515:505–511.

Becker, P.M., Stottmeister, U., 1998. General (Biolog GN) versus site-relevant (pollutant-dependent) sole-carbon-source utilization patterns as a means to approaching community functioning. Canadien Journal of Microbiology 44:913–919.

Belnap, J., Lange, O.L., 2003. Biological Soil Crusts: Structure, Function, and Management. Springer Berlin Heidelberg.

Blagodatskaya, E. and Kuzyakov, Y., 2013. Active microorganisms in soil: Critical review of estimation criteria and approaches. Soil Biology & Biochemistry 67:192–211.

Blumenberg M, Seifert R, Nauhaus K, Pape T, Michaelis W., 2005. In vitro study of lipid biosynthesis in an anaerobically methaneoxidising microbial mat. Applied and Environmental Microbiology. 71:4345–4351.

Boschker, H.T.S. and Middelburg, J.J., 2002. Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology 40:85–95.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkes, R.J., Cappenberg, T.E., 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C labelling of biomarkers. Nature 392:810–805.

Boshker, H.T.S., Kromkamp, J.C., Middelburg, J.J., 2005. Biomarker and carbon isotopic constraints on bacterial and algal community structure and functioning in a turbid, tidal estuary. Limnology and Oceanography 50:70–80.

Bossio, D.A., Scow, K.M., 1998. Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilisation patterns. Microbiology Ecology 35:265–278.

Boutton, T.W., Archer, S.R., Midwood, A.J., Zitzer, S.F., and Bol, R., 1998. $\delta^{13}C$ values of soil organic matter and their use in documenting vegetation change in subtropical savannah ecosystems. Geoderma 82:5–41.

Brant, J.B., Myrold, D.D., Sulzman, E.W., 2006. Root controls on soil microbial community structure in forest soils. Oecologia 148:650–659.

Canuel, E.A. and Martens, C.S.. 1993. Seasonal-variations in the sources and alteration of organic-matter associated with recently deposited sediments. Organic Geochemistry 20:563–577.

Chaudhary, D.R., Saxena, J., Lorenz, N., Dick, L.K., Dick, R.P., 2012. Microbial Profiles of rhizosphere and bulk soil microbial communities of biofuel crops switchgrass (*Panicum virgatum* L.) and Jatropha

(*Jatropha curcas* L.). Applied and Environmental Soil Science, http://dx.doi.org/10.1155/2012/906864.

Christie, W.W., 1982. Lipid Analysis, Pergamon Press, Oxford.

Cook, P.L. M., Butler, E.C.V., and Eyre, B.D., 2004. Carbon and nitrogen cycling on intertidal mudflats of a temperate Australian estuary. I. Benthic metabolism. Marine Ecology Progress Series 280:25–38.

Crossman, Z.M., Ineson, P., Evershed, R.P., 2005. The use of ^{13}C labelling of bacterial lipids in the characterisation of ambient methane-oxidising bacteria in soils. Organic Geochemistry 36:769–778.

Diefenbach, R. and Keweloh, H., 1994. Synthesis of *trans* unsaturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. Archives of Microbiology 162:120–125.

Diefenbach, R., Heipieper, H.J., Keweloh, H., 1992. The conversion of *cis* into *trans* unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity. Applied Microbiology and Biotechnology 38:382–387.

Drenovsky, R.E., Elliott, G.N., Graham, K.J., Scow, K.M., 2004. Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. Soil Biology & Biochemistry 36:1793–1800.

Dunstan, G.A., Volkman, J.K., Barrett, S.M., LeRoi, J.-M., Jeffrey, S.W., 1994. Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35:155–161.

Edlund, A., Nichols, P.D., Roffey, R., White, D.C., 1985. Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. Journal of Lipid Research 26:982–988.

Ekblad, A. and Hoögberg, P., 2000. Analysis of $\delta^{13}C$ of CO_2 distinguishes between microbial respiration of added C4-sucrose and other soil respiration in a C3-ecosystem. Plant and Soil 219:197–209.

Elbert, W., Weber, B., Burrows, S., Steinkamp, J., Büdel, B., Andreae, M.O., Pöschl, U., 2012. Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. Nature Geoscience 5:459–462.

Elhottová, D., Koubová, A., Šimek, M., Cajthaml, T., Jirout, T., Esperschuetz, J., 2012. Changes in soil microbial communities as affected by intensive cattle husbandry. Applied Soil Ecology 58:56–65.

Esperschütz, J., Buegger, F., Winkler, J.B., Munch, J.C., Schloter, M., Gattinger, A., 2009. Microbial response to exudates in the rhizosphere of young beech

trees (*Fagus sylvatica* L.) after dormancy. Soil Biology & Biochemistry 41:1976–1985.

Evershed, R.P., Crossman, Z.M., Bull, I.D., Mottram, H., Dungait, J.A.J., Maxfield, P.J., Brennand, E.L., 2006. ^{13}C-labelling of lipids to investigate microbial communities in the environment. Current Opinion in. Biotechnology 17:72–82.

Freschet, G.T., Cornwell, W.K., Wardle, D.A., Elumeeva, T.G., Liu, W., Jackson, B.G., Onipchenko, V.G., Soudzilovskaia, N.A., Tao, J., Cornelissen, J.H.C.. 2013. Linking litter decomposition of above and below-ground organs to plant-soil feedbacks worldwide. Journal of Ecology 101:943–52.

Frostegård Å, Bååth E (1996) The use of phospholipid analysis to estimate bacterial and fungal biomass in soils. Biol Fertil Soils 22:59–65

Frostegård, Å., Bååth, E., Tunlid, A., 1993a. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology & Biochemistry 25:723–730.

Frostegård, Å., Tunlid, A., Bååth, E., 2003b. Phospholipids fatty acid composition, biomass and activity of microbial communities from two soil types exposed to different heavy metals. Soil Biology & Biochemistry 25:723–730.

Frostegård, Å., Tunlid, A., Bååth, E., 2011. Use and misuse of PLFA measurements in soils. Soil Biology & Biochemistry 43:1621–1625.

Garland, J.L., 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biology & Biochemistry 28:213–221.

Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source-utilization. Applied and Environmental Microbiology 57:2351–2359.

Gattinger, A., Gunthner, A., Schloter, M., Munch, J.C., 2003. Characterization of Archaea in soils by polar lipid analysis. Acta Biotechnologica 23:21–28.

Gattinger, A., Schloter, M., Munch, J.C., 2002. Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. FEMS Microbiolology Letters 213:133–139.

Geyer, R., Peacock, A.D., Miltner, A., Richnow, H.H., White, D.C., Sublette, K.L., Kästner, M., 2005. In situ assessment of biodegradation potential using biotraps amended with ^{13}C-labeled benzene or toluene. Environmental Science and Technology 39:4983–4989.

Graham, J.H., Hodge, N.C., Morton, J.B., 1995. Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae. Applied and Environmental Microbiology 61:58–64.

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., Griffiths, B.S., Rodwell, J.S., Edwards, S.J., Davies, W.J., Elston, D.J., Millard, P., 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA, and community DNA techniques. Applied Soil Ecology 25:63–84.

Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C., 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiology Ecology 31:147–158.

Guckert, J.B., Hood, M.A., White, D.C., 1986. Phospholipids esterlinked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: Increases in the ratio and proportions of cyclopropyl fatty acids. Applied and Environmental Microbiology 52:794–801.

Guckert, J.B., Ringleberg, D.B., White, C.C., Hanson, R.S., Bratina, B.J., 1991. Membrane fatty acids as phenotypic markers for the polyphasic approach to taxonomy of methylotrophs within the Proteobacteria. Journal of General Microbiology 137:2631–2641.

Gunina, A., Dippold, M., Glaser, B., Kuzyakov, Y., 2017. Turnover of microbial groups and cell components in soil: ^{13}C analysis of cellular biomarkers. Biogeosciences 14:271–283.

Handelsman, J., 2009. Metagenetics: spending our inheritance on the future. Microbial Biotechnology 2:138–9.

Hanson, R.S. and Hanson, T.E., 1996. Methanotrophic Bacteria. Microbiological reviews 60:439–471.

Hardison, A.K., Canuel, E.A., Anderson, I.C., Tobias, C.R., Veuger, B., Waters, M.N., 2013. Microphytobenthos and benthic macroalgae determine sediment organic matter composition in shallow photic sediments. Biogeosciences 10:5571–5588.

Harwood, J.L. and Russel, N.J., 1984. Lipids in plants and microbes. Allen and Unwin, London.

Hatzenpichler R., 2012. Diversity, physiology and niche differentiation of ammonia-oxidizing archaea. Applied and Environmental Microbiology 78:7501-7510.

Heipieper, H.J., Loffeld, B., Keweloh, H. and de Bont, J.A.M., 1995. The *trans*/*cis* isomerization of unsaturated fatty acids in *Pseudomonas putida* S12 an

indicator for environmental stress due to organic compounds. Chemosphere 30:1041–1051.

Heipieper, H.J., Meulenbeld, G., Oirschot, Q.V., de Bont, J.A.M., 1996. Effect of environment factors on *trans*/*cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Applied and Environmental Microbiology 62:2773–2777.

Hoffman, L., 1989. Algae of terrestrial habitats. Botanical Review. 55:77–105.

Högberg, M.N., 2006. Discrepancies between ergosterol and the phospholipid fatty acid 18:2ω6,9 as biomarkers for fungi in boreal forest soils. Soil Biology & Biochemistry 38:3431–3435.

Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C., Crowley, D.E., 2002. Microbial diversity along a transect of agronomic zones. FEMS Microbiology Ecology 39:183–191.

Jenkinson, D.S. and Ladd, J.N., 1981. Microbial biomass in soil: measurement and turnover, *In:* Paul, E.A. and Ladd, J.N. (eds.), Soil biochemistry, vol.5., Dekker, New York.

Joergensen, R.G. and Wichern, F., 2008. Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biology & Biochemistry 40:2977–2991.

Kaiser, C., Frank, A., Wild, B., Koranda, M., Richter, A., 2010a. Negligible contribution from roots to soil-borne phospholipid fatty acid fungal biomarkers 18:2ω6,9 and 18:1ω9. Soil Biology & Biochemistry 42:1650–1652.

Kaiser, Ch., Kilburn, M.R., Clode, P.L., Fuchslueger, L., Koranda, M., Cliff, J.B., Solaiman, Z.M., Murphy, D.V., 2015. Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation. New Phytologist 205:1537–1551.

Kaiser, Ch., Koranda, M., Kitzler, B., Fuchslueger, L., Schnecker, .J, Schweiger, P., Rasche, F., Zechmeister-Boltenstern, S., Sessitch, A., Richter, A., 2010. Belowground carbon allocation by trees drives seasonal patterns of extracellular enzyme activities by altering microbial community composition in a beech forest soil. New Phytologist 187:843–58.

Kaneda, T., 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiological Reviews 55:288–302.

Kaštovská, K., Stibal, M., Šabacká, M., Černá, B., Šantrůčková, H., Elster, J., 2007. Microbial community structure and ecology of subglacial sediments in

two polythermal Svalbard glaciers characterized by epifluorescence microscopy and PLFA. Polar Biology 30:277–287.

Kates, M., 1964. Bacterial lipids. Advances in Lipid Research 2:17–90.

Kates, M., 1993. Lipids of archaea. *In:* Kates, M., Kushner, D.J., Matheson, A.T. (eds.), The Biochemistry of Archaea (Archaebacteria). Elsevier, New York, pp. 261–295.

Khotimchenko, S.V., Vaskovsky, V.E., Titlyanova, T.V., 2002. Fatty acids of marine algae from the pacific coast of North California, Botanica Marina 45:17–22.

Kieft, T.L., 2000. Size matters: dwarf cells in soil and subsurface terrestrial environments. *In:* Colwell, R.R., Grimes, D.J. (Eds.), Nonculturable Microorganisms in the Environment. ASM Press, Washington, DC, pp. 19–46.

Kieft, T.L., Ringelberg, D.B., White, D. C.. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in porous medium. Applied and Environmental Microbiology 60:3292–3299.

Kindler, R., Miltner, A., Thullner, M., Richnow, H.-H., Kästner, M., 2009. Fate of bacterial biomass derived fatty acids in soil and their contribution to soil organic matter. Organic Geochemistry 40:29–37.

Kirk, J.L., Beaudette L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T., 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods 58:169–188.

Klamer, M. and Bååth, E., 2004. Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biology & Biochemistry 36:57–65.

Kohring, L.L., Ringelberg, D.B., Devereux, R., Stahl, D.A., Mittelman, M.W., White, D.C., 1994. Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA sequence similarities among dissimilatory sulfate-reducing bacteria. FEMS Microbiology Letters 119:303–308.

Kong, A.Y.Y., Scow, K.M., Córdova-Kreylos, A.L., Holmes, W., Six, J., 2011. Microbial community composition and carbon cycling within soil microenvironments of conventional, low-input, and organic cropping systems. Soil Biology & Biochemistry 42:20–30.

Kotas. P., Cardenas, J., Edwards, K., Bárta, J., Kaštovská, E. Fertilization of wetland plants stimulates fungal utilization of root exudates and their incorporation to soil organic matter. *In preparation*

Kroppenstedt, R.M., 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. *In:* Goodfellow, M., Minikin, D.E., (Eds.), Chemical methods in bacterial systematics. Academic Press, London, pp. 173–194.

Kuzyakov, Y., 2010. Priming effects: interactions between living and dead organic matter. Soil Biology & Biochemistry 42:1363–71.

Lechevalier, H. and Lechevalier, M.P., 1988. Chemotaxonomic use of lipids – an overview. *In:* Ratledge, C., Wilkinson, S.G. (eds.), Microbial lipids, vol. 1., Academic Press, London, pp 869–902.

Lechevalier, M.P., 1989. Lipids in bacterial taxonomy. *In:* O'Leary, W.M. (ed.), Practical handbook of microbiology. CRC, Boca Raton, pp. 455–561

Lerch, T.Z., Dignac, M.-F., Nunan, N., Berdoux, G., Barriuso, E., Mariotti, A., 2009. Dynamics of soil microbial populations involved in 2,4-D biodegradation revealed by FAME-based Stable Isotope Probing. Soil Biology & Biochemistry 41:77–85.

Mancuso, C.A., Odham, G., Westerdahl, G., Reeve, J.N., White, D.C., 1985. C15, C20, and C23 isoprenoid homologues in glycerol diether phospholopids of methanogenic archaebacteria. Journal of Lipid Research 26**:**1120–1125.

Mansour, M.P., Holdsworth, D.G., Forbes, S.E., Macleod, C.K., Volkman, J.K., 2004. High contents of 24:6(n-3) and 20:1(n-13) fatty acids in the brittle star *Amphiura elandiformis* from Tasmanian coastal sediments. Biochemical systematics and Ecology 33:659–674.

Metting, B., 1981. The systematics and ecology of soil algae. Botanical Review. 47:195–312.

Miltner, A., Richnow, H. H., Kopinke, F.D., Kastner, M., 2004. Assimilation of CO2 by soil microorganisms and transformation into soil organic matter. Organic Geochemistry 35:1015–1024.

Miltner, A., Richnow, H.H., Kopinke, F.D., Kastner, M., 2004. Assimilation of CO_2 by soil microorganisms and transformation into soil organic matter. Organic Geochemistry 35:1015–1024.

Muyzer, G. and Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Narure Reviews Microbiology 6:441–454.

Nichols, P.D., Henson, J.M., Antworth, C.P., Parsons, J., Wilson, J.T., White, D.C., 1987. Detection of a microbial consortium, including type II methanotrophs, by use of phospholipid fatty acids in an aerobic halogenated hydrocarbon degrading soil column enriched with natural gas. Environmental Toxicology and Chemistry 6:89–97.

Nichols, P.D., Smith, G.A., Antworth, C.P., Hanson, R.S., White, D.C., 1985. Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria. FEMS Microbiology Ecology 31:327–335.

O´Donnel, A.G., Goodfellow, M., Minnikin, D.F., 1982. Lipids in the Classification of *Nocardioides:* Reclassification of *Arthrobacter simplex* (Jensen) Loehhead in the Genus *Nocardioides* (Prauser) emend. O'Donnell et al. as *Nocardioides simplex* comb. nov. Archives of Microbiology 133:323–329.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303–310.

Olsson, P.A., Bååth, E., Jakobsen, I., 1997. Phosphorus effects on mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by fatty acid signatures. Applied and Environmental Microbiology 63:3531–3538.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The u;se of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal mycelium in soil. Mycological Research 99:623–629.

Olsson, P.A., Francis, R., Read, D.J. Söderström, B., 1998. Growth of arbuscular mycorrhizal mycelium in calcareous dune sand and its interactions with other soil microorganisms as estimated by measurement of specific fatty acids. Plant and Soil 201:9–16.

Oulas, A., Pavloudi, Ch., Polymenakou, P., Pavlopoulos, G.A., Papanikolaou, N., Kotoulas, G., Arvanitidis, Ch., Iliopoulos, I., 2015. Metagenomics: Tools and Insights for Analyzing Next-Generation Sequencing Data Derived from Biodiversity Studies. Bioinformatics and Biology Insights 9:75–88.

Parker, J.H., Smith, G.A., Fredrickson, H.L., Vestal, J.R., White, D.C., 1982. Sensitive assay, based on hydroxy fatty acids from lipopolysaccharide lipid A, for Gram-negative bacteria in sediments. Applied and Environmental Microbiology 44:1170–1177.

Parker, J.H., Smith, G.A., Fredrickson, H.L., Vestal, J.R., White, D.C., 1982. Sensitive assay, based on hydroxy fatty acids from lipopolysaccharide lipid A, for Gram-negative bacteria in sediments. Applied and Environmental Microbiology 44:1170–1177

Parkes, R.J. and Calder, A.G., 1985. The cellular fatty acids of three strains of *Desulfobulbus,* a propionate-utilising sulphate-reducing bacterium. FEMS Microbiology Ecology 31:361–363.

Parkes, R.J. and Taylor, J., 1983. The relationship between fatty acid distributions and bacterial respiratory types in contemporary marine sediments. Estuarine, Coastal and Shelf Science 16:173–189.

Petersen, S.O. and Klug, M.J., 1994. Effect of sieving, storage, and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. Applied and Environmental Microbiology 62:2421–2430.

Peterson, M. and Bååth, E., 2003. Temperature-dependent changes in the soil bacterial community in limed and unlimed soil. FEMS Microbiology Ecology 45:13–21.

Pinkart, H.C., Ringelberg, D.B., Piceno, Y.M., MacNaughton, S.J., White, D.C*., 2002. Biochemical approaches to biomass measurements* and community structure analysis. *In:* Hurst, C.J., Crawford, R.L., Knudsen, R., McInerney, M.J., Stetzenbach, L.D. (eds.), Manual of Environmental Microbiology (Second Edition), Washington, DC: ASM Press. pp. 101–103.

Potthoff, M., Steenwerth, K.L., Jackson, L.E., Drenovsky, R.E., Scow, K.M., Joergensen, R.G., 2006. Soil microbial community composition as affected by restoration practices in California grassland. Soil Biology & Biochemistry 38:1851–1860.

Potts, M., Olie, J.J., Nickels, J.S., Parsons, J., White, D.C., 1987. Variations in phospholipid ester-linked fatty acids and carotenoids of desiccated *Nostoc commune* (cyanobacteria) from different geographic locations. Applied and Environmental Microbiology 53:4–9.

Prescott, L.M., Harley, J.P., and Klein, D.A., 2005. Microbiology, Sixth edition, McGraw Hill International edition, New York.

Prosser, J.I., 2002. Molecular and functional diversity in soil microorganisms. Plant Soil 244:9–17.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Holben, W.E., Gannon, J.E., 2006. Choice of methods for soil microbial community analysis: PLFA maximizes power compared to CLPP and PCR-based approaches. Pedobiologia 50:275–280.

Ratledge, C. and Wilkinson, S.G., 1988. Microbial lipids. Academia Press Ltd., London, U.K.

Reichardt, W., Mascarina, G., Padre, B., Doll, J., 1997. Microbial communities of continuously cropped, irrigated rice fields. Applied and Environmental Microbiology 63:233–238.

Rice, S.A. and Oliver, J.D., 1992. Starvation response of the marine barophile CNPT-3. Applied and Environmental Microbiology 58:2432–2437.

Robinson, P.M., Smith, J.M., 1979. Development of cells and hyphae of *Geotrichum candidum* in chemostat and batch culture. Transactions of the British Mycological Society 72:39–47.

Rues, L. and Chamberlain, P.M., 2010. The fat that matters: Soil food web analysis using fatty acids and their carbon stable isotope signature. Soil Biology & Biochemistry 42:1898–1910.

Šantrůčková, H., Bird, M.I., Elhottová, D., Novák, J., Picek, T., Šimek, M., Tykva, R., 2005. Heterotrophic fixation of CO_2 in soil. Microbial Ecology 49:218–225.

Šantrůčková, H., Kotas, P., Bárta, j., Urich, T., Čapek, P., Palmtag, J., Eloy Alves, R. J., Biasi, Ch., Diáková, K., Gentsch, N., Gittel, A., Guggenberger, G., Hugelius, G., Laschinsky, N., Martikainen, P. J., Mikutta, R., Schleper, Ch., Schnecker, J., Schwab, C., Shibistova, O., Wild, B., Richter, A. Significance of dark CO2 fixation in arctic soil. Soil Biology & Biochemistry, in review.

Schoug, Å, Fischer, J., Heipieper, H.J., Schnürer, J., Håkansson, S., 2008. Impact of temperature on the phospholipid fatty acid profile of a soil microbial community. Applied and Environmental Microbiology 62:2421–2430.

Sikkema, J., de Bont, J.A.M., Poolman, B., 1995. Mechanism of membrane toxicity of hydrocarbons. Microbiological Reviews 59:201–222.

Souffreau, C., Vanormelingen, P., Verleyen, E., Sabbe, K., Vyverman, W., 2010. Tolerance of benthic diatoms from temperate aquatic and terrestrial habitats to experimental desiccation and temperature stress. Phycologia 49:309–324.

Taylor, J., Parkes, R.J., 1983. The cellular fatty acids of the sulphate-reducing bacteria, *Desulfobacter* sp., *Desulfobulbus* sp. and *Desulfovibrio desulfuricans.* Journal of General Microbiology 129:3303–3309.

Tollefson, T.S. and McKercher, R.B., 1983. The degradation of ^{14}C-labelled phosphatidyl choline in soil. Soil Biology & Biochemistry 15:145–148.

Tringe, S.G., von Mering, Ch., Kobayashi, A., Salamov, A., Chen, K., Chang, H.W., Podar, M., Short, J.M., Marthur, E.J., Detter, J.C., Bork, P., Hugenholtz, P., Rubin, E.M., 2005. Comparative metagenomics of microbial communities. Science 308:554–557.

Tunlid, A. and White, D.C., 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. *In:* Stotzky, G., Bollag, J.M. (eds), Soil biochemistry, vol .7., Dekker, New York, pp. 229–262.

Uhlířová, E., Elhottová, D., Tříska, J., Šantrůčková, H., 2005. Physiology and microbial community structure in soil at extreme water content. Folia Microbiologica 50:161–166.

van de Heijden, M.G.A., Bardgett, R.D., and van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant 644 diversity and productivity in terrestrial ecosystems, Ecology Letters, 11:296–310.

Vestal, J.R. and White, D.C., 1989. Lipid analysis in microbial ecology. Bioscience 39:535–541.

Volkman, J.K., Barrett, S.M., Blackburn, S.I., Mansour, M.P., Sikes, E.L., Gelin, F., 1998. Microalgal biomarkers: a review of recent research developments. Organic Geochemistry 29:1163–1179.

Volkman, J.K., Jeffrey, S.W., Nichols, P.D., Rogers, G.I., Garland, C.D., 1989. Fatty acid and lipid-composition of 10 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology 128:219–240.

Waldrop, M.P. and Firestone, M.K., 2004. Microbial community utilization of recalcitrant and simple carbon compounds: Impact of oak-woodland plant communities. Oecologia 138:1432–1439.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., van der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304:1629–1633.

Weckwerth, W., 2011. Green systems biology - From single genomes, proteomes and metabolomes to ecosystems research and biotechnology. Journal of Proteomics 75:284–305.

White, D.C. and Tucker, A.N., 1969. Phospholipid metabolism during bacterial growth. Journal of Lipid Research 10:220-233.

White, D.C., 1986. Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity. Archiv für Hydrobiologie-Beiheft Ergebnisse der Limnologie 31:1–18.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences 95:6578–6583.

Widmer, F., Fliessbach, A., Laczko, E., Schulze-Aurich, J., Zeyer, J., 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PLFA-, and biolog-analyses. Soil Biology & Biochemistry 33:1029–1036.

Williams, M.A., Myrold, D.D., Bottomley, P.J., 2006. Carbon flow from ^{13}C-labeled straw and root residues into the phospholipid fatty acids of soil

microbial community under field conditions. Soil Biology & Biochemistry 38:759–768.

Yao, H., He, Z., Wilson, M.J., Campbell, C.D., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microbial Ecology 40:223–237.

Yu, C.-P., Sayler, R.A.G., Chu, K.-H., 2005. Quantitative molecular assay for fingerprinting microbial communities of wastewater and estrogen-degrading consortia. Applied and Environmental Microbiology 71:1433–1444.

Yuan, H., Zhu, Z., Liu, S., Ge, T., Jing, H., Li, B., Liu, Q., Lynn, T.M., Wu, J., Kuzyakov, Y., 2016. Microbial utilization of rice root exudates: ^{13}C labeling and PLFA composition. Biology and Fertility of Soils 52:615–627.

Zak, D.R. and Kling, G.W., 2006. Microbial community composition and function across an arctic tundra landscape. Ecology 87:1659–1670.

Zancan, S., Trevisan, R., Paoletti, M.G., 2006. Soil algae composition under different agro-ecosystems in North-Eastern Italy. Agriculture, Ecosystems and Environment 112:1–12.

Zechmeister-Boltenstern, S., Keiblinger, K.M., Mooshammer, M., Penuelas, J., Richter, A., Sardans, J., Wanek, W., 2015. The application of ecological stoichiometry to plant-microbial-soil organic matter transformations. Ecological Monographs 85:133–155.

Zelles, L. and Bai, Q.Y., 1993. Fractionation of fatty acids derived from soil lipids by solid phase extraction and their quantitative analysis by GC-MS. Soil Biology & Biochemistry 25:495–507.

Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35:275–294.

Zelles, L., 1999a. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29:111–129.

Zelles, L., 1999b. Identification of single cultured micro-organisms based on their whole-community fatty acid profiles, using extended extraction procedure. Chemosphere 39:665–682.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology & Biochemistry 24:317–323.

Zenova, G.M., Shtina, E.A., Dedysh, S.N., Glagoleva, O.B., Likhacheva, A.A., Gracheva, T.A., 1995. Ecological relations of algae in biocenoses. Microbiology 64:121–133.

Research articles

Study I

Linking above- and belowground responses to 16 years of fertilization, mowing, and removal of the dominant species in a temperate grassland

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ECOSYSTEMS

Linking Above- and Belowground Responses to 16 Years of Fertilization, Mowing, and Removal of the Dominant Species in a Temperate Grassland

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Abstract

Species-rich oligotrophic meadows are affected by a wide range of management interventions that influence their functioning and capacity to deliver ecosystem services, but long-term studies on the above- and belowground adaptations to different management tools are still scarce. We focused on the interactive effects of NPK fertilization, mowing, and removal of the initially dominant species (*Molinia caerulea*) on plant, soil, and microbial responses in wet oligotrophic grassland in a 16-year full-factorial manipulative experiment. Changes in vegetation composition, soil pH, and nutrient availability were accompanied by altered microbial phospholipid fatty acid (PLFA) composition, whereas treatment effects on soil microbial biomass and carbon (C) mineralization were mainly related to changes in soil organic matter (SOM) content and nutrient availability. Fertilization decreased plant species richness aboveground and lowered SOM storage and microbial activity belowground. Mowing preserved high plant diversity and led to more efficient recycling of N within the grassland, whereas *Molinia* removal significantly affected only plant community composition. Mowing combined with fertilization maintained high species richness only in the short term. Belowground, mowing reduced N leaching from the fertilized system but did not prevent SOM depletion, soil acidification, and concomitant adverse effects on soil microbes. We conclude that annual mowing is the appropriate type of extensive management for oligotrophic species-rich meadows, but the concomitant nutrient depletion should not be compensated for by regular NPK fertilization due to its adverse effects on soil quality.

Key words: microbial community structure; PLFA; grassland; mowing; fertilization; dominant removal; pH.

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Author contributions All authors conceived the study; PK, HS, and EK analyzed the data; PK performed the PLFA analysis; MC analyzed the physico-chemical soil properties and performed microbial biomass measurements; JL designed and managed the long-term experiment and monitored the plant community; PK wrote the paper with editorial assistance from all authors.

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Introduction

Traditionally managed oligo- and mesotrophic species-rich meadows are among the ecosystems most endangered by current land use changes throughout Europe (Kaligaric and others 2006). For economic reasons, their traditional management of mowing once or twice a year is either intensified by fertilization and increasing mowing frequency, leading to significant changes in the above- and belowground components of the ecosystem (Bardgett and others 1999; Hedlund and others 2003), or the management is completely abandoned.

The two compartments of the system closely interact via top-down effects through plant-litter inputs (Bardgett and Shine 1999; Fornara and others 2011) and rhizodeposition (Hartmann and others 2009; Doornbos and others 2012), and bottom-up feedback effects on plant nutrient supply and resource partitioning via the microbial community associated with roots (van der Heijden and others 2008; Friesen and others 2011). These plant–microbe interactions are particularly strong in oligotrophic ecosystems (Bardgett and others 1999; Paterson 2003; van der Heijden and others 2008), while nutrient enrichment weakens the co-determinacy of plants and microbes (Wei and others 2013; Liu and others 2014). Therefore, the type of management with its specific impacts on the above- and belowground compartments of the system exerts an important influence on the functioning and services provided by the ecosystem.

Traditional regular mowing is a convenient management system for maintaining the species richness of grasslands (for example, Silvertown and others 2006; Galvánek and Lepš 2008). Long-term hay harvesting affects nutrient availability by decreasing soil potassium (K) and nitrogen (N) contents (Venterink and others 2009) and increases C allocation belowground (Bardgett and others 1998; Kuzyakov and others 2002). Enhanced belowground C inputs commonly stimulate the biomass and activity of the soil biotic community (Guitian and Bardgett 2000; Uhlířová and others 2005; Zhang and others 2013) and affects N cycling within the ecosystem (Hamilton and Frank 2001; Kaštovská and others 2014). Grassland fertilization potentially has the opposite effects of mowing in many cases. Application of mineral N and phosphorus (P) improves forage production and its nutritional quality but commonly causes loss of plant species richness (Hejcman and others 2014; Lepš 2014). Direct impacts of fertilization on soil include acidification (Liu and others 2014), increased N (Bardgett and McAlister 1999), and decreased base cation availability (Clark and others 2007). These changes are usually accompanied by reduced soil microbial biomass (Treseder 2008; Sillen and Dieleman 2012) and shifts in microbial community composition (Rousk and others 2010a; Zhang and others 2013; Legay and others 2016) including decreased fungal abundance (Bardgett and McAlister 1999; Bååth and Anderson 2003; Treseder 2008). It has been suggested that simultaneous application of fertilization and mowing may partly balance the negative effects of fertilization on plant diversity in semi-natural meadows (Lepš 2014). Whether mowing also compensates for the negative fertilization effects belowground is, however, unknown.

Management effects on the structure and functioning of species-rich wet meadows are dependent on the temporal scale (Hedlund and others 2003; Lepš 2014). Short-term experiments might underestimate the response of vegetation and soil and could thus provide erroneous conservation recommendations. Therefore, long-term studies combining aboveground–belowground approach to study ecosystem responses to management practices are desirable, but rare. To increase our understanding of plant–microbe–soil linkages, we examined a wet temperate grassland subjected for 16 years to mowing, fertilization, and dominant plant species removal in a full-factorial design. Changes in plant community composition in this experiment have previously been described (Lepš 1999, 2004, 2014). We complemented the existing data by studying plant productivity, stoichiometry, and soil microbial and physico-chemical properties. Our aim was to document the adaptation of this grassland to long-term management and any coupling or uncoupling of above- and belowground responses. We hypothesized that (1) fertilization would exert the strongest influence on plant and soil properties due to its direct effect on nutrient availability and soil acidity; the influence of mowing would be less pronounced than the influence of fertilization and mediated through regular biomass removal and a concomitant decrease in nutrient availability; dominant removal would have the weakest effect on belowground characteristics. (2) Combined application of fertilization and mowing would balance the effects of the individual treatments. Fertilization of mown plots would replenish the nutrients withdrawn with the harvested biomass, while mowing would reduce the amount of available nutrients and concomitant soil acidification compared to plots that are only fertilized. We expected that mown fertilized plots would have

intermediate nutrient availability and pH compared to mown-only and fertilized-only plots. (3) The resulting changes in microbial biomass, activity, and community composition in response to management would be mainly driven by the treatment effects on SOM content, nutrient availability, and soil acidity.

Materials and Methods

Experimental Site and Sampling

The study site is a species-rich oligotrophic wet meadow situated in south Bohemia, Czech Republic (48°57′11″ N, 14°35′34″ E, at 510 m a.s.l.). Mean annual temperature and precipitation are 7.8°C and 620 mm, respectively (nearby meteorological station České Budějovice, 400 m a.s.l.). The meadow was traditionally mown once or twice a year until the end of the 1980s and then mowing was re-introduced at the start of the experiment in 1994. Before the treatments were imposed, the plant community was highly similar among plots and characterized as Molinion with some elements of Violion caninae (Chytrý 2012) (dominant species *Molinia caerulea* with 35% cover). The soil is a cambisol with pH 4.1 ± 0.02, total soil C (C_{tot}) and N (N_{tot}) concentrations of 28 ± 0.6 and 2.5 ± 0.05 mg g^{-1}, respectively, and a bulk density of 1.4 ± 0.05 g cm^{-3} (mean values ± SEM for the upper 20 cm of soil in mown, unfertilized, and non-removal plots).

The experiment combined mowing, fertilization, and dominant species removal in a full-factorial design giving eight treatment combinations, each in three replications (plots 2 m × 2 m square with central 1 m^2 used for sampling (see Lepš 1999, 2004 and Table 6 in Online Appendix A for more details). Briefly, fertilization was conducted using commercial NPK fertilizer (78 kg N ha^{-1} y^{-1}, 123 kg P ha^{-1} y^{-1}, and 123 kg K ha^{-1} y^{-1}) applied annually in spring. Mowing was performed annually in late June, and the harvested biomass was removed. *Molinia* individuals were manually removed in April 1995, new individuals were removed annually when needed. The soil samples were collected in October 2010 after plant senescence to eliminate the direct influence of root exudation on the soil microbial community and to assess only permanent changes in soil characteristics. From each of the 24 plots, composite samples were prepared from four subsamples gathered with a soil corer (3-cm diameter) to a depth of 20 cm. Fresh soil samples were homogenized and sieved through a 2-mm mesh. Sample aliquots for enzyme activities and PLFA analyses were stored at −80°C. Samples for the determination of available nutrients and microbial biomass and activity were stored at 4°C for a maximum of 14 days. Subsamples for CEC, C_{tot}, and N_{tot} determinations were air-dried.

Abiotic Soil Properties

The pH of fresh soil samples was measured in 1 M KCl with a pH meter (1:5, w:v; pH 315i, WTW, Germany). The cation exchange capacity (CEC) was determined as the sum of base cations (BC: Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) and exchangeable acidity (Al^{3+} and H^{+}). BC content in soil was measured by atomic absorption spectrophotometry (Varian, Australia) in a 1 M NH_4Cl extract, and Al^{3+} and H^{+} ions were extracted in 1 M KCl and determined by titration according to Thomas (1982). C_{tot} and N_{tot} were measured using an elemental analyzer (vario MICRO cube, Elementar GmbH, Germany); total soil P (P_{tot}) was determined according to Kopáček and others (2001). Soluble organic C and N (C_{ex} and N_{ex}), representing pools of dissolved organic matter available for microorganisms (Wagai and Sollins 2002), were extracted in duplicates from fresh soil (0.5 M K_2SO_4, 1:4, w/v; Rennert and others 2007) within 48 h after sampling and determined with a LiquiTOC II analyzer (Elementar, Germany). The concentrations of NH_4^+ and NO_3^- in sulfate extracts were determined with a Flow Injection Analyzer (FIA Lachat QC8500, Lachat Instruments, USA). Extractable P (P_{ex}) was determined in 0.5 M $NaHCO_3$ (1:5, w/v) and measured with an absorption spectrophotometer (Genesys 10S, UV–Vis, Thermo Scientific, USA).

Plant Biomass and Stoichiometry

To measure the amount of aboveground plant biomass, three non-contiguous 20 cm × 20 cm squares were cut with scissors on the edges of the central 1 m^2 of each plot in June 2010. The litter was separated first, and then the biomass was sorted into species, dried, and weighed. The biomass mean C, N, and P contents were calculated as community-weighted averages of individual species (see Lepš and others 2011 for detailed description and analysis).

Microbial Biomass and Activity

Microbial C (C_{mic}), N (N_{mic}), and P (P_{mic}) were determined by chloroform fumigation-extraction

using k_{EC} = 0.45 (Vance and others 1987), k_{EN} = 0.54 (Brookes and others 1985), and k_{EP} = 0.4 (Brookes and others 1982). Net N mineralization rate (sum of nitrification and ammonification; N_{min}) was measured as the difference in concentrations of NH_4^+ and NO_3^- that occurred between day 7 and day 21 of incubation at 20°C, divided by the number of days. C mineralization (C_{min}) was measured with a gas chromatograph (Agilent 6850 Series, Agilent, USA) as the increase in CO_2 concentration during 48 h of soil incubation at 20°C in bottles sealed with rubber covers.

To characterize the microbial potential to release C, N, and P from organic substrates, activities of six hydrolytic enzymes responsible for the acquisition of C (*β*-glucosidase, cellobiosidase), N (Ala-aminopeptidase, Leu-aminopeptidase), and P (phosphatase, phosphodiesterase) were determined (Bárta and others 2013; Sinsabaugh and others 2009). Fresh soil subsamples (1 g) were homogenized in distilled water (100 ml) and enzyme activities were measured with standard fluorometric techniques (Marx and others 2001). Based on the preliminary measurements of saturation concentration of each 4-methylumbelliferone/7-amino-4-methylcoumarin-labeled substrate, we used 50 µl of 50 µM substrate solutions for the determination of *β*-glucosidase and cellobiosidase activities, 200 µM substrate solution for determination of phosphatase activity and 300 µM substrate solutions for determination of phosphodiesterase, Ala- and Leu-aminopeptidase activities. All fluorescence measurements were carried out using the microplate reader INFINITE F200 (TECAN, Germany) at an excitation wavelength of 365 nm and emission wavelength of 450 nm.

Microbial Community Structure: Phospholipid Fatty Acid Profile

The determination of phospholipid fatty acids (PLFAs) was based on the method of Frostegård and others (1993). Briefly, 2 g of soil (dry weight) was extracted twice with a single-phase extraction mixture consisting of chloroform, methanol, and phosphate buffer and consequently purified on silica columns (SPE-SI 1 g/6 ml; Phenomenex®, CA, USA) using chloroform, acetone, and methanol. The polar fraction was trans-esterified to the fatty acid methyl esters (FAME) through mild alkaline methanolysis (Bossio and Scow 1998). All PLFAs were quantified by an internal standard calibration procedure using methyl nonadecanoate (19:0) as the internal standard and six calibration levels of the following FAME standards: Bacterial Acid Methyl Ester (BAME), the 37-component FAME Mix, PUFA-2 and PUFA-3 (Supelco, Bellefonte, USA), BR3 Mixture, methyl nonadecanoate, 10-methyloctadecanoic acid and methyl 9(R),10(S)-methyleneoctadecanoate (Larodan lipids, Malmö, Sweden). To identify the FAMEs, retention times and mass spectra were compared with those obtained from standards. The PLFA profile comprised 27 individual PLFAs and was used to compare the microbial community composition between experimental plots. The fatty acids i15:0, a15:0, i16:0, i17:0, a17:0, i18:0 were used as markers of gram-positive bacteria (*G*+) and 16:1n11, 16:1n9, 16:1n7, 16:1n5, cy17:0, 18:1n7, cy19:0 as markers of gram-negative bacteria (*G*−) (Kaiser and others 2010); 10Me16:0, 10Me17:0 were considered as actinobacterial (Kroppenstedt 1985) and 18:1n9, 18:2n6,9 as fungal markers (Frostegård and Bååth 1996). Total bacterial biomass is represented by the sum of general bacterial markers 15:0, 17:0, 18:1n5, 18:1n11 and markers for *G*+ and *G*− bacteria. The total amount of PLFAs was calculated as the sum of all lipid markers mentioned above and non-specific PLFAs 14:0, 16:0, 17:1n6, 18:0, 20:0 and 22:0 (Kaiser and others 2010). The fungi-to-bacteria ratio (F/B) was calculated from the respective sums of the above bacterial and fungal PLFA markers. The PLFA profiles were compared as mol% of PLFA for detection of structural changes in the microbial community.

Statistical Analysis

We measured numerous biotic and abiotic soil variables, some of which were significantly correlated. To achieve the best statistical power while keeping the global Type I error low, we carried out separate multivariate analyses for logical groups of variables, allowing us to describe the treatment effect on (a) soil physico-chemical properties, (b) plant biomass and stoichiometry, (c) plant community composition, (d) microbial activity, (e) microbial biomass and stoichiometry, (f) microbial community structure (the PLFA profile), and (g) activity of C, N, and P hydrolyzing enzymes. We used Redundancy Analysis (RDA) to test the effect of all treatments together and partial RDA to test three main effects (that is, the significance of fertilization, mowing or dominant removal using the remaining treatments as covariates) without standardization by samples, centering and standardization by variables (because the variables were not always measured at the same scale, see Šmilauer and Lepš 2014) and a Monte Carlo simulation with 4999 permutations. We then examined each variable separately with three-way ANOVA with

interactions, followed by Tukey HSD tests. Principal component analyses (PCA) on the physico-chemical properties, plant community composition, and PLFA profile were used to identify particular trends connected with the applied treatments. To find the best candidates among soil physico-chemical properties for explaining shifts in the PLFA profile, RDA with forward selection of explanatory variables was used. We further correlated the sample scores of the first three principal components of PCA on the microbial PLFA composition and plant community composition (see Table 8 in Online Appendix C) to determine whether the microbial PLFA profiles were related to plant community composition. Consequently, the decomposition of explained variation (Šmilauer and Lepš 2014) was used on selected parameters to quantify their unique and shared effects on the PLFA profile.

Before applying the statistical analyses, data were checked for normality and log-transformed if necessary. All C:N:P stoichiometric ratios were calculated on a molar basis. Statistical tests with a *P* value lower than 0.05 were considered significant. Multivariate statistical analyses were performed with CANOCO for Windows version 5.0 (Ter Braak and Šmilauer 2012). ANOVA and Tukey HSD tests were performed with Statistica 10 (StatSoft, USA).

Results

Comparison of Treatment Effects on Ecosystem Properties

Annual spring application of mineral NPK fertilizer and regular mowing once a year were associated with significant shifts in ecosystem properties (Table 1). Both treatments significantly affected plant community composition, productivity and biomass C:N:P stoichiometry, with the largest proportion of data variability explained by fertilization. Belowground, the soil physico-chemical properties and mineralization rates were significantly influenced, again more by fertilization than by mowing (Table 1), but the microbial biomass and its C:N:P stoichiometry remained unchanged. Fertilization was the only treatment that affected the microbial PLFA profile. Removal of the dominant plant species explained only a small fraction of the variability in plant and soil data (Table 1) and its effect on the examined ecosystem parameters was not significant (shown by three-way ANOVAs with interactions). Therefore, we present below the results of two-way ANOVAs, comprising the effects of fertilization, mowing, and their interactions.

Influence of Treatments on Soil Physico-Chemical Properties

Fertilization significantly acidified soil and decreased soil CEC and C_{tot} content, while significantly increasing soil P_{tot} and P_{ex}. It also lowered C/P and N/P ratios of both bulk SOM and the pools of available nutrients (Table 2). The pronounced fertilization effect is shown on the PCA ordination diagram, separating unfertilized (left) from fertilized (right) plots along the first principal component (PC1) (Figure 1). Unfertilized plots were further separated along the second principal component (PC2) according to the presence or absence

Table 1. The Response of Grouped Plant, Soil Physico-Chemical and Microbial Variables to Experimental Treatments

Group of response variables	Percentage of variation explained by all treatments	Percentage of overall explained variation explained by individual treatments		
		Fertilization	Mowing	Dominant removal
Soil physico-chemical properties	**42.4*****	**68.7*****	**26.7****	4.6
Plant biomass and stoichiometry	**56.4*****	**54.3*****	**37.7*****	8.0
Plant community composition	**30.8*****	**57.6*****	**38.5*****	3.9
C and net N mineralization	**31.1***	**55.1***	**44.7***	0.2
Microbial biomass and stoichiometry	7.7	75.3	24.7	0.0
Microbial community composition—PLFA profile	17.2	**59.1***	27.3	13.6
Activity of C, N, P hydrolyzing enzymes	12.2	30.9	48.8	20.3

The figures shown are proportions of data variability explained by all treatments together and by individual treatments separately (that is, using the remaining two treatments as covariates; values represent the portion of total explained variability). Results of RDA.
*Statistically significant tests are indicated by: *P < 0.05, **P < 0.01, ***P < 0.001.*

Table 2. Soil Physico-Chemical Characteristics (pH, CEC, Contents of C, N, and P and Their Molar Ratios), Contents and Molar Ratios of Extractable C, N, and P Pools and Contents of NO_3^-, NH_4^+ in Plots Under Different Experimental Regimes

Treatment F	Treatment M	pH	CEC (meq g^{-1})	C_{tot} (mg g^{-1})	N_{tot} (mg g^{-1})	P_{tot} (mg g^{-1})	C_{tot}/N_{tot}	C_{tot}/P_{tot}	N_{tot}/P_{tot}
No	No	$^{b}4.0 \pm 0.03$	$^{b}63 \pm 2.8$	$^{b}29 \pm 0.9$	$^{b}2.6 \pm 0.1$	$^{a}0.48 \pm 0.02$	$^{a}13.3 \pm 0.2$	$^{c}157 \pm 9.2$	$^{b}12.1 \pm 0.7$
No	Yes	$^{b}4.1 \pm 0.02$	$^{b}66 \pm 4.2$	$^{b}30 \pm 1.0$	$^{b}2.6 \pm 0.1$	$^{b}0.58 \pm 0.03$	$^{a}13.7 \pm 0.2$	$^{bc}135 \pm 4.3$	$^{a}9.9 \pm 0.3$
Yes	No	$^{a}3.9 \pm 0.03$	$^{a}53 \pm 4.5$	$^{a}27 \pm 0.7$	$^{a}2.4 \pm 0.1$	$^{b}0.62 \pm 0.02$	$^{a}13.2 \pm 0.3$	$^{ab}113 \pm 4.9$	$^{a}8.6 \pm 0.3$
Yes	Yes	$^{a}3.9 \pm 0.02$	$^{a}53 \pm 2.1$	$^{a}26 \pm 1.3$	$^{a}2.3 \pm 0.1$	$^{b}0.64 \pm 0.03$	$^{a}13.2 \pm 0.2$	$^{a}106 \pm 4.5$	$^{a}8.1 \pm 0.4$

Treatment F	Treatment M	C_{ex} (μg g^{-1})	N_{ex} (μg g^{-1})	P_{ex} (μg g^{-1})	C_{ex}/N_{ex}	C_{ex}/P_{ex}	N_{ex}/P_{ex}	NO_3^- (μg g^{-1})	NH_4^+ (μg g^{-1})
No	No	$^{a}86 \pm 8.0$	$^{b}36 \pm 3.1$	$^{a}2.6 \pm 0.2$	$^{a}2.8 \pm 0.2$	$^{b}92 \pm 14$	$^{b}33 \pm 4.3$	$^{b}0.43 \pm 0.03$	$^{a}3.8 \pm 0.1$
No	Yes	$^{a}80 \pm 4.5$	$^{a}22 \pm 1.5$	$^{a}1.5 \pm 0.2$	$^{b}4.3 \pm 0.3$	$^{c}153 \pm 18$	$^{b}38 \pm 6.7$	$^{a}0.31 \pm 0.07$	$^{a}3.6 \pm 0.2$
Yes	No	$^{a}75 \pm 3.3$	$^{b}33 \pm 3.0$	$^{b}11.9 \pm 1.8$	$^{a}2.7 \pm 0.2$	$^{a}18 \pm 2$	$^{a}6.5 \pm 0.6$	$^{b}0.38 \pm 0.05$	$^{a}3.7 \pm 0.3$
Yes	Yes	$^{a}80 \pm 4.9$	$^{ab}30 \pm 2.2$	$^{b}13.4 \pm 1.3$	$^{a}3.2 \pm 0.2$	$^{a}16 \pm 2$	$^{a}5.2 \pm 0.6$	$^{a}0.24 \pm 0.03$	$^{a}3.2 \pm 0.3$

Treatment	d.f.	pH	CEC (meq g^{-1})	C_{tot} (mg g^{-1})	N_{tot} (mg g^{-1})	P_{tot} (mg g^{-1})	C_{tot}/N_{tot}	C_{tot}/P_{tot}	N_{tot}/P_{tot}
F	1	**20.03****	**8.9***	**8.43***	4.64	**12.93***	0.55	**30.65*****	**27.39*****
M	1	0.50	0.20	0.01	0.22	4.66	2.15	5.11	**7.42***
F × M	1	0.58	0.14	1.08	0.05	2.43	1.78	1.30	2.89

Treatment	d.f.	C_{ex} (μg g^{-1})	N_{ex} (μg g^{-1})	P_{ex} (μg g^{-1})	C_{ex}/N_{ex}	C_{ex}/P_{ex}	N_{ex}/P_{ex}	NO_3^- (μg g^{-1})	NH_4^+ (μg g^{-1})
F	1	0.91	0.69	**78.94*****	7.18	**69.66*****	**45.51*****	1.35	1.12
M	1	0.01	**9.16***	0.02	**17.11****	**5.71***	0.23	**6.48***	2.87
F × M	1	0.87	3.77	1.11	**5.25***	**6.36***	0.62	0.05	0.39

Results of two-way ANOVAs (F values) of the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns ($P < 0.05$). Statistically significant differences are indicated by: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (lower part of the table).*

of mowing (Figure 1; left part of the diagram), pointing to the significant interaction of fertilization and mowing. Mowing of unfertilized plots significantly lowered N_{ex} and increased the C_{ex}/N_{ex} and C_{ex}/P_{ex} ratios (Table 2; Figure 1), but this negative effect of mowing on N_{ex} and P_{ex} was compensated when mown plots were fertilized. The fertilized mown and fertilized unmown plots thus overlaid in the right part of the PCA diagram (Figure 1). The soil NO_3^- content was reduced by mowing, regardless of fertilization (Table 2).

Plant Response to Treatments

Fertilization stimulated aboveground plant productivity, while mowing decreased the amount of litter on experimental plots due to biomass removal (Table 3). Mowing was the only management, which significantly affected plant N content. It decreased N concentration in plant biomass, with concomitant increase of plant C/N ratios independent of fertilization (Table 3). Mowing and fertilization further significantly interacted in their effects on plant stoichiometry. Fertilization of unmown plots increased plant N and P concentrations compared to unfertilized plots; therefore, vegetation in fertilized unmown plots had the lowest C/N ratio from all treatments and lower C/P and N/P ratios than non-fertilized plants. Fertilization combined with mowing lowered plant C content and increased plant P but not N content, resulting in the lowest plant biomass N/P ratio of all treatments. The highest C/P ratio was determined in plant biomass from unfertilized mown plots (Table 4).

Microbial Response to Treatments

Neither the microbial biomass nor the microbial C:N:P stoichiometry reflected significant changes in the elemental stoichiometry of SOM, available nutrients, and plant biomass caused by the treatments. Among other microbial variables, only C mineralization rate decreased in fertilized versus unfertilized plots. Mowing strongly decreased net N mineralization rate in unfertilized plots, but this was balanced by fertilization of mown plots (Table 5). Generally, the net N mineralization was negatively correlated with the soil C_{ex}/N_{ex} ratio ($r = -0.69$, $P < 0.001$) and positively related with soil N_{ex} and NH_4^+ contents ($r = 0.62$ and 0.43, $P = 0.001$ and 0.035, respectively). The potential activities of extracellular enzymes for the acquisition of C, N, and P were spatially variable and not significantly influenced by the management interventions.

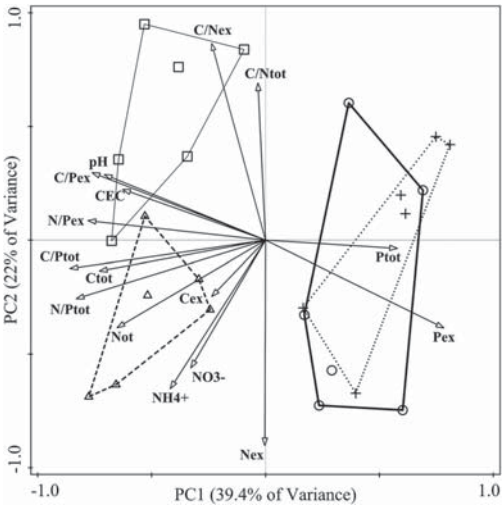

Figure 1. Separation of treated plots by the PCA based on soil physico-chemical properties. The *thin solid* and *dashed lines* represent unfertilized mown (*square*) and unmown (*triangle*) plots, the *bold solid* and *dotted lines* represent fertilized unmown (*circle*) and mown (*plus*) plots, respectively ($n = 6$). The portion of variance explained by the respective axis is given in the axis title.

The relative abundance of *G*+ and *G*− bacteria, actinobacteria, and fungi within microbial community as well as the F/B ratio was unaffected. All experimental manipulations accounted only for 17.2% of the variation in the microbial PLFA profile and their combined effect was not significant (Table 1; the alignment of PLFAs with experimental manipulations shown in Figure 3 in Online Appendix D). Therefore, the first two PCA components of the microbial PLFA composition did not depict any trends connected with the treatments (PC1 and PC2 accounted for 34.6 and 18.7% of variation, respectively; see Table 8 in Online Appendix C). However, the third principal component (PC3, 13.8% of data variation) significantly separated microbial PLFA profiles in fertilized and unfertilized plots (Figure 2A), which corresponds with a significant fertilization effect on the microbial PLFA profile (Table 1, results of the partial

Table 3. Plant Aboveground Productivity, Litter Amount, and Plant Biomass C, N, P Stoichiometry in Plots Under Different Experimental Regimes

Treatment F	Treatment M	Productivity (g m^{-2} DW)	Litter (g m^{-2} DW)	C (mg g^{-1} DW)	N (mg g^{-1} DW)	P (mg g^{-1} DW)	C:N	C:P	N:P
No	No	$^{b}414 \pm 52$	$^{b}650 \pm 134$	$^{b}435 \pm 2$	$^{a}20.1 \pm 0.4$	$^{a}1.9 \pm 0.1$	$^{b}25.4 \pm 0.4$	$^{b}611 \pm 25$	$^{c}24.1 \pm 1.1$
No	Yes	$^{a}270 \pm 22$	$^{a}134 \pm 18$	$^{b}436 \pm 2$	$^{a}18.3 \pm 0.4$	$^{a}1.4 \pm 0.0$	$^{b}27.9 \pm 0.5$	$^{c}788 \pm 12$	$^{c}28.3 \pm 0.6$
Yes	No	$^{b}504 \pm 47$	$^{b}735 \pm 154$	$^{b}438 \pm 1$	$^{b}23.3 \pm 1.0$	$^{b}2.9 \pm 0.2$	$^{a}22.2 \pm 1.0$	$^{a}407 \pm 31$	$^{b}18.5 \pm 1.6$
Yes	Yes	$^{b}500 \pm 50$	$^{ab}299 \pm 74$	$^{a}427 \pm 2$	$^{a}18.7 \pm 0.7$	$^{c}3.3 \pm 0.1$	$^{b}26.9 \pm 0.9$	$^{a}335 \pm 11$	$^{a}12.5 \pm 0.7$

Treatment	d.f.	Productivity (g m^{-2} DW)	Litter (g m^{-2} DW)	C (mg g^{-1} DW)	N (mg g^{-1} DW)	P (mg g^{-1} DW)	C:N	C:P	N:P
F	1	**10.5****	1.10	3.03	5.91	**136.41*****	**6.86***	**194.61*****	**84.77*****
M	1	2.25	**15.88****	**7.53***	**18.16****	0.01	**19.99*****	4.90	0.60
F × M	1	2.02	0.11	**10.99****	3.65	**12.74****	1.81	**28.24*****	**19.22*****

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns ($P < 0.05$). Statistically significant differences are indicated by: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (lower part of the table).*

Table 4. Microbial Biomass C, N, P Stoichiometry and Soil PLFA Content in Plots Under Different Experimental Regimes

Treatment F	Treatment M	C_{mic} ($\mu g\ g^{-1}$)	N_{mic} ($\mu g\ g^{-1}$)	P_{mic} ($\mu g\ g^{-1}$)	C_{mic}/N_{mic}	C_{mic}/P_{mic}	N_{mic}/P_{mic}	PLFAtot (nmol g^{-1} DW)
No	No	$^{a}896 \pm 41$	$^{a}113 \pm 8$	$^{a}44 \pm 6$	$^{a}9.4 \pm 0.4$	$^{a}56.5 \pm 5.3$	$^{a}6.2 \pm 0.7$	$^{a}152 \pm 8$
No	Yes	$^{a}1003 \pm 74$	$^{a}139 \pm 11$	$^{a}45 \pm 4$	$^{a}8.5 \pm 0.5$	$^{a}60.3 \pm 5.9$	$^{a}7.3 \pm 1.0$	$^{a}157 \pm 14$
Yes	No	$^{a}822 \pm 47$	$^{a}104 \pm 14$	$^{a}35 \pm 2$	$^{a}10.1 \pm 1.3$	$^{a}62.0 \pm 4.2$	$^{a}6.6 \pm 0.9$	$^{a}148 \pm 14$
Yes	Yes	$^{a}827 \pm 70$	$^{a}102 \pm 15$	$^{a}42 \pm 5$	$^{a}10.1 \pm 0.8$	$^{a}52.5 \pm 3.0$	$^{a}5.3 \pm 0.3$	$^{a}139 \pm 13$

Treatment	d.f.	C_{mic} ($\mu g\ g^{-1}$)	N_{mic} ($\mu g\ g^{-1}$)	P_{mic} ($\mu g\ g^{-1}$)	C_{mic}/N_{mic}	C_{mic}/P_{mic}	N_{mic}/P_{mic}	PLFAtot (nmol g^{-1} DW)
F	1	3.69	2.77	1.45	1.65	0.05	0.86	0.01
M	1	0.72	0.76	0.72	0.23	0.31	0.01	0.64
F x M	1	0.60	1.03	0.38	0.20	1.65	2.25	0.12

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented.
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns ($P < 0.05$). Statistically significant differences are indicated by: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (lower part of the table).*

Table 5. The C and Net N Mineralization Rates and Activities of C, N, P Hydrolyzing Enzymes in Plots Under Different Experimental Regimes

Treatment F	Treatment M	C mineralization ($\mu g\ C\text{-}CO_2\ g^{-1}\ day^{-1}$)	Net N mineralization ($\mu g\ N\text{-}NH_4^+ + N\text{-}NO_3^-\ g^{-1}\ day^{-1}$)	C hydrolysing enzymes (nmol $g^{-1}\ h^{-1}$)	N hydrolysing enzymes (nmol $g^{-1}\ h^{-1}$)	P hydrolysing enzymes (nmol $g^{-1}\ h^{-1}$)
No	No	$^{ab}7.9 \pm 0.5$	$^{b}0.184 \pm 0.035$	$^{a}191 \pm 46$	$^{a}107 \pm 13.0$	$^{a}548 \pm 90$
No	Yes	$^{b}8.8 \pm 0.4$	$^{a}0.051 \pm 0.013$	$^{b}426 \pm 119$	$^{a}168 \pm 29.9$	$^{a}794 \pm 94$
Yes	No	$^{a}7.2 \pm 0.3$	$^{b}0.223 \pm 0.040$	$^{b}458 \pm 159$	$^{a}121 \pm 19.9$	$^{a}971 \pm 323$
Yes	Yes	$^{a}7.3 \pm 0.5$	$^{b}0.167 \pm 0.028$	$^{b}448 \pm 106$	$^{a}158 \pm 29.8$	$^{a}798 \pm 46$

Treatment	d.f.	C mineralization ($\mu g\ C\text{-}CO_2\ g^{-1}\ day^{-1}$)	Net N mineralization ($\mu g\ N\text{-}NH_4^+ + N\text{-}NO_3^-\ g^{-1}\ day^{-1}$)	C hydrolysing enzymes (nmol $g\ h^{-1}$)	N hydrolysing enzymes (nmol $g\ h^{-1}$)	P hydrolysing enzymes (nmol $g\ h^{-1}$)
F	1	**5.61***	**5.22***	1.31	0.01	1.23
M	1	1.19	**7.82***	0.79	3.45	0.04
F × M	1	0.73	1.33	0.94	0.21	1.19

Results of two-way ANOVAs (F values) on the effects of fertilization (F), mowing (M), and their interaction (F × M) are also presented
*Mean ± SE (n = 6) are given in the upper part of the table. Different letters indicate significant differences between values within columns ($P < 0.05$). Statistically significant differences are indicated by: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (lower part of the table)*

RDA). Loadings of individual PLFAs revealed that particularly the bacterial markers were aligned along the PC3 (Figure 2B). The fungal PLFAs 18:2n6,9 and 18:1n9 were among the specific markers least correlated with PC3 and thus their abundances were not directly associated with experimental manipulations and changes in soil properties.

The RDA with forward selection of explanatory physico-chemical soil variables highlighted the influence of soil acidification and C/P_{ex} ratio on the PLFA profile ($P = 0.004$ and 0.015, respectively). These parameters affected mainly abundances of bacterial and actinobacterial PLFAs—relative abundance of markers 16:1n9 and 10Me17:0 significantly increased in acidified plots, while the bacterial PLFAs a15:0, i17:0, 16:1n5, cy17:0, and 18:1n7 were more abundant in unfertilized plots with higher soil pH. The PLFAs showing a relative increase with higher C/P_{ex} ratios were bacterial

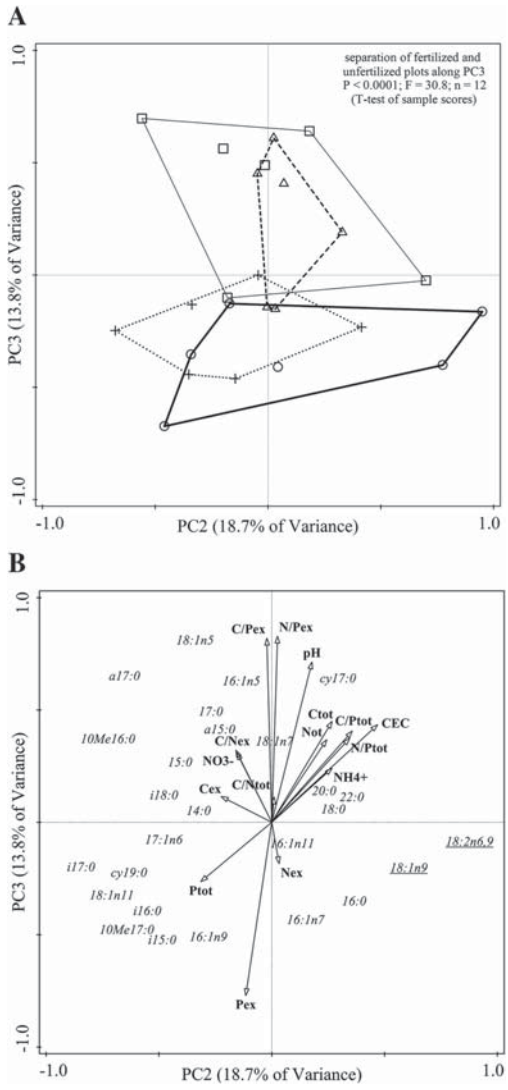

Figure 2. Sample scores on second and third axes from a PCA on the microbial PLFA composition (**A**) and loading of individual PLFAs (*italics*) together with loading of physico-chemical soil properties (*bold*) (**B**). Both figures display the same ordination space. The *thin solid* and *dashed lines* represent unfertilized mown (*square*) and unmown (*triangle*) plots, the *bold solid* and *dotted lines* represent fertilized unmown (*circle*) and mown (*plus*) plots, respectively ($n = 6$; **A**). *Arrows* indicate the direction in which the respective soil parameter value increases. Fungal PLFAs are underlined (**B**). The portion of variance explained by the respective axis is given in the axis title.

markers 16:1n5, a17:0, 17:0, and 18:1n5, whereas i15:0 and 16:1n7 were more abundant in fertilized soils with lower C/P_{ex} ratios (see Table 7 in Online Appendix B).

Microbial PLFA profiles were also related to plant community composition. This was indicated by significant correlations between sample scores on PC1 and PC3 of the microbial PLFA profiles and the corresponding sample scores on PC3 and PC1 of the plant community composition ($r = 0.6$ and 0.69, $P = 0.027$ and 0.003, respectively). The markers responsive to altered plant community composition were saturated, mainly non-specific PLFAs (15:0, 16:0, 17:0, 18:0, 20:0, 22:0), both fungal (18:1n9, 18:2n6,9) and bacterial PLFAs (16:1n5, 18:1n7, a15:0, a17:0) (see Table 7 in Online Appendix B). The plant community PC1 and PC3 together with soil pH and C/P_{ex} explained 22% of the variability in the PLFA profiles ($P < 0.001$). The results from variation partitioning show that 7.5% of the total variation in the PLFA profiles was explained by pH and the C/P_{ex} ratio ($P = 0.017$) and 5.5% solely by PC1 and PC3 of the plant community composition ($P = 0.034$). The rest of the explained variability (9%) was shared by these variables.

Discussion

Fertilization Effects on Vegetation, Soil, and Microbial Characteristics

In agreement with our first hypothesis, regular spring application of NPK fertilizer (78 kg N ha^{-1} y^{-1}, 123 kg P ha^{-1} y^{-1} and 123 kg K ha^{-1} y^{-1}) for 16 years exerted the strongest influence on both above- and belowground ecosystem characteristics of all treatments. Long-term fertilization brought about negative changes in basic soil physico-chemical properties such as decreased soil pH and CEC, as commonly reported in grassland fertilization experiments (for example, Silvertown and others 2006; Clark and others 2007; Liu and others 2014), and increased nutrient availability in the soil. This was reflected aboveground in the vegetation composition, where nutrient loading increased the abundance of competitive species like *Scirpus sylvaticus* L. and nitrophilous *Urtica dioica* L. in unmown plots, and of grasses (for example, *Agrostis capillaris* L., *A. canina* L., *Festuca rubra* L.) in mown plots. These plants outcompeted other species, including the initially dominant *Molinia*, and caused a sharp decrease of species richness (see Lepš 1999, 2004, 2014 for details).

Even though the new plant communities were more productive, the amount of soil C_{tot} decreased, indicating an overall loss of SOM. The only element found in greater amounts in the fertilized soil was P, but its presence mainly in exchangeable forms indicated physico-chemical sorption rather than incorporation into stabilized SOM. Our data showing a negative fertilization effect on SOM content are in agreement with the results of a meta-analysis of fertilization effects on grasslands by Sillen and Dieleman (2012). However, other studies have shown varying effects of N fertilization on SOM content, from the absence of any significant change in C content in mineral soils across biomes (Liu and Greaver 2010) to an increase in soil C sequestration (Conant and others 2001; Rousk and others 2011). The ambiguous image arising from these studies indicates that the fertilization effect on SOM content depends on many factors, such as the fertilization dose, the type of fertilizer, the longevity of fertilization, the ecosystem type, and its initial conditions. While an increase in SOM after grassland fertilization is commonly explained by enhanced input of organic matter due to increased primary production (Nyborg and others 1997; Rousk and others 2011), a decrease in SOM used to be ascribed to an enhanced decomposition of new, nutrient-rich organic matter and its priming effect on the old SOM (Bengston and others 2012; Kuzyakov 2010) as well as a declining plant belowground allocation (Bardgett and others 1999; Eisenhauer and others 2012). In our case, exoenzymatic activity and microbial biomass were not changed and microbial respiration even decreased, which indicated a decrease rather than an increase in potential SOM decomposition after 16 years of NPK fertilization. We thus suggest that the decrease in soil C_{tot} should be ascribed to lower root production, which is commonly observed after fertilization (Bardgett and others 1999; Eisenhauer and others 2012; Kaštovská and others 2012). The significantly increased aboveground productivity could not avert the loss of SOM, because the aboveground litter did not significantly contribute to soil C sequestration in this grassland. This was shown by the absence of differences in soil C_{tot} contents between mown plots, where the aboveground biomass is removed, and unmown plots with significantly more litter left on site (see Tables 2, 3), independent of fertilization. A likely explanation is the significantly faster decomposition of aboveground litter compared with belowground litter (Freschet and others 2013). Our results show that the effect of long-term fertilization on SOM content is a function of belowground rather than aboveground plant production. These findings are in line with the prominent role of belowground plant production in SOM formation suggested by Freschet and others (2013).

We further hypothesized that the resulting changes in microbial biomass and activity in response to management would be mainly driven by the treatment effects on SOM content and nutrient availability (Hypothesis 3). This was supported by the significant correlations across treatments between the SOM content and microbial biomass C as well as C mineralization ($r = 0.754$ and 0.674, respectively, both $P < 0.0001$). Similar relationships were found in other fertilization studies (Treseder 2008; Sillen and Dieleman 2012). Furthermore, C mineralization was also controlled by the relative C availability, as shown by its significant correlations with C_{ex}/N_{ex} and C_{ex}/P_{ex} ratios ($r = 0.512$ and 0.433, $P = 0.011$ and 0.035, respectively). Across all plots, C mineralization decreased together with decreasing availability of C relative to N and P, whereas absolute C availability (C_{ex} content) was not affected. This decrease cannot be ascribed to more efficient microbial metabolism when nutrient availability is higher, as suggested, for example by Agren and others (2001), because the ratios of mineralized to total C and mineralized to microbial C did not significantly change (data not shown). Instead, we conclude that long-term nutrient loading decreased C availability relative to N and P and thus strengthened the C limitation of microbial metabolism, as suggested by Kamble and others (2013). In summary, we suggest that fertilization significantly not only decreased the inputs of belowground plant litter to the soil but also decreased mineralization of preexisting SOM due to a stronger C limitation of microbial activity. In the long-term, however, the lower microbial activity could not compensate for the lower input of root-derived compounds, which resulted in reduced C sequestration in the soil of this grassland.

Mowing Effects on Vegetation, Soil, and Microbial Characteristics

In agreement with our first hypothesis, mowing had a weaker influence on ecosystem properties than fertilization. In contrast to fertilization, mowing increased plant species richness and spatial homogeneity of species composition (Lepš 2014). The long-term annual removal of aboveground plant biomass decreased soil nutrient availability compared with soil from unmown and fertilized

grasslands, which corroborates our hypotheses. This was indicated by lower availability of N and P relative to C, lower concentration of soil-available N (both N_{ex} and NO_3^-) and increased C/N and C/P ratios in plant biomass in comparison with unmown plots. Nutrient depletion was further indicated by significantly decreased plant aboveground productivity in plots that were only mown. Besides soil N availability, net N mineralization also significantly decreased in mown plots, suggesting more efficient N recycling between plants and microbes (Kaštovská and others 2014) and thus tighter plant–microbe interactions in the mown grassland (Bardgett and others 1999; Paterson 2003; van der Heijden and others 2008).

Some grassland studies have reported increased C allocation belowground and stimulation of soil microbial biomass and activity following mowing (Bardgett and others 1998; Guitian and Bardgett 2000; Uhlířová and others 2005; Zhang and others 2013). We found no permanent mowing effect on the biomass or activity of microbes in this long-term experiment, although such an effect could potentially be considerable in the short term following plant defoliation. In summary, mowing (on its own) decreased nutrient availability in the system, leading to more closely coupled plant–microbe relations driving a more efficient N cycling. These changes did not affect soil microbial biomass and its activity and had no effect on the SOM content in the long term. Considering the definitely positive effects on plant community composition and diversity, annual mowing is the most convenient management for sustaining species-rich wet grasslands.

Combined Effects of Mowing and Fertilization

It was recently shown that mowing can partially prevent the decline of plant species richness caused by fertilization (Lepš 2014). This led us to formulate Hypothesis 2 that combination of mowing and fertilization could compensate for a slow depletion of soil nutrients connected with long-term biomass removal (Venterink and others 2009). In support of our hypothesis, we found that fertilization of mown plots enhanced relative nutrient availability, as evidenced by significantly lower C/N and C/P ratios of soil extractable pools. The avoidance of nutrient depletion was further documented by significantly higher plant aboveground productivity and strongly increased plant P content in comparison with plots that were only mown. We also documented a positive effect of mowing on N recycling within the fertilized system, similar to that described above in mown-only plots. In plots under combined management, mowing decreased soil nitrate concentration as compared with fertilized-only plots and thus decreased the potential for N losses from the fertilized grassland.

However, mowing did not counterbalance the soil acidification and decrease in CEC directly connected with the application of mineral NPK fertilizer. Mowing also did not change the trend of decreasing SOM content and concomitant decrease in C mineralization and soil microbial biomass. These soil characteristics are among soil attributes adversely connected with maintaining soil quality and sustainable ecosystem productivity (Tóth 2008). Therefore, we summarize that mowing combined with annual application of NPK fertilizer at the given dose compensated only for nutrient deficiency caused by long-term grassland mowing but did not balance out negative changes in the soil caused by fertilization. Combination of these two management forms thus does not preserve soil functioning and the existing diversity of plant community in the longer term.

Effects of Dominant Species Removal on Vegetation and Soil Characteristics

The removal of *Molinia caerulea* had a positive effect on plant species richness, but the changes were much less pronounced than the effects of fertilization and mowing (Lepš 2014). However, *Molinia* removal did not cause any significant changes belowground. The evaluation of removal effects on soil properties was further complicated by the fact that *Molinia* was found to retreat spontaneously after fertilization and mowing, being replaced by other species in a way similar to the plots where it was selectively removed (Lepš 2004). Even though studies of microcosms (for example, Bardgett and others 1999; Kaštovská and others 2014; Legay and others 2016) and complex plant communities (Lange and others 2015) demonstrated regulation of microbial abundance and activity as well as C and N turnover by plant species traits, we found no evidence that removal of a single plant species, although dominant, induced a permanent effect on the soil environment in species-rich grassland.

Shifts in Microbial Community Composition as an Adaptation to Soil and Vegetation Changes

Microbial community composition was significantly affected only by NPK fertilization. The

changes we found within PLFA profiles were related mainly to soil acidification and decreasing C/P ratio of the soil solution, which corroborates with our third hypothesis. Soil pH was previously noted as a key factor driving microbial community composition (Fierer and others 2009; Rousk and others 2010a,b; Rousk and others 2011) and fungi-to-bacteria ratio (Bååth and Anderson 2003). In agreement with this, soil acidification significantly altered the microbial PLFA composition even though the overall pH range in our experiment stretched only from 4.18 to 3.85. These findings emphasize the need to consider carefully even very slight changes of soil pH when evaluating fertilization effects on microbial community composition. In accordance with observations by Bååth and Anderson (2003) and Rousk and others (2010a), only PLFA specific to bacteria were sensitive to changes in soil physico-chemical properties (see Table 7 in Online Appendix B), but these changes within bacterial PLFA had no effect on the fungi-to-bacteria ratio. High sensitivity of bacteria to soil pH was previously demonstrated also by gene-based molecular techniques (Fierer and others 2009; Rousk and others 2010a) and can be ascribed to the narrow pH ranges for optimal growth of bacteria (Fernández-Calviño and Bååth 2010). The specific bacterial PLFAs 18:1n7, 16:1n7, 16:1n5, cy19:0, and i16:0 have been noted previously as sensitive indicators of direct pH effects on the microbial community (Bååth and Anderson 2003; Rousk and others 2010b; Rousk and others 2011). Among those, only 18:1n7 and 16:1n5 responded significantly to soil pH changes in our study and we can thus recommend them as widely applicable sensitive indicators of the microbial response to changing pH. The likely explanation of the relatively weak response could be the narrow range of acidification in our experiment.

Shift in microbial PLFA profiles was not only related to soil pH and soil solution C/P ratio but also to plant community composition. The proportions of variability in the PLFA profiles explained by soil characteristics (7.5%) and by altered plant community composition (5.5%) were comparable. However, unlike the pH-related changes, shifts in vegetation composition affected the whole PLFA profile, including specific bacterial and fungal markers and many non-specific PLFAs, with the only exception of actinobacterial markers (see Table 7 in Online Appendix B). Such a widespread response of the microbial community to shifts in plant species composition is likely connected with potential variation in the quantity and quality of biochemical compounds provided by different plants (De Deyn and others 2008) and varying responses of different microorganisms to the altered plant inputs into the soil (Orwin and others 2010). The relation between plant and microbial community compositions indicates mutual adaptation of plant and microbial communities to particular grassland management types and supports the suggestion of specific plant–microbe interactions (for example, Bardgett and others 1999; Hartmann and others 2009; Orwin and others 2010; Doornbos and others 2012).

Conclusions

Long-term annual NPK fertilization of oligotrophic species-rich grasslands had a negative effect on the whole system. Aboveground, plant species richness decreased, and belowground, the soil was acidified and depleted of cations, and although C mineralization decreased, total SOM storage was reduced. Mowing as the single form of management has the potential to preserve plant diversity and decrease nutrient losses through microbial activity and leaching. Management combining fertilization with mowing effectively maintained the high plant species richness only in the short term. In the long term, it significantly reduced N leaching from the system but did not prevent SOM depletion, acidification and concomitant adverse effects on soil microbes. Our results show that making decisions about sustainable grassland management should be based on long-term studies and take into consideration responses above as well as belowground. Here, our results indicate that belowground plant production, driving an input of organic matter directly into the soil profile, had a more important role in affecting soil C storage in the long term than aboveground plant production. The conservation efforts on species-rich oligotrophic meadows should target extensive use by low-frequency mowing but the concomitant nutrient depletion should not be compensated for by regular NPK fertilization due to its adverse effects on soil quality.

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References

Agren GI, Bosatta E, Magill AH. 2001. Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. Oecologia 128:94–8.

Bååth E, Anderson T-H. 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. Soil Biol Biochem 35:955–63.

Bardgett RD, McAlister E. 1999. The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. Biol Fertil Soils 29:282–90.

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS, Davies WJ. 1999. Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Funct Ecol 19:650–60.

Bardgett RD, Shine A. 1999. Linkages between plant litter diversity, soil microbial biomass and ecosystem function in temperate grasslands. Soil Biol Biochem 31:317–21.

Bardgett RD, Wardle DA, Yeates GW. 1998. Linking above-ground and below-ground interactions: how plant responses to foliar herbivory influence soil organisms. Soil Biol Biochem 30:1867–78.

Bárta J, Šlajsová P, Tahovská K, Picek T, Šantrůčková H. 2013. Different temperature sensitivity and kinetics of soil enzymes indicate seasonal shifts in C, N and P nutrient stoichiometry in acid forest soil. Biogeochemistry 117:525–37.

Bengston P, Barker J, Grayston J. 2012. Evidence of a strong coupling between root exudation, C and N availability, and stimulated SOM decomposition caused by rhizosphere priming effect. Ecol Evol 2:1843–52.

Bossio DA, Scow KM. 1998. Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. Microb Ecol 35:265–78.

Brookes PC, Landman A, Pruden G, Jenkinson DS. 1985. Chloroform fumigation and release of soil nitrogen: a rapid and direct extraction method to measure microbial biomass nitrogen. Soil Biol Biochem 17:837–47.

Brookes PC, Powlson DS, Jenkinson DS. 1982. Measurement of microbial biomass phosphorus in soil. Soil Biol Biochem 14:319–29.

Chytrý M. 2012. Vegetation of the Czech Republic: diversity, ecology, history and dynamics. Preslia 84:427–504.

Clark CM, Cleland EE, Collins SL, Fargione JE, Gough L, Gross KL, Pennings SC, Suding KN, Grace JB. 2007. Environmental and plant community determinants of species loss following nitrogen enrichment. Ecol Lett 10:596–607.

Conant RT, Paustian L, Elliot ET. 2001. Grassland management and conversion into grassland: effects on soil carbon. Ecol Appl 11:343–55.

De Deyn GB, Cornelissen JHC, Bardgett RD. 2008. Plant functional traits and soil carbon sequestration in contrasting biomes. Ecol Lett 11:516–31.

Doornbos RF, van Loon LC, Bakker PAHM. 2012. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agron Sustain Dev 32:227–43.

Eisenhauer N, Cesarz S, Koller R, Worm K, Reich PB. 2012. Global change belowground: impacts of elevated CO_2, nitrogen, and summer drought on soil food webs and biodiversity. Glob Change Biol 18:435–47.

Fernández-Calviño D, Bååth E. 2010. Growth response of the bacterial community to pH in soils differing in pH. FEMS Microbiol Ecol 73:149–56.

Fierer N, Strickland MS, Liptzin D, Bradford MA, Cleveland CC. 2009. Global patterns in belowground communities. Ecol Lett 12:1238–49.

Fornara DA, Bardgett R, Steinbeiss S, Zak DR, Gleixner G, Tilman D. 2011. Plant effects on soil N mineralization are mediated by the composition of multiple soil organic fractions. Ecol Res 26:201–8.

Freschet GT, Cornwell WK, Wardle DA, Elumeeva TG, Liu W, Jackson BG, Onipchenko VG, Soudzilovskaia NA, Tao J, Cornelissen JHC. 2013. Linking litter decomposition of above- and below-ground organs to plant-soil feedbacks worldwide. J Ecol 101:943–52.

Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E. 2011. Microbially mediated plant functional traits. Ann Rev Ecol Evol Syst 42:23–46.

Frostegård Å, Bååth E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol Fertil Soils 22:59–65.

Frostegård Å, Bååth E, Tunlid A. 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol Biochem 25:723–30.

Galvánek D, Lepš J. 2008. Changes of species richness pattern in mountain grasslands: abandonment versus restoration. Biodivers Conserv 17:3241–53.

Guitian R, Bardgett RD. 2000. Plant and soil microbial responses to defoliation in temperate semi-natural grassland. Plant Soil 220:271–7.

Hamilton EW, Frank DA. 2001. Plant defoliation promotes microbial nitrogen cycling through increased root exudation of carbon. Ecology 82:2397–402.

Hartmann A, Schmid M, van Tuinen D, Berg G. 2009. Plant-driven selection of microbes. Plant Soil 321:235–57.

Hedlund K, Regina IS, Van der Putten WH, Lepš J, Díaz T, Korthals GW, Lavorel S, Brown VK, Gormsen D, Mortimer SR, Barrueco CR, Roy J, Smilauer P, Smilauerova M, Van Dijk C. 2003. Plant species diversity, plant biomass and responses of the soil community on abandoned land across Europe: idiosyncracy or above-belowground time lags. Oikos 103:45–58.

Hejcman M, Sochorova L, Pavlu V, Strobach J, Diepolder M, Schellberg J. 2014. The Steinach grassland experiment: soil chemical properties, sward height and plant species composition in three cut alluvial meadow after decades-long fertilizer application. Agric Ecosyst Environ 184:76–87.

Kaiser Ch, Koranda M, Kitzler B, Fuchslueger L, Schnecker J, Schweiger P, Rasche F, Zechmeister-Boltenstern S, Sessitch A, Richter A. 2010. Belowground carbon allocation by trees drives seasonal patterns of extracellular enzyme activities by altering microbial community composition in a beech forest soil. New Phytol 187:843–58.

Kaligaric M, Ciluberg M, Kramberger B. 2006. Recent vegetation history of the North Adriatic grasslands: expansion and decay of an anthropogenic habitat. Folia Geobotanica 41:241–58.

Kamble PN, Rousk J, Frey SD, Bååth E. 2013. Bacterial growth and growth-limiting nutrients following chronic nitrogen additions to a hardwood forest soil. Soil Biol Biochem 59:32–7.

Kaštovská E, Edwards K, Picek T, Šantrůčková H. 2014. A larger investment into exudation by competitive versus conservative plants is connected to more coupled plant-microbe N cycling. Biogeochemistry 122:47–59.

Kaštovská E, Picek T, Bárta J, Mach J, Cajthaml T, Edwards K. 2012. Nutrient addition retards decomposition and C immobilization in two wet grasslands. Hydrobiologia 692:67–81.

Kopáček J, Borovec J, Hejzlar J, Porcal P. 2001. Spectrophotometric determination of iron, aluminium, and phosphorus in soil and sediment extracts after their nitric and perchloric acid digestion. Commun Soil Sci Plant Anal 32:1431–43.

Kroppenstedt RM. 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: Goodfellow M, Minikin DE, Eds. Chemical methods in bacterial systematics. London: Academic Press. p 173–94.

Kuzyakov Y, Biryukova OV, Kuznetsova TV, Molter K, Kandeler E, Stahr K. 2002. Carbon partitioning in plant and soil, carbon dioxide fluxes and enzyme activities as affected by cutting ryegrass. Biol Fertil Soils 35:348–58.

Kuzyakov Y. 2010. Priming effects: interactions between living and dead organic matter. Soil Biol Biochem 42:1363–71.

Lange M, Eisenhauer N, Sierra CA, Bessler H, Engels C, Griffiths RI, Mellado-Vázquez PG, Malik AA, Roy J, Scheu S, Steinbeiss S, Thomson BC, Trumbore SE, Gleixner G. 2015. Plant diversity increases soil microbial activity and soil carbon storage. Nat Commun 6:6707. doi:10.1038/ncomms7707.

Legay N, Grassein F, Binet MN, Arnoldi C, Personeni E, Perigon S, Poly F, Pommier T, Puissant J, Clément JC, Lavorel S, Mouhamadou B. 2016. Plant species identities and fertilization influence on arbuscular mycorrhizal fungal colonization and soil bacterial activities. Appl Soil Ecol 98:132–9.

Lepš J. 1999. Nutrient status, disturbance and competition? An experimental test of relationships in a wet meadow. J Veg Sci 10:219–30.

Lepš J. 2004. Variability in population and community biomass in a grassland community affected by environmental productivity and diversity. Oikos 107:64–71.

Lepš J. 2014. Scale- and time-dependent effects of fertilization, mowing and dominant removal on a grassland community during a 15-year experiment. J Appl Ecol 51:978–87.

Lepš J, de Bello F, Šmilauer P, Doležal J. 2011. Community trait response to environment: disentangling species turnover vs intraspecific trait variability effects. Ecography 34:856–63.

Liu L, Greaver TL. 2010. A global perspective on belowground carbon dynamics under nitrogen enrichment. Ecol Lett 13:819–28.

Liu W, Jiang L, Hu S, Li L, Liu L, Wan S. 2014. Decoupling of soil microbes and plants with increasing anthropogenic nitrogen inputs in a temperate steppe. Soil Biol Biochem 72:116–22.

Marx MC, Wood M, Jarvis SC. 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biol Biochem 33:1633–40.

Nyborg M, Molina-Ayala M, Solberg ED, Izaurralde EC, Malhi SS, Janzen HH. 1997. Carbon storage in grassland soils as related to N and S fertilizers. In: Lal R, Ed. Management of carbon sequestration in soil. Boca Raton: CRC Press. p 421–32.

Orwin KH, Buckland SM, Johnson D, Turner BL, Smart S, Oakley S, Bardgett RD. 2010. Linkages of plant traits to soil properties and the functioning of temperate grassland. J Ecol 98:1074–83.

Paterson E. 2003. Importance of rhizodeposition in the coupling of plant and microbial productivity. Eur J Soil Sci 54:741–50.

Rennert T, Gockel KF, Mansfeld T. 2007. Extraction of water-soluble organic matter from mineral horizons of forest soils. J Plant Nutr Soil Sci 170:514–21.

Rousk J, Bååth E, Brooks PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N. 2010a. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J 4:1340–51.

Rousk J, Brookes PC, Bååth E. 2010b. The microbial PLFA composition as affected by pH in an arable soil. Soil Biol Biochem 42:516–20.

Rousk J, Brookes PC, Bååth E. 2011. Fungal and bacterial growth responses to N fertilization and pH in the 150-year Park Grass UK grassland experiment. FEMS Microbiol Ecol 76:89–99.

Sillen WMA, Dieleman WIJ. 2012. Effects of elevated CO_2 and N fertilization on plant and soil carbon pools of managed grasslands: a meta-analysis. Biogeosciences 9:2247–58.

Silvertown J, Poulton P, Johnston E, Edwards G, Heard M, Biss PM. 2006. The park grass experiment 1856–2006: Its contribution to ecology. J Ecol 94:801–14.

Sinsabaugh RL, Hill BH, Shah JJF. 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462:795–8.

Šmilauer P, Lepš J. 2014. Multivariate analysis of ecological data using CANOCO 5. Cambridge: Cambridge University Press.

Ter Braak CJF, Šmilauer P. 2012. Canoco reference manual and user's guide: software for ordination, version 5.0. Ithaca: Microcomputer Power. p 496.

Thomas GW. 1982. Exchangeable cations. In: Page AL, Ed. Methods of soil analysis, Part 2 Chemical and microbiological properties, 2nd edition. Agronomy 9. pp 159–65.

Tóth G. 2008. Soil quality in the European Union. In: Tóth G, Montanarella L, Rusco E, Eds. Threats to soil quality in Europe. JRC Scientific and Technical Reports. pp 11–9.

Treseder KK. 2008. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecol Lett 11:1111–20.

Uhlířová E, Šimek M, Šantrůčková H. 2005. Microbial transformation of organic matter in soils of montane grasslands under different management. Appl Soil Ecol 28:225–35.

van der Heijden MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310.

Vance ED, Brookes PC, Jenkinson DS. 1987. An extraction method for measuring soil microbial biomass-C. Soil Biol Biochem 19:703–7.

Venterink HO, Kardel I, Kotowski W, Peeters W, Wassen MJ. 2009. Long-term effects of drainage and hey-removal on nutrient dynamics and limitation in the Biebrza mires, Poland. Biogeochemistry 93:235–52.

Wagai R, Sollins P. 2002. Biodegradation and regeneration of water-soluble carbon in a forest soil: leaching column study. Biol Fertil Soils 35:18–26.

Wei CZ, Yu Q, Bai E, Lu XT, Li Q, Xia JY, Kardol P, Liang WJ, Wang ZW, Han XG. 2013. Nitrogen deposition weakens plant–microbe interactions in grassland ecosystems. Glob Change Biol 19:3688–97.

Zhang X, Chen Q, Han X. 2013. Soil bacterial communities respond to mowing and nutrient addition in a steppe ecosystem. PLoS ONE 8:e84210.

Appendix A

See Table 6.

Table 6. Spatial Arrangement of Experimental Plots

1 mown removed	2	3 mown removed	4
5 removed	6 mown	7 removed	8 mown
9 mown	10 removed	11 mown	12 removed
13	14 mown removed	15	16 mown removed
17 removed	18 mown	19 removed	20 mown
21 mown removed	22	23 mown removed	24

Grey – fertilized, white - unfertilized, "mown" - mown, "removed" - dominant species removed

Appendix B

See table 7.

Table 7. Correlation of PLFA Markers with pH, C/P_{ex} and Plant Community Composition PC1 and PC3 Sample Scores

PLFA indicator	microbial group	pH	C/P_{ex}	PC1	PC3
			r		
i15:0	*G+*	-0.27	**-0.42**	-0.35	-0.22
a15:0	*G+*	**0.61**	0.12	0.39	**-0.58**
i16:0	*G+*	0.19	-0.33	-0.10	-0.03
i17:0	*G+*	**0.51**	-0.04	-0.11	0.35
a17:0	*G+*	0.38	**0.49**	**0.56**	-0.19
i18:0	*G+*	-0.21	0.21	0.02	0.32
16:1n5	*G-*	**0.71**	**0.49**	**0.52**	**-0.46**
16:1n9	*G-*	**-0.41**	-0.33	-0.35	0.12
16:1n7	*G-*	0.03	**-0.42**	-0.33	-0.28
cy17:0	*G-*	**0.61**	0.40	0.21	-0.28
18:1n7	*G-*	**0.43**	0.25	0.19	**-0.54**
cy19:0	*G-*	-0.38	-0.08	0.04	-0.05
18:1n9	*fungi*	-0.23	-0.03	-0.08	**0.42**
18:2n6,9	*fungi*	-0.19	-0.14	-0.24	**0.41**
10Me16:0	*actinobact.*	0.11	0.29	0.32	-0.18
10Me17:0	*actinobact.*	**-0.55**	-0.34	-0.34	0.29
15:0	*gen. bact.*	-0.18	0.35	0.16	**0.41**
17:0	*gen. bact.*	0.06	**0.48**	**0.45**	**0.55**
17:1n6	*gen. bact.*	-0.29	-0.19	-0.21	-0.05
18:1n5	*gen. bact.*	0.38	**0.75**	**0.59**	-0.02
14:0	*nonspec.*	-0.32	0.13	-0.14	0.20
16:0	*nonspec.*	-0.31	-0.13	-0.26	**0.45**
16:1n11	*nonspec.*	0.40	-0.21	0.00	**-0.69**
18:0	*nonspec.*	-0.29	0.18	-0.08	**0.58**
18:1n11	*nonspec.*	-0.37	-0.30	-0.12	-0.18
20:0	*nonspec.*	-0.23	0.23	-0.50	**0.46**
22:0	*nonspec.*	-0.24	0.14	-0.16	**0.48**

Bold values indicate significant correlation between PLFA and the respective variable ($P < 0.05$).

Appendix C

See Table 8.

Table 8. Sample Scores from the First Three Axes from the PCAs on Microbial PLFA Profile and Plant Community Composition.

	experimental design			microbial community composition			plant community composition		
plot	fertilization	mowing	removal	PC1 (34.6%)	PC2 (18.7%)	PC3 (13.8%)	PC1 (21.4%)	PC2 (10.6%)	PC3 (10.2%)
1	0	1	1	-1.572	-1.409	1.759	1.473	0.407	1.005
2	0	0	0	-1.080	-0.119	1.141	-0.158	-0.171	0.116
3	1	1	1	-1.147	-0.851	-0.330	-0.430	-1.501	0.387
4	1	0	0	-1.356	-0.859	-0.890	-0.497	0.382	0.420
5	0	0	1	-0.347	0.064	1.541	0.130	1.780	-1.695
6	0	1	0	-0.231	0.466	1.617	1.482	0.215	0.462
7	1	0	1	0.256	2.395	-0.410	-0.855	0.575	0.419
8	1	1	0	-1.187	-0.886	-1.114	0.049	-0.641	0.562
9	1	1	0	0.164	-0.110	0.001	-0.615	-0.115	0.966
10	1	0	1	-0.907	0.110	-1.032	-0.888	0.596	1.915
11	0	1	0	-0.651	1.766	-0.061	1.102	0.021	1.542
12	0	0	1	0.955	0.830	0.480	-0.687	1.446	0.011
13	1	0	0	-1.428	1.953	-1.009	-1.233	1.231	0.018
14	1	1	1	-0.683	-0.367	-1.162	-1.048	-1.189	1.437
15	0	0	0	0.793	0.081	-0.379	-0.812	1.558	-0.175
16	0	1	1	0.115	-0.497	1.420	1.723	1.187	-0.228
17	0	0	1	0.924	0.177	1.029	0.371	-0.396	-1.891
18	1	1	0	0.321	1.044	-0.584	-0.348	-0.866	-0.614
19	1	0	1	0.969	-1.158	-1.696	-0.658	-1.031	-1.572
20	0	1	0	1.698	-0.452	-0.254	1.447	-0.642	-0.136
21	0	1	1	0.964	-0.033	1.233	2.253	-0.521	-0.192
22	1	0	0	1.582	-0.429	-0.319	-0.099	-1.395	-1.370
23	1	1	1	0.527	-1.707	-0.623	-0.911	-1.730	-0.127
24	0	0	0	1.320	-0.010	-0.359	-0.792	0.796	-1.256

Appendix D

See Figure 3.

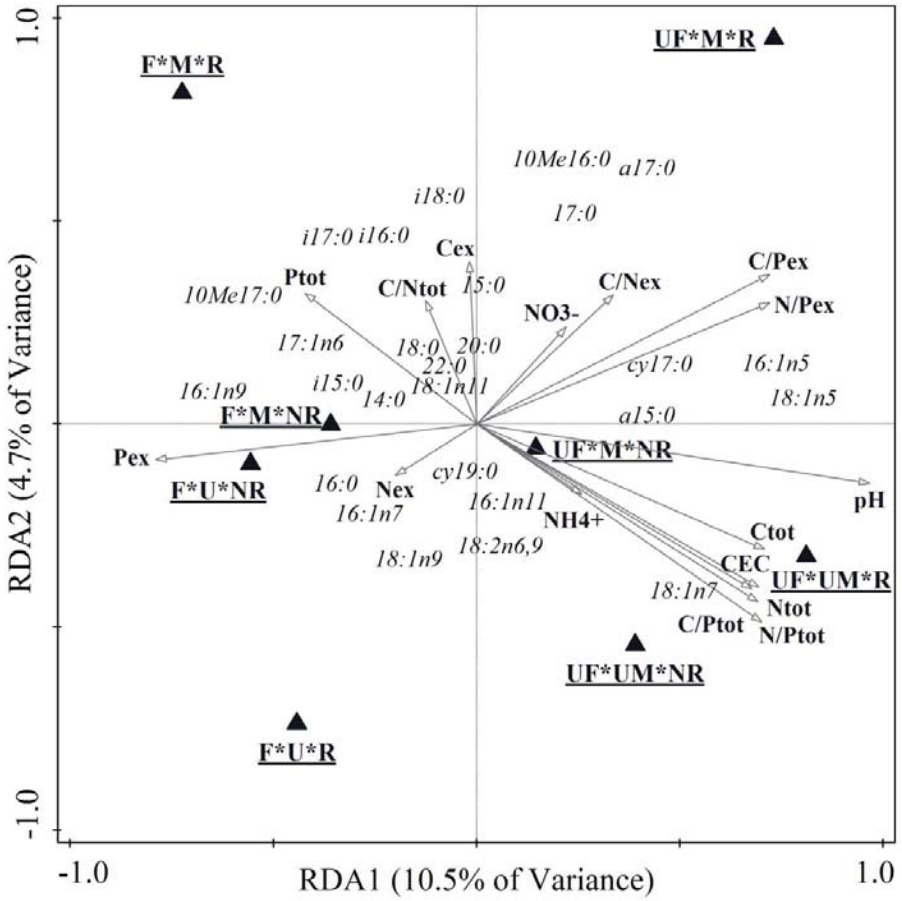

Figure 3. Ordination diagram showing results of the redundancy analysis of PLFA abundance data with management practices combining fertilization, mowing and removal of the dominant plant species (*Molinia cearulea*) as the only explanatory variables. Triangles are centroids (n = 3) of the plots belonging to the same treatment combination (F*M*R – fertilized mown removal, F*M*NR – fertilized mown non-removal, F*UM*R – fertilized unmown removal, F*UM*NR – fertilized unmown non-removal, UF*M*R – unfertilized mown removal, UF*M*NR – unfertilized mown non-removal, UF*UM*R – unfertilized unmown removal, UF*UM*NR – unfertilized unmown non-removal). Arrows indicate loading of physico-chemical soil properties used as supplementary variables. The numbers in parentheses are the proportions of the total variation explained by each axis (both axes are not significant).

Study II

Soil microbial biomass, activity and community composition along altitudinal gradients in the High Arctic (Billefjorden, Svalbard)

Kotas, P., Šantrůčková, H., Elster, J., and Kaštovská, E.

Under major revision in Biogeosciences

Soil microbial biomass, activity and community composition along altitudinal gradients in the High Arctic (Billefjorden, Svalbard)

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Abstract

The unique and fragile High Arctic ecosystems are vulnerable to proceeding global climate warming. Elucidation of factors driving microbial distribution and activity in Arctic soils is essential for comprehensive understanding of the ecosystem functioning and its response to environmental change. The goals of this study were to investigate the microbial biomass, activity, microbial community structure (MCS) and their environmental controls in soils along three elevational transects in coastal mountains of Billefjorden, Central Svalbard. Soils from four different altitudes (25, 275, 525, and 765 m above sea level) were analysed for a suite of characteristics including temperature regimes, organic matter content, base cation availability, moisture, pH, basal respiration, and microbial biomass and community structure using phospholipid fatty acids (PLFA). We observed significant spatial heterogeneity of edaphic properties among transects, resulting in transect-specific effect of altitude on most soil parameters. We did not observed any clear elevation pattern in the microbial biomass and the microbial activity revealed contrasting elevational patterns between transects. We found relatively large

horizontal variability in MCS, mainly due to different composition of bacterial PLFAs, but also systematic altitudinal shift in MCS related with different habitat preferences of fungi and bacteria, resulting in high fungi to bacteria ratios at the most elevated sites. Our data further showed that the biological soil crusts on these most elevated, unvegetated sites can host microbial assemblages of the size and activity comparable with the arctic tundra ecosystem. The key environmental factors determining horizontal and vertical changes in soil microbial properties were soil pH, organic carbon content, soil moisture and Mg^{2+} availability.

1 Introduction

Knowledge about the spatial distribution and activity patterns of soil microbial communities is essential to understand ecosystem functioning as the soil microbes play fundamental role in biogeochemical cycling and drive productivity in terrestrial ecosystems (van de Heijden et al., 2008). The soil microbial diversity in the Arctic is comparable to that in other biomes (Chu et al., 2010) and the spatiotemporal variability in microbial community composition is large (Lipson, 2007; Blaud et al., 2015; Ferrari et al., 2016). However, it is still uncertain which environmental factors drive the heterogeneity of soil microbial properties in the Arctic.

Altitudinal transects offer great opportunity to study a distribution of microbial communities adapted to local habitats and explain the patterns by natural gradients of soil conditions, vegetation occurrence and climate regimes over short spatial distances (Ma et al., 2004; Körner et al., 2007). The proceeding climate change will further affect environmental conditions in the Arctic (Collins et al., 2013) including expected upward migration of the vegetation and increasing plant cover (Vuorinen et al., 2017; Yu et al. 2017). Therefore, the knowledge of current microbial distribution and activity patterns along the altitudinal gradients together with identifying their controlling factors can help to predict future development of ecosystems in this region. However, such studies are scarce despite the fact that Arctic tundra comprises 5% of the land on Earth (Nemergut et al., 2005) and most coastal areas in the northern circumpolar region have mountainous character. So far, only few studies assessing altitudinal trends in soil microbial properties were

conducted in the Scandinavian Arctic (Löffler et al., 2008; Männistö et al., 2007). The research on spatial variation in microbial community composition and activity in polar regions was conducted mainly at narrow elevation range (Oberbauer et al., 2007; Trevors et al., 2007; Björk et al., 2008; Chu et al., 2010; Van Horn et al., 2013; Blaud et al., 2015; Tytgat et al., 2016) or was focused on initial soil development following glacier retreat (Bekku et al., 2004; Yoshitake et al., 2007; Schűtte et al., 2010). Majority of studies on the elevational patterns in microbial community structure (MCS) and activity has been done in mountain regions of lower latitudes from tropics to temperate zone. The studies commonly show that the microbial activity decreases with increasing elevation (Schinner, 1982; Niklińska and Klimek, 2007; Margesin et al., 2009), while there are no general altitudinal patterns in soil microbial diversity and community structure. For example, the microbial community composition did not change along elevational gradients in Swiss Alps (Lazzaro et al., 2015), while other studies have documented decreasing bacterial (Ma et al., 2004; Lipson, 2007; Shen et al., 2013) and fungal (Schinner and Gstraunthaler, 1981) diversity with an increasing altitude, and several studies reported the mid–altitudinal peak in microbial diversity (Fierer et al., 2011; Singh et al., 2012; Meng et al., 2013). Beside the fungal and bacterial diversity, the relative abundance of these main microbial functional groups is also variable. For example, Djukic et al. (2010), Xu et al. (2014) and Hu et al. (2016) found decreasing fungi to bacteria (F/B) ratio with an increasing elevation, while Margesin et al. (2009) reported opposite trend in Central Alps.

The research focusing on environmental controls over microbial communities in polar and alpine regions recognized many significant factors, including vegetation, litter C : N stoichiometry, organic carbon content, soil pH, nutrient availability, microclimatic conditions, and bedrock chemistry. However, the effect of these variables was site- and scale-specific (Van Horn et al., 2013; Blaud et al., 2015; Ferrari et al., 2016), which highlights the need for further research on environmental controls of microbial community size, activity and structure at local and regional scales. To extend our knowledge about microbial ecology and soil functioning in the arctic alpine ecosystems, we conducted study aiming to assess the activity, biomass and structure of soil microbial communities and to determine their controlling environmental factors along three altitudinal transects located in Central Svalbard. These transects spanned from the vegetated tundra habitats at the narrow areas at

the sea level to unvegetated soils at the top of the coastal mountains. The specific objectives of our study were (i) to describe gradients of microclimatic and geochemical soil properties; (ii) to assess microbial activity (soil respiration) and abundance of main microbial groups (fungi, Gram–negative and Gram–positive bacteria, Actinobacteria, phototrophic microorganisms) using phospholipid fatty acid (PLFA) analysis; and (iii) to identify environmental factors explaining the trends in soil microbial parameters along these altitudinal gradients.

2 Materials and methods

2.1 Study area and soil sampling

The Petunia bay (Billefjorden; 78° 40´ N, 16° 35´ E) is located in the center of Svalbard archipelago and represents typical High Arctic ecosystem in the northern circumpolar region. The mean, minimum and maximum air temperatures recorded in the area at 25 m above the sea level (a.s.l.) were –3.7, –28.3 and 17 °C in the period of 2013–2015, respectively, and stayed permanently below 0 °C for eight months a year (Ambrožová and Láska, 2017). The mean annual precipitation in the Central Svalbard area is only 191 mm (Svalbard Airport, Longyearbyen, 1981–2010) and is equally distributed throughout the year (Førland et al., 2010).

In August 2012, we collected soils from three altitudinal transects (Tr1–3) on the east coast of Petunia bay. Each transect was characterized by four sampling sites at altitudes 25, 275, 525 and 765 m a.s.l. (± 5 m). Transects were located on slopes with similar exposition (Tr1 W–E, Tr2 WNW–ESE, Tr3 WSW–ENE; Fig. 1) and lithostratigraphy. Soils at the lowest elevations developed from Holocene slope (Tr1 and Tr3) and marine shore deposits (Tr2), while the bedrock at more elevated sites is formed by dolomite and limestone with units of basal calcareous sandstone (Dallmann et al., 2004). The soils were classified as Leptic Cryosols (Jones et al., 2010) with loamy texture and clay content increasing with altitude (Table 2), and were from 0.15–0.2 m to only few cm deep at 25 and 765 m a.s.l., respectively. The poorly developed organic horizon was present only at the lowest elevation. The sampling locations were selected in geomorphologically stable areas with a similar slope

(20±5°). On each sampling site, nine soil cores (4 cm deep, 5.6 cm diameter) were collected and mixed into three representative samples. Each representative sample was mixed from one soil core taken from the edge of the vegetation tussocks (if vegetation was present) and two other cores taken in increasing distance from the vegetation to maintain the consistency with respect to heterogeneity of vegetation cover and soil surface. The triplicates were collected approximately 5 m apart from each other. Immediately after sampling, the soil was sieved (2 mm) to remove larger rocks and roots, sealed in plastic bags and kept frozen at −20 °C till further processing. Soil subsamples for biomarker analysis were as soon as possible freeze−dried and stored at −80 °C until extraction.

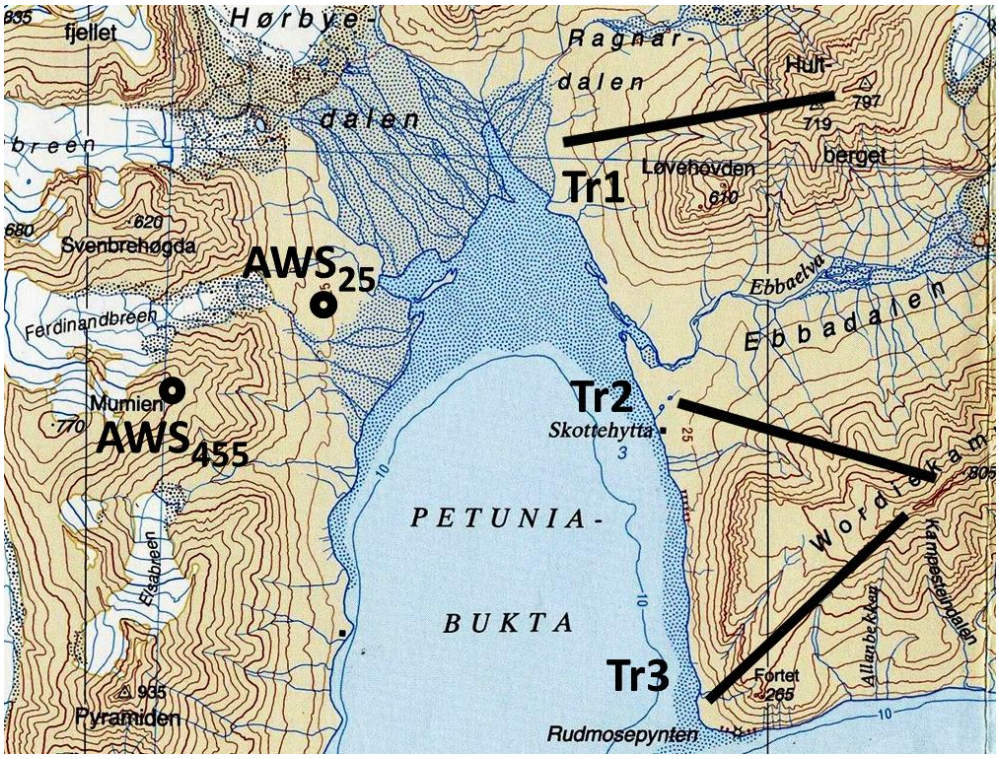

Figure 1. Location of the three investigated transects Tr1−Tr3 and automated weather stations (AWS) in Petunia bay, Billefjorden, Central Spitsbergen. Map source: map sheet C7, Svalbard 1:100 000, Norwegian Polar Institute 2008.

Transects represented climosequences from high Arctic tundra to unvegetated bare soil. Vegetation of two lowest sites was dominated by *Dryas octopetala*, with significant contribution of *Saxifraga oppositifolia,* and variable contribution of *Cassiope tetragona, Salix polaris* and grasses (*Carex nardina, C. rupestris, C. misandra*; Prach et al., 2012; personal observations). The vascular plants species formed scattered vegetation patches at the altitude of 525 m a.s.l. with *Salix polaris* and *Saxifraga oppositifolia* being the most abundant species. The soils at the most elevated sites were covered mainly by soil crusts with scarcely occurring *Saxifraga oppositifolia* and *Papaver dahlianum* (personal observations). The percentage cover of main surface types (i.e. stones, bare soil, vegetation, crusts and mosses) was estimated on each sampling site from approximately 1m^2 area in a close vicinity of coring sites (Table S1, Fig. S6).

2.2 Monitoring of microclimatic characteristics

To describe the soil microclimatic conditions along the altitudinal transects, we continuously measured soil temperature at –5 cm from 2012–2013 directly at the sampling sites of Tr1 using dataloggers (Minikin Ti Slim, EMS Brno, CZ). The soil water content at the time of sampling was determined in soil subsamples by drying to constant weight at 105 °C. The temperature regimes at particular altitudinal levels were characterized by 10 climatic variables (Table 1). The period of above–zero daily mean ground temperatures is referred to as summer season throughout the text. We also considered number of days with daily mean ground temperatures above 5 °C, characterizing a period with conditions suitable for vascular plant growth (Kleidon and Mooney, 2000). The positive soil surface energy balance was calculated as a sum of daily mean summer temperatures. The records from three years (2011–2013) continuous measurements at two automated weather stations located at 25 and 455 m a.s.l. approximately 3 km apart from the observed transects (hereafter referred as AWS_{25} and AWS_{455}, respectively; Fig. 1; see Ambrožová and Láska, 2017 for detailed description) were used to evaluate seasonal variation of soil temperature and moisture regimes (Figs. S2, S3, respectively), and coupling of soil and atmospheric

temperatures (measured at -5 cm and 2 m above terrain, respectively; Fig. S2). Even though we were not able to continuously measure soil moisture directly at the sampling sites, we regarded data from both AWS locations as representative for the evaluation of seasonal moisture regimes.

2.3 Soil characteristics

The particle size distribution was assessed using aerometric method (Lovelland and Whalley, 2001), the soil type was classified according to U.S. Department of Agriculture. The soil pH was determined in soil–water mixture (1:5, w/v) using glass electrode. The cation exchange capacity (CEC) was considered to be equal to the sum of soil exchangeable base cations Mg^{2+}, Ca^{2+}, Na^{+}, K^{+} extracted with 1M NH_4Cl (Richter et al., 1992). The amount of H^{+} and Al^{3+} ions was neglected due to the high soil pH. Base cations accessible for plant and microbial uptake (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}) were extracted by the Mehlich 3 reagent (Zbíral and Němec, 2000). Cations were measured by atomic absorption spectroscopy (AA240FS instrument, Agilent Technologies, USA). Total soil organic carbon (TOC) and nitrogen (TN) contents were measured in HCl fumigated samples (Harris et al., 2001) using elemental analyser (vario MICRO cube, Elementar, Germany).

2.4 Microbial respiration

Since we were not able to measure soil respiration on site or immediately after soil collection, we measured the potential respiratory activity (soil CO_2 production) in the laboratory incubation experiment. We stored and transported the soils frozen because it was previously demonstrated that freezing-thawing has a weaker effect on microbial activity than long-term refrigeration (Stenberg et al., 1998) and comparable effect as drying-rewetting (Clein and Schimel, 1994). We then measured microbial respiration in slowly melted field-moist soils twice during the adaptation period (day 4 and 12), which allowed stabilization of the microbial activity after a respiratory flush following freeze-thaw events (Schimel and Clein, 1996), and at day 13, when we expected a stabilized microbial activity. Briefly, soil subsamples (10 g) were incubated in 100 mL flasks at 6 °C, which corresponds to the mean summer soil temperature of all sites along Tr1. At days 4, 12 and 13, a cumulative CO_2 production from the soils was measured using Agilent 6850 GC system (Agilent

technologies, CA, USA). The flasks were then thoroughly ventilated and sealed again. Due to high soil pH, the total amount of produced CO_2 was corrected for its dissolution and dissociation in soil solution according to Henderson-Hasselbach equation (Sparling and West, 1990) and expressed as the microbial respiration rate per day. The daily microbial respiration rates measured between days 4-12 and after stabilization (day 13) were not significantly different in any soil samples, therefore, we present only the later one.

2.5 Microbial biomass and community structure

The soil microbial community structure was defined using PLFA analysis according to modified protocol of Frostegård et al. (1993). Briefly, 1-3 g (according to TOC content) of freeze–dried soil samples was extracted twice with a single–phase extraction mixture consisting of chloroform, methanol and citrate buffer. After overnight phase separation achieved by adding more chloroform and buffer, the organic phase was purified on silica columns (SPE–SI Supelclean 250mg/3 mL; Supelco®, PA, USA) using chloroform, acetone and methanol. The polar fraction was trans–esterified to the fatty acid methyl esters (FAME) (Bossio and Scow, 1998). All FAMEs were quantified by an internal standard calibration procedure using methyl-nonadecanoate (19:0) as an internal standard. To identify the FAMEs, retention times and mass spectra were compared with those obtained from standards (Bacterial Acid Methyl Esters standard, the 37–component FAME Mix, PUFA–2, and PUFA–3; Supelco, USA). The ISQ mass spectrometer (MS) equipped with Focus gas chromatograph (GC) (Thermo Fisher Scientific, USA) was used for chromatographic separation and detection.

Only specific PLFAs were used to assess the microbial community structure: a14:0, i15:0, a15:0, i16:0, i17:0, a17:0 were used as markers of Gram–positive bacteria (G+); 16:1ω9, 16:1ω5, cy17:0, 18:1ω11, 18:1ω7, cy19:0 as markers of Gram–negative bacteria (G–); 10Me16:0 and 10Me18:0 as markers of Actinobacteria (Kroppenstedt, 1985), 18:1ω9, 18:2ω6,9 as fungal markers (Frostegård and Bååth, 1996) and polyunsaturated fatty acids 18:4ω3, 20:5ω3 were used as markers of phototrophic microorganisms (Hardison et al., 2013; Khotimchenko et al., 2002). A sum of Actinobacterial markers, PLFAs specific to G+ and G– bacteria and general bacterial markers 15:0, 17:0 and 18:1ω5 was used to calculate bacterial biomass and fungi to

bacteria (F/B) ratio. The sum of all lipid markers mentioned above and nonspecific PLFAs 14:0, 16:0, 18:0 and 16:1ω7 was used as proxy for microbial biomass ($PLFA_{tot}$).

2.6 Sterol analyses

The β–sitosterol and brassicasterol were used as biomarkers of plant (Sinsabaugh et al., 1997) and microalgal (Volkman, 1986; 2003) residues in organic matter (OM), respectively. Sterols were simultaneously determined using microwave assisted extraction adapted from Montgomery et al. (2000) and GC/MS (ISQ MS equipped with Focus GC, Thermo Fisher Scientific, USA) analysis. Briefly, 0.5 g of freeze–dried soil was treated with 6 mL of methanol and 2 mL of 2 M NaOH. Vials were heated twice at the centre of a microwave oven (2450 MHz and 540 W output) for 25 s. After cooling, the contents were neutralized with 1 M HCl, treated with 3 mL of methanol and extracted with hexane (3×4 mL). Extracts were spiked by an internal standard (cholesterol), evaporated and derivatized by adding of pyridine and 1 % BSTFA at 60 °C for 30 min prior analysis. Sterols were quantified by an internal standard calibration procedure.

2.7 Statistical analyses

All data were checked for normality and homoscedasticity, and log–transformed if necessary. The relative PLFA data (mol%) were log-transformed in all statistical tests. The significance of environmental gradients and corresponding shifts in MCS (mol% of summed PLFA specific for fungi, G– and G+ bacteria, Actinobacteria and soil phototrophic microorganisms) in horizontal direction (ie. effect of transect) and vertical direction (ie. effect of altitude) were tested using the partial redundancy analyses (RDA) with covariates. Variation partitioning was subsequently performed to quantify the unique and shared effects of transect and altitude on variability of MCS. Forward selection procedure was used to identify the soil geochemical parameters best explaining the shifts in MCS. During the forward selection procedure, only *P* values adjusted by Holms correction were considered. This procedure is slightly less conservative compared to the often recommended Bonferroni correction, but it is a sequential procedure and takes into account

that the candidate predictors with stronger effect were selected first (Holm, 1979). The multivariate tests were performed without standardization by samples, but with centering and standardization by variables (because the variables were not always measured at the same scale, see Šmilauer and Lepš 2014) and Monte Carlo test with 1999 permutations. Only adjusted explained variation is referred throughout the text. Since the samples from each triplicate cannot be considered as independent observations due to relatively low inter-sample distance (otherwise we had 9 independent transects), only the sampling sites were freely permuted while the individual samples were exchangeable only within the sampling sites. The differences in particular soil and microbial parameters between respective transects and altitudes were addressed by ANOVA complemented with Tukey–HSD post hoc test. To find out how tightly were variables related to each other, Pearson correlation coefficient was used. All statistical tests were considered significant at $P < 0.05$. Multivariate statistical analyses were performed with CANOCO for Windows version 5.0 (Ter Braak and Šmilauer 2012), for ANOVA, Tukey-HSD test and correlations between soil and/or microbial parameters, Statistica 13 was used (StatSoft, USA).

3 Results

3.1 Altitudinal changes in soil microclimate

The soil microclimate at the studied sites was characterized by two distinct periods respecting the air temperature dynamics (compare Fig. S2a with S2b). The winter period lasted typically from the middle of September to early June. The winter soil temperatures were stratified according to the elevation and the temperature means decreased from −4 °C at 25 m a.s.l. to −10 °C at 765 m a.s.l. (Table 1, Fig. S2). In contrast, a short summer period was characterized by a significant diurnal fluctuation of soil temperatures and weak altitudinal temperature stratification (Fig. S2). The length of the summer season more than doubled at the lowest elevations compared to the most elevated study sites, while the period with daily mean soil temperatures above 5 °C shortened almost four times. Correspondingly, the positive surface energy balance gradually decreased with an increasing altitude (Table 1).

Table 1. Climatic variables; temperatures given in °C

Sites	Sites [m a.s.l.]	Means Summer	Means Winter	Means Year	Min daily means Winter	Max daily means Summer	Mean daily amplitude Summer	Max daily amplitude Summer	Number of days with daily mean > 0 °C	Number of days with daily mean > 5 °C	Positive soil surface energy balance
Alt 1	25	5.8	-3.6	-0.8	-7.0	11.2	5.2	10.9	110	62	615
Alt 2	280	7.1	-5.7	-2.7	-10.3	14.5	8.5	18.2	96	54	571
Alt 3	520	5.8	-8.9	-4.9	-15.8	14.7	8.1	17.7	91	40	480
Alt 4	765	5.3	-9.5	-6.6	-17.1	11.6	5.5	14.0	51	11	290

The maximum daily mean temperatures and diurnal temperature fluctuation were highest at the mid–elevated sites, with the highest mean summer soil temperature reached at 275 m a.s.l. In contrast, the least and most elevated sites experienced lower summer maximum daily means and soil temperature amplitudes (Table 1). The effect of altitude on soil moisture was significant along Tr1 and Tr3 ($P < 0.001$ and 0.01, F = 22.76 and 7.39, respectively) with soil moisture content decreasing along with increasing elevation, but nonsignificant along Tr2. Continual volumetric measurements of soil water content at AWS_{25} and AWS_{455} showed that the soil moisture was relatively stable during the summer season and desiccation events did not occur during the summer periods 2011–2013 (for more information, see Fig. S3).

3.2 Gradients of soil geochemical properties and surface vegetation cover

Both factors, transect and altitude, significantly affected soil geochemical properties (partial RDA, pseudo–F = 8.3, $P < 0.001$) and explained 61% of the total variation in soil characteristics. The RDA ascribed most of the explained variability (73%) to vertical zonation. Accordingly, the effect of altitude was significantly reflected in all soil parameters (Table 2, 3, Fig. S4), but the significant interactive effect between transect and altitude indicated that the elevational trends were in most cases specific for particular transects (Tables 2, 3). Especially the CEC and availabilities of Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} were spatially variable, reflecting complicated geology of the Petunia bay area. The soils along Tr1 were significantly richer in available Mg^{2+} and K^{+} than soils from other two transects (Table 2). The Mg^{2+} availability also significantly increased with increasing elevation along the Tr1 (Table 2). Other soil properties showed more systematic altitudinal patterns. The mean soil pH ranged from 7.8 to 9.0 and increased with altitude along all transects (Table 2,

Table 2. Geochemical characteristics of soils along the studied altitudinal transects (Tr1-Tr3). Means ± SD (n = 3) are given in the upper part of the table. Results of two–way ANOVAs (F–values) of the effects of transect (Tr), altitude (Alt) and their interaction (Tr x Alt) are presented in the lower part of the table.

transect	altitude [m a.s.l]	soil type	soil moisture [%]	pH	CEC [meq/100g^{-1}]	Ca^{2+} [mg g^{-1}]	Mg^{2+} [mg g^{-1}]	K^{+} [µg g^{-1}]	Na^{+} [µg g^{-1}]
Tr1	25	sandy loam	^{a} 28.4 ± 2.5	^{b} 7.8 ± 0.1	^{a} 35.8 ± 0.4	^{b} 4.9 ± 0.2	^{c} 0.50 ± 0.03	^{b} 104 ± 2.3	^{a} 16.0 ± 1.4
	275	sandy loam-loam	^{b} 18.0 ± 0.5	^{b} 7.9 ± 0.2	^{b} 27.4 ± 2.3	^{b} 5.2 ± 0.6	^{c} 0.55 ± 0.08	^{b} 81 ± 8.8	^{bc} 8.4 ± 1.3
	525	loam	^{b} 18.6 ± 2.5	^{b} 8.1 ± 0.1	^{b} 30.3 ± 0.7	^{b} 4.3 ± 0.4	^{b} 0.85 ± 0.04	^{a} 160 ± 18.1	^{b} 11.3 ± 1.1
	765	clay-loam	^{c} 12.1 ± 1.8	^{a} 9 ± 0.0	^{b} 26.8 ± 2.3	^{a} 19.8 ± 1.0	^{a} 1.25 ± 0.06	^{c} 11 ± 2.7	^{c} 7.3 ± 0.0
Tr2	25	sandy loam	^{a} 21.1 ± 2.4	^{c} 7.8 ± 0.1	^{b} 25.6 ± 2.7	^{b} 14.7 ± 2.6	^{c} 0.19 ± 0.01	^{ab} 52 ± 4.0	^{a} 13.2 ± 1.7
	275	sandy loam-loam	^{a} 21.1 ± 2.4	^{c} 7.9 ± 0.1	^{b} 30.3 ± 1.7	^{ab} 16.5 ± 1.1	^{b} 0.26 ± 0.01	^{a} 59 ± 4.3	^{ab} 10.1 ± 1.7
	525	sandy loam-loam	^{a} 21.7 ± 5.3	^{b} 8.4 ± 0.1	^{b} 30.8 ± 1.1	^{c} 7.8 ± 1.6	^{a} 0.34 ± 0.01	^{a} 69 ± 3.3	^{ab} 9.6 ± 1.8
	765	loam	^{a} 22.5 ± 1.7	^{a} 8.8 ± 0.1	^{a} 45.1 ± 0.5	^{a} 27.9 ± 9.3	^{b} 0.25 ± 0.01	^{b} 41 ± 8.8	^{b} 8.1 ± 1.4
Tr3	25	sandy loam	^{a} 39.5 ± 1.4	^{b} 8.1 ± 0.1	^{a} 49.4 ± 2.1	^{c} 7.7 ± 0.3	^{a} 0.20 ± 0.03	^{b} 52 ± 5.3	^{a} 17.1 ± 1.1
	275	sandy loam-loam	^{ab} 31.9 ± 2.9	^{b} 8.1 ± 0.1	^{b} 39.2 ± 5.4	^{b} 10.8 ± 0.6	^{a} 0.21 ± 0.01	^{ab} 59 ± 1.9	^{a} 18.5 ± 0.5
	525	loam	^{ab} 28.2 ± 6.5	^{b} 8 ± 0.1	^{b} 34.9 ± 3.0	^{ab} 13.0 ± 4.6	^{a} 0.22 ± 0.00	^{a} 66 ± 6.6	^{a} 18.4 ± 3.1
	765	loam	^{b} 22.5 ± 1.7	^{a} 8.8 ± 0.1	^{b} 30.6 ± 3.9	^{a} 14.2 ± 0.1	^{b} 0.16 ± 0.00	^{b} 52 ± 1.6	^{b} 9.9 ± 0.2
		d.f.							
Tr		2	**31.4** ***	0.10	**22.1** ***	**6.43** **	**634** ***	**51.7** ***	**36.2** ***
Alt		3	**11.1** ***	**98** ***	**4.61** *	**14.1** ***	**66.9** ***	**74.9** ***	**18.7** ***
Tr x Alt		6	**5.07** **	**5.6** ***	**20.5** ***	0.83	**60.6** ***	**31.6** ***	**3.94** **

Different letters indicate significant differences between sampling sites along particular transects (P < 0.05; upper part of the table). Statistically significant differences are indicated by: * P < 0.05, ** P < 0.01, *** P < 0.001 (lower part of the table).

Fig. S4). Oppositely, the soil TOC and TN contents declined towards higher elevations along all transects; the exception was the lowest site along Tr2 with lower soil OM content compared to the respective sites from Tr1 and Tr3. The OM poorest soil occurred at the highest site of Tr1 (Table 3). The soil C/N ratio, sitosterol content in TOC and the ratio between plant–derived sitosterol and brassicasterol of algal origin were solely affected by the altitude.

Table 3. Total soil carbon (TOC) and nitrogen (TN) contents, their molar ratios, contents of sitosterol in TOC and sitosterol / brassicasterol ratios and soil PLFA contents in soils along the altitudinal transects (Tr1-Tr3). Means ± SD (n = 3) are given in the upper part of the table. Results of two–way ANOVAs (F–values) of the effects of transect (Tr), altitude (Alt) and their interaction (Tr x Alt) are presented in the lower part of the table.

transect	altitude [m a.s.l.]	TOC [mg g^{-1}]	TN [mg g^{-1}]	TOC/TN	Sitosterol [µg g^{-1} TOC]	Sitosterol / Brassicasterol
Tr1	25	^{c} 70.6 ± 13.4	^{b} 5.0 ± 1.01	^{b} 12.1 ± 0.2	^{c} 534 ± 62.8	^{b} 5.5 ± 0.4
	275	^{b} 21.1 ± 1.9	^{a} 2.0 ± 0.29	^{ab} 9.0 ± 0.7	^{bc} 521 ± 140	^{b} 5.3 ± 0.8
	525	^{b} 18.5 ± 4.2	^{a} 1.8 ± 0.31	^{ab} 8.8 ± 0.7	^{ab} 293 ± 66.5	^{b} 4.7 ± 1.0
	765	^{a} 4.4 ± 1.5	^{a} 0.5 ± 0.07	^{a} 7.9 ± 2.6	^{a} 81.1 ± 2.7	^{a} 2.3 ± 0.4
Tr2	25	^{ab} 30.6 ± 4.8	^{a} 1.9 ± 0.40	^{c} 13.7 ± 0.9	^{bc} 515 ± 44.9	^{b} 6.7 ± 0.7
	275	^{b} 37.2 ± 5.0	^{a} 3.0 ± 0.26	^{b} 10.7 ± 0.7	^{c} 616 ± 143	^{b} 5.6 ± 1.2
	525	^{a} 24.4 ± 7.8	^{a} 1.9 ± 0.64	^{b} 9.8 ± 1.2	^{ab} 299 ± 73.3	^{a} 2.9 ± 0.4
	765	^{a} 21.6 ± 3.6	^{a} 2.8 ± 0.20	^{a} 6.7 ± 0.6	^{a} 161 ± 36.9	^{a} 2.7 ± 0.7
Tr3	25	^{c} 81.1 ± 8.7	^{b} 6.1 ± 0.38	^{b} 11.5 ± 0.7	^{b} 587 ± 144	^{b} 6.4 ± 2.1
	275	^{b} 62.2 ± 9.1	^{ab} 4.8 ± 0.32	^{b} 11 ± 0.7	^{ab} 370 ± 42.9	^{a} 4.2 ± 0.7
	525	^{ab} 39.6 ± 11.4	^{a} 4.8 ± 0.32	^{b} 10.6 ± 0.6	^{a} 270 ± 112	^{a} 3.3 ± 1.0
	765	^{a} 23.1 ± 3.9	^{a} 2.5 ± 0.37	^{a} 7.9 ± 0.2	^{a} 151 ± 37.8	^{a} 3.1 ± 0.9
	d.f.					
Tr	2	**27.8** ***	**31.5** ***	1.57	0.79	1.04
Alt	3	**42.4** ***	**26.4** ***	**23.6** ***	**28.4** ***	**14.4** ***
Tr x Alt	6	**8.33** ***	**11.3** ***	1.96	1.34	2.17

Different letters indicate significant differences between sampling sites along particular transects ($P < 0.05$; upper part of the table). Statistically significant differences are indicated by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (lower part of the table).

Their values systematically decreased with an increasing elevation irrespective of the soil OM content (Table 3), indicating an altitudinal shift in the OM quality

and origin. The percentage of plant cover also continuously decreased with an increasing elevation along Tr1 and Tr3 but was comparable on the three lower sites along Tr2 (Fig. S5), which significantly resembled the trends in soil OM content (r = 0.53; P = 0.001). The lichenized soil crusts were predominant type of soil surface cover at all sites, while mosses covered very small proportion of surface area. The bare surface without any vegetation (bare soil) occurred only at the two most elevated sites (Fig. S5, Table S1).

3.3 Soil microbial biomass and activity

The soil PLFA content, used here as a measure of soil microbial biomass, was significantly correlated with soil TOC and TN contents (r = 0.773 and 0.719, respectively; both P < 0.0001) and soil moisture (r = 0.772; P < 0.0001), and negatively affected by Mg^{2+} availability (r = -0.775; P < 0.0001). Despite these relations, the soil PLFA content did not show any altitudinal pattern. The soil PLFA amounts were comparable among differently elevated sites along particular transect (Fig. 2a). Only the most elevated site of Tr1 had significantly lower soil PLFA content than other sites, which corresponded with its very low stock of OM (Table 3). Similarly, neither the flush of microbial respiration measured after soil thawing (day 4 of incubation) nor the respiration measured after stabilization (day 12, not shown, and day13) showed any systematic altitudinal pattern (Fig. 3b, c). Generally, the flush respiration rate was closely related (r = 0.74, P < 0.0001, n = 36) to microbial respiration after stabilization and ca 2.3 ± 0.3 times faster, showing similar freezing-thawing effect on the whole set of samples independently of altitude and transect. Along each transect, the three lower sites (from 25 to 525 m a.s.l.) had after stabilization comparable microbial respiration rates, but the most elevated sites always differed - along Tr1 had the most elevated site significantly lower microbial respiration rate, whilst the most elevated sites along Tr2 and Tr3 produced markedly more CO_2 compared to remaining sites along these transects (Fig. 2b). The respiration rate was related neither to PLFA nor to TOC contents, but significant positive correlation with soil Ca^{2+} availability and F/B ratio, and negative correlation with Mg^{2+} availability (r = 0.489, 0.661and -0.545; P = 0.003, < 0.001 and 0.001, respectively) was observed.

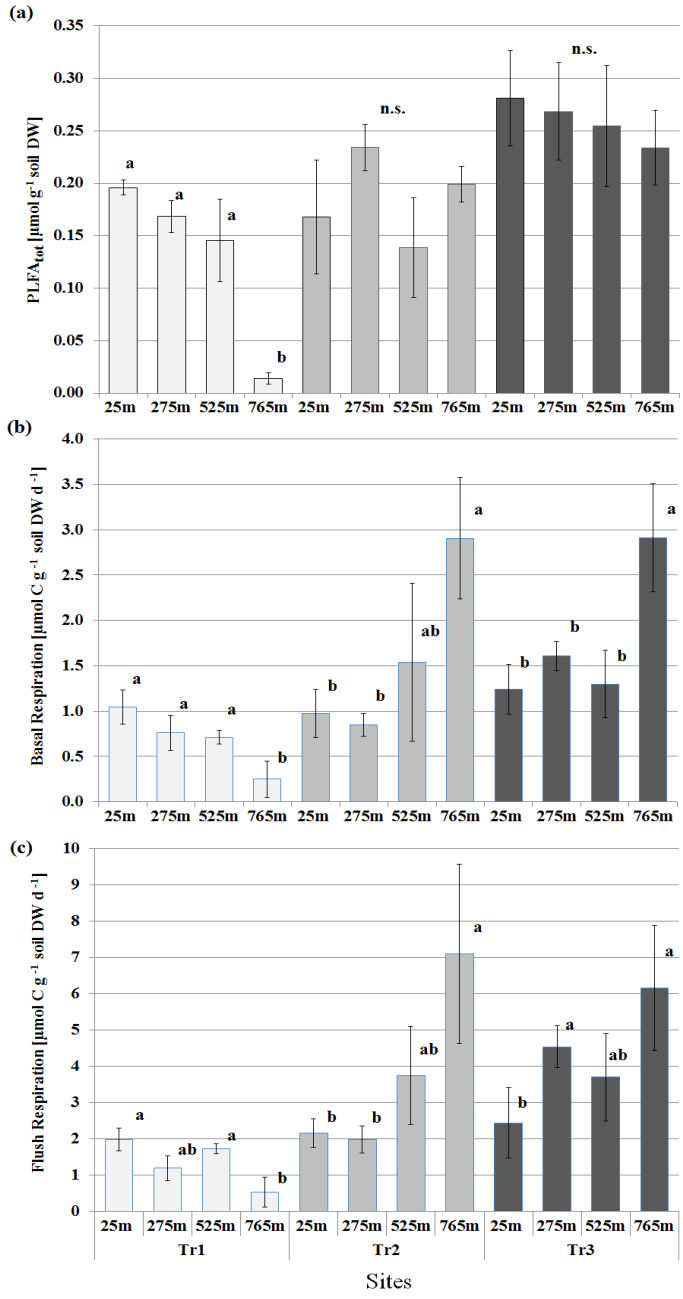

Figure 2. The soil PLFA contents (a), the potential respiration rates (b) and the flush respiration rates (c) in the soils along altitudinal transects (Tr1-Tr3). Error bars indicate mean ± SD (n = 3). Small case letters denote significant differences among altitudes within particular transects ($P < 0.05$; One–way ANOVA combined with Tukey post hoc test).

3.4 Microbial community structure

The partial RDA revealed significant interactive effect of altitude and transect on MCS (pseudo–F = 4.8, P < 0.001). Both factors explained 51% of the total variation in the MCS, with 66 % of explained variability ascribed to altitude, 26% to transect, and 8% of explained variability shared by both factors.

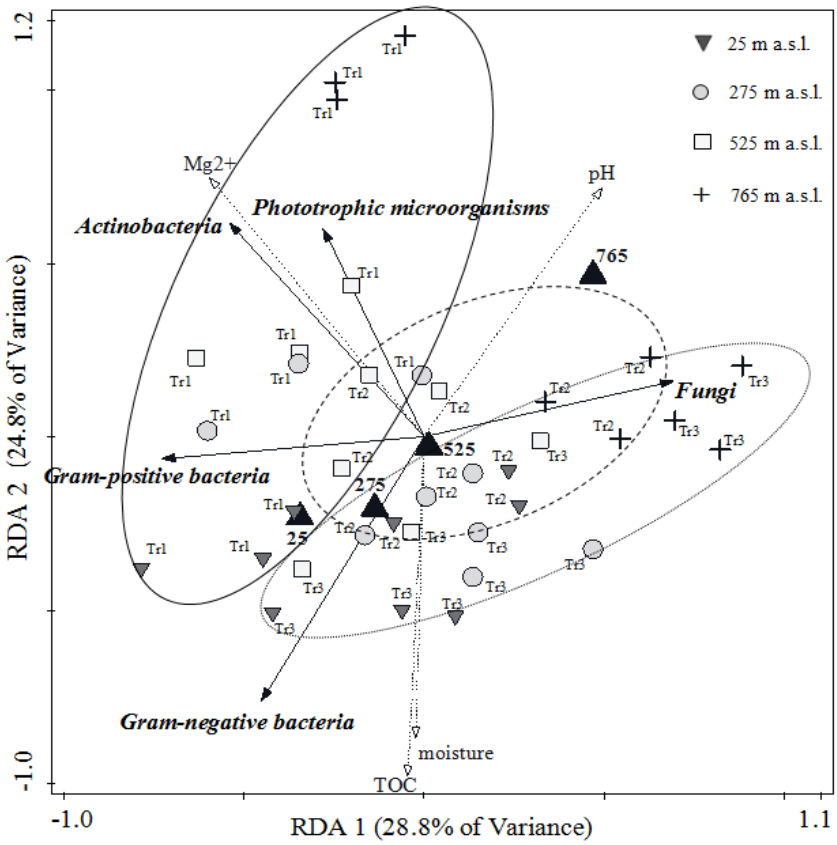

Figure 3. The correlation between abundance of main microbial groups (bold italic) and soil geochemical parameters retained by forward selection of explanatory variables. Results of RDA. Altitude of sampling sites was used as supplementary variable. *Arrows* indicate the direction in which the respective parameter value increases, *solid* lines indicate microbial groups, *dotted* lines indicate selected environmental variables. *Up triangles* are centroids of sites with corresponding elevation (n = 9), numbers indicate elevation (m a.s.l.). The thin solid line encases sites along the Transect 1 (Tr1), the dashed line encases sites along the Transect 2 (Tr2), and the dotted line encases sites along the Transect 3 (Tr3). The numbers in parentheses are the portions of the variation explained by each axis.

The soil geochemical variables explained 72% of the variation in the MCS (pseudo–F = 7.1; P < 0.001) indicating that the interactive effect of altitude and transect on MCS was largely driven by vertical and horizontal variability in soil properties. The forward selection of explanatory variables retained four geochemical parameters: Mg^{2+} availability, pH, moisture and TOC content, all together accounting for 55% of variation in the data (pseudo–F = 11.6, P < 0.001). The most pronounced shift in the MCS was given by different altitudinal preferences of bacteria and fungi.

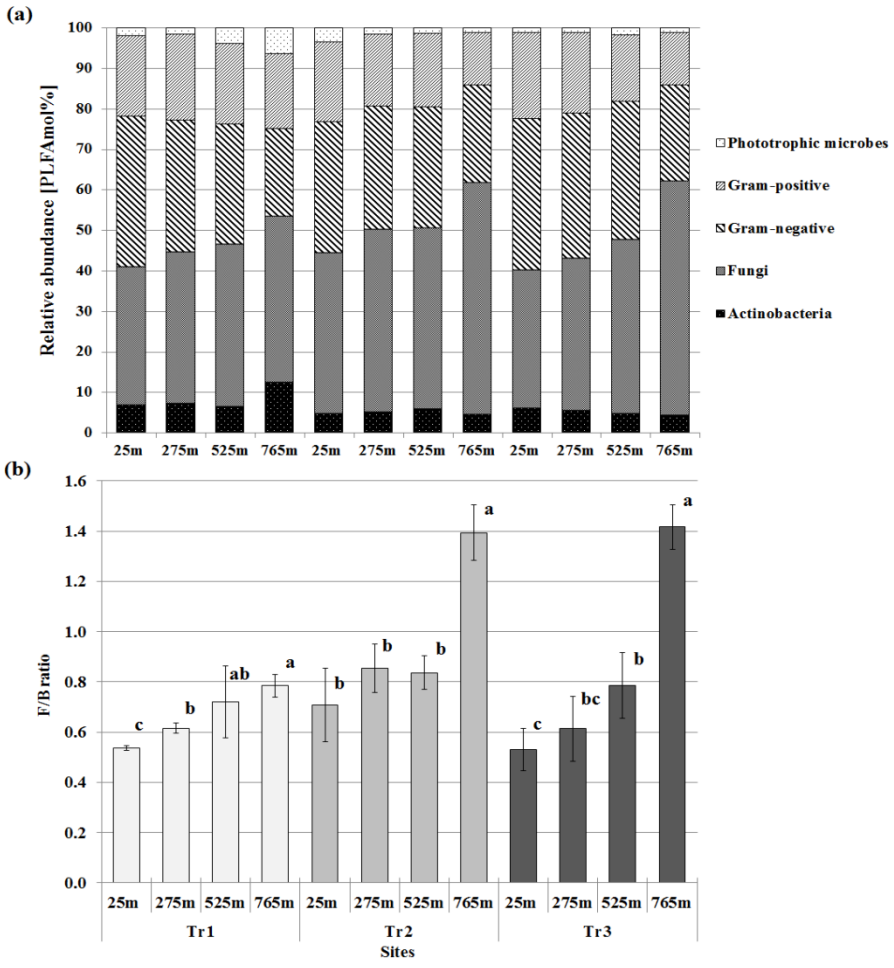

Figure 4. Relative abundance of specific PLFAs within the microbial community (a), and fungi to bacteria (F/B) ratios (b) along altitudinal transects (Tr1-Tr3). Error bars indicate mean ± SD (n = 3). Small case letters denote significant differences between altitudes within particular transects (P < 0.05; One–way ANOVA combined with Tukey post hoc test).

The bacteria were consistently more abundant in the soils from lower elevations, having lower pH and higher TOC and moisture contents (Fig. 3). In general, PLFAs specific to G− bacteria were more abundant than PLFAs of G+ bacteria (Fig. 4a; mean G−/G+ ratio ± SD = 1.76 ± 0.17; n = 36). Oppositely, the fungal contribution to microbial community increased with an increasing altitude, at the sites having TOC poorer soils and higher pH (Fig. 3). Therefore, the F/B ratio gradually increased with an increasing altitude along all three transects (Fig. 4b). The significant interactive effect of altitude and transect on MCS was mainly connected with a strong effect of soil Mg^{2+} availability, which was higher along the whole Tr1 and differentiated its microbial communities from sites located along Tr2 and Tr3, where microbial communities of respective sites were more similar. The differences in MCS among the respective sites along Tr1 and other two transects further increased towards higher elevations in coincidence with an increasing soil Mg^{2+} availability along Tr1 (Fig. 3). In result, the TOC poorest and Mg^{2+} richest soil at the highest site on Tr1 had the most distinct MCS from all the sites. Its microbial community was characterized by higher abundance of Actinobacteria and PLFAs of phototrophic microorganisms and much lower contribution of G− bacteria compared to communities of all other sampling sites (Fig. 3, 4a).

4 Discussion

4.1 Climatic and soil edaphic conditions along altitudinal transects

The coastal area of the Petunia Bay in Svalbard is characteristic by ca four months lasting summer, long winter period (Ambrožová and Láska, 2017) and very low precipitations (Førland et al., 2010). Our measurements in this area further showed that soils along an elevation gradient from 25 to 765 m a.s.l. face significantly different microclimatic regimes. During winter, when the air temperatures varied a lot in time but less with elevation (Fig. S2b, data from AWS_{25} and AWS_{455}), the soil temperatures were relatively stable but significantly stratified with altitude (Fig. S1, S2a). The mean winter soil temperatures decreased from −4 to −10 °C along the elevation gradient from 25 to 765 m a.s.l. (Table 1), which can strongly reduce winter soil microbial activity at high altitudes (Drotz et al., 2010; Nikrad et al., 2016). In contrast, the mean summer soil temperatures did not reflect the site elevation (Table 1) and

the comparison of temperature fluctuations, mean and maximum daily mean temperatures showed that the lowest and highest sites experienced during summer on average colder, but more stable soil microclimate compared to the mid–elevated sites (Table 1). However, the summer season prolonged with decreasing elevation and the increasing number of days with mean temperature above 5 °C and a rising positive surface energy balance (Table 1) positively affected the occurrence and spreading of vascular plants (Kleidon and Mooney, 2000; Klimeš and Doležal, 2010), which had strong implications for a transition of edaphic conditions along studied elevation transects. Together with increased litter inputs and stocks of soil OM with lower C/N ratio (Table 3) was the plant growth associated with root respiration, cation uptake, and release of H^+ and organic acids from roots, all together accounting for decreased soil pH (van Breemen at al., 1984). The increasing soil OM content was further positively related to soil moisture (Fig. 3). Interestingly, the soils in general did not suffer from desiccation (Fig. S3), commonly identified among the most stressing factors in polar and alpine ecosystems (Ley et al., 2004; Van Horn et al., 2013; Tytgat et al., 2016), probably due to high cloudiness and fog occurrence (Sawaske and Freyberg, 2015) in the maritime climate.

The alkaline bedrock material resulted in high soil pH (7.8–9) and high availabilities of basic cations, which were, however, spatially variable due to diverse geology of the studied area (Dallmann et al., 2004; Table 2). Beside clear altitudinal trends in soil edaphic conditions connected mostly with the soil OM content, the Mg^{2+} availability was recognized as main factor driving differences in soil microbial properties between transects (Fig. 3). In result, the character of the parent substrate mostly controlled soil microbial properties at the most elevated sites, which had generally low OM content and the most divergent MCS compared to lower located sites (Fig. 3). The highest site along Tr1 was the most extreme habitat among all the chosen sites, with the highest proportion of bare unvegetated soil surface (Fig. S5), the lowest OM and moisture contents, highest Mg^{2+} availability and soil pH, and consequently also the most distinct microbial characteristics (Fig. 2, 3). Towards lower elevations, the soil OM content became increasingly important and the microbial characteristics of the sites on different transects were more similar.

4.2 Soil microbial properties along altitudinal transects

The altitudinal shifts in soil edaphic properties were not significantly reflected in the soil microbial biomass and potential microbial respiration. Generally, the

soil PLFA contents were comparable between all the sites along particular elevation transects, with the exception of very low soil PLFA concentration on the highest site of the Tr1 (Fig. 2a). There are no other studies from the High Arctic ecosystems reporting about altitude effect on soil microbial biomass. However, other studies conducted on alpine gradients in the temperate and boreal zones documented weak or absent altitudinal trends in the microbial biomass (Djukic et al. 2010, and Xu et al., 2014 using PLFA; Löffler et al., 2008 using cell counts) but also a negative effect of elevation in the Alps (Margesin et al., 2009) and northewestern Finland (Väre et al., 1997). Importantly, none of the studies considered unvegetated habitats and all of them were conducted in soils with acidic or neutral soil pH.

Microbial respiration also did not change systematically with increasing elevation. The three lowest sites along each transect always had comparable soil microbial respiration rates (Fig. 2b), while soil microbial activities of the highest sites differed. The most elevated site on the Tr1 showed significantly lower respiration rates than the lower sites on this transect, which was in line with the lowest OM content as well as soil PLFA content. However, the soils from the highest sites on both Tr2 and Tr3 respired significantly more than the soils from lower sites on these transects, irrespective of relatively stable microbial biomass. This is in contrast to other studies, which reported decreasing microbial activity with increasing elevation (Schinner, 1982; Väre et al., 1997; Niklińska and Klimek, 2007). However, these studies were conducted in lower latitudes and the studied altitudinal gradients did not include unvegetated habitats. To comment on and justify our results, we are aware that microbial activities were measured in freeze-stored and not fresh samples (see section 2.3 for details) and, therefore, the respiration rates measured after thawing show the potential activity of soil microbial communities in the soils. However, the respiration rates in three subsequent measurements (after flush, during adaptation and after stabilization) were positively correlated ($r = 0.93$ and 0.74, both $P < 0.0001$, $n = 36$), the ratios between the flush and stabilized respiration rates were comparable across all the soils (compare Fig. 2b and c) and the above-described differences in microbial activities among the sites were consistent. Our data are in accord with the study of Larsen et al., (2002), who found comparable response to freeze-thaw events between two different arctic ecosystem types. We thus suggest that the soils responded similarly to the storage treatment independently of site location and that observed differences in soil microbial activities are representative for the studied transects. Therefore, the higher soil microbial respiration at the most elevated sites point to a higher lability of

the present OM (Lipson et al., 2000; Uhlířová et al., 2007) and/or to a shift in microbial communities towards groups with higher potential to mineralize the OM (Gavazov, 2010; Djukic et al., 2013). Previous studies, considering either bare soil or vegetated habitats, reported rather increasing complexity of soil OM with elevation (Ley et al., 2004; Xu et al., 2014). However, in this study was majority of OM and microbial biomass at the most elevated sites associated with biological soil crusts with high algal and cyanobacterial abundance (Table S1, Fig. S5), known for their high microbial activity (Pushkareva et al., 2017; Bastida et al., 2014). The high microbial activity in the most elevated sites could be ascribed to prevalence of compounds of algal/cyanobacterial origin with very low portion of complex and slowly decomposable lignin and lignified compounds and protective waxes (like cutin and suberin) mainly derived from vascular plants. In accord, the sitosterol to brassicasterol ratio gradually decreasing with increasing elevation (Table 3) and increasing sitosterol content in the TOC pool at lower elevations pointed to growing importance of microalgal sources of OM in high elevation habitats (Sinsabaugh et al., 1997; Rontani et al., 2012). Even though both sterols can be found in higher plants and microalgae, the changing ratio indicates shift in the origin of OM (reviewed by Volkman, 1986, see also Volkman, 2003). Changes within microbial communities, which can also help to explain higher soil microbial respiration at the most elevated sites are discussed below.

Although the soil PLFA content did not change along the studied elevation transects, we have found a systematic altitudinal shift in the PLFA composition, resulting in significantly increasing F/B ratio towards higher elevations. This shift was best explained by a decreasing soil OM content and soil moisture and increasing pH (Fig. 3). Reports about soil F/B ratios and their altitudinal changes from the High Arctic are missing, but studies from lower latitudes showed either a similar trend of increasing F/B ratio with an altitude in the Alps (Margesin et al., 2009) or the opposite altitudinal effect in the Alps (Djukic et al., 2010) and Himalayas (Xu et al., 2014; Hu et al., 2016). Such divergent results indicate that altitude alone is not the key driving factor of the soil F/B ratio. In contrast to our observation, these studies reported very low soil F/B ratios of 0.05-0.2, which may indicate important role of fungi in functioning of the Arctic habitats. Soil pH was previously identified as the main driver of fungal-bacterial dominance in the soil (Baath and Anderson, 2003; Högberg et al., 2007; Rousk et al., 2009; Siles and Margesin, 2016). Fungi have been found more acid tolerant than bacteria, leading to higher F/B ratio in acidic soils (Högberg et al., 2007; Rousk et al., 2009; reviewed by Strickland and Rousk, 2010). However, here we report high F/B ratios in the alkaline soils (pH 7.8-

9.0) and increasing F/B ratios with an increasing soil pH. Similar trend was reported also by Hu et al., (2016), but the authors found F/B ratios one order of magnitude lower compared to our study. The possible explanation of generally high fungal abundance and increasing F/B ratio at more elevated sites, which are typical by unfavourable edaphic conditions and severe winter microclimate, could be higher competitiveness of fungi compared to bacteria in suboptimal conditions due to their wider pH (Wheeler et al., 1991) and lower temperature (Margesin et al., 2003) growth optima. We further found that the increasing F/B ratio was significantly coupled with an increasing soil respiration ($r = 0.649$; $P < 0.001$). Indeed, such relationship can be related to higher fungal ability either to prosper in the soil conditions at the most elevated sites, or to utilize more efficiently available C sources (Ley et al., 2004; Bardgett et al., 2005; Nemergut et al., 2005; van der Heijden et al., 2008). In turn, the higher bacterial contribution at lower elevations may be associated with more benign soil conditions and bacterial preference for utilization of labile root exudates released by vascular plants (Lipson et al., 1999; Lipson et al., 2002). Since the projected warming in the Arctic (Collins et al., 2013) will likely cause an upward migration of the vegetation and increasing plant cover in detriment of lichens and biological soil crusts (Vuorinen et al., 2017; Yu et al. 2017; de Mesquita et al., 2017), the soil microbial communities will likely respond by decreasing F/B ratios at higher elevations.

Apart from the systematic altitudinal shift in the F/B ratio, we observed a strong shift in the bacterial composition, which differentiated the altitudinal trends in the soil MCS along Tr1 from trends along Tr2 and Tr3. This difference between transects increased towards higher elevations and was best explained by Mg^{2+} availability (Fig. 3). The soils from Tr1, except the lowest site, had a lower G– to G+ bacterial ratios within microbial communities than soils from other two transects. Further, the microbial community of the most elevated site along Tr1 was significantly more contributed by actinobacteria and phototrophic microorganisms compared to all other sites (Fig. 3, 4a). It is known that the high Mg^{2+} availability inhibits growth of many soil bacterial species. The observed inhibitive Mg^{2+} levels were 5 and 50 p.p.m for G– and G+ bacteria, respectively (Webb 1949), indicating that these bacterial groups significantly differ in their tolerance for enhanced Mg^{2+} levels. Considering half of available Mg^{2+} in soil solution and average soil moisture content 20%, the Mg^{2+} concentrations ranged approximately from 16-140 p.p.m,, which could explain decreased abundance of G- bacteria in sites with high Mg^{2+} availability. This inhibitive Mg^{2+} effect further corresponds with the negative correlations between Mg^{2+} availability and soil microbial biomass and

respiration found in our study, and could explain the lower microbial biomass and respiration in the soils from Tr1. Our data thus indicate that beside the traditionally identified drivers of microbial activity and MCS such as soil OM content, moisture and pH, Mg^{2+}availability is in important factor shaping the microbial environment along the arctic altitudinal transects on dolomitic parent materials.

5 Conslusions

The results obtained in this study have shown significant altitudinal zonation of most edaphic properties, but also significant spatial heterogeneity in horizontal direction, resulting in transect-specific effect of altitude on abiotic soil properties. Our data demonstrated that soils on the most elevated, unvegetated sites around the Petunia Bay can host microbial assemblages comparable in size and activity with the tundra ecosystem. The high microbial biomass and activity at the most elevated sites were almost exclusively associated with biological soil crusts, largely contributed by fungi. However, their development was retarded on some sites by high pH, low moisture and high Mg availability, resulting in pronouncedly low OM content, microbial biomass and distinct MCS. Despite the ubiquitous occurrence of soil crusts, the gradually increasing plant productivity and litter inputs down along transects were associated with decreasing soil pH, increasing OM content and soil moisture. Concurrently, the soil edaphic and microbial properties become more uniform. As the rise in temperatures and humidity predicted by climatic models will likely cause an upward migration of the vegetation and increasing plant cover, the higher plant litter inputs will overreach the influence of parent material and entail an increasing abundance of bacteria and decreasing F/B ratio in the summer microbial assemblages.

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Competing interests

The authors declare that they have no conflict of interest.

References

Ambožová, K. and Láska, K.: Air temperature variability in the vertical profile over the coastal area of Petuniabukta, central Spitsbergen, Pol. Polar Res., 38, 41–60, 2017.

Bååth, E. and Anderson, T.–H.: Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA based techniques. Soil Biol. Biochem., 35, 955–63, 2003.

Bardgett, R. D., Bowman, W. D., Kaufmann, R., and Schmidt, S.K.: A temporal approach to linking aboveground and belowground ecology, Trends Ecol. Evol., 20, 634–641, 2005.

Bastida, F., Jehmlich, N., Ondoño, S., von Bergen, M., García, C., and Moreno, J. L.: Characterization of the microbial community in biological soil crusts dominated by Fulgensia desertorum (Tomin) Poelt and Squamarina cartilaginea (With.) P. James and in the underlying soil, Soil Biol. Biochem., 76, 70–79, 2014.

Bekku, Y. S., Kume, A., Masuzawa, T., Kanda, H., Nakatsubo, T., and Koizumi, H.: Soil respiration in a high arctic glacier foreland in Ny-Ålesund, Svalbard, Polar Bioscience, 17, 36–46, 2004.

Björk, R. G., Björkman, M. P., Andersson M. X., and Klemedtsson L.: Temporal variation in soil microbial communities in Alpine tundra, Soil Biol. Biochem., 40, 266–268, 2008.

Blaud, A., Lerch, T. Z., Phoenix, G. K., and Osborn M. A.: Arctic soil microbial diversity in a changing world, Res. Microbiol., 166, 796–813, 2015.

Bossio, D.A. and Scow, K.M.: Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns, Microb. Ecol., 35, 265–278, 1998.

Clein, J. S. and Schimel, J. P.: Reduction in microbial activity of litter due to drying and rewetting events, Soil Biol. Biochem., 26, 403–406, 1994.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J. L., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W. J., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A. J., and Wehner, M.: Long-term climate change. Projections, commitments and irreversibility. In: Stocker, T. F., Qin, D., Plattner, G. K., Tignor, M. M. B., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V., and Midgley, P. M. (eds.).: Climate Change 2013. The physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press. Cambridge. p. 1029-1136, ISBN: 9781107057991, 2013.

Chu, H., Fierer, N., Lauber, Ch. L., Caporaso, J. G., Knight, R., and Grogan, P.: Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes, Environ. Microb., 12, 2998–3006, 2010.

Dallmann, W. K., Piepjohn, K., and Blomeier, D.: Geological map of Billefjorden, Central Spitsbergen, Svalbard, Temakart Nr. 36, Norsk Polarinstitutt, 2004.

de Mesquita, C. P. B., Knelman, J. E., King, A. J., Farrer, E. C., Porazinska, D. L., Schmidt, S. K., and Suding, K. N.: Plant colonization of moss-dominated soils in the alpine: Microbial and biogeochemical implications, Soil Biol. Biochem., 111, 135–142, 2017.

Djukic, I., Zehetner, F., Mentler, A., and Gerzabek, M. H.: Microbial community composition and activity in different Alpine vegetation zones, Soil Biol. Biochem., 42, 155–161, 2010.

Djukic, I., Zehetner, F., Watzinger, A., Horacek, M., and Gerzabek, M. H.: In situ carbon turnover dynamics and the role of soil microorganisms therein: a climate warming study in an Alpine ecosystem, FEMS Microbiol. Ecol., 83, 112–124, 2013.

Drotz, S. H., Sparrman, T., Nilsson, M. B., Schleucher, J., and Öquist, M. G.: Both catabolic and anabolic heterotrophic microbial activity proceed in frozen soils, PNAS, 107, 21046–21051, 2010.

Ferrari, B. C., Bissett, A., Snape, I., van Dorst, J., Palmer, A. S., Ji, M., Siciliano, S. D., Stark, J. S., Winsley, T., and Brown, M. V.: Geological connectivity drives microbial community structure and connectivity in polar, terrestrial ecosystems, Environ. Microb., 18, 1834–1849, 2016.

Fierer, N., McCain C. M., Meir, P., Zimmermann, M., Rapp, J. M., Silman, M. R., and Knight, R.: Microbes do not follow the elevational diversity patterns of plants and animals, Ecology, 92, 797–804, 2011.

Førland, E.J., Benestad, R., Hanssen-Bauer, I., Haugen, J.E., and Skaugen, T.: Temperature and Precipitation Development at Svalbard 1900-2100, Advances in Meterology, doi:10.1155/2011/893790, 2010.

Frostegård, A. and Bååth, E.: The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil, Biol. Fert. Soils, 22, 59–65, 1996.

Frostegård, Å., Bååth, E., and Tunlid, A.: Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis, Soil Biol. Biochem., 25, 723–730, 1993.

Gavazov, K. S.: Dynamics of alpine plant litter decomposition in a changing climate, Plant Soil, 337, 19–32, 2010.

Hardison, A. K., Canuel, E. A., Anderson, I. C., Tobias, C. R., Veuger, B., and Waters, M. N.: Microphytobenthos and benthic macroalgae determine sediment organic matter composition in shallow photic sediments, Biogeosciences, 10, 5571–5588, 2013.

Harris, D., Horwáth, W. R., and van Kessel, Ch.: Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis, Soil Sci. Soc. Am. J., 65, 1853–1856, 2001.

Holm, S.: *A simple sequentially rejective multiple test procedure.* Scand. J. Stat., 6, 65–70, 1979.

Högberg, M. N., Högberg, P., and Myrold, D. D.: Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia, 150, 590–601, 2007.

Hu, L., Xiang, Z., Wang, G., Rafique, R., Liu W., and Wang, C.: Changes in soil physicochemical and microbial properties along elevation gradients in two forest soils, Scandinavian Journal of Forest Research, 31, 242–253, 2016.

Jones, A, Stolbovoy, V., Tarnocai, C., Broll, G., Spaargaren, O., and Montanarella, L. (eds.): Soil Atlas of the Northern Circumpolar Region. European Commission, Publications Office of the European Union, Luxembourg, 144pp., 2010.

Khotimchenko, S. V., Vaskovsky, V. E., and Titlyanova, T. V.: Fatty acids of marine algae from the pacific coast of North California, Bot. Mar., 45, 17–22, 2002.

Kleidon, A. and Mooney, H. A.: A global distribution of biodiversity inferred from climatic constraints: results from a process-based modelling study, Global Change Biol., 6, 507–523, 2000.

Klimeš, L. and Doležal J.: An experimental assessment of the upper elevational limit of flowering plants in the western Himalayas, Ecography, 33, 590–596, 2010.

Körner, Ch.: The use of 'altitude' in ecological research, Trends in Ecol. And Evol., 22, 569–574, 2007.

Kroppenstedt, R. M.: Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: Goodfellow, M., Minikin, D. E., Eds. Chemical methods in bacterial systematics. Academic Press, London, pp. 173–194, 1985.

Larsen, K. S., Jonasson, S., and Michelsešn, A.: Repeated freeze–thaw cycles and their effects on biological processes in two arctic ecosystem types, Appl. Soil Ecol., 21, 187–195, 2002.

Lazzaro, A., Hilfiker, D., and Zeyer, J.: Structure of microbial communities in alpine soils: seasonal and elevational effects, Frontiers in Microbiology, doi: 10.3389/fmicb.2015.01330, 2015.

Ley, R. E., Williams, M. W., and Schmidt, S. K.: Microbial population dynamics in an extreme environment: controlling factors in talus soils at 3750m in the Colorado Rocky Mountains, Biogeochemistry, 68, 313–335, 2004.

Lipson, D. A.: Relationships between temperature responses and bacterial community structure along seasonal and altitudinal gradients, FEMS Microbiol. Ecol., 59, 418–427, 2007.

Lipson, D. A., Schmidt, S. K., and Monson, R. K.: Links between microbial population dynamics and nitrogen availability in an alpine ecosystem, Ecology, 80, 1623–1631, 1999.

Lipson, D. A., Schmidt, S. K., and Monson, R. K.: Carbon availability and temperature control the post-snowmelt decline in alpine soil microbial biomass, Soil Biol. Biochem., 32, 441–448, 2000.

Lipson, D. A., Schadt, C. W., and Schmidt, S. K.: Changes in soil microbial community structure and function in an alpine dry meadow following spring snow melt, Microb. Ecol., 43, 307–314, 2002.

Lovelland P. J. and Whalley W. R.: Particle size analysis. In: Soil and Environmental Analysis Physical Method (Eds Smith, K.A. and Mullins, Ch.E.), Dekker Press, New York, USA, 2001.

Löffler, U. C. M., Cypionka, H., and Löffler, J.: Soil microbial activity along an arctic-alpine altitudinal gradient from a seasonal perspective, Eur. J. Soil Sci., 59, 842–854, 2008.

Ma, X., Chen, T., Zhang, G., and Wang R.: Microbial community structure along an altitude gradient in three different localities, Folia Microbiol., 49, 105–111, 2004.

Männistö, M., Tiirola, M., and Häggblom, M. M.: Bacterial communities in Arctic fjelds of Finnish Lapland are stable but highly pH-dependent, FEMS Microbiol. Ecol., 59, 452–465, 2007.

Margesin, R., Gander, S., Zacke, G., Gounot, A. M., and Schinner, F.: Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts, Extremophiles, 7, 451–458, 2003.

Margesin, R., Jud, M., Tscherko, D., and Schinner, F.: Microbial communities and activities in alpine and subalpine soils, FEMS Microbiol. Ecol., 67, 208–218, 2009.

Meng, H., Li, K., Nie, M.,Wan, J.-R., Quan, Z.-X., Fang, C.-M., Chen, J.-K., Gu, J.-D., and Li, B.: Responses of bacterial and fungal communities to an elevation gradient in a subtropical montane forest of China, Appl. Microbiol. Biot., 97, 2219–2230, 2013.

Montgomery, H. J., Monreal, C. M., Young, J. C., and Seifert, K. A.: 2000. Determination of soil fungal biomass from soil ergosterol analyses, Soil Biol. Biochem., 32, 1207–1217, 2000.

Nemergut, D. R., Costello, E. K., Meyer, A. F., Pescador, M. Y., Weintraub, M. N., and Schmidt, S. K.: Structure and function of alpine and arctic soil microbial communities, Res. Microbiol., 156, 775–784, 2005.

Niklińska, M. and Klimek, B.: Effect of temperature on the respiration rate of forest soil organic layer along an elevation gradient in the Polish Carpathians, Biol. Fert. Soils, 43, 511–518, 2007.

Nikrad, M. P., Kerkhof, L. J., and Häggblom, M. M.: The subzero microbiome: microbial activity in frozen and thawing soils, FEMS Microbiol. Ecol., 92, doi:10.1093/femsec/fiw08, 2016.

Oberbauer, S. F., Tweedie, C. E., Welker, J. M., Fahnestock, J. T., Henry, G. H. R., Webber, P. J., Hollister, R. D., Walker, M. D., Kuchy, A., Elmore, E., and Starr, G.: Tundra CO2 fluxes in response to experimental warming across latitudinal and moisture gradients. Ecol. Monogr., 77, 221–238, 2007.

Prach, K., Klimešová, J., Košnar, J., Redčenko O., and Hais M.: Variability of contemporary vegetation around Petuniabukta, central Spitsbergen, Pol. Polar Res., 33, 383–394, 2012.

Pushkareva, E., Kvíderová, J., Šimek, M., and Elster, J.: Nitrogen fixation and diurnal changes of photosynthetic activity in Arctic soil crusts at different development stage, Europ Journal of Soil Biology, 79, 21–30, 2017.

Richter, D. D., Johnson, D. W., and Dai, K. H.: Cation exchange reactions in acid forested soils: effects of atmospheric pollutant deposition. In: Johnson, D.W., Lindberg, S.E. (eds.), Atmospheric Deposition and Nutrient Cycling in Forest Ecosystems, Spring-Verlag, New York, pp 339–358, 1992.

Rontani, J.–F., Charriere, B., Petit, M., Vaultier, F., Heipieper, H. J., Link, H., Chaillou, G., and Sempéré, R.: Degradation state of organic matter in

surface sediments from the Southern Beaufort Sea: a lipid approach, Biogeosciences, 9, 3513–3530, 2012.

Rousk, J., Brookes, P. C., and Bååth, E.: Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization, Appl. Environ. Microb., 75, 1589–1596, 2009.

Sawaske, S. R. and Freyberg, D. L.: Fog, fog drip, and streamflow in the Santa Cruz Mountains of the California Coast Range, Ecohydrology, 8, 695–713, 2015.

Schimel, J. P. and Clein, J. S.: Microbial response to freeze-thaw cycles in tundra and taiga soils, Soil Biol. Biochem., 28, 1061–1066, 1996.

Schinner, F.: Soil microbial activities and litter decomposition related to altitude, Plant Soil, 65, 87–94, 1982.

Schinner, F. and Gstraunthaler, G.: Adaptation of microbial activities to the environmental conditions in alpine soils, Oecologia, 50, 113–116, 1981.

Schűtte, U. M. E., Abdo, Z., Foster, J., Ravel, J., Bunge, J., Solheim, B., and Forney, L. J.: Bacterial diversity in a glacier foreland of the high Arctic, Mol. Ecol., 19, 54–56, 2010.

Shen, C., Xiong, J., Zhang, H., Feng, Y., Lin, X., Li, X., Liang, W., and Chu, H.: Soil pH drives the spatial distribution of bacterial communities along elevation on Changbai Mountain, Soil Biol. Biochem., 57, 204–211, 2013.

Siles, J. S. and Margesin, R.: Abundance and diversity of bacterial, archaeal, and fungal communities along an altitudinal gradient in alpine forest soils: What are the driving factors? Soil Microbiology, 72, 207–220, 2016.

Singh, D., Takahashi, K., Kim, M., Chun, J., and Adams, J. M.: A hump-backed trend in bacterial diversity with elevation on Mount Fuji, Japan, Microb. Ecol. 63, 429–437, 2012.

Sinsabaugh, R. L., Antibus, R. K., Jackson, C. R., Karpanty, S., Robinson, M., Liptak, M., and Franchini, P.: A β-sitosterol assay for fine-root mass in soil, Soil Biol. Biochem., 29, 39–44, 1997.

Sparling, G. P. and West, A. W.: A comparison of gas chromatography and differential respirometer methods to measure soil respiration and to estimate the soil microbial biomass, Pedobiologia, 34, 103–112, 1990.

Stenberg, B., Johanssson, M., Pell, M., Sjödahl-Svensson, K., Stenström, J., and Torstensson, L.: Microbial biomass and activities in soil as affected by frozen and cold storage, Soil Biol. Biochem., 30, 393–402, 1998.

Strickland, M. S. and Rousk, J.: Considering fungal:bacterial dominance in soils – Methods, controls, and ecosystem implications, Soil Biol. Biochem., 42, 1385–1395, 2010.

Šmilauer, P. and Lepš, J.: Multivariate analysis of ecological data using CANOCO5. Cambridge University Press, Cambridge, 2014.

Ter Braak, C. J. F. and Šmilauer, P.: Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power, Ithaca, USA, 496 pp, 2012.

Trevors, J. T., Kevan, P. G., and Tam, L.: Microbial diversity across a Canadian sub-Arctic, isostatically rebounding, soil transect. Polar Science, 4, 81–91, 2010.

Tytgat, B., Verleyen, E., Sweetlove, M., D'hondt, S., Clercx, P., Van Ranst, E., Peeters, K., Roberts, S., Namsaraev, Z., Wilmotte, A., Vyverman, W., and Willems, A.: Bacterial community composition in relation to bedrock type and macrobiota in soils from the Sør Rondane Mountains, East Antarctica, FEMS Microbiol. Ecol., 92, doi: 10.1093/femsec/fiw126, 2016.

Uhlířová, E., Šantrůčková, H., and Davidov, S. P.: Quality and potential biodegradability of soil organic matter preserved in permafrost of Siberian tussock tundra, Soil Biol. Biochem., 39, 1978–1989, 2007.

van Breemen, N., Driscoll, C. T., and Mulder, J.: Acidic deposition and internal proton sources in acidification of soils and waters, Nature, 307, 599–604, 1984.

van de Heijden, M. G. A., Bardgett, R. D., and van Straalen, N. M.: The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems, Ecol. Lett., 11, 296–310, 2008.

Van Horn, D. J., Van Horn, M. L., Barrett, J. E., Gooseff, M. N., Altrichter, A. E., Geyer, K. M., Zeglin, L. H., and Takacs-Vesbach, K.: Factors controlling soil microbial biomass and bacterial diversity and community composition in a cold desert ecosystem: Role of geographic scale, PLoS One, 8, doi: 10.1371/journal.pone.0066103, 2013.

Väre, H., Vestberg, M., and Ohtonen, R.: Shifts in mycorrhiza and microbial activity along an oroarctic altitudinal gradient in northern Fennoscandia, Arctic Alpine Res., 29, 93–104, 1997.

Volkman, J. K.: Sterols in microorganisms, Appl. Microbiol. Biot., 60, 495–506, 2003.

Volkman, J. K.: A review of sterol markers for marine and terrigenous organic matter, Org. Geochem., 9, 83–99, 1986.

Vuorinen, K. E. M., Oksanen, L., Oksanen, T., Pyykönen, A., Olofsson, J., and Virtanen, R.: Open tundra persist, but arctic features decline–Vegetation changes in the warming Fennoscandian tundra, Global Change Biol., 23, 3794–3807, 2017.

Webb, M.: The influence of magnesium on cell division. 2. The effect of magnesium on the growth and cell division of various bacterial species in complex media, Microbiology, 3, 410–417, 1949a.

Wheeler, K. A., Hurdman, B. F., and Pitt, J. I.: Influence of pH on the growth of some toxigenic species of *Aspergillus*, *Penicillium* and *Fusarium,* Int. J. Food Microbiol., 12, 141–150, 1991.

Xu, M., Li, X., Cai, X., Gai, J., Li, X., Christie, P., and Zhang, J.: Soil microbial community structure and activity along a montane elevational gradient on the Tibetan Plateau, Eur. J Soil Biol., 64, 6–14, 2014.

Yoshitake, S., Uchida, M., Koizumi, H., and Nakatsubo, T.: Carbon and nitrogen limitation of soil microbial respiration in a High Arctic successional glacier foreland near Ny-Ålesund, Svalbard, Pol. Res., 26, 22–30, 2007.

Yu, Q., Epstein, H., Engstrom, R., and Walker, D.: Circumpolar arctic tundra biomass and productivity dynamics in response to projected climate change and herbivory, Global Change Biol., 23, 3895–3907, 2017.

Zbíral, J. and Němec, P.: Data presentation, interpretation, and communication: Integrating of Mehlich 3 extractant into the Czech soil testing scheme, Communications in Soil Science and Plant Analysis, 31, 2171–2182, 2000.

Supplementary information

Figure S1 Time series of soil temperatures at –5 cm from sampling sites located along Tr1 in the period June 2012 – May 2013.

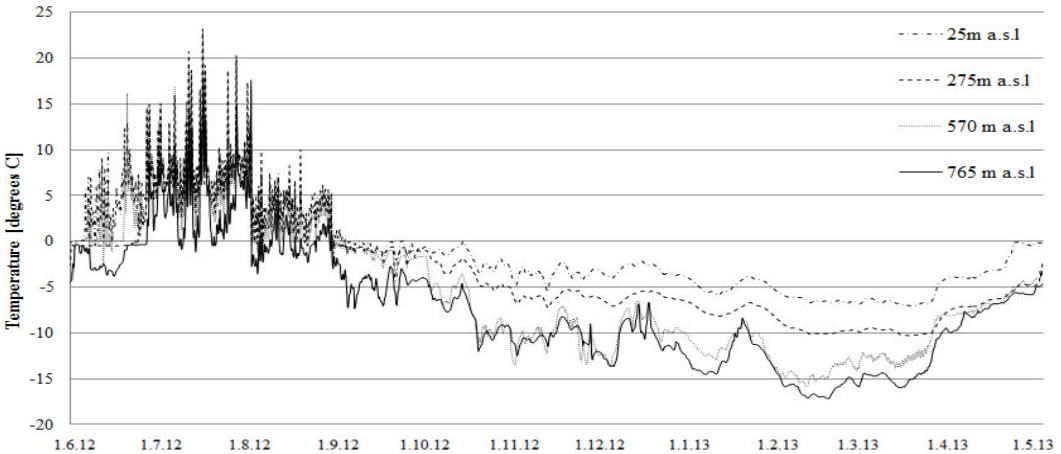

Figure S3 Time series of mean ± SD (n = 3) soil water content at -5 cm from AWS_{25} (25 m a.s.l.) and AWS_{455} (455 m a.s.l.) for three consecutive summer seasons (2011–2013). The grey area indicates time of sample collection in 2012. Note the stable soil water content from July until September. The volumetric measurement enable to determine soil water content only in a liquid state, which explains the very low water content in frozen soils during the winter period. These technical limitations explain also conspicuous fluctuations of soil water content during transient periods between winter and summer season, resulting from freeze–thaw cycles when temperature oscillated around zero.

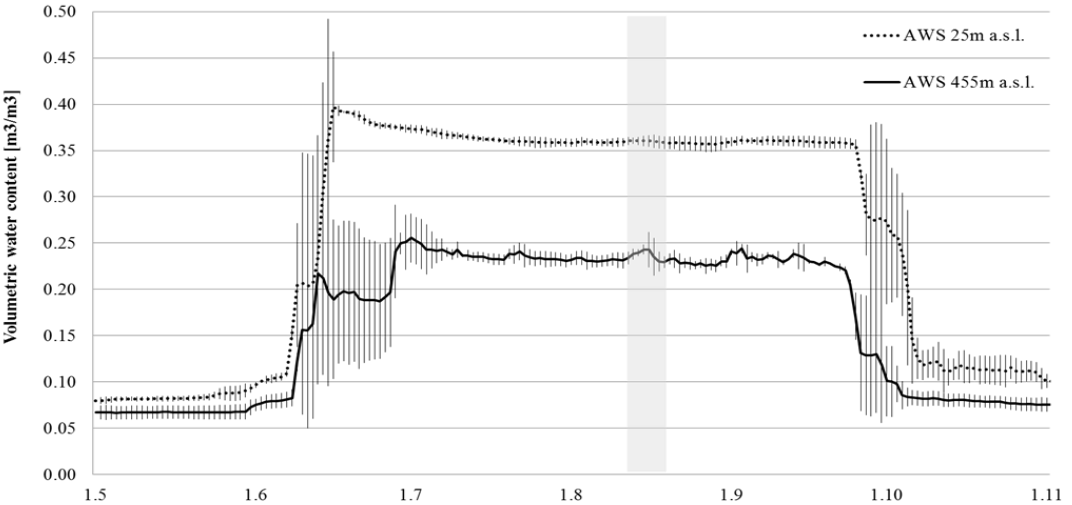

Figure S2 Time series of daily means of soil temperatures at -5 cm **(a)** and of air temperatures at 2 m **(b)** from AWS_{25} (25m a.s.l.) and AWS_{455} (455m a.s.l.) meteorological stations from May 2012 to September 2013.

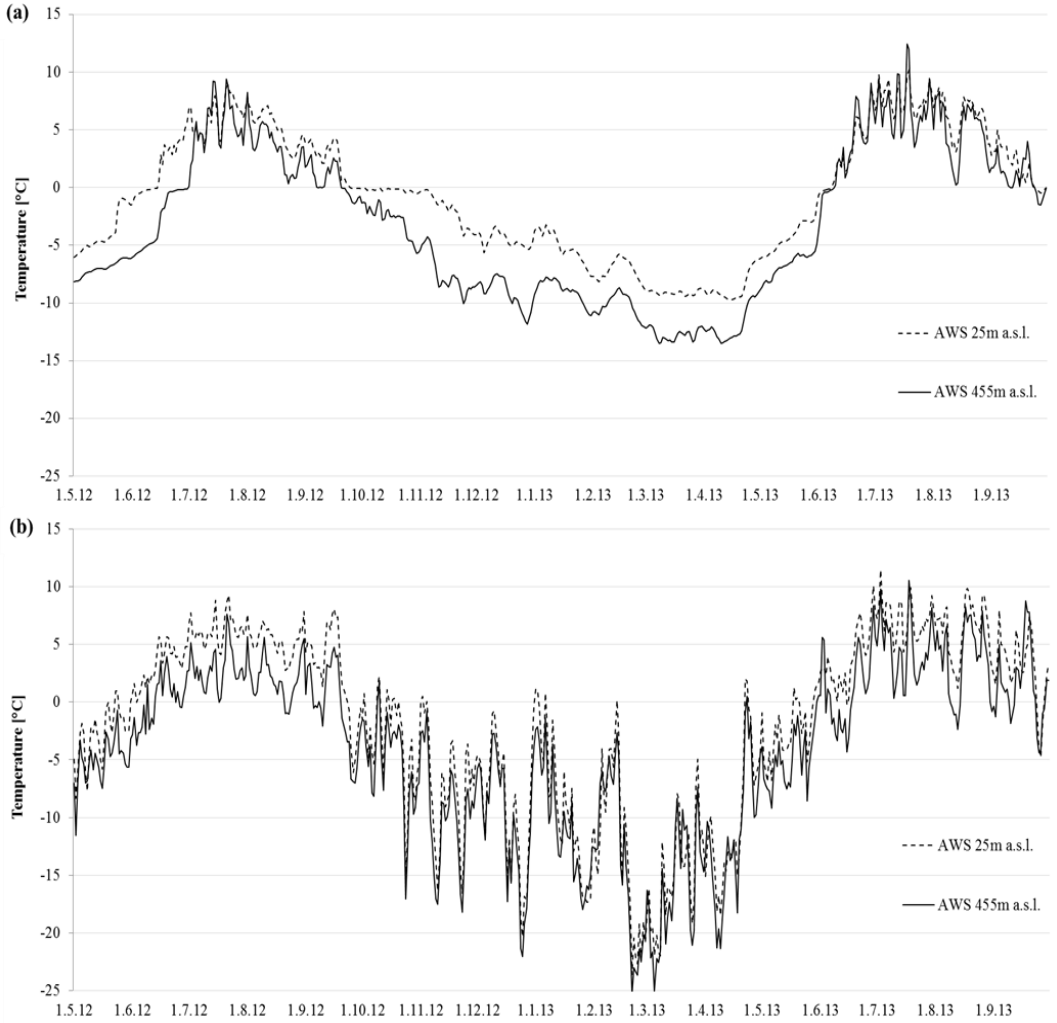

Figure S4 Loading of soil geochemical properties determined in soils along the altitudinal gradients. Ordination diagrams show results of the redundancy analyses constrained by the effect of altitude. *Arrows* indicate the direction in which the respective parameter value increases. *Triangles* are centroids (n = 9) of the sites with corresponding elevation. The portion of variance explained by the respective axis is given in the axis title. The orientation of majority of arrows and site centroids in parallel with RDA 1 indicate strong altitudinal effect on soil properties.

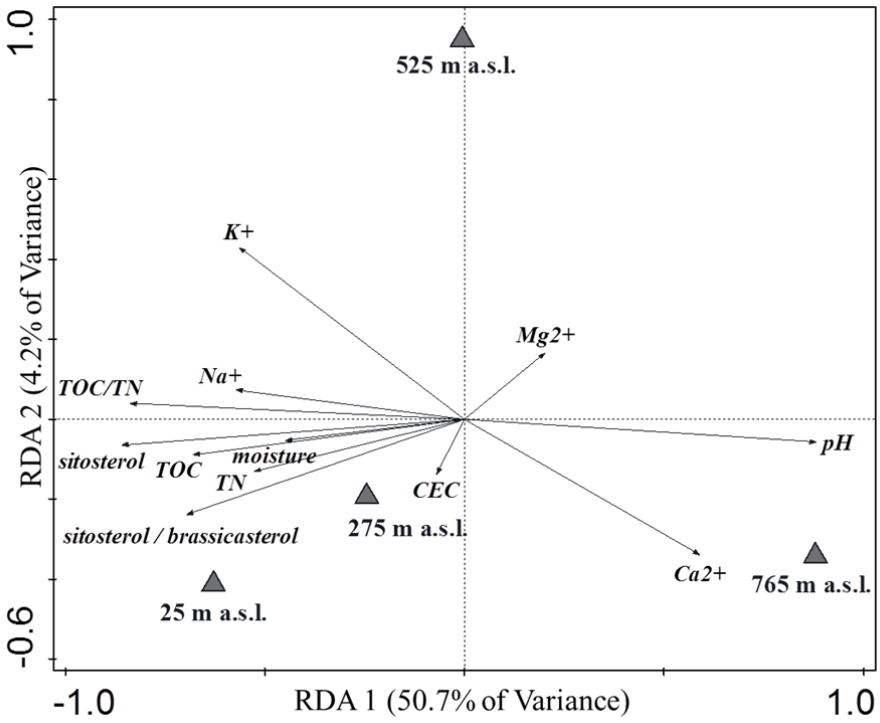

Figure S5 Mean contribution of various types of soil surface cover at particular sampling sites. The areas covered solely by stones were not considered in this figure in order to describe the surface cover in areas suitable for soil sampling (see Table S1 for data including the stony areas).

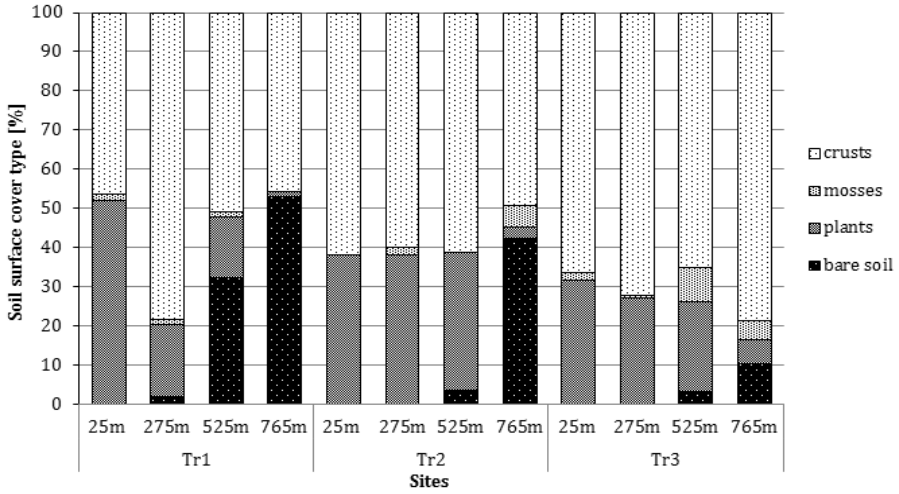

Table S1 Mean contribution of various types of soil surface cover and brief description of soils crusts predominant at particular sampling sites. Means ± SE (n = 3) are given in the upper part of the table. Results of two–way ANOVAs (F–values) of the effects of transect (Tr), altitude (Alt) and their interaction (Tr x Alt) are presented in the lower part of the table.

transect	altitude	soil surface cover type [%]					
	[m a.s.l.]	stones	bare soil	plants	mosses	crusts	crust type
Tr1	25	^{c} 1.0 ± 0.8	n.o.	^{a} 52 ± 6.2	^{a} 1.7 ± 0.9	^{b} 46 ± 6.3	lichenized
	275	^{b} 11 ± 3.9	^{b} 1.7 ± 0.9	^{b} 17 ± 2.4	^{a} 1.0 ± 0.0	^{a} 70 ± 3.7	poorly lichenized
	525	^{b} 17 ± 4.7	^{a} 27 ± 6.2	^{b} 13 ± 5.6	^{a} 1.3 ± 1.2	^{b} 43 ± 15	poorly lichenized
	765	^{a} 47 ± 2.4	^{a} 28 ± 2.4	^{c} 0.7 ± 0.5	n.o.	^{c} 24 ± 4.1	dark cyanobacterial
Tr2	25	^{b} 4.0 ± 2.2	n.o.	^{a} 37 ± 3.4	n.o.	^{a} 59 ± 4.8	lichenized
	275	^{b} 1.3 ± 0.9	n.o.	^{ab} 38 ± 7.1	^{b} 2.0 ± 0.0	^{a} 59 ± 6.5	lichenized
	525	^{a} 38 ± 17	^{b} 2.0 ± 0.8	^{b} 22 ± 9.0	n.o.	^{b} 38 ± 9.2	poorly lichenized
	765	^{a} 49 ± 4.2	^{a} 14 ± 2.9	^{c} 2.0 ± 0.8	^{a} 3.7 ± 0.9	^{b} 33 ± 3.1	dark cyanobacterial
Tr3	25	^{c} 0.7 ± 0.9	n.o.	^{a} 32 ± 9.4	^{b} 1.7 ± 0.9	^{a} 66 ± 9.9	lichenized
	275	^{b} 13 ± 2.4	n.o.	^{a} 23 ± 8.5	^{b} 0.7 ± 0.9	^{a} 63 ± 10	poorly lichenized
	525	^{a} 33 ± 9.4	^{b} 2.0 ± 1.4	^{b} 15 ± 0.0	^{a} 5.7 ± 0.9	^{b} 44 ± 11	poorly lichenized
	765	^{a} 46 ± 6.5	^{a} 5.7 ± 0.9	^{c} 3.3 ± 1.2	^{b} 2.7 ± 1.7	^{b} 42 ± 3.8	dark cyanobacterial
	d.f.						
Tr	2	0.01	**55.6** ***	**4.48** *	2.33	**8.13** **	
Alt	3	**36.1** ***	**58.7** ***	**69.4** ***	**18.5** ***	**3.09** *	
Tr x Alt	6	**5.32** **	**17.6** ***	**3.09** *	1.65	**9.69** ***	

Different letters indicate significant differences between sampling sites along particular transects (P < 0.05; upper part of the table). Statistically significant differences are indicated by: * P < 0.05, ** P < 0.01, *** P < 0.001 (lower part of the table). n.o. – surface cover type was not observed.

Study III

Significance of dark CO_2 fixation in arctic soils

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Significance of dark CO_2 fixation in arctic soils

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Abstract

The occurrence of dark fixation of CO_2 by heterotrophic microorganisms in soil is generally accepted, but its importance for microbial metabolism and soil organic carbon (C) sequestration is unknown, especially under C-limiting conditions. To fill this knowledge gap, we measured dark $^{13}CO_2$ incorporation into soil organic matter and conducted a ^{13}C-labelling experiment to follow the ^{13}C incorporation into phospholipid fatty acids as microbial biomass markers across soil profiles of four tundra ecosystems in the northern circumpolar region, where net primary productivity and thus soil C inputs are low. We further determined the abundance of various carboxylase genes and identified their microbial origin with metagenomics. The microbial capacity for heterotrophic CO_2 fixation was determined by measuring the abundance of carboxylase genes and the incorporation of ^{13}C into soil C following the augmentation of bioavailable C sources. We demonstrate that dark CO_2 fixation occurred ubiquitously in arctic tundra soils, with increasing importance in deeper soil horizons, presumably due to increasing C limitation with soil depth. Dark CO_2 fixation accounted on average for 0.4, 1.0, 1.1, and 16 % of net respiration in the organic, cryoturbated organic, mineral and permafrost horizons, respectively. Genes encoding anaplerotic enzymes of heterotrophic microorganisms comprised the majority of identified carboxylase genes. The genetic potential for dark CO_2 fixation was spread over a broad taxonomic range. The results suggest important regulatory function of CO_2 fixation in C limited conditions. The measurements were corroborated by modelling the long-term impact of dark CO_2 fixation on soil organic matter. Our results suggest that increasing relative CO_2 fixation rates can explain, at least in part,

the soil internal C cycling and the isotopic enrichment of soil organic matter with soil depth.

Keywords: anaplerotic enzymes, carboxylase genes, microbial community composition, permafrost soils, ^{13}C enrichment of soil profile

1 Introduction

Terrestrial ecosystems represent a major sink of CO_2 through fixation by plants but they have been shown to mitigate the rise of atmospheric CO_2 also via microbial CO_2 fixation (Ge et al., 2016; Yuan et al., 2012). Microbial CO_2 fixation has been mostly ascribed to autotrophic microorganisms (Ge et al., 2016), but fundamentally all microorganisms may use inorganic C (IC; i.e. CO_2 or bicarbonate) in their metabolism. All these fixations require energy generated by phototrophic, autotrophic or heterotrophic energy sources. IC is the main or even the only C source for chemoautotrophs and photoautotrophs, while heterotrophs and mixotrophs rely on organic C (OC) but also incorporate IC via a variety of carboxylation reactions that are part of their central or peripheral metabolic pathways (for review see Erb, 2011; Wood and Stjernholm, 1962). The importance of carboxylases in heterotrophic metabolism increases whenever microorganisms experience C limitation through a disproportion between C demand for energy generation and growth and its availability, caused by deficiency or complexity of OC sources, or fast growth (Alonso-Saez et al., 2010; Feisthauer et al., 2008; Merlin et al., 2003). Even though the occurrence of dark and largely heterotrophic CO_2 fixation in soils is generally accepted, very few studies have assessed its relevance for soil microorganisms (Miltner et al., 2004, 2005a, b; Šantrůčková et al., 2005). Estimates of the importance of soil CO_2 fixation for the C balance in certain ecosystems or within an entire soil profile are rare (Ge et al., 2016; Yuan et al., 2012) and analyses of diversity and abundance of carboxylases are missing entirely.

Soil OC becomes progressively enriched in ^{13}C with increasing soil depth (Bird et al., 2002; Gentsch et al., 2015; Nadelhoffer and Fry, 1988; Torn et al., 2002). There are several explanations but no one can fully explain the measured isotopic shift. The enrichment of soil OC with depth can be connected with decrease of $\delta\ ^{13}C$ of atmospheric CO_2 by 1.3 ‰ due to Suess effect (McCarroll and Loader, 2004), with preferential decomposition of different organic compounds and microbial fractionation during litter

decomposition or mixing of new C input with old soil OC (Buchmann et al., 1997; Ehleringer et al., 2000, Šantrůčková et al., 2000). Another hypothesis that has been discussed but never supported experimentally states that soil microbes should be isotopically heavier as a result of carboxylation reactions (Ehleringer et al., 2000). Whenever carboxylation reactions are involved, CO_2 molecules used in the reactions likely originate from the soil atmosphere, which is isotopically heavier than the organic materials being decomposed (Cerling et al., 1991). The $^{13}CO_2$ enrichment of bulk soil atmosphere is highest in the uppermost soil horizons, where CO_2 originates mostly from the atmospheric air. In deeper horizons of the soil profile, CO_2 originates from organic matter decomposition and carries the isotopic signal of decomposed material. But still CO_2 remaining in the soil that surrounds microbes is 4.4 ‰ heavier than organic matter at the location due to slower diffusion of heavier $^{13}CO_2$ than lighter $^{12}CO_2$ (Cerling et al., 1991). CO_2 hydrogenation causes further enrichment of ^{13}C in HCO_3^- by 8 to 12 ‰, depending on temperature (Mook et al., 1974). HCO_3^- is accepted by many carboxylases operating in a variety of carboxylation reactions, including PEP and biotin carboxylases (Berg et al., 2010; Supplement Appendix B Table SB1), while CO_2 is used as an active species by Rubisco, the most abundant autotrophic carboxylase. Accordingly, incorporation of IC through microbial processes and accumulation of microbial products in soil theoretically might increase the isotopic signal ($\delta^{13}C$) of OC.

In arctic permafrost soils, high soil moisture, the presence of a permafrost layer and accumulation of fine particles on the interface between active and permafrost layers (Bockheim and Tarnocai, 1998; Makeev and Kerzhentsev, 1974) restrict air diffusion through the soil profile. Arctic permafrost soils are also a large reservoir of OC whose bioavailability is limited, among other factors, by the OC subduction into subsoil via cryoturbation and the subsequent formation of mineral-organic associations (Gentsch et al., 2015). High moisture content and the presence of a permafrost horizon restrict air diffusion through the soil profile, which may favor pockets and microsites with elevated CO_2 concentration. Under such conditions, CO_2 fixation might play a more important role than in well-aerated temperate soils. In addition, net primary production and soil carbon input are known to be low in northern ecosystems.

The aim of this study was to elucidate the role of dark CO_2 fixation in arctic soils. We postulated that (i) dark CO_2 fixation is a common attribute of arctic

soils and occurs across the whole soil profile. We further hypothesized that (ii) various pathways of CO_2 fixation are operative in soil and distributed among different members of the soil microbial community, including heterotrophs, and (iii) CO_2 incorporation increases ^{13}C enrichment of organic carbon with soil age. To test the hypotheses, we measured isotopic signal $\delta^{13}C$ in OC, IC incorporation into OC, and abundances and taxonomic affiliations of carboxylase genes by shotgun metagenomics in soils across a range of tundra ecosystems from Eastern Siberia to Greenland, covering entire soil profiles. A simple model based on measured data was employed to elucidate a possible effect of IC incorporation on $\delta^{13}C$ of OC. In addition, ^{13}C-labelling experiments with soil from one location were performed under aerobic and anaerobic conditions and the incorporation $^{13}CO_2$ into OC was addressed by analyzing the ^{13}C incorporation into phospholipid fatty acids (PLFA) as microbial biomarkers. To gain supporting evidence of heterotrophic CO_2 fixation, CO_2 incorporation into OC, abundance of carboxylase genes and changes in microbial community composition after augmentation of bioavailable C were measured as well.

2 Material and methods

2.1 Soil sampling

We sampled soils from four different arctic tundra types (heath tundra, tussock tundra, shrub-moss tundra and graminoid tundra) that belong to the bioclimatic subzones E and D (Walker et al., 2005), also called southern tundra and typical tundra subzone in the Russian classification: (i) The heath tundra site was located in eastern Greenland close to the Zackenberg Research Station (ZK; 74° 29’ N, 20° 32’ W). (ii) The tussock tundra site was located approximately 80 km north of Cherskii (CH; 69° 26’ N, 161° 44’ E). iii) The shrubby moss tundra site was on the Taymyr peninsula in the north of central Siberia (Ari Mas, AM; 72° 30’ N, 101° 39’ E). (iiii) The graminoid (moss) tundra was also on the Taymyr peninsula, a little bit north of AM (Logata, LG; 73° 25’ N, 98° 16’ E). All areas are in the continuous permafrost zone and thaw depth during sampling reached 65-90 cm (samples were collected in late summer, close to the time of maximum active layer depth). All soils were classified as Turbic Cryosols according to World Reference Base (IUSS Working Group WRB

2007) and as Turbels according to Soil Survey Staff (2010). Two types of soil samples were used in this study, one for the general screening of dark CO_2 fixation and a second one for more detailed microbial and molecular biological analyses.

(i) Soil samples for measuring natural abundance of bulk soil ^{13}C and dark CO_2 fixation (see section 2.2) were obtained on each site from extensive soil sampling for assessment of C storage and distribution (Palmtag et al., 2015). Briefly, soil pits were excavated down to the permafrost and the active layer was sampled using a fixed volume cylinder. Samples from permafrost were collected by coring with a steel pipe (5 cm in diameter) that was hammered into the soil at 5-10 cm depth increments. Samples representative of the uppermost organic, cryoturbated organic (pockets of cryoturbated topsoil material), and adjacent active mineral layers and permafrost horizons were quickly dried in thin layers and kept at 4 °C until analyzed (in total, 149 samples from all sites). For detailed soil characteristics see Palmtag et al. (2015).

(ii) Soil samples for more detailed microbial and molecular analyses were taken from 5-m long active layer pits on Cherskii, Ari Mas and Logata sites. Soil samples were taken from uppermost organic, mineral and cryoturbated horizons, as well as from the uppermost permafrost layer. One part of the samples was immediately stabilized with RNAlater and kept cold. After transporting the samples to the laboratory within 20 days, RNAlater was washed out with PBS buffer (Gittel et al., 2014) and samples were deep-frozen and later used for DNA extraction and subsequent metagenomics. From the remaining material, living roots were carefully removed and the soil was kept at 4 °C until analyzed for ^{13}C. Soils from the AM site were also used for the microbial ^{13}C incorporation and C supplementation experiments (see sections 2.3 and 2.4). For basic soil properties we kindly refer to Table 1, while details are given in Gentsch et al. (2015).

2.2 Screening of CO_2–C incorporation into OC across sampling sites

Soil (0.2 g) was moistened to 80 % water holding capacity (WHC) in 10 ml vacutainers, which were covered by Parafilm and conditioned for 2 weeks at 12 °C. The vacutainers were then hermetically closed and flushed with CO_2-free air. Thereafter, the headspace was enriched with $^{13}CO_2$ (99 at% [atomic %] of

^{13}C) to a final CO_2 concentration of about 1 % v/v, which is realistic for soil pores. In fact, soil CO_2 concentration can fluctuate widely, and values of 1-5 % v/v CO_2 are typical, although 10 % v/v and higher have also been recorded (Nobel and Palta, 1989). The soil was incubated at 12 °C for 5 days in the dark under the same conditions as in the conditioning phase. At the end of the incubation period, the CO_2 concentration in the headspace was analyzed, the soil was immediately dried at 60 °C and analyzed for total C and N and $\delta^{13}C$. All analyses were run in four replicates.

2.3 CO_2–C incorporation into microbial biomass

Soil taken from pits in the uppermost organic, mineral and cryoturbated horizons of the AM site was used in the $^{13}CO_2$ incorporation experiment. Soil moisture was adjusted to 80 % WHC for incubation under aerobic conditions and to 100 % WHC for incubation under anaerobic conditions. Before incubation with ^{13}C-labelled CO_2, the soil was conditioned either for 2 weeks (aerobic incubation, four replicates for each horizon, 5 g soil) or 4 weeks (anaerobic conditions, four replicates for each horizon, 5 g soil) in hermetically closed 100 mL bottles at 12 °C in the dark to allow microbial communities to stabilize. After soil conditioning, half of the samples were used for initial soil analyses (controls used for determination of natural abundance of ^{13}C in microbial biomass, extractable C and N pools, PLFA and bulk soil). The remaining bottles were flushed with CO_2-free air, and the headspace of each was enriched with $^{13}CO_2$ (99 at% of ^{13}C) to a final CO_2 concentration of about 1 % v/v. The soil was incubated for 5 days under the same conditions as used for the conditioning. At the end of the incubation period, respiration was measured and the soil was used for further analyses; one part of the soil sample was immediately dried at 60 °C and used for chemical and isotopic analyses and the other part was deep-frozen and used for PLFA determination.

2.4 Effect of organic C supplement on CO_2 incorporation and carboxylase genes

As in the previous experiment, soil taken from pits in the mineral and cryoturbated horizons of the AM site was used. The soil was incubated only in aerobic conditions and conditioned in the same way as in the previous experiment. After conditioning, soils were amended with either sucrose or

lipids extracted from soil (see below) as energy and C sources as follows: sucrose and lipids, respectively, were mixed with C-free silica sand and the mixture was then mixed with soil (sand/soil 1:2, w/w) to get a final concentration of the added C source of approximately 300 µg C per g dry soil. Control soil was mixed with sand only. The final soil mixture was moistened to 80 % WHC with Veldkamp nutrient solution containing biotin (Veldkamp, 1970). An aliquot of the soil mixture (of all treatments) was dried to determine the natural abundance of ^{13}C before the incubation. The incubation with $^{13}CO_2$ was carried out in four replicates for each treatment as described above. After 5 days of incubation, soil respiration was measured, 1 g of soil mixture from each replicate was immediately dried (60 °C) for bulk C and ^{13}C analyses and the remaining soil was deep-frozen (-80 °C) for DNA extraction and subsequent metagenomics. The lipid mixture used for the soil C supplement had been extracted from soil slurry (equivalent of 450 g of dry soil supplemented by Veldkamp nutrient solution; Veldkamp, 1970) incubated on a shaker for 5 days. The slurry was subsequently centrifuged to remove excess of water and lipids were extracted according to Bligh and Dyer (1959). A part of the resulting extract was fractionated using SPE (Strata SI-1000mg/6mL, Phenomenex, Torrance, CA, USA) to characterize the extracted lipids. We found that 25, 22 and 23 % belonged to neutral-, glyco- and phospholipid fractions, respectively, and 30 % was not held by the SPE sorbent and considered as non-lipid fraction.

2.5 Analytical methods

Microbial biomass was estimated by chloroform-fumigation and extraction with 0.5 M K_2SO_4, and calculated as the difference in soluble C between the extracts from fumigated and non-fumigated soils, using $K_{EC} = 0.38$ (Vance et al., 1987). Extractable organic C was analyzed on a LiquicTOC II (Elementar, Germany). Total CO_2 concentration in the headspace was measured with an HP 5890 gas chromatograph (Hewlett-Packard, East Norwalk, CT, USA), equipped with a thermal conductivity detector, at the beginning and end of the experiment (after the addition of $^{13}CO_2$ and after the incubation, respectively). The total amount of CO_2 in the bottles ($totCO_2$, µmol) was calculated as the sum of the amount of CO_2 in the headspace and the amount of CO_2 dissolved in the soil solution (Sparling and West, 1990). Net respiration rate was estimated as

the difference between totCO_2 at the beginning and the end of the experiment divided by the number of days of incubation. Analyses of total C and N and ^{13}C contents of dried soil material were conducted with an NC Elemental analyzer (ThermoQuest, Bremen, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Bremen, Germany). Prior to carrying out the analyses, all samples were tested for their carbonate content. No carbonates were detected (data not shown).

A binary mixing model was used to estimate the amount of the pulse-derived ^{13}C immobilized in the various C pools (bulk soil, PLFA):

$$^{13}C\ (\mu g\ g^{-1}) = [(at\%_{sample} - at\%_{control}) / (99.90 - 1.10)] \times C\ pool\ size\ (\mu g\ C\ g^{-1})$$

where $at\%_{control}$ is the natural abundance in the control samples, $at\%_{sample}$ is the ^{13}C abundance in the samples after labeling, 99.9 is the pulse ^{13}C at% and 1.10 is the at% of the ambient atmosphere. All results were normalized to total C content in order to eliminate differences in C contents of the soils.

PLFA were extracted from subsamples of 0.3 to 2 g dry soil containing comparable amounts of OC according to Frostegård et al. (1993), with minor modifications. Purification of phospholipids was conducted on silica columns (SPE-SI Supelclean 250mg/3 mL; Supelco, PA, USA) using chloroform, acetone and methanol. Following trans-esterification (Bossio and Scow, 1998), the concentration and isotopic composition of individual PLFAs was determined on a GC-IRMS system consisting of a Trace GC coupled to a Delta V Advantage IRMS via a GC Isolink interface (Thermo Fisher Scientific, Waltham, MA, USA); see Wild et al. (2014) for a detailed description of the instrument setup. Concentration and isotopic composition of each PLFA were corrected for C added during methylation. The microbial community composition was described using PLFAs i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 as markers of Gram-positive bacteria, 16:1ω9, 16:1ω7, 16:1ω5, cy17:0, 18:1ω7, cy19:0 as markers of Gram-negative bacteria and 18:1ω9, 18:2ω6,9 as markers of fungi (Frostegård and Bååth, 1996). Total bacterial biomass was calculated as the sum of general bacterial markers 15:0, 17:0, 18:1ω5 and markers for Gram-positive and negative bacteria. The PLFAs 14:0, 16:0, 16:1ω11, 18:0, 19:1ω8, and 20:0 were considered nonspecific markers (Kaiser et al., 2010).

DNA was extracted from samples of cryoturbated (4 samples), mineral (2) and top soil (4) from Logata, AriMas and Cherskiy sites using bead-beating and

the phenol-chloroform method (Gittel et al., 2014; Urich et al., 2008). Total DNA was quantified using SybrGreen (Leininger et al., 2006). In the case of the incubation experiment with added substrates (sucrose or lipids), only the DNA from cryoturbated horizons (9 samples) contained a reasonable amount of DNA of high quality (Table 4). Sequencing of DNA from in-situ and incubation experiments was performed on an IonTorrent PGM (Thermo Fisher Scientific, Waltham, MA, USA) sequencer at the Department of Archaea Biology and Ecogenomics (University of Vienna). Barcoded, adapter-ligated DNA libraries were generated and sequenced using 200 bp sequencing chemistry and 318 chips according to the manufacturer's instructions. Sequence reads were quality-trimmed (Phred score >20) and size-selected (>100 bp) before further processing. For the identification of carboxylase genes, all metagenome reads were translated into all six frames, with each frame into separate open reading frames (ORFs), avoiding any '*' characters marking stop codons in a resulting ORF. All ORFs equal to 30 amino acids or larger were screened for assignable conserved protein domains using reference hidden Markov models (HMMs) of the PfamA database (Punta et al. 2012; PfamA release 25, http://Pfam.janelia.org) with HMMER tools (http://hmmer.janelia.org/). All database hits with *e*-values below a threshold of 10^{-4} were considered significant (Tveit et al., 2015). To obtain taxonomic information of reads with Pfam code, a BLASTX search implemented in diamond software (Buchfink et al., 2015) was performed (-minscore 50, -maxhits 25) and the resulting hits in sam format were analyzed by MEGAN 5.11.3 (Huson et al. 2007) using the last common ancestor (LCA) algorithm (LCA parameters: MinScore 50, MaxExpect = 0.01, TopPercent = 10, MinSupport = 1).

The calculations of carboxylase gene abundances per g soil were done by combining relative abundance of SSU rRNA genes of bacteria in the metagenomes with the number of bacterial SSU rRNA genes per g soil as determined by qPCR in the same DNA sample (Table SB2). The absolute number of carboxylase genes per g of soil was used for normalizing their abundance to soil C_{mic} and/or OC contents. The molecular data were processed as follows: metagenome sequences encoding fragments of SSU rRNA genes were extracted with the program SortMeRNA (Kopylova et al., 2012), applying default parameters and the reference databases therein. Extracted reads were compared with BLASTN (Astchul et al., 1990) against the ARB Silva SSUref database v. 119 (Quast et al., 2012) and analyzed in MEGAN 5.11.3.

Carboxylase reads were recalculated to absolute number of gene copies per g of soil by combining the absolute quantity of bacterial SSU rRNA genes and the amount of bacterial SSU rRNA reads determined by the MEGAN LCA algorithm in each metagenome, using the following formula:

$$q(carboxylase) = \frac{seqs(carboxylase)}{seqs(bacterial\ SSU\ rRNA\ gene)} \cdot q(bacterial\ SSU\ gene)$$

where *seqs(carboxylase)* is the amount of metagenome carboxylase gene sequences (assigned by hmmer algorithm using Pfam database), *seqs(bacterial SSU rRNA gene)* is the amount of bacterial SSU rRNA gene sequences and *q(bacterial SSU rRNA gene)* is the quantity of bacterial SSU rRNA genes (gene copies per g soil) determined by qPCR.

2.6 Statistics and modeling

A general linear model, followed by Newman-Keuls post-hoc testing, was used to determine the differences in C incorporation, respiration rate, bulk C content and isotopic signal between sites and horizons at a significance level of $P \leq 0.05$. To compare total PLFA contents between control and $^{13}CO_2$-incubated samples in the laboratory experiment, one-way ANOVA and Newman-Keuls post-hoc test was used. Data were log-transformed in all cases except for total PLFA. Statistical evaluation of data was carried out with STATISTICA 13.

The statistical analysis of carboxylase genes was done using the statistical program R (Team 2016). Because data were not normally distributed, generalized linear models with gamma distribution were used to test the significance of the effects of lipids or sucrose addition and soil horizon, respectively, on the abundance of carboxylase genes. Soil horizons had unequal numbers of replicated measurements of carboxylase gene abundance. Therefore, we calculated the type-II F statistic using the package car (Fox and Weisberg, 2011). Post-hoc multiple comparisons were carried out based on least-square means using the package lsmeans (Lenth, 2016).

In order to estimate tentatively the effect of CO_2 fixation on the isotopic signal of OC ($\delta^{13}C_{tot}$), we applied a simple model of microbial OC decomposition (Fig. 1; for details see Supplementary Information Appendix A). Briefly,

decomposition of OC by heterotrophic soil microorganisms (Mic) is a process with first-order kinetics. Organic C from decomposing organic matter with an initial isotopic signal of -27 ‰ is consumed by soil microorganisms and respired or assimilated into microbial biomass. Microbial biomass is dying over time and becomes part of OC. For the sake of simplicity, we assume that no isotopic discrimination occurs during decomposition and microbial dying. Heterotrophic microorganisms largely depend on organic matter as C and energy source but under certain circumstances they use IC as additional C source (see introduction of this article for more details). We assume that microbes are capable of assimilating part of the respired CO_2 back and incorporate it into microbial biomass. Before CO_2 is fixed by soil microorganisms, an isotopic discrimination of 4 ‰ occurs because of the faster diffusion of $^{12}CO_2$ out of the soil. By fixing heavier CO_2, microbial biomass is becoming more enriched in ^{13}C. When this microbial biomass is dying and becomes part of OC, OC becomes enriched in ^{13}C as well. In the model, CO_2 fixation is set to be proportional to respiration. Four scenarios were modelled, with CO_2 fixation making up 0, 0.1, 1 and 5 % of respiration, respectively.

3 Results

3.1 Screening for CO_2 fixation in arctic soils

Sampling site and soil horizon significantly affected soil OC content and respiration rate (Table 2) while natural abundance of ^{13}C in the soil (bulk $\delta^{13}C$) and CO_2 incorporation were affected only by the type of soil horizon. Consequently, data from the particular horizons were averaged across sampling sites (Table 2). Across all sites, average bulk $\delta^{13}C$ in the upper organic horizon was -27.3 ‰ at the start of incubation. In comparison with the upper organic horizon, the mineral, cryoturbated and permafrost horizons were all ^{13}C-enriched by 1.2, 1.1, and 2.7 ‰, respectively (Table 2). Incorporation of $^{13}CO_2$-derived C per unit total OC was lowest in the upper organic horizon (4.9 µmol ^{13}C mol $C_{tot}^{-1}d^{-1}$) and increased two- to three-fold in cryoturbated and mineral horizons (Table 2). In the permafrost horizon, ^{13}C incorporation was higher by almost two orders of magnitude (Table 2). ^{13}C incorporation reached 0.4, 1.0, 1.1 and 16 % of net respiration in the organic, cryoturbated organic,

mineral and permafrost horizons, respectively. When ^{13}C incorporation was expressed per mol C of soil OM, it also increased with soil depth, and it rose exponentially with the $\delta^{13}C$ value of OC ((R^2 = 0.44, n = 149, P <0.001; C_{incorp} = e^(21.09+0.72 $\delta^{13}C_{OC}$)).

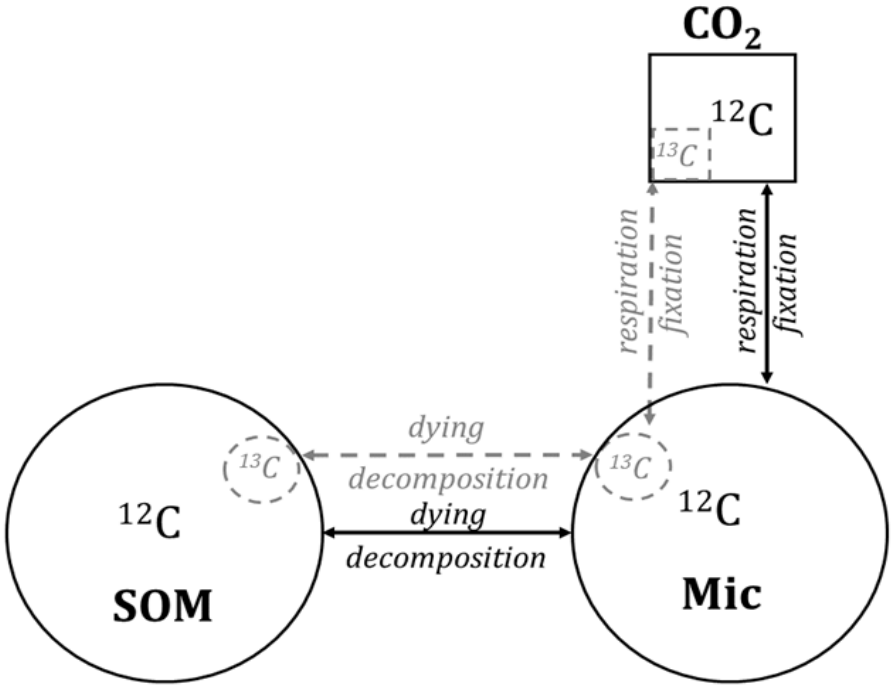

Fig. 1. Scheme of the soil organic matter (SOM) decomposition model that was used to estimate the effect of long-term CO_2 incorporation into SOM on its isotopic signal (for details of the model see Supplementary information, Appendix A).

Table 1 Basic chemical (pH; total soil organic carbon – OC; total soil C/N ratio; extractable C – CEX; natural abundance of soil ^{13}C – bulk $\delta^{13}C$) and biochemical characteristics (microbial biomass – CMB; microbial C/N - CMB/NMB, net respiration; total microbial phospholipids – PLFAtot) of three different soil layers from Ari Mas site. Mean values and standard deviations (in brackets) are given (n = 4); different letters in superscript denote significant differences between layers (ANOVA).

soil layer	pH_{H2O}	C_{tot} mmol g^{-1}	C/N	C_{EX} µmol g^{-1}	C_{MB} µmol g^{-1}	C_{MB}/N_{MB}	bulk $d^{13}C$ ‰	net respiration mmol C-CO_2 mol C^{-1} d^{-1}	$PLFA_{tot}$ mmol C mol C_{tot}^{-1}	$PLFA_{tot}$ mol C mol C_{MB}^{-1}
upper organic	6.2	13.4(1.1)^{a}	20.4(0.21)^{b}	28.2(2.4)^{a}	174.1(3.2)^{a}	18.7(0.47)^{a}	27.5(0.10)^{b}	0.796(0.08)^{a}	0.339 (0.05)^{a}	0.022^{c}
subducted	6.3	3.8(0.23)^{b}	26.2(0.32)^{a}	3.8(0.76)^{b}	10.6(0.05)^{b}	16.4(1.9)^{a}	27.5(0.17)^{b}	0.135 (0.10)^{b}	0.284(0.021)^{a}	0.085^{b}
mineral	6.7	0.8(0.14)^{c}	18.7(0.67)^{b}	1.22(0.73)^{c}	2.2(0.36)^{c}	11.7(4.3)^{b}	26.1(0.51)^{a}	0.082(0.02)^{c}	0.352(0.013)^{a}	0.106^{a}

Table 3 Bulk inorganic 13C incorporation into soil organic carbon (OC) and PLFA in three different soil layers from Ari Mas site incubated under aerobic conditions. Mean values and standard deviations (in brackets) are given (n = 3). Different letters in superscript denote significant differences between layers (Newman-Keuls post-hoc test).

soil layer	bulk ^{13}C incorporation		^{13}C incorpotration to PLFA	PLFA ^{13}C to bulk ^{13}C	PLFA ^{13}C to PLFA C	^{13}C fungi to ^{13}C bacteria	fungi to bacteria	relative contribution of specific PLFAs to total ^{13}C incorporated into PLFA [%]				
	µmol ^{13}C mol OC^{-1} d^{-1}	% respiration rate	nmol ^{13}C mol OC^{-1} d^{-1}	%	%			G-	G+	fungi	bacteria	nonspecific
upper organic	6.13(0.62)^{b}	0.78(0.05)^{c}	19.8(2.65)^{a}	0.377(0.04)^{a}	0.041(0.004)^{a}	0.21(0.02)^{b}	0.44(0.02)^{a}	53	12	14	68	18
subducted	2.40(0.21)^{b}	1.88(0.25)^{b}	11.2(0.75)^{b}	0.536(0.11)^{a}	0.028(0.002)^{b}	0.22(0.004)^{b}	0.23(0.01)^{c}	57	9	16	67	17
mineral	7.61(0.79)^{a}	13.57(3.23)^{a}	7.26(0.20)^{c}	0.153(0.02)^{b}	0.015(0.001)^{c}	0.45(0.06)^{a}	0.37(0.02)^{b}	39	11	24	54	22

Table 2 Soil organic carbon (OC), natural abundance of 13C, soil respiration, CO2 incorporation into OC pool and residence time of OC of four different soil layers from four different arctic tundra sites (upper part of the table). Dark CO2 immobilization was measured in a laboratory experiment in which soil was incubated for 5 days in an atmosphere enriched with 13CO2 to a final concentration of about 1 % v/v (see Methods for details). Mean values from Cherskii, Ari Mas, Logata and Zackenberg are given. The effects of locality, soil layer, and their interaction were calculated by factorial ANOVA (lower part of the table). Different letters in superscript denote significant differences between soil layers (Newman-Keuls post-hoc test).

locality	soil layer	number of samples	OC	^{13}C of bulk soil	net soil respiration	^{13}C -incorporation	
			mmol g^{-1}	‰	mmol CO_2 mol $C_{tot}^{-1}d^{-1}$	µmol ^{13}C mol$C_{tot}^{-1}d^{-1}$	% of respiration
Cherskii	upper organic	16	21 ± 9.2	-27.3 ± 0.85	1.3 ± 0.9	7.1 ± 5.1	0.6 ± 0.5
	Mineral	17	1.9 ± 1.7	-26.1 ± 0.85	2.1 ± 1.5	11.5 ± 6.9	0.9 ± 0.8
	cryoturbated	13	4.5 ± 2.5	-25.8 ± 0.36	1.0 ± 0.8	5.2 ± 4.0	0.6 ± 0.2
	Permafrost	17	1.5 ± 0.9	-24.6 ± 2.58	2.0 ± 1.4	310 ± 496	13.4 ± 20.6
Ary Mass	upper organic	4	17.8 ± 5.7	-27.8 ± 0,32	2.8 ± 1.7	3.8 ± 1.3	0.2 ± 0.2
	Mineral	11	0.9 ± 0.4	-25.8 ± 1.02	3.8 ± 1.7	17.2 ± 15.4	1.0 ± 1.3
	cryoturbated	4	1.0 ± 0.2	-26.3 ± 0.16	2.2 ± 0.6	8.7 ± 0.6	0.4 ± 0.1
	Permafrost	15	1.4 ± 1.6	-24.0 ± 1.67	0.9 ± 0.7	81.5 ± 42.3	14.4 ± 13.3
Logata	upper organic	3	18.6 ± 7.4	-27.3 ± 0.65	3.7 ± 1.1	5.4 ± 4.4	0.1 ± 0.1
	Mineral	7	2.5 ± 1.4	-27.4 ± 0.18	3.9 ± 1.0	7.1 ± 2.4	0.2 ± 0.1
	cryoturbated	6	3.1 ± 2.3	-27.1 ± 0.71	2.9 ± 1.2	21.3 ± 27.2	2.1 ± 4.0
	Permafrost	9	2.0 ± 0.3	-25.5 ± 1.50	1.3 ± 1.0	102 ± 83.9	20.1 ± 18.9
Zackenberg	upper organic	8	16.4 ± 5.8	-27.3 ± 0.75	1.9 ± 1.1	0.8 ± 0.6	0.1 ± 0.1
	Mineral	8	2.0 ± 1.0	-25.6 ± 1.04	1.2 ± 0.4	15.8 ± 27.2	2.0 ± 4.0
	cryoturbated	9	6.4 ± 1.6	-25.2 ± 0.66	0.9 ± 0.5	8.9 ± 17.5	1.3 ± 2.8
	Permafrost	2	0.9 ± 0.1	-26.0 ± 1.13	0.9 ± 0.6	74.0 ± 69.7	32.1 ± 31.8
all sites	upper organic	31	19.0 ± 8.18^{a}	-27.3 ± 0.78^{c}	1.88 ± 1.36^{ab}	4.86 ± 4.86^{c}	0.37 ± 0.44^{c}
	Mineral	43	1.77 ± 1.44^{c}	-26.1 ± 1.06^{b}	2.62 ± 1.94^{a}	13.1 ± 15.4^{b}	1.00 ± 2.14^{b}
	cryoturbated	32	4.33 ± 2.72^{b}	-26.2 ± 0.73^{b}	1.47 ± 1.20^{b}	9.71 ± 16.6^{c}	1.08 ± 2.38^{b}
	Permafrost	43	1.55 ± 1.18^{c}	-24.7 ± 2.13^{a}	1.46 ± 1.34^{b}	175.7 ± 338^{a}	16.1 ± 19.5^{a}
	effect of locality		****	n.s.	****	n.s.	n.s.
	effect of layer		****	****	****	****	****
	layer*locality		****	n.s.	****	n.s.	n.s.

Carboxylase genes employed in autotrophic as well as heterotrophic metabolism were detected in metagenomes of all horizons and were not affected by site. However, the variability in absolute carboxylase gene counts between Siberian sites was large (Fig. 2a). Their abundances were higher in mineral horizons than in organic and cryoturbated horizons when normalized to microbial biomass ($F = 4.2$, $df = 2$, $P = 0.02$; Fig. 2a). However, deeper active layer horizons (mineral and cryoturbated) had lower abundances than organic horizons when carboxylase genes were normalized to total C ($F = 4.1$, $df = 2$, $P = 0.02$; Fig. SB1a). Genes encoding Rubisco contributed about 5 % (4.3-5.3 %)

to the total abundance of carboxylase genes, whilst anaplerotic carboxylase genes (pyruvate and PEP carboxylase genes) were the most abundant carboxylase genes and contributed more than 30 % (31-42 %).

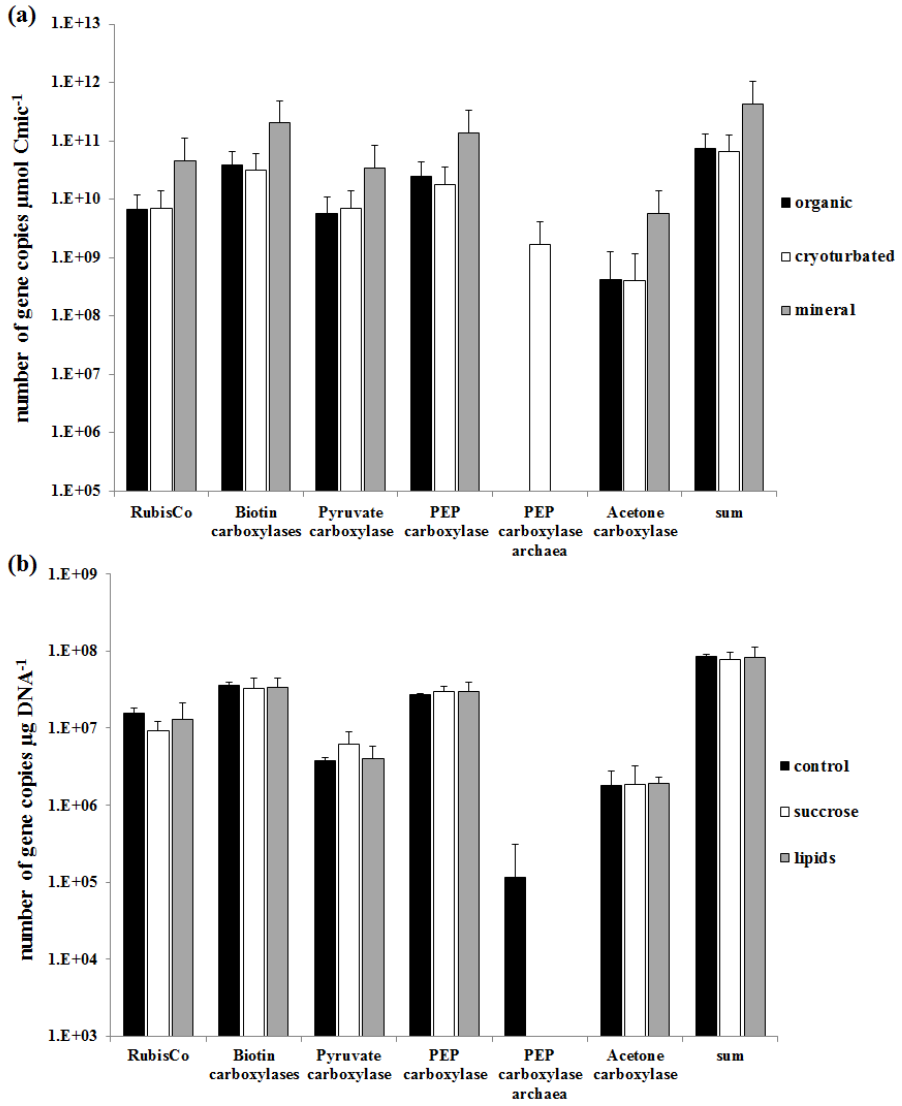

Fig. 2. Abundance of carboxylase genes normalised to microbial biomass in uppermost organic, cryoturbated and mineral soil layers from Cherskii, Ari Mas and Logata sites **(a)**, and in the cryoturbated layer from Ari Mas site, either unamended (control) or amended with organic C sources **(b)**. Note that the scale on *y* axis is logarithmic. The standard deviation of absolute carboxylase gene counts was in some cases larger than average, only positive error bars are thus visualized.

The ^{13}C data of OC within the soil profile corresponds well with the model predictions (Fig. 4b). The estimates indicate that dark fixation of C released from decomposed OC could, after 1000 years, cause an increase of the isotopic signal of OC from an initial -27 ‰ to -26.7 or -15.2 ‰ when IC incorporation represents 0.1 or 5 % of net respiration, respectively (Fig. SA3). C sequestration via dark CO_2 fixation of 0.1, 1 and 5 % of net respiration, respectively, would add 0.1, 1.4 and 7.4 mg C per g soil, respectively, over a time frame of 250 years (Fig. SA6).

3.2 C incorporation into microbial biomass at the AM site

Under anaerobic conditions, CO_2 fixation was below the detection limit in the cryoturbated and mineral horizons and negligible in the upper organic horizon (0.251 µmol ^{13}C mol C_{tot}^{-1} day^{-1}, corresponding to 0.1 % of net respiration). The anaerobic incubation is thus not discussed any further. During aerobic incubation neither microbial functioning (respiration rate) nor microbial biomass (total PLFA) ($P > 0.3$ for all horizons, $df = 1$, $n = 3$) or PLFA profiles were changed (Table SB3).

Bulk ^{13}C incorporation ranged from 2.4 µmol ^{13}C mol OC^{-1} day^{-1} in cryoturbated horizons to 7.6 µmol ^{13}C mol OC^{-1} day^{-1} in the mineral horizons (Table 3), which corresponds well with ^{13}C incorporation observed during the screening across all study sites. It represented less than 2 % of net respiration in the organic and cryoturbated horizons but exceeded 13 % in the mineral horizons. Of the total amount of ^{13}C incorporated into the soil, generally less than 1 % of bulk ^{13}C was found in PLFA biomarkers (^{13}C PLFA to bulk ^{13}C) and less than 0.05 % of total PLFA-C was newly incorporated ^{13}C (^{13}C PLFA to PLFA C; Table 3). All detected PLFAs were enriched in ^{13}C , but the distribution of ^{13}C among PLFA markers of individual functional groups showed that more ^{13}C was incorporated into bacterial than fungal PLFA. The ratio of ^{13}C in fungi to ^{13}C in bacteria increased from 0.20 in organic and cryoturbated horizons to 0.45 in the mineral horizons. The significantly higher fungal to bacterial PLFA ratio in the organic layers and lower ratio in mineral layers compared to the ^{13}C in fungal to ^{13}C in bacterial PLFA (Table 3) indicate, that bacteria were more active in ^{13}C incorporation per unit of biomass compared to fungi in organic layers, but less active in mineral layers. Within bacteria, gram-negative

bacteria incorporated three to four times more ^{13}C into PLFA than gram-positive bacteria (Table 3). The proportion of bulk ^{13}C built into PLFA was higher in the organic and cryoturbated horizons than in the mineral horizons. Across all horizons, ^{13}C incorporated into PLFA was closely correlated with net respiration rate RR ($R^2 = 0.89$, n = 9; $^{13}C_{(PLFA/OC)} = 7.36 + 20.5\ RR_{(CO2/OC)}$).

3.3 Effect of C addition on dark CO_2 fixation

Addition of sucrose significantly increased net respiration rate in the mineral and cryoturbated horizons while addition of lipids had a positive effect only in the mineral soil. ^{13}C immobilization ranged from 3.1 µmol ^{13}C mol OC^{-1} day^{-1} (cryoturbated horizon; control) to 46.8 µmol ^{13}C mol OC^{-1} day^{-1} (mineral horizon; sucrose addition) and increased in both the cryoturbated and mineral horizons in the order control < lipids < sucrose (Table 4).

Table 4 DNA concentration, net respiration and inorganic carbon incorporation into soil organic carbon (OC) of cryoturbated and mineral soil layers from Ari Mas site incubated under aerobic conditions without carbon source addition (control) and with sucrose or mixture of lipids as added carbon source. Mean values and standard deviations are given (n = 3). Different letters in superscript denote significant differences between layers (ANOVA).

soil layer	treatment	DNA concentration	net respiration	^{13}C immobilization	
		ng DNA mol OC^{-1}	mmol C mol OC^{-1} d^{-1}	µmol ^{13}C mol OC^{-1} d^{-1}	% net respiration
cryoturbated	control	8.61(0.13)	$0.168(0.074)^{b}$	$3.06(0.59)^{c}$	$2.08(1.59)^{c}$
	sucrose	32.8(15.7)	$0.541(0.141)^{a}$	$21.7(6.26)^{b}$	$4.32(2.16)^{b}$
	lipids	22.8(6.20)	$0.106(0.032)^{b}$	$13.0(2.89)^{b}$	$13.2(4.99)^{a}$
mineral	control	nd	$0.144(0.027)^{c}$	$7.37(1.02)^{c}$	$5.16(0.79)^{ab}$
	sucrose	nd	$1.254(0.240)^{a}$	$46.8(4.34)^{a}$	$3.82(0.74)^{b}$
	lipids	nd	$0.333(0.120)^{b}$	$18.3(2.70)^{b}$	$5.81(1.85)^{a}$

nd - not detected

Across all treatments, IC incorporation was closely correlated with respiration rate ($R^2 = 0.91$, n = 18; $^{13}C_{(incorp/OC)} = 4.86 + 31.9\ RR_{(CO2/OC)}$). Both C substrates induced microbial growth (measured as an increase in DNA amount per gram soil; Table 4, Fig. 3a) in the cryoturbated horizon. Unfortunately, the amount of extracted DNA from the mineral soil was too small to be evaluated or used for further analyses. The increase of microbial biomass in the cryoturbated horizon was accompanied by a shift in the composition of the microbial community. Analysis of the SSU rRNA gene fragments in the metagenomes

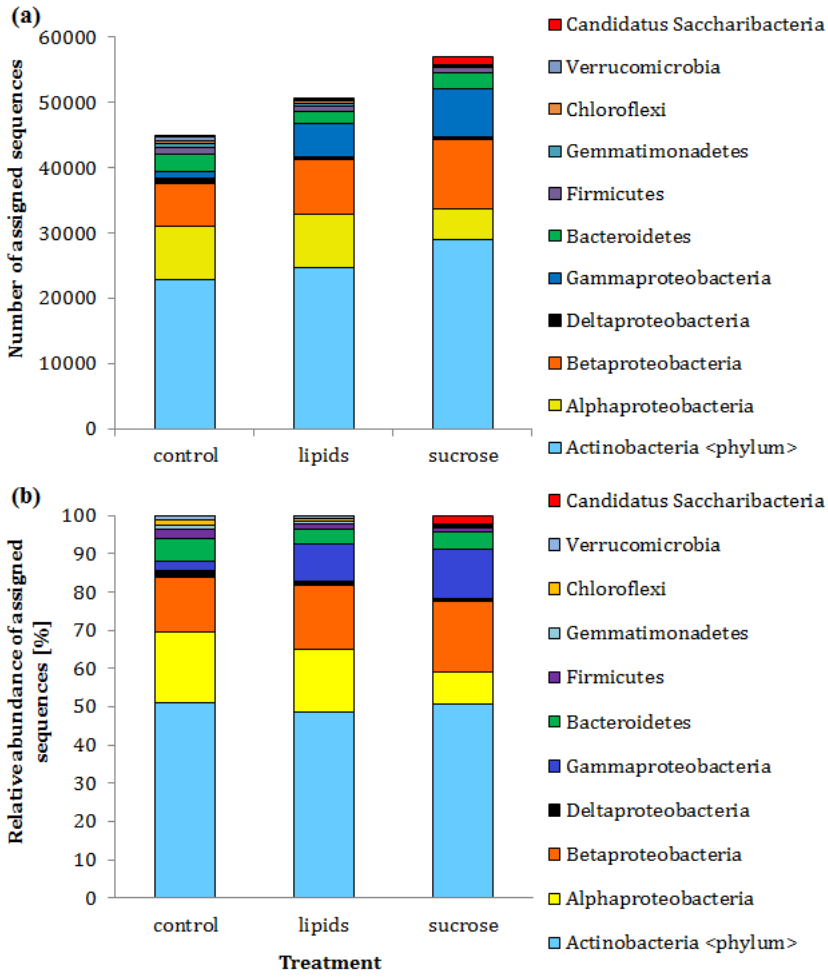

Fig. 3. Composition of the bacterial community (phylum level) based on absolute (a) and relative (b) abundance of assigned sequences in the cryoturbated layer, either unamended (control) or amended with organic C sources from Ari Mas site. Taxonomic assignment is based on LCA classifiers implemented in MEGAN software package.

showed that the relative abundance of *Beta-*, *Gammaproteobacteria* and *Saccharibacteria* increased due to substrate addition, whereas the relative abundance of *Alphaproteobacteria, Deltaproteobacteria and Firmicutes* rather decreased, mainly in the sucrose addition treatment (Fig. 3). The abundance of carboxylase genes normalized to microbial biomass was not affected by C source addition (Fig. 2b) and increased along with microbial biomass growth, thus a significant increase of carboxylase genes per unit of total C was

observed with C source addition (Fig. SB1b). Rubisco accounted for 13 to 18 % of total carboxylase and anaplerotic carboxylase genes (pyruvate and PEP carboxylases) accounted for about 40 % (37-46 %). Taxonomic analysis of carboxylase genes revealed a link between PEP carboxylases and the composition of microbial community (Fig. SB3).

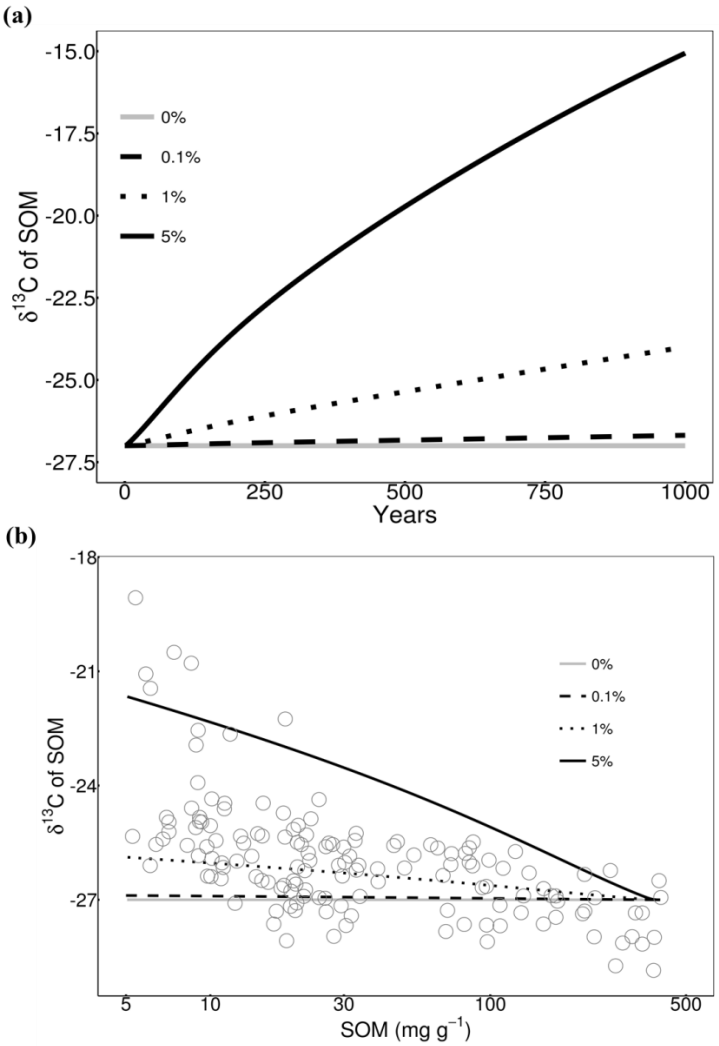

Fig. 4. The modelled shift of SOM isotopic signal over time caused by heterotrophic CO_2 fixation **(a)**, and relationship between the soil SOM concentration and $\delta\ ^{13}C$ ratio **(b)** (please note that x-axis is in logarithmic scale). Open circles represent measured data and lines the model predictions. The solid grey line represents control (0 % - no fixation), the dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of net respiration rate, respectively.

4 Discussion

4.1 General relevance of dark CO_2 fixation in arctic soils

The available knowledge about dark CO_2 fixation is still very limited and can be summarized as follows: It is a relevant process in the soil mediated by soil microorganisms, it is correlated with microbial respiration rate, it can be enhanced by addition of bioavailable OC, and it is related to microbial heterotrophic activity (Miltner et al., 2004; 2005a; Šantrůčková et al., 2005). Previous studies estimated that about 2 to 7 % of fixed CO_2 can be incorporated into microbial biomass (Miltner et al., 2004; Šantrůčková et al., 2005). In addition, this study documents that (i) IC incorporation increases with depth in the soil profile and is higher in the permafrost layers by more than one order of magnitude due to higher CO_2 fixation rate, lower OC and higher CO_2 concentrations with depth; (ii) genes encoding Rubisco, the only enzyme operating strictly in autotrophic metabolism, account for a small part of all carboxylase genes only, while anaplerotic carboxylase genes are several times more abundant; (iii) a wide spectrum of soil microorganisms contain genes encoding PEP carboxylases; (iv) IC incorporation may lead to ^{13}C enrichment of soil OC in the long-term and the increase is more pronounced in the deeper soil horizons. Across all study sites, IC incorporation expressed per unit of total C was higher in the mineral than in the organic horizons. The larger C incorporation in mineral horizons was accompanied by the highest abundance of all detected carboxylase genes per unit microbial biomass, suggesting that both the chemoautotrophic and heterotrophic pathways accounted for the larger IC incorporation in the mineral horizons as compared with the top soil. The significant proportion of anaplerotic carboxylase genes (pyruvate and PEP carboxylases) further emphasizes the importance of reactions replenishing central metabolic pathways under conditions where microbial C demand exceeds C supply or where microorganisms catabolize complex hydrocarbons, the dominant fractions of OC in mineral horizons (Alonso-Saez et al., 2010; Feisthauer et al., 2008; Merlin et al., 2003). It implies an important regulatory function of CO_2 fixation, which may enable microbes to keep activity in harsh conditions of C limitation.

4.2 Link between dark CO_2 fixation and microbial communities

We found less than 1% of fixed C in PLFA (0.2-0.5 %), which is much less than it was found in bulk microbial cells (Miltner et al., 2004; Šantrůčková et al., 2005). The low values can be explained by the fact that membranes constitute only about 4 % of cellular material and, therefore, bulk microbial biomass should contain more fixed C. This suggestion is supported by Feisthauer et al. (2008) who found that the amount of fixed C in bulk microbial biomass is higher by one order of magnitude than that of fatty acids, indicating greater incorporation of fixed C into other cell components. Proteins, the most abundant component of cytoplasm, contain four to eight times more fixed C than PLFA (Miltner et al., 2004).

The variety of detected carboxylase genes, the wide spectrum of bacteria linked to PEP carboxylase genes and significant ^{13}C incorporation into all detected PLFAs including fungal markers imply a general importance of CO_2 incorporation for the microbial community. This matches existing knowledge that, apart from chemoautotrophic bacteria and archaea, which use CO_2 as the only source of C (for review see Berg et al., 2010; Saini et al., 2011) a wide spectrum of heterotrophic bacteria and fungi employ carboxylases to (i) assimilate various organic substrates such as acetone, phenolics, propionate, or leucine, (ii) replenish the citric acid cycle in anaplerotic reactions and, finally, (iii) synthesize cellular compounds (e.g. Erb, 2011; Hesselsoe et al., 2005). The significant increase in the amount of genes involved in CO_2 fixation belonging to predominantly heterotrophic genera (*Arthrobacter*, *Nocardioides* and *Pseudomonas*; Fig. SB2) after addition of sucrose or lipids indicated increased potential of heterotrophic metabolism for additional IC incorporation into the bacterial biomass.

We found a positive effect of bioavailable C addition on IC incorporation, which contradicts our expectation that the importance of heterotrophic carboxylases would increase with any imbalance between C demand and supply (Alonso-Saez et al., 2010; Feisthauer et al., 2008; Merlin et al., 2003). This apparent discrepancy might be partly explained by the increase of chemoautotrophic C fixation because of increasing CO_2 concentration in the system. A more likely explanation is that the added C initiated fast microbial growth as indicated by increased DNA concentration (Table 4), but was exhausted within days, microbes quickly became substrate limited and

microbial C demand exceeded C supply at the end of the experiment when the analyses were performed. Therefore, IC had to replenish C for microbial metabolism.

Even though we detected genes for key enzymes in various autotrophic pathways (e.g. Calvin Benson Bassham cycle, reductive citric acid and reductive acetyl-CoA cycles, 3-hydroxypropionate cycle, 4-hydroxybutyrate cycle), we assume a negligible contribution of autotrophs to overall IC incorporation into OC. These chemoautotrophic pathways are, except for the Calvin Benson Bassham cycle and 4-hydroxybutyrate cycle, operated by strictly anaerobic prokaryotes, and in our study the anaerobic CO_2 incorporation was below the detection limit. We are aware that the contribution of the chemoautotrophic IC incorporation may be higher under natural conditions, but heterotrophy should be prevalent. This is supported by the generally lower growth efficiency of chemolithotrophic compared to heterotrophic growth, because the former requires more energy to produce a unit of biomass. In addition, carboxylases have low substrate affinity and need a high concentration of IC for its effective incorporation into organic compounds (Bar-Even et al., 2012). The most kinetically favorable carboxylases are anaplerotic PEP carboxylases, the main carboxylase for replenishing the citric acid cycle, and pyruvate carboxylases (Bar-Even et al., 2012).

4.3 Importance of dark CO_2 fixation for C sequestration

We assessed the importance of dark CO_2 fixation for C sequestration in the soils from two different perspectives: (i) promotion of microbial metabolism and (ii) role in soil C balance. With respect to the promotion of the heterotrophic metabolism, IC may be used as an additional C source to replenish central metabolic cycles (anaplerotic reactions) in situations when organic carbon is limiting and primarily used for energy production. In such a case, microbes may re-fix CO_2 that has been respired for energy production and thus minimize the overall loss of carbon. Carboxylation is also a vital step of fatty acid biosynthesis in general, and it enables the utilization of acetone by nitrate reducing bacteria (Acosta et al., 2014). Inorganic C has a stimulatory effect, which is most apparent under low metabolic activity in resting aerobic and facultative cells (Harris, 1954) and in the metabolism of resource-depleted

bacteria (Alonso-Saez et al., 2010). Inorganic C also serves as the only C source for a variety of obligate chemoautotrophs, either bacteria or archaea, which provide many metabolic pathways that are indispensable for soil functioning, such as oxidation of sulphur, ammonia, reduced metal ions, and methane production (Badger and Bek, 2008; Berg et al., 2007; Konneke et al., 2014). With respect to the role in the soil C balance, we consider a mean CO_2 release from tundra soil by soil respiration of about 76 g m^{-2} yr^{-1} (Fahnestock et al., 1999). Our data thus indicate that re-use of 0.1 to 5 % of net respiration may account for dark fixation of 0.08 to 3.8 g C m^{-2} yr^{-1}. Above-ground net primary production (i.e. IC incorporation into plant material) ranges from 10 to 500 g C m^{-2} yr^{-1} (Gould et al., 2003). Thus microbial CO_2 fixation may correspond in the long term from 0.016 to 38 % of plant C fixation.

4.4 Effect of dark CO_2 fixation on isotopic signal of OC

In general, the $\delta^{13}C$ of organic matter mirrors the $\delta^{13}C$ of plant input in the uppermost horizons. However it increases with soil depth to values 1-3 ‰ higher than in the uppermost organic horizon (e.g. Bird et al., 2002; Buchmann et al., 1997). This was also observed for our arctic tundra soils (Tables 1, 2; Gentsch et al., 2015). The mechanisms behind this enrichment are still unclear and none of the following potential causes can fully explain it: isotopic change of atmospheric CO_2 and microbial processing of OC (Boström et al., 2007), mixing of new C input with old soil organic matter (SOM) and microbial fractionation during litter decomposition (Ehleringer et al., 2000), increase of leaf internal to ambient CO_2 concentration (ci/ca) due to global change during the last 40 years (Betson et al., 2007). Similarly, the causes of the ^{13}C-enrichment of microbial biomass (Dijkstra et al., 2006; Šantrůčková et al., 2000) have not been satisfactorily resolved yet. Our results suggest that IC incorporation into microbial biomass and OC can contribute to soil microbial biomass and OC enrichment. Our model of CO_2 fixation, which was parameterized using data from the same locations, indicates that in the long term, IC incorporation into OC can result in similar ^{13}C-OC enrichments as measured for bulk soils in the deep soil. The model assumes CO_2 enrichment in the soil profile corresponding to 0.1-5 % of net respiration, which is within the range of measured values. Unfortunately, the uncertainty connected to changes in isotopic signal of various soil pools during decomposition process is high.

Our model is therefore largely simplified and the predicted change of SOM isotopic signature is only tentative. We used several assumptions, which may affect model prediction. More specifically, we assumed that there is one pool of well mixed SOM with unique isotopic signal in soil which is decomposed at a constant rate. Including additional, more resistant SOM pool with the same isotopic signal would lead to lesser enrichment of SOM by heterotrophic CO_2 fixation in long-term. It is, however, uncertain whether chemically different pools of SOM have the same isotopic signal. The predicted change of SOM isotopic signal would largely depend on the signal of the resistant fraction at the start of decomposition process if accounted for. It might be also argued that all initial SOM is in fact decomposable in long-term and the resistant SOM fraction, represented by microbial products, builds up during decomposition. If this was the case, the enrichment of SOM would be similar as predicted but it would take longer. We further assumed that no other process lead to isotopic discrimination. I opposite case, our predictions might be affected positively or negatively depending on the particular process, its rate and the connected discrimination.

However, four lines of evidence support our suggestion that IC incorporation can importantly contribute to ^{13}C enrichment of soil OC. **First**, across all sites, inorganic ^{13}C incorporation into OC, expressed on a total C basis, occurred throughout the soil profile and increased with soil depth. The isotopic signal of OC exponentially increased accordingly (R^2 = 0.44, n = 149). **Second**, IC available in the soil profile is enriched relative to associated SOM and plant material. Soil CO_2 may be more enriched in ^{13}C by up to 5 ‰ than SOM because of diffusion of lighter $^{12}CO_2$ out of the soil profile, leaving the heavier $^{13}CO_2$ behind (Cerling et al., 1991). Carbon dioxide dissolution (CO_2^*) causes only negligible fractionation (around 1 ‰) and CO_2^* entering carboxylation reactions should be enriched by 4 ‰ relative to the surrounding OC. Carbon dioxide hydrogenation comes with a huge positive discrimination, and HCO_3^- entering carboxylation reactions is enriched by 9 ‰ at 25 °C. The discrimination increases with decreasing temperature to 12 ‰ at 0 °C (equilibrium fractionation factor; Mook et al., 1974). Thus, IC entering carboxylation as HCO_3^- should be enriched relative to the surrounding organic material by 13 to 16 ‰ (4 ‰ plus 9 ‰ - 12 ‰)). Methanogenesis in anaerobic microsites and deeper parts of the soil profile can cause further CO_2 enrichment as methanogens strongly discriminate against heavier ^{13}C

(difference between CO_2 and CH_4 [$\Delta_{CO2/CH4}$] from 5 ‰ to 93 ‰; Penger et al., 2012), producing relatively light CH_4 and leaving behind much heavier CO_2 (Han et al., 2007). **Third,** genes for anaplerotic, assimilatory and biosynthetic carboxylase enzymes accounted for the major part of detected carboxylase genes. The majority of those enzymes accept HCO_3^- instead of CO_2 (Table SB1). Thus the initial reactant entering the carboxylation reaction is substantially enriched relative to SOM. Although HCO_3^- entering carboxylation can originate from HCO_3^- hydrated in soil solution (see above) or from CO_2^* that is transported from soil solution into the cell, it will always be enriched relative to OC, as intracellular CO_2^* hydration is catalysed by intracellular carbonic anhydrase which prefers ^{13}C. The reaction causes an enrichment of HCO_3^- by 7 ‰. Carbonic anhydrase is widespread among autotrophs but also among heterotrophic eukaryota and prokaryota (Merlin et al., 2003; Nafi et al., 1990; Smith and Ferry, 2000) and is indispensable for the HCO_3^- concentrating mechanism. If there were no CO_2 leak from cells, every bicarbonate ion pulled into the cell should end up in organic compounds, and the isotopic signal of biomass would be determined by carbonic anhydrase fractionation (Hayes, 1993). Even though there is always a leakage, cells utilizing bicarbonate should be enriched relative to CO_2 (Hayes, 1993). It has been documented in plant cells that initial hydration of CO_2^* to bicarbonate and subsequent PEP carboxylation causes enrichment of the resulting OC by 5.7 ‰ at 25 °C relative to gaseous CO_2. The enrichment is dependent on temperature and the amount of carbonic anhydrase present (Cousins et al., 2006; Farquhar, 1983). **Finally,** while autotrophs mostly discriminate against heavier ^{13}C and mean C discrimination of various autotrophic pathways (ΔIC/cell) ranges from zero to 26.7 ‰ when measured in pure cultures (House et al. 2003 and reference therein), the cells utilizing HCO_3^- will not always be depleted relative to CO_2 due to fractionation in the hydration of CO_2 to HCO_3^-. In addition, the discrimination can be decreased by limited gas diffusion in water and the soil environment (Descolasgros and Fontugne, 1990) and low cell density (House et al., 2003). The autotrophs employing Rubisco, the most common autotrophic carboxylase accepting CO_2^* and discriminating against $^{13}CO_2$ (-11 to -30 ‰; see Table SB1), should be depleted in ^{13}C relative to soil CO_2. The proportion of autotrophs in bulk microbial biomass is, however, generally very low. In our experiment, genes encoding Rubisco represented at most 18 % of all carboxylase genes, implying that autotrophic prokaryotes should not

determine the isotopic signal of total microbial biomass.

5 Conclusion

Our results demonstrate that dark CO_2 fixation is common in all arctic soils investigated and anaplerotic reactions are mainly responsible for this. Many anaplerotic pathways in heterotrophic CO_2 incorporation do not lead to any net C assimilation and biomass production (Alonso-Saez et al., 2010). Microbial biomass did not increase in soils without addition of bioavailable substrate either, which further suggests that dark IC fixation may only enable microorganisms to maintain metabolic activity even in C poor conditions. Inorganic C incorporation into OC only corresponds to a few percent of net soil respiration, but still it can play an important role in supporting microbial metabolism and organic matter transformation. We further demonstrate a positive impact of bioavailable soil organic compounds on inorganic C incorporation, implying that increases in plant litter decomposition induced by projected warming and input of root-derived compounds may also enhance C incorporation via dark C fixation.

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References

Acosta, O.B.G., Schleheck, D., Schink, B., 2014. Acetone utilization by sulfate-reducing bacteria: draft genome sequence of Desulfococcus biacutus and a proteomic survey of acetone-inducible proteins. BMB Genomics 15, doi 10.1186/1471-2164-15-584.

Alonso-Saez, L., Galand, P.E., Casamayor, E.O., Pedros-Alio, C., Bertilsson, S., 2010. High bicarbonate assimilation in the dark by Arctic bacteria. ISME Journal 4, 1581-1590.

Astchul, S.F., Gish, W., Miller, W., Myers, E.V., Lipman, D., 1990. Basic local alignment tool. Journal of Molecular Biology 215, 403-410.

Badger, M.R., Bek, E.J., 2008. Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. Journal of Experimental Botany 59, 1525-1541.

Bar-Even, A., Noor, E., Milo, R., 2012. A survey of carbon fixation pathways through a quantitative lens. Journal of Experimental Botany 63, 2325-2342.

Berg, I.A., Kockelkorn, D., Buckel, W., Fuchs, G., 2007. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea. Science 318, 1782-1786.

Berg, I.A., Kockelkorn, D., Ramos-Vera, W.H., Say, R.F., Zarzycki, J., Hugler, M., Alber, B.E., Fuchs, G., 2010. Autotrophic carbon fixation in archaea. Nature Reviews Microbiology 8, 447-460.

Betson, N.R., Johannisson, C., Lofvenius, M.O., Grip, H., Granstrom, A., Hogberg, P., 2007. Variation in the $\delta^{13}C$ of foliage of Pinus sylvestris L. in relation to climate and additions of nitrogen: analysis of a 32-year chronology. Global Change Biology 13, 2317-2328.

Bird, M.I., Šantrůčková, H., Arneth, A., Grigoriev, S., Gleixner, G., Kalaschnikov, Y.N., Lloyd, J., Schulze, E.D., 2002. Soil carbon inventories and carbon-13 on a latitude transect in Siberia. Tellus Series B-Chemical and Physical Meteorology 54, 631-641.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Bockheim, J.G., Tarnocai, C., 1998. Recognition of cryoturbation for classifying permafrost-affected soils. Geoderma 81, 281-293.

Bossio, D.A., Scow, K.M., 1998. Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35, 265-278.

Boström, B., Comstedt, D., Ekblad, A., 2007. Isotope fractionation and ^{13}C enrichment in soil profiles during the decomposition of soil organic matter. Oecologia 153, 89-98.

Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nature Methods 12, 59-60.

Buchmann, N., Kao, W.Y., Ehleringer, J., 1997. Influence of stand structure on carbon-13 of vegetation, soils, and canopy air within deciduous and evergreen forests in Utah, United States. Oecologia 110, 109-119.

Cerling, T.E., Solomon, D.K., Quade, J., Bowman, J.R., 1991. On the Isotopic Composition of Carbon in Soil Carbon-Dioxide. Geochimica et Cosmochmica Acta 55, 3403-3405.

Cousins, A.B., Badger, M.R., Von Caemmerer, S., 2006. Carbonic anhydrase and its influence on carbon isotope discrimination during C-4 photosynthesis. Insights from antisense RNA in Flaveria bidentis. Plant Physiology 141, 232-242.

Descolasgros, C., Fontugne, M., 1990. Stable carbon isotope fractionation by marine-phytoplankton during photosynthesis. Plant Cell and Environment 13, 207-218.

Dijkstra, P., Ishizu, A., Doucett, R., Hart, S.C., Schwartz, E., Menyailo, O.V., Hungate, B.A., 2006. C-13 and N-15 natural abundance of the soil microbial biomass. Soil Biology and Biochemistry 38, 3257-3266.

Ehleringer, J.R., Buchmann, N., Flanagan, L.B., 2000. Carbon isotope ratios in belowground carbon cycle processes. Ecological Applications 10, 412-422.

Erb, T.J., 2011. Carboxylases in natural and synthetic microbial pathways. Applied and Environmental Microbiology 77, 8466-8477.

Fahnestock, J.T., Jones, M.H., Welker, J.M., 1999. Wintertime CO2 efflux from arctic soils: implications for annual carbon budgets. Global Biogeochemical Cycles 13, 775-779.

Farquhar, G.D., 1983. On the nature of carbon isotope discrimination in C-4 species. Australian Journal of Plant Physiology 10, 205-226.

Feisthauer, S., Wick, L.Y., Kastner, M., Kaschabek, S.R., Schlomann, M., Richnow, H.H., 2008. Differences of heterotrophic 13CO2 assimilation by *Pseudomonas knackmussii* strain B13 and Rhodococcus opacus 1CP and potential impact on biomarker stable isotope probing. Environmental Microbiology 10, 1641-1651.

Fox, J., Weisberg, S., 2011. Multivariate linear models in R. An R companion to applied regression, 2nd edn. SAGE Publications.

Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to

estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723-730.

Ge, T.D., Wu, X.H., Liu, Q., Zhu, Z.K., Yuan, H.Z., Wang, W., Whiteley, A.S., Wu, J.S., 2016. Effect of simulated tillage on microbial autotrophic CO_2 fixation in paddy and upland soils. Scientific Reports 6.

Gentsch, N., Mikutta, R., Alves, R.J.E., Barta, J., Čapek, P., Gittel, A., Hugelius, G., Kuhry, P., Lashchinskiy, N., Palmtag, J., Richter, A., Šantrůčková, H., Schnecker, J., Shibistova, O., Urich, T., Wild, B., Guggenberger, G., 2015. Storage and transformation of organic matter fractions in cryoturbated permafrost soils across the Siberian Arctic. Biogeosciences 12, 4525-4542.

Gittel, A., Barta, J., Kohoutova, I., Mikutta, R., Owens, S., Gilbert, J., Schnecker, J., Wild, B., Hannisdal, B., Maerz, J., Lashchinskiy, N., Čapek, P., Šantrůčková, H., Gentsch, N., Shibistova, O., Guggenberger, G., Richter, A., Torsvik, V.L., Schleper, C., Urich, T., 2014. Distinct microbial communities associated with buried soils in the Siberian tundra. ISME Journal 8, 841-853.

Gould, W., Raynolds, M., Walker, D., 2003. Vegetation, plant biomass, and net primary productivity patterns in the Canadian Arctic. Journal of Geophysical Research, doi: 10.1029/2001JD000948.

Han, G.H., Yoshikoshi, H., Nagai, H., Yamada, T., Ono, K., Mano, M., Miyata, A., 2007. Isotopic disequilibrium between carbon assimilated and respired in a rice paddy as influenced by methanogenesis from CO2. Journal of Geophysical Research, doi:10.1029/2006JG000219.

Harris, J.O., 1954. The influence of carbon dioxide on oxygen uptake by resting cells of bacteria. Journal of Bacteriology 67, 476-479.

Hayes, J.M., 1993. Factors controlling ^{13}C contents of sedimentary organic-compounds - Principles and evidence. Marine Geology 113, 111-125.

Hesselsoe, M., Nielsen, J.L., Roslev, P., Nielsen, P.H., 2005. Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$. Applied and Environmental Microbiology 71, 646-655.

House, C.H., Schopf, J.W., Stetter, K.O., 2003. Carbon isotopic fractionation by Archaeans and other thermophilic prokaryotes. Organic Geochemistry 34, 345-356.

Kaiser, C., Koranda, M., Kitzler, B., Fuchslueger, L., Schnecker, J., Schweiger, P., Rasche, F., Zechmeister-Boltenstern, S., Sessitsch, A., Richter, A., 2010.

Belowground carbon allocation by trees drives seasonal patterns of extracellular enzyme activities by altering microbial community composition in a beech forest soil. New Phytologist 187, 843-858.

Konneke, M., Schubert, D.M., Brown, P.C., Hugler, M., Standfest, S., Schwander, T., von Borzyskowski, L.S., Erb, T.J., Stahl, D.A., Berg, I.A., 2014. Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO2 fixation. Proceedings of the National Academy of Sciences of the United States of America 111, 8239-8244.

Kopylova, E., Noé, L., Touzet, H., 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics 28, 3211-3217.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806-809.

Lenth, R.V., 2016. Least-squares means: the R package lsmeans. Journal of Statistical Software 69, 1-33.

Makeev, O.W., Kerzhentsev, A.S., 1974. Cryogenic processes in the soils of northern Asia. Geoderma 12, 101-109.

McCarroll, D., Loader, N.J., 2004. Stable isotopes in tree rings. Quaternary Science Reviews 23, 771-801.

Merlin, C., Masters, M., McAteer, S., Coulson, A., 2003. Why is carbonic anhydrase essential to Escherichia coli? Journal of Bacteriology 185, 6415-6424.

Miltner, A., Kopinke, F.D., Kindler, R., Selesi, D.E., Hartmann, A., Kastner, M., 2005a. Non-phototrophic CO2 fixation by soil microorganisms. Plant and Soil 269, 193-203.

Miltner, A., Richnow, H.H., Kopinke, F.D., Kastner, M., 2004. Assimilation of CO2 by soil microorganisms and transformation into soil organic matter. Organic Geochemistry 35, 1015-1024.

Miltner, A., Richnow, H.H., Kopinke, F.D., Kastner, M., 2005b. Incorporation of carbon originating from CO2 into different compounds of soil microbial biomass and soil organic matter. Isotopes in Environmental and Health Studies 41, 135-140.

Mook, W.G., Bommerson, J.C., Staverman, W.H., 1974. Carbon Isotope Fractionation between Dissolved Bicarbonate and Gaseous Carbon-Dioxide. Earth and Planetary Science Letters 22, 169-176.

Nadelhoffer, K.F., Fry, B., 1988. Controls on natural nitrogen-15 and carbon-13 abundances in forest soil organic-matter. Soil Science Society of America Journal 52, 1633-1640.

Nafi, B.M., Miles, R.J., Butler, L.O., Carter, N.D., Kelly, C., Jeffery, S., 1990. Expression of carbonic-anhydrase in Neisseriae and other heterotrophic bacteria. Journal of Medical Microbiology 32, 1-7.

Nobel, P.S., Palta, J.A., 1989. . Soil O_2 and CO_2 effects on root respiration of cacti. Plant and Soil 120, 263-271.

Palmtag, J., Hugelius, G., Lashchinskiy, N., Tamstorf, M.P., Richter, A., Elberling, B., Kuhry, P., 2015. Storage, landscape distribution, and burial history of soil organic matter in contrasting areas of continuous permafrost. Arctic Antarctic and Alpine Research 47, 71-88.

Penger, J., Conrad, R., Blaser, M., 2012. Stable Carbon Isotope Fractionation by Methylotrophic Methanogenic Archaea. Applied and Environmental Microbiology 78, 7596-7602.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research, 41, 590-596.

Saini, R., Kapoor, R., Kumar, R., Siddiqi, T.O., Kumar, A., 2011. CO2 utilizing microbes - A comprehensive review. Biotechnology Advances 29, 949-960.

Šantrůčková, H., Bird, M.I., Elhottová, D., Novák, J., Picek, T., Šimek, M., Tykva, R., 2005. Heterotrophic fixation of CO_2 in soil. Microbial Ecology 49, 218-225.

Šantrůčková, H., Bird, M.I., Lloyd, J., 2000. Microbial processes and carbon-isotope fractionation in tropical and temperate grassland soils. Functional Ecology 14, 108-114.

Smith, K.S., Ferry, J.G., 2000. Prokaryotic carbonic anhydrases. FEMS Microbiology Reviews 24, 335-366.

Sparling, G.P., West, A.W., 1990. A comparison of gas chromatography and differential respirometer methods to measure soil respiration and to estimate the soil microbial biomass. Pedobiologia 34, 103-112.

Team, R., 2016. R development core team. RA Lang Environ Stat Comput, 55, 275-286.

Torn, M.S., Lapenis, A.G., Timofeev, A., Fischer, M.L., Babikov, B.V., Harden, J.W., 2002. Organic carbon and carbon isotopes in modern and 100-year-old-soil archives of the Russian steppe. Global Change Biology 8, 941-953.

Tveit, A.T., Urich, T., Frenzel, P., Svenning, M.M., 2015. Metabolic and trophic interactions modulate methane production by Arctic peat microbiota in response to warming. Proceedings of the National Academy of Sciences 112, E2507-E2516.

Urich, T., Lanzén, A., Qi, J., Huson, D.H., Schleper, C., Schuster, S.C., 2008. Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. PLoS One 3, e2527.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry 19, 703-707.

Veldkamp, H., 1970. Chapter V - Enrichment cultures of prokaryotic organisms. Methods in Microbiology 3, 305-361.

Walker, D.A., Raynolds, M.K., Daniëls, F.J.A., Einarsson, E., Elvebakk, A., Gould, W.A., Katenin, A.E., Kholod, S.S., Markon, C.J., Melnikov, E.S., 2005. The circumpolar Arctic vegetation map. Journal of Vegetation Science 16, 267-282.

Wood, H.G., Stjernholm, R.L., 1962. Assimilation of carbon dioxide by heterotrophic organisms. The Bacteria 3, 41-117.

Yuan, H.Z., Ge, T.D., Chen, C.Y., O'Donnell, A.G., Wu, J., S., 2012. Significant role for microbial autotrophy in the sequestration of soil carbon. Applied and Environmental Microbiology 78, 2328-2336.

Supplementary information

Appendix A - model of microbial SOM decomposition

Model structure

A schematic representation of the model is given in Fig. 1 in the main text. Here, we specify details of the model. There are two carbon pools in the soil – soil organic matter (SOM) and soil microorganisms (Mic). At time zero, SOM and Mic are mainly composed of ^{12}C (400 mg ^{12}C and 4 mg ^{12}C in SOM and Mic, respectively, per g soil). Both pools have $\delta^{13}C$ -27 ‰, therefore the amounts of ^{13}C per g soil are 4.37 mg and 0.0437 mg for SOM and Mic, respectively. $\delta^{13}C$ of each pool is changing as the decomposition process proceed. Over time, SOM and Mic change. SOM changes as a result of decomposition and microbial dying:

$$\frac{dSOM}{dt} = -decomposition + dying. \quad [1]$$

Mic also changes as a result of decomposition and dying:

$$\frac{dMic}{dt} = decomposition \times CUE - dying. \quad [2]$$

In eq. 2, CUE defines carbon use efficiency, i.e. the proportion of assimilated C in the overall consumed C. Its value is set to 0.55 (see below). Accordingly, respiration (CO_2 production) by the microbial community is:

$$respiration = decomposition \times (1 - CUE). \quad [3]$$

Decomposition follows first-order kinetics with the rate constant k (d^{-1}):

$$decomposition = k \times SOM. \quad [4]$$

Microbial dying also follows first-order kinetics, but with the rate constant d (d^{-1}):

$$dying = d \times Mic. \quad [5]$$

a) Isotopic signal

In order to account for tentative changes in the isotopic signals of SOM and Mic, the fates of ^{12}C and ^{13}C in SOM and Mic are modelled separately. For SOM, the change in ^{12}C is:

$$\frac{dSOM_{12_C}}{dt} = -decomposition_{12_C} + dying_{12_C}, \quad [6]$$

and the change in ^{13}C is:

$$\frac{dSOM_{13_C}}{dt} = -decomposition_{13_C} + dying_{13_C}. \quad [7]$$

Accordingly, the changes in ^{12}C and ^{13}C in Mic are:

$$\frac{dMIC_{12_C}}{dt} = decomposition_{12_C} \times CUE - dying_{12_C}, \quad [8]$$

$$\frac{dMic_{13_C}}{dt} = decomposition_{13_C} \times CUE - dying_{13_C}. \quad [9]$$

We assume that during decomposition and dying, no isotopic discrimination occurs. We use this simplification because the available literature is inconclusive in this sense (see the Discussion section in the main text). Therefore, $decomposition_{13_C}$ and $dying_{13_C}$ are related to $decomposition_{12_C}$ and $dying_{12_C}$ according to the ratio of the lighter and heavier C isotopes in SOM and Mic, respectively:

$$decomposition_{13_C} = decomposition_{12_C} \times \frac{SOM_{13_C}}{SOM_{12_C}}, \quad [10]$$

$$dying_{13_C} = dying_{12_C} \times \frac{Mic_{13_C}}{Mic_{12_C}}. \quad [11]$$

$decomposition_{12_C}$ and $dying_{12_C}$ are modelled according to equations 4 and 5 respectively.

b) CO_2 fixation

For the sake of simplicity, we assume that dark CO_2 fixation represents a constant part of net microbial respiration:

$$fixation = a \times \left[decomposition_{12_C} \times (1 - CUE) + decomposition_{13_C} \times (1 - CUE)\right]. \quad [12]$$

The term in square brackets represents overall respiration and parameter a defines the ratio between fixation and respiration. It can theoretically range from 0 (no fixation) to 1 (all respired CO_2 is fixed). We modelled four scenarios with a set to 0, 0.001, 0.01 or 0.05.

During CO_2 fixation, discrimination occurs because CO_2 is dissolved in soil solution first and then taken up by soil microorganisms. The amounts of $^{12}CO_2$ and $^{13}CO_2$, respectively, fixed by soil microorganisms are modelled as:

$$fixation_{12_C} = \frac{fixation}{1+Df}, \quad [13]$$

$$fixation_{13_C} = \frac{fixation \times Df}{1+Df}. \quad [14]$$

In eqs. 13 and 14, Df is the discrimination term. It represents the proportion of $^{13}CO_2$ in fixed CO_2. Df is defined to be consistent with the terminology used across isotopic studies:

$$Df = \left(\frac{\delta^{13}C_{resp} - \Delta}{(\Delta+1) \times 1000} + 1\right) \times PDB. \quad [15]$$

$\delta^{13}C_{resp}$ is the isotopic signature of respired CO_2 that becomes dissolved and is consumed by soil microorganisms. Δ is the isotope discrimination factor. For CO_2 fixation, its value is set to 4 ‰. PDB stands for the isotopic signature of pee dee belemnite.

Accounting for CO_2 fixation, eqs. 8 and 9 can be rearranged to:

$$\frac{dMIC_{12_C}}{dt} = decomposition_{12_C} \times CUE - dying_{12_C} + fixation_{12_C}, \quad [16]$$

$$\frac{dMic_{13_C}}{dt} = decomposition_{13_C} \times CUE - dying_{13_C} + fixation_{13_C}. \quad [17]$$

Because fixed CO_2 is enriched in the heavier isotope, Mic becomes enriched over time. When microbes die, they become part of SOM and SOM becomes enriched too.

During decomposition, SOM decreases and the abundance of microbial products within SOM increase. We assume that SOM is not well mixed and therefore the probabilities that soil microorganisms consume SOM with an isotopic signature of -27 ‰ or enriched microbial products depend on their abundance. Microbial products accumulate within SOM as microbial biomass is dying:

$$\frac{dMIC_{products}}{dt} = dying_{12_C} + dying_{13_C}. \quad [18]$$

The higher is the proportion of microbial products within SOM, the higher the probability that they decompose and are consumed by soil microorganisms. Therefore, the isotopic signature of decomposition flux changes with time:

$$decomposition_{13_C} = decomposition_{12_C} \times \left(\frac{SOM_{13_{C-t0}}}{SOM_{12_{C-t0}}} \times f_{SOM} + \frac{SOM_{13_{C-t}}}{SOM_{12_{C-t}}} \times f_{MIC_{products}}\right) \quad [19]$$

$\frac{SOM_{13_{C-t0}}}{SOM_{12_{C-t0}}}$ is the isotopic signature of SOM at time 0 and $\frac{SOM_{13_{C-t}}}{SOM_{12_{C-t}}}$ the isotopic signature of SOM at time t. SOM at time t includes some microbial products.

The parameters f_{SOM} and $f_{MIC_{products}}$ define the abundances of original SOM and microbial products in the SOM pool at time t, respectively:

$$f_{SOM} = \frac{SOM_t}{SOM_t + MIC_{products-t}}, \quad [20]$$

$$f_{MIC_{products}} = \frac{MIC_{products-t}}{SOM_t + MIC_{products-t}}, \quad [21]$$

According to eq. 19, the isotopic signature of decomposition flux is close to -27 ‰ in the early stages of decomposition and then steadily increases as the enriched microbial products accumulate in SOM.

Model parameterization

There are three unknown parameters in our model – k, d and CUE. Parameter k was experimentally estimated in a separate long-term incubation experiment (Gentsch et al., in prep). Its mean value for different topsoil organic horizons across the arctic tundra is 3.5×10^{-4} d^{-1}. Parameters d and CUE were defined according to Allison and collective (2010). The value of parameter d is 4.8×10^{-3} d^{-1}. Under steady-state conditions, it would correspond to the turnover time of microbial biomass, which is 208 days. This is a rather conservative estimate. According to Allison et al. (2010), CUE changes with temperature. When the temperature increases, CUE decreases, and vice versa. We assumed that SOM is decomposing only during the vegetative season, which in arctic tundra lasts approximately 90 days. The average temperature during that period is approximately 5°C. At that temperature, CUE should be 0.55 (Allison et al., 2010). The model was run with these parameter values in daily steps for 1000 years (90 000 days).

Model results

According to our model, SOM exponentially decreases over time, in accordance with what is usually observed when there is no SOM input.

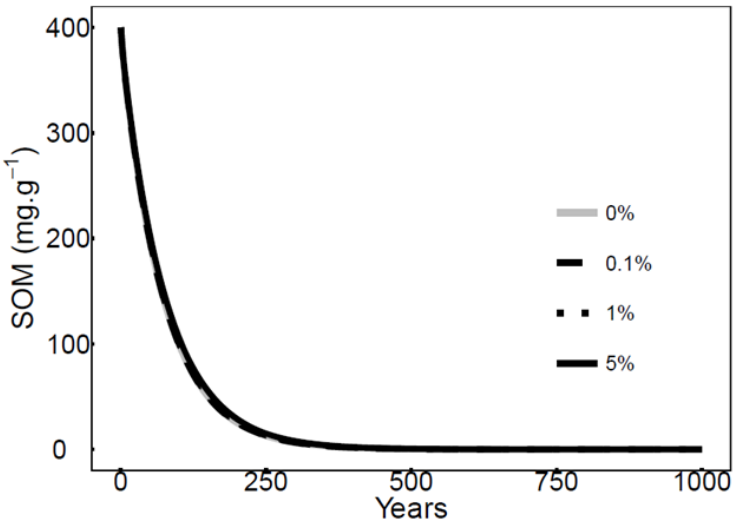

Fig. SA1. Modelled change of SOM over time. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively.

At the same time, Mic decreases accordingly. The proportion between SOM and Mic steadily increases with time and equilibrates after the first 10 years. When no fixation occurs, microbial biomass makes up 4.2 % of SOM. This is a somewhat higher number than is usually observed. The proportion of microbial biomass increases up to 4.4 % of SOM when fixation occurs at 5 % fixation rate.

The isotopic signatures of SOM and Mic increase over time when fixation occurs. As expected, the change in isotopic signature of Mic is faster than in SOM.

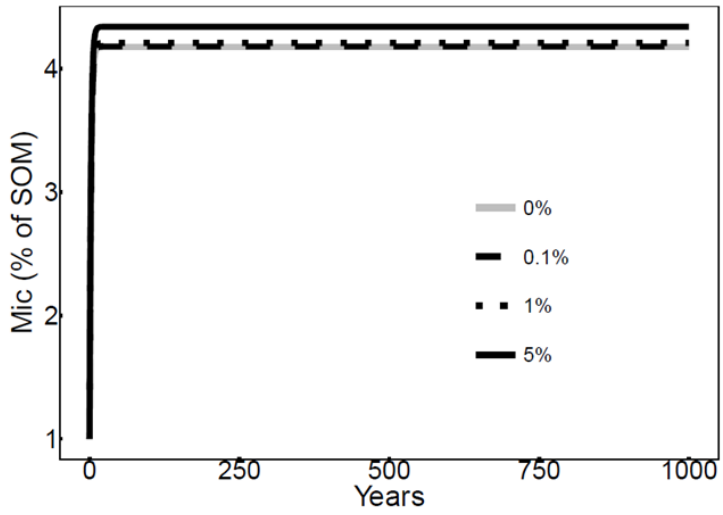

Fig. SA2. Modelled abundance of soil microorganisms (Mic) in SOM over time. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively.

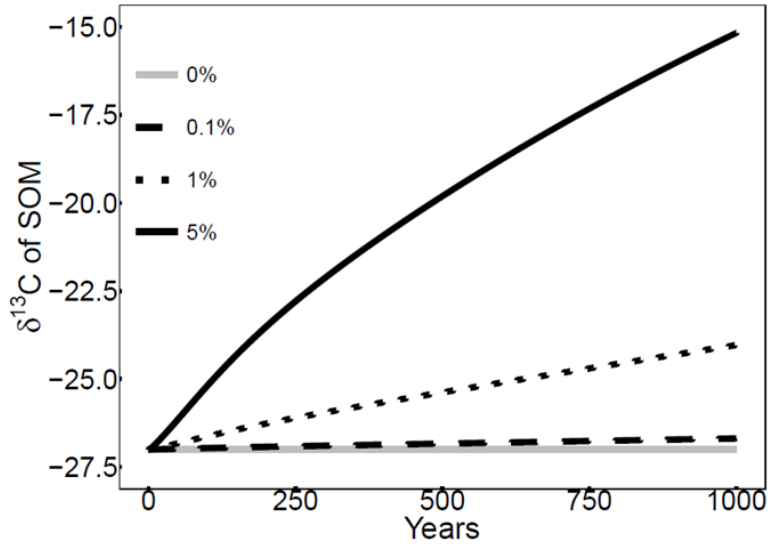

Fig. SA3. Modelled shift of SOM isotopic signature over time caused by heterotrophic CO_2 fixation. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively.

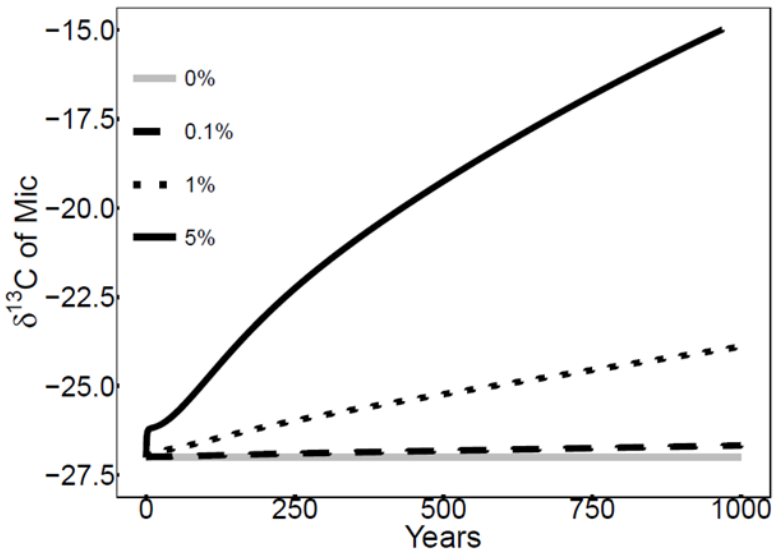

Fig. SA4. Modelled shift of isotopic signature of soil microorganisms (Mic) over time caused by heterotrophic CO_2 fixation. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively.

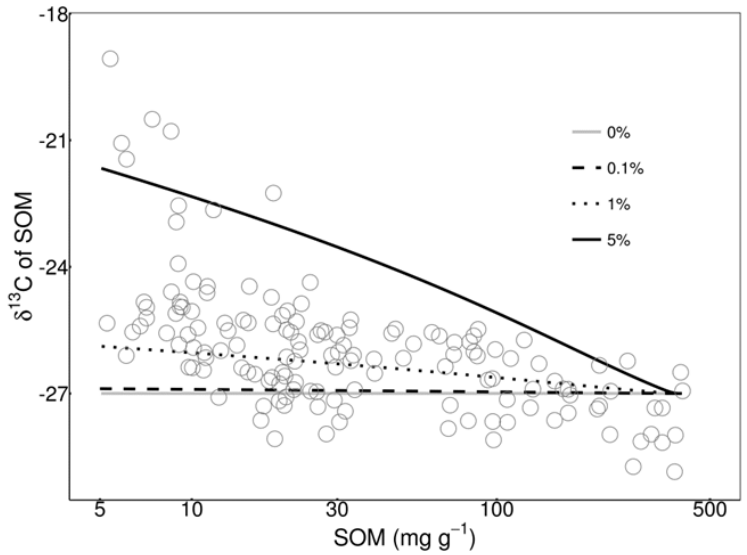

Fig. SA5. The relationship between concentration of SOM and SOM isotopic signature. Open circles show measured data and lines model predictions. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively. Please note that x-axis is in logarithmic scale.

After 1,000 years, the isotopic signature of SOM changes from -27 ‰ to -26.69, -24.03 and -15.17 ‰ at CO_2 fixation rates of 0.1, 1 and 5% of respiration, respectively. As SOM decreases over time, its isotopic signature increases. This leads to a relationship between SOM concentration and its isotopic signature that is similar to the one we observed in our measurements.

Because CO_2 fixation reduces the amount of CO_2 released to the atmosphere, there is a net difference in SOM between the control and soils where CO_2 fixation has occurred. The difference steadily increases until it reaches maximum. The maximum depends on fixation rate, at 5% fixation rate, the net difference in SOM pool makes 7.4 mg carbon per gram of dry soil, which is equivalent to 1.8% of initial SOM pool. After reaching maximum, the net difference decreases as the SOM pool becomes depleted. After 1000 years, the difference in SOM pool between the control and soils where CO_2 fixation has occurred is almost non-existing.

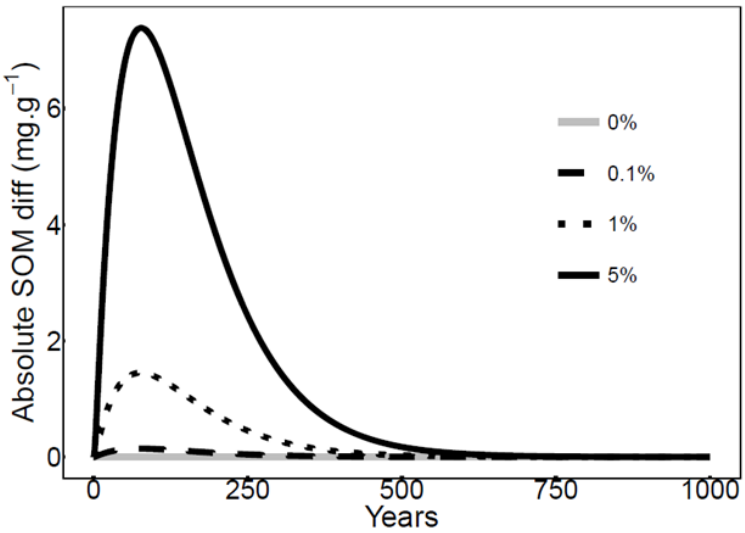

Fig. SA6. Absolute difference in SOM concentration between control (0 % - no fixation) and soils with occurrence of heterotrophic CO_2 fixation over time. The solid grey line represents control (0 % - no fixation), dashed, dotted and solid black lines represent CO_2 fixation rates equal to 0.1, 1 and 5 % of respiration rate, respectively.

Appendix B - supplementary tables and figures

Table SB1 Carboxylases detected in analyzed soil samples, active inorganic carbon (IC) species, carboxylases functions and known fractionation factor Δ.

Detected enzyme	Active IC species	Function	Δ	Reference
Acyl CoA carboxylases	HCO_3^-	biosynthesis of long chain fatty acids (mycolic acid), biotin-dependent, mycobacteria, other actinobacteria, Corynebacteriales,		Gago et al. (2011); Marrakchi et al. (2014); Tran et al. (2015)
Acetyl CoA carboxylases	HCO_3^-	catalyzes the first step in the synthesis of long chain fatty acids, autotrophic CO_2 fixation (3-hydroxypropionate pathway/malyl-CoA autotrophic pathway, 3HP), biotin dependent plants, bacteria and fungi, not in archaea		Cronan and Waldrop (2002); Marrakchi et al. (2014)
Pyruvate carboxylases	HCO_3^-	carboxylation of pyruvate to oxaloacetate, anaplerotic enzyme, also 5-dicarboxylate/4-hydroxybutyrate cycle, reductive TCA (autotrophic CO_2 fixation and assimilation of organic carbon) bacteria (epsilon and delta proteobacteria) and archaea, yeasts		Hügler et al. (2003); Jitrapakdee et al. (2008)
Rubisco - different variants	CO_2	autotrophic CBB pathway higher plants, prokaryotes (cyanobacteria, symbionts, alpha and beta proteobacteria, diatoms, archaea, ammonia oxidisers	-30‰ to -11‰	Badger and Bek (2008); McNevin et al. (2007); Robinson and Cavanaugh (1995)
Acetone carboxylases	CO_2, CO, HCO_3^-	key enzyme of bacterial acetone metabolism, catalyzes the condensation of acetone and CO_2 to acetonacetate denitrifying bacteria, dissimilatory sulphate reducing bacteria, aerobic alpha proteobacteria		Acosta et al. (2014); Birks and Kelly (1997); Boyd et al. (2004); Schühle and Heider (2012)
Biotin carboxylases	HCO_3^-	the first step of fatty acid synthesis, 3-hydroxypropionate pathway/malyl-CoA autotrophic strictly anaerobic pathway (3HP), 3-hydroxypropionate/4-hydroxybutyrate autotrophic cycle (also in aerobic conditions)		Tong (2013)
PEP carboxylases	HCO_3^-	anaplerotic enzyme, supplying acetate to TCA in bacteria and fungi, N_2 fixation, reductive TCA	~-2‰	González et al. (2008)
PEP carboxylase - Archaea	HCO_3^-	reductive TCA, 5 dicarboxylate/4-hydroxybutyrate cycle archaea		Berg et al. (2010); Ettema et al. (2004)
Pyruvates ferredoxin oxidoreductases	CO_2	autotrophic CO_2 fixation (reductive TCA, reductive acetyl-CoA, dicarboxylate/hydroxybutyrate), fermentative CO_2 assimilation (monocarboxylic acid cycle)		Erb (2011); Yoon et al. (2016)

Table SB2 Sequencing summary of 10 in-situ metagenomes and 9 metagenomes from the incubation experiment. Total amount of sequences, amount of ribosomal small subunit (SSU) sequences, sequences with assigned Pfam code, sequences with Pfam code corresponding to one and/or several carboxylase families (see Table SB1), and the amount of SSU copies and carboxylase genes copies per gram of soil are presented.

	Sample ID	Site	Soil horizon	Total seqs	SSU seqs.[a]	Seqs. w Pfam code	Carboxylase seqs.[b]	qPCR SSU copies per g soil	Carboxylase genes copies per g soil [c]
In-situ metagenomes	AMD14-O	AriMas	upper organic	1 474 397	1 374	39 991	95	3.9E+10	2.7E+09
	AMA10-OE	AriMas	upper organic	719 830	894	21 780	46	1.6E+10	8.3E+08
	CHI12-O	Cherskiy	upper organic	561 543	1 028	19 042	42	2.3E+10	9.5E+08
	LgC16-O	Logata	upper organic	2 629 002	2 706	86 881	209	1.4E+10	1.1E+09
	AMA1-AJJ	AriMas	cryoturbated	790 424	1 127	26 888	68	3.1E+08	1.9E+07
	AMD5-AJJ	AriMas	cryoturbated	1 207 723	1 848	57 990	117	4.1E+08	2.6E+07
	CHI3-AJJ	Cherskiy	cryoturbated	1 018 852	1 482	32 580	62	7.0E+09	2.9E+08
	LGC4-AJJ	Logata	cryoturbated	3 234 580	3 598	98 871	248	3.4E+09	2.3E+08
	CHI8-BCG	Cherskiy	mineral	772 685	570	10 946	29	1.8E+05	9.2E+03
	LGC11-BCG	Logata	mineral	713 207	1 251	30 177	74	7.7E+08	4.6E+07
Incubation experiment metagenomes	control1	AriMas	cryoturbated	700 247	291	92 250	553	1.7E+08	2.3E+08
	control2	AriMas	cryoturbated	520 405	255	74 260	442	9.1E+07	1.6E+08
	control3	AriMas	cryoturbated	680 465	286	82 242	482	1.9E+08	2.3E+08
	succrose1	AriMas	cryoturbated	312 486	252	15 294	131	5.7E+08	3.0E+08
	succrose2	AriMas	cryoturbated	430 516	539	17 740	142	1.1E+09	3.8E+08
	succrose3	AriMas	cryoturbated	607 566	454	67 993	442	4.9E+08	4.1E+08
	lipids1	AriMas	cryoturbated	548 327	570	70 842	439	3.8E+08	4.8E+08
	lipids2	AriMas	cryoturbated	399 030	542	25 432	170	4.1E+08	2.7E+08
	lipids3	AriMas	cryoturbated	680 777	846	104 316	568	2.9E+08	3.5E+08

[a] bacterial SSU sequences were extracted by sortmerna algorithm, identified by blastn and extracted by MEGAN

[b] based on Pfam assignment

[c] recalculated by equation 1 (see material and methods)

Table SB3 The effect of incubation in a 13CO2-amended atmosphere on microbial PLFA profiles in soil samples from Ari Mas site. The figures shown are significances of an incubation effect on microbial community structure and percentages of variability explained by the incubation in samples from particular soil horizons (control vs 13CO2 treatment, n = 3). Results are from redundancy analyses on the microbial PLFA composition (mol% of PLFA). Analyses were performed using CANOCO for Windows version 5.0 (Ter Braak and Šmilauer 2012).

soil layer	p	pseudo-F	% of explained variability
upper organic	0.104	3.6	47.3
mineral	0.199	1.2	23.6
cryoturbated	0.606	0.5	11.1

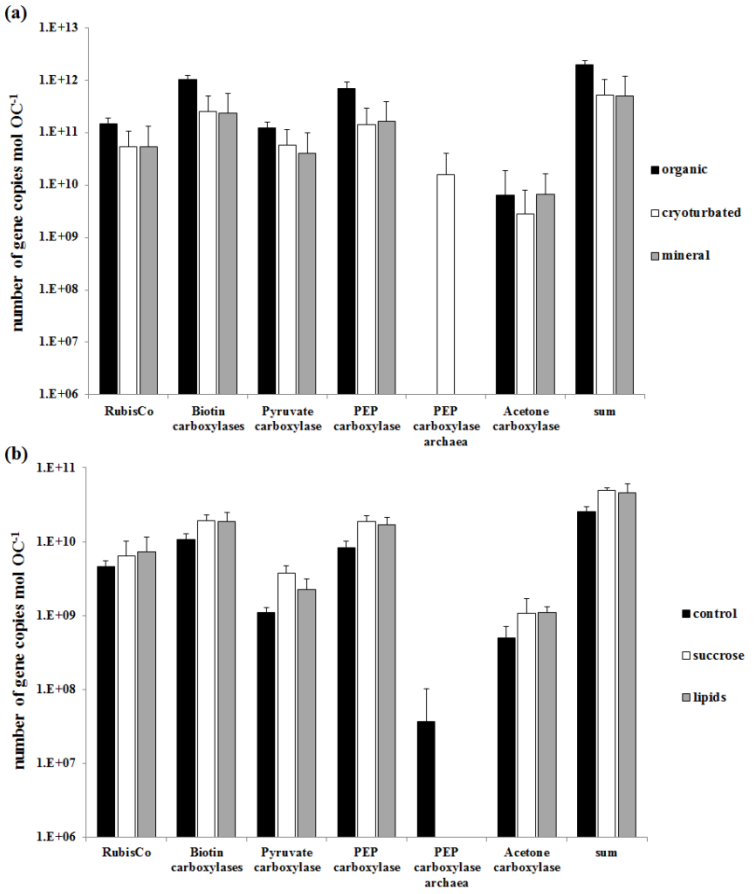

Fig. SB1. Abundance of carboxylases normalised to C content in uppermost organic, cryoturbated and mineral soil layers from Cherskii, Ari Mas and Logata sites **(a)**, and in the cryoturbated layer from Ari Mas site, either unamended (control) or amended with organic carbon sources **(b)**. Note that the scale on *y* axis is logarithmic. The standard deviation of absolute carboxylase gene counts was in some cases larger than average, only positive error bars are thus visualized.

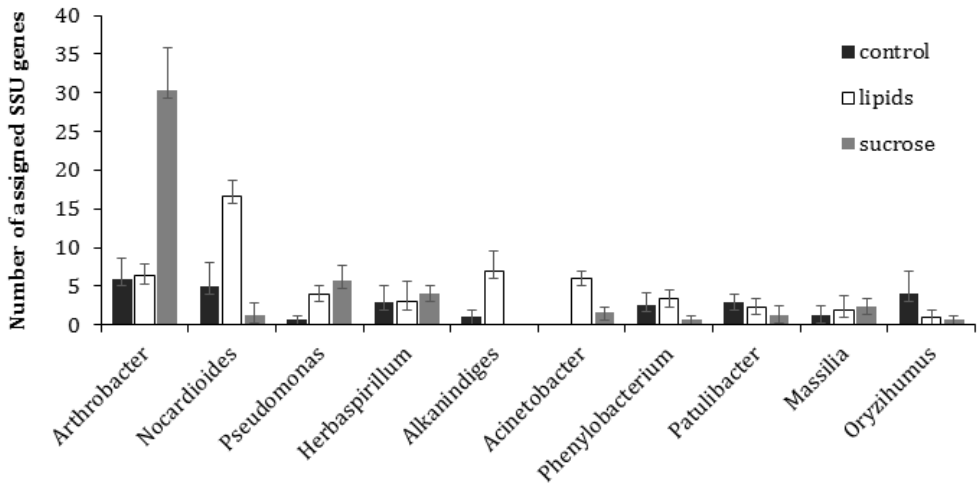

Fig. SB2. Top 10 most abundant bacterial genera determined from extracted SSU reads from metagenomes in the cryoturbated layer, either unamended (control) and after sucrose and lipid addition from Ari Mas site.

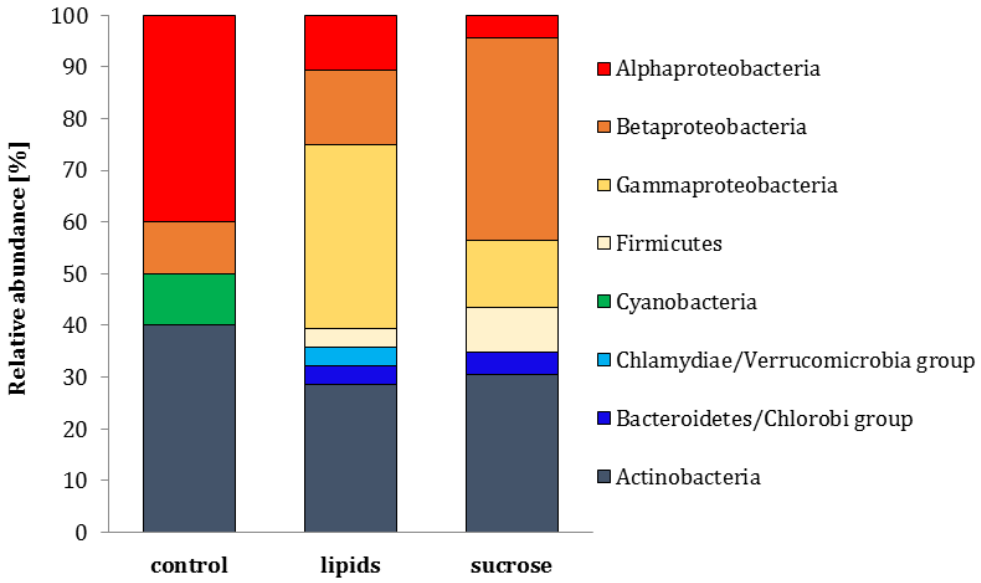

Fig. SB3. Link between the PEP carboxylase and microbial community in the cryoturbated horizon from Ari Mas site. Taxonomy of PEP carboxylase was assigned by LSA classifiers (151 of 189 sequences). Data were normalized to 25 sequences per sample.

References

Acosta, O.B.G., Schleheck, D., Schink, B., 2014. Acetone utilization by sulfate-reducing bacteria: draft genome sequence of Desulfococcus biacutus and a proteomic survey of acetone-inducible proteins. BMB Genomics 15, doi 10.1186/1471-2164-15-584.

Allison, S.D., Wallenstein, M.D., Bradford, M.A., 2010. Soil-carbon response to warming dependent on microbial physiology. Nature Geoscience 3, 336-340.

Badger, M.R., Bek, E.J., 2008. Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO2 acquisition by the CBB cycle. Journal of Experimental Botany 59, 1525-1541.

Berg, I.A., Kockelkorn, D., Ramos-Vera, W.H., Say, R.F., Zarzycki, J., Hugler, M., Alber, B.E., Fuchs, G., 2010. Autotrophic carbon fixation in archaea. Nature Reviews Microbiology 8, 447-460.

Birks, S.J., Kelly, D.J., 1997. Assay and properties of acetone carboxylase, a novel enzyme involved in acetone-dependent growth and CO2 fixation in Rhodobacter capsulatus and other photosynthetic and denitrifying bacteria. Microbiology 143, 755-766.

Boyd, J.M., Ellsworth, H., Ensign, S.A., 2004. Bacterial acetone carboxylase is a manganese-dependent metalloenzyme. Journal of Biological Chemistry 279, 46644-46651.

Cronan, J.E., Waldrop, G.L., 2002. Multi-subunit acetyl-CoA carboxylases. Progress in lipid research 41, 407-435.

Erb, T.J., 2011. Carboxylases in natural and synthetic microbial pathways. Applied and Environmental Microbiology 77, 8466-8477.

Ettema, T.J., Makarova, K.S., Jellema, G.L., Gierman, H.J., Koonin, E.V., Huynen, M.A., de Vos, W.M., van der Oost, J., 2004. Identification and functional verification of archaeal-type phosphoenolpyruvate carboxylase, a missing link in archaeal central carbohydrate metabolism. Journal of Bacteriology 186, 7754-7762.

Gago, G., Diacovich, L., Arabolaza, A., Tsai, S.C., Gramajo, H., 2011. Fatty acid biosynthesis in actinomycetes. FEMS Microbiology Reviews 35, 475-497.

González, J.M., Fernández-Goméz, B., Fernández-Guerra, A., Goméz-Consarnau, L., Sánchez, O., Coll-Lladó, M., del Campo, J., Escudero, L., Rodriguéz-Martínez, R., Alonso-Sáez, L., Latasa, M., Paulsen, I., Nedashkovskaya, O., Lekunberri, I., Pinhassi, J., Pedrós-Alió, C., 2008. Genome analysis of the proteorhodopsin-containing bacterium *Polibacter* sp. MED152 (Flavobacteria). PNAS 105, 8724-8729.

Hügler, M., Huber, H., Stetter, K.O., Fuchs, G., 2003. Autotrophic CO2 fixation

pathways in archaea (Crenarchaeota). Archives of Microbiology 179, 160-173.

Jitrapakdee, S., St Maurice, M., Rayment, I., Cleland, W.W., Wallace, J.C., Attwood, P.V., 2008. Structure, mechanism and regulation of pyruvate carboxylase. Biochemical Journal 413, 369-387.

Marrakchi, H., Lanéelle, M.-A., Daffé, M., 2014. Mycolic acids: structures, biosynthesis, and beyond. Chemistry & biology 21, 67-85.

McNevin, D.B., Badger, M.R., Whitney, S.M., von Caemmerer, S., Tcherkez, G.G.B., Robinson, J.J., Cavanaugh, C.M., 1995. Expression of form I and form II Rubisco in chemoautotrophic symbioses: implications for the interpretation of stable carbon isotope values. Limnology and Oceanography 40, 1496-1502.

Schühle, K., Heider, J., 2012. Acetone and butanone metabolism of the denitrifying bacterium “Aromatoleum aromaticum” demonstrates novel biochemical properties of an ATP-dependent aliphatic ketone carboxylase. Journal of Bacteriology 194, 131-141.

Ter Braak, C.J.F., Šmilauer, P., 2012. Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power, Ithaca, USA, 496 pp.

Tong, L., 2013. Structure and function of biotin-dependent carboxylases. Cellular and Molecular Life Sciences 70, 863-891.

Tran, T.H., Hsiao, Y.-S., Jo, J., Chou, C.-Y., Dietrich, L.E.P., Walz, T., Tong, L., 2015. Structure and function of a single-chain, multi-domain long-chain acyl-CoA carboxylase. Nature 518, 120-124.

Yoon, K.-S., Ishii, M., Kodama, T., Igarashi, Y., 2016. Carboxylation reactions of pyruvate: ferredoxin oxidoreductase and 2-oxoglutarate: ferredoxin oxidoreductase from Hydrogenobacter thermophilus TK-6. Bioscience, Biotechnology, and Biochemistry, 61, 510-513.

Study IV

Drivers of phosphorus limitation across soil microbial communities

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Drivers of phosphorus limitation across soil microbial communities

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Summary

1. Nutrient limitation of soil microbial communities controls the rates of plant litter and soil organic matter decomposition and nutrient mineralization, and as such, it is central to soil and ecosystem models.

2. According to ecological stoichiometry theory, when the carbon (C)-to-nutrient (E) ratio of resources used by a microbial community is higher than a critical ratio (C : E_{CR}), that nutrient is limiting. The C-to-phosphorus (P) critical ratio (C : P_{CR}) that determines P limitation is largely unknown for soils, and thus, it is the subject of our study.

3. Our results show that the C : P_{CR} in widely different soils ranges from 26·6 to 465·1 or from 20·9 to 740·7 when accounting for 95% confidence intervals. Using constant or narrowly fluctuating C : P_{CR} in ecosystem models is therefore inaccurate.

4. The C : P_{CR} cannot be simply predicted from microbial community C : P or available soil P. C : P_{CR} was only related to relative abundance of phospholipid fatty acids, which reflects microbial community structure and physiology. Our data suggest complex controls over microbial community C : P_{CR}.

5. We further propose that using P storage compounds that allow the microbial community to temporarily buffer variability in available P can represent a widely adopted strategies across soils.

Key-words: ecological stoichiometry, Monod equation, nutrient limitation, phosphorus mineralization and immobilization, phospholipid fatty acid, soil microbial community

Introduction

Since the 1990s, important ecosystem processes, such as plant litter and soil organic matter decomposition and nutrient mineralization, became widely framed in ecological stoichiometry theory (Enriquez, Duarte & Sandjensen 1993; Sterner & Elser 2002; Manzoni & Porporato 2009; Hall *et al.* 2010). One central paradigm of ecological stoichiometry is the constant or narrowly fluctuating (homeostatic) stoichiometry of three macronutrients in living organisms [carbon (C), nitrogen (N) and phosphorus (P), thereafter expressed on a molar basis]. In the soil, the degree of the microbial homeostasis is supposed to be strong (Makino *et al.* 2003; Cleveland & Liptzin 2007) and the variability in bulk microbial community stoichiometry is low (6·3 < C : N < 8·3, 31·6 < C : P < 130·7; Xu, Thornton & Post 2013). Comparing to soil microbial communities, plants are less homeostatic (Elser *et al.* 2010) and therefore plant litter stoichiometry displays much higher variability (5 < C : N < 100, 250 < C : P, 3500; Elser *et al.* 2010), primarily reflecting differences among plant organs (e.g. leaves are richer in P than wood), as well as species composition and stand conditions. The stoichiometry of soil organic matter also varies greatly (10·5 < C : N < 31·4, 6·6 < C : P < 1347·0; Xu, Thornton & Post 2013), depending on plant litter input, edaphic conditions, geology, microbial transformations, etc. Thus, the variable stoichiometry of plant litter and to a lesser degree of soil organic matter often does not meet the nutritional requirements of homeostatic microbial communities and causes nutrient limitation of microbial growth. In turn, nutrient limitation may slow down the rate of plant litter decomposition and decrease nutrient mineralization rate (Enriquez, Duarte & Sandjensen 1993; Schade *et al.* 2005; Manzoni & Porporato 2009). Therefore, with abiotic factors and C availability, nutrient limitation is considered an

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important factor controlling growth of soil microbial communities and ultimately the above-mentioned ecosystem processes (Brown *et al.* 2004; Cross *et al.* 2015).

Under controlled conditions, microbial growth is a non-linear function of available substrate, usually modelled by Monod equation (Sinsabaugh, Shah & Follstad Shah 2012). This equation is hyperbolic; that is, it has saturating character and thus reflects substrate-saturated growth at high substrate concentration and substrate limited growth at low substrate concentration. Substrates are complex mixtures of compounds including C, N and P and other nutrients including micronutrients. When one of these nutrients is in shortage with respect to the others and in relation to the decomposer nutritional requirements, microbial growth becomes limited by that nutrient and follows the linear part of the Monod equation (Schmidt 1992; Boer *et al.* 2009).

To determine the most limiting nutrients, which ultimately drive microbial growth rate, the principle of critical ratio was formulated – growth-limiting nutrients are those whose C-to-nutrient ratio is higher than the critical ratio. This concept has a simple mathematical origin (Bosatta & Berendse 1984; Ågren & Bosatta 1998; Sinsabaugh *et al.* 2013): it is the ratio at which the maximum rate of supply of a given nutrient is equal to the nutrient demand. For phosphorus, all P is supplied by an inorganic P pool because mineralization is extracellular process ensured by phosphatases (McGill & Cole 1981). It can be shown (see Appendix S1, Supporting information) that in this case the critical ratio of organic C-to-inorganic P can be approximated as,

$$(\mathrm{C:P})_{\mathrm{CR}} = \frac{k_{\mathrm{I}}}{k_{\mathrm{D}}}\frac{(\mathrm{C:P})_{\mathrm{m}}}{\mathrm{CUE}} \qquad \text{eqn 1}$$

where k_{I} and k_{D} are kinetic constants for inorganic P and organic C uptake, respectively, $(\mathrm{C:P})_{\mathrm{m}}$ is the microbial community C : P ratio, and CUE is the carbon use efficiency. When the C : P ratio of the resource is lower than $\mathrm{C:P_{CR}}$, P is in excess of demand and is mineralized; otherwise, the rates of P immobilization and microbial growth become limited by inorganic P. The origin of $\mathrm{C:P_{CR}}$ as the breakpoint between C-limited and P-limited regimes is illustrated in Fig. 1a (dots). Figure 1b shows how $\mathrm{C:P_{CR}}$ varies as a function of the key stoichiometric traits $(\mathrm{C:P})_{\mathrm{m}}$ (on the abscissa) and CUE (higher for the black curves), and the ratio of k_{I} to k_{D}, which indicates how effectively the microbial community takes up inorganic P vs. organic C.

Empirical studies estimated the C : N ratio above which microbes require net N immobilization. For terrestrial microbial communities, this threshold varies within ≈20 and 200 (Ågren *et al.* 2013; Zechmeister-Boltenstern *et al.* 2015), with higher values in N-poor litter and woody residues (Moore *et al.* 2006; Manzoni *et al.* 2010). These results suggest that microbial community C : N ratio and/or CUE might adapt to the available substrates. However, the critical C : P ratio has not been estimated for soils yet,

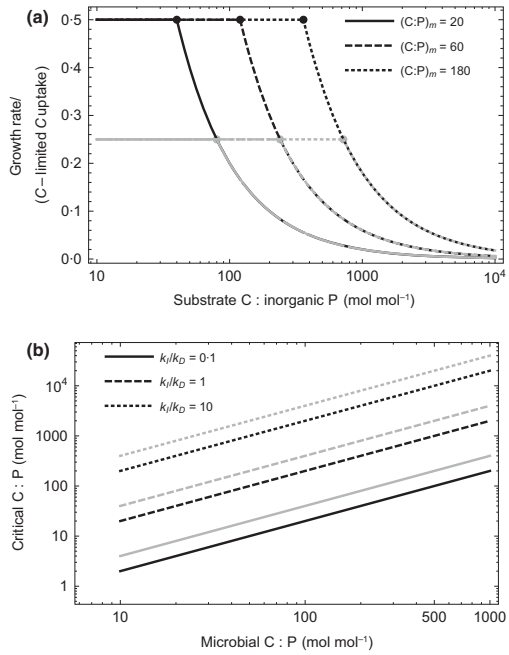

Fig. 1. Definition of critical C : P ratio and its variability as a function of microbial community characteristics. a) Microbial growth rate, normalized by the C uptake rate in C-limited conditions (eqn A4 in Appendix S1), as a function of organic C-to-inorganic P ratio, for different microbial C : P ratios [$(\mathrm{C:P})_{\mathrm{m}}$, indicated by different dashing styles) and CUE (black curves, CUE = 0·5; grey curves, CUE = 0·25; $k_{\mathrm{I}}/k_{\mathrm{D}} = 1$]. The critical C : P is the C : P value at which the microbial community switches from C-to-P limitation – that is, the breakpoints of the curves (dots). b) Critical C : P (eqn 1), as a function of $(\mathrm{C:P})_{\mathrm{m}}$, for different values of $k_{\mathrm{I}}/k_{\mathrm{D}}$, where k_{I} and k_{D} are the kinetic constants for inorganic P and organic C uptake, respectively (different dashing styles); CUE values are indicated as in panel a.

and thus, it is difficult to recognize when P limitation occurs and whether microbial communities can adapt to contrasting stoichiometric conditions.

It could be argued that the flexibility of critical ratios reflects an underlying strategy adopted by soil microbial communities to cope with the large variability of plant litter and soil organic matter stoichiometry and thus avoid nutrient limitation. Therefore, it is reasonable to expect that the $\mathrm{C:P_{CR}}$ increases with declining P availability, if microbial communities adapt their stoichiometric traits to alleviate P limitation. However, it could be also argued that the microbial community adapts to abiotic factors other than P limitation and thus their stoichiometric traits do not primarily reflect P limitation. In this case, the $\mathrm{C:P_{CR}}$ would not increase with declining P availability and strong effects of environmental and edaphic conditions could be expected.

The aim of the present study was to investigate the $\mathrm{C:P_{CR}}$ of soil microbial communities and its variability

across ecosystems. We first present a methodological approach to calculate the C : P_{CR}, and then, we assess its variability among 18 soils from a variety of geographic areas and ecosystems and sampled at different depths. We expect the C : P_{CR} to vary widely among soils and generally increase in P-poor soils as a result of microbial community adaptation to P limitation. Microbial community can adapt by change in species composition [change of community level $(C : P)_m$] or physiology (change of CUE or k_I/k_D). We further expect a direct link between C : P_{CR} and $(C : P)_m$ as result of prior community-level $(C : P)_m$ adaptation to P limitation.

Materials and methods

EXPERIMENTAL SET-UP

Soil samples collected from diverse localities and ecosystems were used in the experiment. Basic site and soil characteristics are given in Table S1; detailed methods are described in Appendix S2.

Soils were sieved on 3-mm mesh and stored wet at 4 °C in dark until start of the incubation. Before incubation, soils were conditioned for 1 week at 20 °C in the dark. Soils were then mixed with sterile C-free silica sand (sterilized by heating: 550 °C for 5 h) with grains <2 mm (25 : 1, w/w; sand : soil) to minimize the background effect of the original soil organic C and nutrients. All organic C and nutrients were supplied to the mixture in basal medium. The mixture was moistened to 75% water holding capacity by supplying basal medium (8 g of sucrose, 0·1 g of yeast extract, 0·2 g of $MgSO_4.7H_2O$, 0·02 g of $FeSO_4$, 1 g of NH_4Cl, 0·02 g of CaCl in 500 mL of deionized H_2O; Veldkamp 1970). The amount of basal medium added was 1 mL to 5.2 g of the mixture and the medium concentration was appropriately manipulated to reach the concentration 20 mol of organic C per mol of soil microbial biomass C in all soils. N and other nutrients except for P were in excess. P was supplied in the form of K_2HPO_4 to achieve concentrations from 0 to 5 g P per litre (11 concentration levels for each soil). Samples were incubated in 100 mL vials with oxitop heads on top (WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) for 7 days in the dark at 20 °C in three replicates for each treatment. Oxitop system was set to measure oxygen consumption continuously every 30 min during the whole incubation period.

The microbial growth rate was calculated from oxygen consumption. The slope of increase of O_2 consumption is directly related to microbial community growth rate (Hill & Robinson 1974). For data fitting, the R package GROFIT was employed (Fig. S1; Kahm *et al.* 2010; R Core Team 2014). When the slope of increase was statistically undistinguishable from 0, the microbial community was considered not growing.

MICROBIAL AND SOIL ANALYSIS

Microbial C (Vance, Brookes & Jenkinson 1987), N (Brookes *et al.* 1985) and P (Brookes, Powlson & Jenkinson 1982) were measured in 3 replicates in all soils by the chloroform fumigation extraction method before the start of the incubation. Soil available C, N and P were measured simultaneously with microbial C, N and P as 0·5 M K_2SO_4 extractable C and N and 0·5 M $NaHCO_3$ (pH = 8·5) extractable P. Details on the method are given in Appendix S2.

PHOSPHOLIPID FATTY ACID IDENTIFICATION AND QUANTIFICATION

The determination of phospholipid fatty acid (PLFA) was done in 6 representative soils with the low [Siberia (Ajj) and (BCg)], intermediate [Plesne, Certovo, Siberia (O)] and high (Pop Ivan) C : P_{CR} (see Appendix S2) according to Frostegard, Baath & Tunlid (1993), with minor modifications. Soils were analysed in three replicates at two different stages of sample processing: (i) after a pre-incubation period before mixing with the sand and (ii) after 40 h of soil/sand mixture incubation (at the time of exponential growth of the microbial community). The analysis was done at the P supply value corresponding to incipient P limitation (i.e. at the critical C : P ratio, see Mathematical description). Such sampling design was chosen in order to answer two questions: (i) Does soil manipulation affect soil microbial community structure? (ii) What is the structure of growing microbial community at the critical C-to-P ratio? Details on the method are given in Appendix S2.

MATHEMATICAL DESCRIPTION

The response of microbial community growth rate to the amount of available P in the soil/sand mixture (for more details see Appendix S2) was described by a modified Monod equation,

$$\mu = \frac{V_{MAX} P}{K_M + P} + \mu_0 \qquad \text{eqn 2}$$

where μ is the biomass-specific growth rate of the bulk microbial community (h^{-1}; see also Appendix S2), P is the total amount of available phosphorus in the soil/sand mixture (μmol), K_M is the half saturation constant, and V_{MAX} is the maximum growth rate. In contrast to the classic Monod equation, equation (2) has an additional parameter μ_0 that represents the growth rate at zero P supply in the basal medium and shifts the curve along the y-axis (Fig. S2). When μ_0 is positive, the soil microbial community is able to temporarily grow without P supply, but solely relying on its own P storage compounds – as commonly observed among microbial species (Kulaev, Vagabov & Kulakovskaya 1999; Cotner, Makino & Biddanda 2006; Nikel *et al.* 2013, see below). Negative values mean instead that microbes cannot grow without external P supply and only respire. They do not grow until certain amount of P is provided. The sum of μ_0 and V_{MAX} defines the asymptotic or maximum growth rate attainable by a microbial community at defined substrate-saturated conditions.

Equation (2) predicts a steep increase of growth rate with increasing amount of P followed by a slower approach to the asymptotic value (Fig. S2). These two 'stages' of growth rate response to P amount can be mathematically separated at a critical amount of P, at which the growth rate increase slows down. To calculate this critical P amount (P_{CR}), first the fraction of the asymptotic growth rate after all P storage has been consumed (R) is defined based on equation (2),

$$R = \frac{\mu - \mu_0}{V_{MAX}} \qquad \text{eqn 3}$$

Combining equations (2) and (3) allows us rewriting R as a function of K_M and the P amount:

$$R = \frac{P}{P + K_M} \qquad \text{eqn 4}$$

The critical value of P amount, P_{CR}, is chosen so that $R = ¾$ (three-quarters of asymptotic growth rate), corresponding to the point where the sensitivity of μ to P availability starts decreasing. This is the point that most closely resembles the theoretical critical ratio when C and P limitation switch as described in Appendix S1. Rearranging equation (3), we obtain P_{CR} as a function of K_M,

$$P_{CR} = 3 \times K_M \quad \text{eqn 5}$$

As nitrogen and other nutrients in the growth medium were in excess, the calculated P_{CR} is not affected by other nutrient limitations. Finally, the critical C : P ratio was calculated as the ratio of available organic C in the growth medium and P_{CR}.

STATISTICAL ANALYSIS

The relationship between microbial community stoichiometry and soil stoichiometry was tested using simple linear regression. To evaluate microbial community growth rate response to phosphorus availability, the modified Monod equation was fitted and equation parameters were estimated (with 95% confidence intervals) using nonlinear least square regression for each soil separately. Nonlinear mixed-effect models (using R package NLME; Pinhero *et al.* 2015) were used for further statistical evaluation. First, the null model was defined as nonlinear function of P availability according to modified Monod equation. Equation parameters were allowed to randomly vary across samples. Secondly, the null model was improved by forward selection of the best equation parameters predictors. The best predictors were chosen from all ancillary data we obtained. These included microbial and soil C, N, P, microbial and soil stoichiometric ratios (C : N, C : P, N : P), pH, mean annual temperature and precipitation, latitude, altitude, sampling depth, ecosystem type, land management and microbial community structure.

Because the characterization of the microbial community structure by relative abundance of PLFA markers in the sample lead to an extensive data set, this information was collapsed into vectors using principal components analysis (PCA). We retained the first three principal components and used scores for each soil on these axes as predictor variables in the nonlinear mixed-effect model. Before PCA analysis, data were normalized. Permutational multivariate analysis of variance (using R package VEGAN; Oksanen *et al.* 2015) was further used to check the difference between microbial community structure of each soil before and after manipulation. All statistical analyses were done using the statistical program R (R Core Team 2014).

Results

SOIL AND MICROBIAL COMMUNITY STOICHIOMETRY

The C : N ratio of the soil microbial communities from our 18 samples was between 5·5 to 34·6 (Table S1). The $(C : P)_m$ ranged from 24·7 to 346·6 (Table S1). The stoichiometric ratios of available nutrients were more variable spanning more than one order in magnitude (C : N from 1·0 to 29·5; C : P from 22·8 to 788·7, Table S1). There was no linear relationship between soil and microbial stoichiometry (C : N and C : P, Fig. S3), and the slopes of both relationships were statistically undistinguishable from 0.

MICROBIAL COMMUNITY GROWTH RATE RESPONSE TO PHOSPHORUS

All microbial communities showed a clear trend of increasing microbial community growth rate with increasing amount of available P according to the modified Monod equation (Fig. 2), but parameters were distinctly different among soils (Table S2). Variations of the equation parameters from their mean values are interpreted as indicators of differences among soils. The highest variability occurred in the μ_0 parameter (mean value 0·53, coefficient of variation CV = 187%) and K_M parameter, which defines P_{CR}, showed second highest variability (mean value 1·59, CV = 56%). P_{CR} for individual soils varied from 1·29 to 20·55 μmol (Table S2), resulting in a range of more than one order in magnitude for the critical C : P ratio (Fig. 3; 26·6–465·1). Accounting for uncertainty in K_M estimates using 95% confidence intervals, the range of C : P_{CR} increased (Fig. 3; 20·9–740·7).

MODIFIED MONOD EQUATION PARAMETERS

After quantifying the variability of the parameters in the modified Monod equation, we continued with the null regression model build up (Fig. 2).

μ_0 parameter

Growth rate at zero P concentration in the soil, μ_0, was inversely and significantly correlated with microbial C : P at the beginning of the incubation (Fig. 4a), except for deep soil horizons. This linear relationship was significant only for the topsoil horizons ($F = 31·6$, d.f. = 1, $P < 0·001$); therefore, it did not lead to significant improvement of the whole nonlinear mixed-effect model (LR = 4·16, d.f. = 12, $P = 0·125$) as deep soil horizons fell out of the linear relationship.

Asymptotic growth rate

We found a significant linear relationship between microbial C : N and asymptotic growth rate (Fig. 4b, $F = 8·8$, d.f. = 1, $P = 0·009$). The lower was microbial C : N, and the higher was the asymptotic growth rate. Incorporation of this linear relationship into the mixed-effect model improved the model at 10% level of significance (LR = 2·84, d.f. = 13, $P = 0·092$).

K_M parameter

The half saturation constant, K_M, was driven by microbial community structure of the soil, as assessed by PLFA analysis on 6 representative soil samples. Microbial community structure explained most of the variability in K_M (78%; Fig. 5). The relationship between microbial species composition and K_M was significant ($F = 21·2$, d.f. = 1, $P < 0·001$) and led to significant improvement of the non-

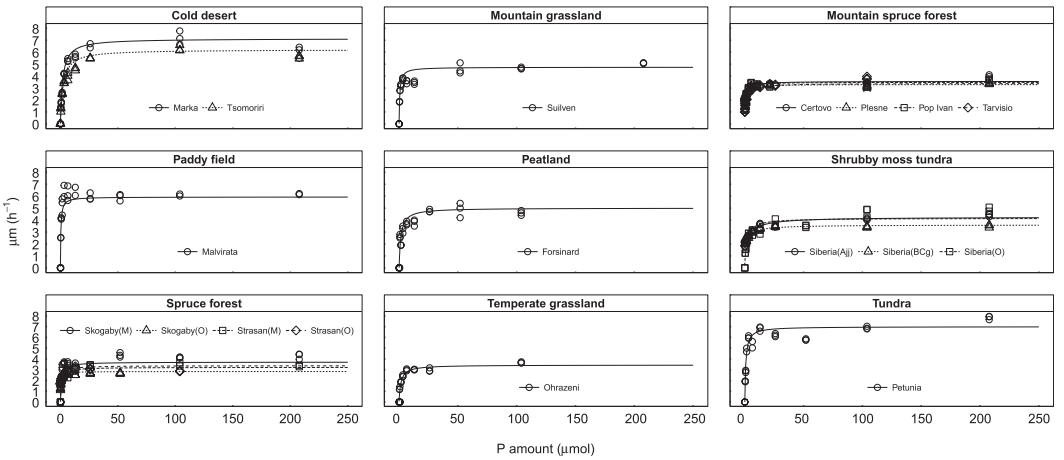

Fig. 2. Growth rate response of microbial communities to the amount of P in our 18 soils. Soils are grouped in the boxes according to ecosystem type. Open symbols represent raw data and curves represent the best fit of the modified Monod equation to the data. When more than one soil per ecosystem type is present, different soils are distinguished by different symbols and line dashing styles.

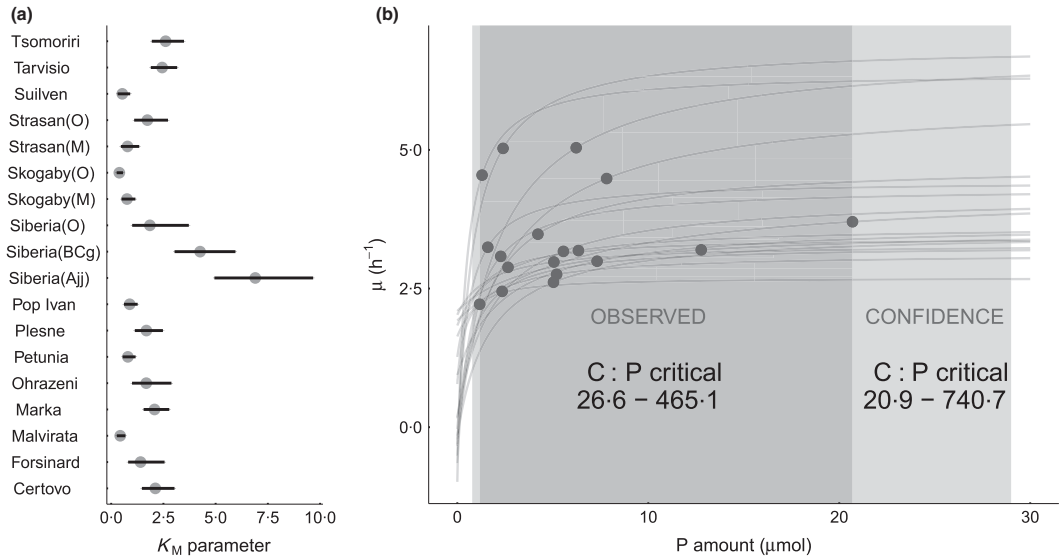

Fig. 3. (a) Estimates of the half saturation constant K_M for the 18 soils with its 95% confidence interval. (b) Mean values of the critical P amount ($P_{CR} = 3 \times K_M$) are plotted as a dots on lines representing the fit of the modified Monod equation to the data. The critical carbon-to-phosphorus ratio ($C : P_{CR}$) is calculated as ratio between the amount of organic C and P_{CR}. Mean and 95% confidence of K_M estimates were used to calculate P_{CR} and $C : P_{CR}$.

linear mixed-effect model (LR = 12·2, d.f. = 14, $P = 0{\cdot}015$).

MICROBIAL PLFA COMPOSITION

Soil manipulation and incubation significantly affected microbial community structure of the original soil (Fig. S4; *pseudo* $F = 194{\cdot}9$, d.f. = 1, $P < 0{\cdot}001$). Microbial community structure of all soils from spruce forest ecosystems (Certovo, Plesne and Pop Ivan), even though geographically distant, changed in the same way (Fig S4). The same was true for Siberia(Ajj) and Siberia(BCg) soil samples, which are next to each other in the soil profile. In spruce forest ecosystem soils, the shift of microbial community structure was mainly characterized by an increase of relative abundance of two fungal markers (18:1n9, 18:2n6,9) and parallel decrease of G+ and G− bacteria-specific markers (a15:0, 16:1n7, cy17 and 18:1n7; Fig. S4).

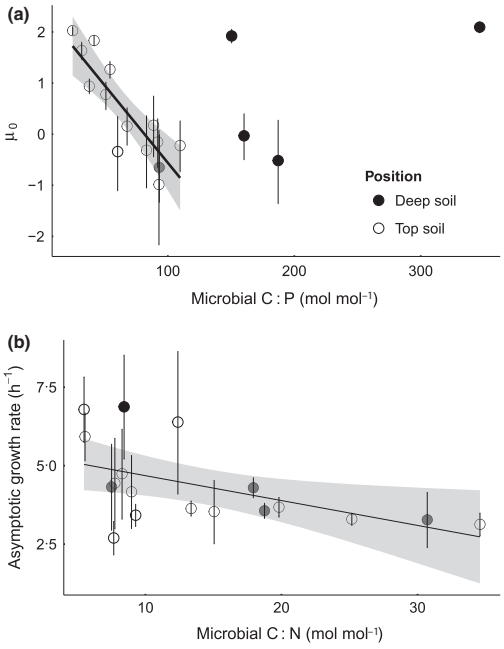

Fig. 4. Negative linear relationship between microbial community C : P and mean estimate of μ_0 parameter (a) and between microbial community C : N and asymptotic growth rate (b). Error bars represent 95% confidence intervals of parameter estimates. Open circles refer to topsoil horizons, whereas black dots refer to deep soil horizons. Solid lines represent linear regression fitted to the topsoil horizon data (a) or to all samples (b). The grey area along the solid lines shows the 95% confidence bands of the linear regression.

The opposite was true for Siberia(Ajj) and Siberia(BCg) soil samples (Fig. S4). However, other G– and G+ bacteria-specific markers (cy19:0 and i16:0) decreased in both Siberian samples as well as actinobacterial and general bacterial markers (10Me16:0 and i15:0). Therefore, the simple measure fungi to bacteria ratio could not characterize the shift of microbial community structure after incubation and thus was a poor predictor of K_M.

Discussion

MODIFIED MONOD EQUATION PARAMETERS

μ_0 parameter

Among our soil samples, some had extremely low microbial community C : P, around or below 30 (Plesne, Strasan, Tarvisio). Those values are lower than the global average, which is according to different studies around 60 (Cleveland & Liptzin 2007), 88 (Mouginot *et al.* 2014) or 42 (Xu, Thornton & Post 2013). The high concentration of P in microbial cells relative to C can be reached either by increase of ribosomes in the cell (Sterner & Elser 2002; Elser *et al.* 2003) or by accumulation of P storage compounds, mainly polyphosphates (Kulaev, Vagabov &

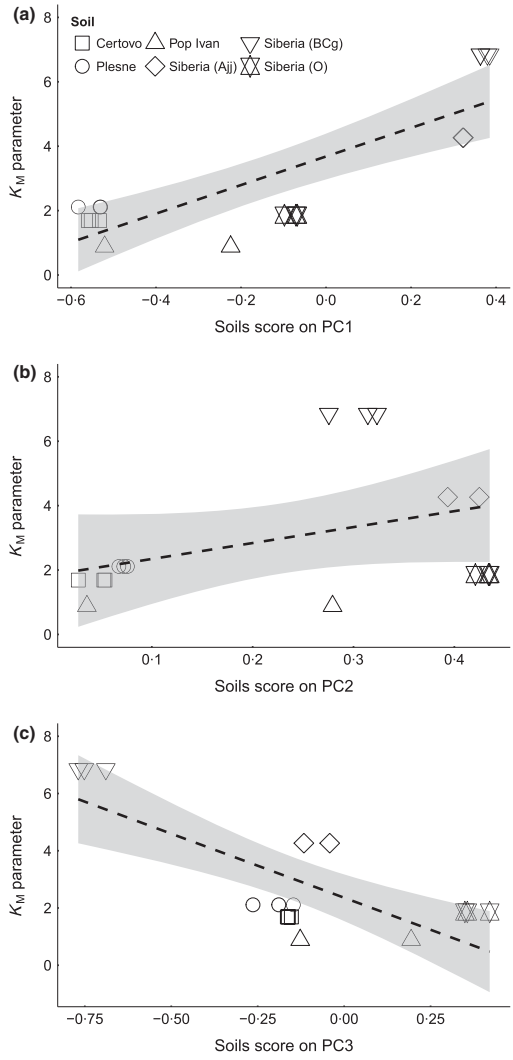

Fig. 5. Relationship between the half saturation constant K_M and principal components 1 (a), 2 (b) and 3 (c), which represent collapsed information from phospholipid fatty acid analysis done on 6 representative soil samples [Certovo, Plesne, Pop Ivan, Siberia (Ajj), Siberia(BCg) and Siberia(O)]. Dashed lines represent the linear relationship between K_M and the score of particular soils on the PC axis. The grey area along the dashed lines shows the 95% confidence bands of the linear relationship.

Kulakovskaya 1999; Cotner, Makino & Biddanda 2006; Nikel *et al.* 2013). While ribosomes can make up to 1·5% of cell dry weight (Elser *et al.* 2003), polyphosphates can make as much as 30% of cell dry weight (Kulaev, Vagabov & Kulakovskaya 1999) and thus might be responsible for observed variation in cellular P concentrations. Polyphosphates serve the microbes as an internal P source when external sources are scarce (Kulaev, Vagabov & Kulakovskaya 1999). The negative linear relationship we found

between microbial community C : P and growth rate at zero P supply (μ_0) across all topsoil horizons (Fig. 4a) confirms this view. It suggests that P reserves stored as polyphosphates were utilized to allow growth of the microbial community in presence of abundant C, but at zero P supply. It might be argued that residual soil P could have also been used by the microbial community at zero P in the basal medium. However, we found no relationship between μ_0 and soil P. Moreover, the dilution rate of our soils by a sterilized sand was high enough to reduce the amount of residual soil P (see Appendix S2).

While the trend of decreasing μ_0 with increasing microbial C : P was strong across all surface soils, the deep soil horizons behaved differently. These soils were characterized by extremely high microbial community C : P ratios, almost two to four times higher than global average (Cleveland & Liptzin 2007; Xu, Thornton & Post 2013; Mouginot *et al.* 2014). However, the same communities also exhibited high μ_0 [especially Siberia(Ajj) – C : P = 150·5, μ_0 = 2·09; Siberia(BCg), C : P = 346·6, μ_0 = 1·92]. The two most extremes – the Siberian deep soil horizons – were taken from depths of 50 and 70 cm, respectively. We suggest that the reason why deep soil horizon falls out of the linear relationship is the high abundance of dormant microbial cells in their microbial community, which decouple microbial community C : P and μ_0:

1. It is documented that the microbial community of deep soil horizons is characterized by high abundance of dormant cells (up to 98%; Blagodatskaya & Kuzyakov 2013), and thus, it is expected that community C : P reflects the C : P of dormant cells. Microbial dormancy is accompanied by unique physiological changes (Herbert 1961; Oliver 1993; Lennon & Jones 2011) that increase cell C : P (Linder & Oliver 1989; Kieft *et al.* 1997; Wang & Bakken 1998; Sterner & Elser 2002; Kadouri *et al.* 2005). Assuming that the C : P of dormant cells is near the highest known level (409; Cleveland & Liptzin 2007), we can infer that the proportion of dormant cells needed to compensate the C : P of active cells [27·2 and 28·7 for Siberia(Ajj) and Siberia(BCg) soils, respectively, using surface values] has to be 87·6% and 96·5% for Siberia(Ajj) and Siberia(BCg) soil, respectively. Such an amount of dormant microbial cells in deep soil horizons is realistic and comparable to previous estimates (Blagodatskaya & Kuzyakov 2013). Thus, we surmise that the linear relationship between microbial C : P and μ_0 is generally valid for active microbial cells, which can store and use polyphosphates.
2. Dormant cells cannot start their reactivation and grow unless carbon and nutrients became available (Blagodatskaya & Kuzyakov 2013). At zero P amount, the only microbial cells that can grow are the already active ones with low C : P (dormant cells with high C : P cannot grow). However, at higher P amounts (and also around the critical P amount), the dormant cells reactivate and thus the growth response to P amount can be explained by the characteristics of the whole community.

These hypothesized mechanisms appear to explain the deviation of deep soil horizons from the behaviour of the top soils, but more data would be needed to properly test the mechanism.

Asymptotic growth rate

According to the modified Monod equation, the asymptotic growth rate is the maximum growth that the microbial community can reach under substrate-saturated conditions. This value was negatively related to microbial community C : N (Fig. 4b), in accordance with empirical evidence (Mouginot *et al.* 2014) and the theoretical predictions of Vrede *et al.* (2004), who showed that the maximum attainable growth rate increases with increasing total amount of catalytic macromolecules in the cell (mainly rRNA, see above) or with increasing ribosome-specific protein synthesis rate. The amount of catalytic macromolecules change cellular C : N or C : P, because they are N and P rich (Elser *et al.* 2003; Vrede *et al.* 2004). The higher the amount of catalytic macromolecules, the lower the cellular C : N and C : P, thus explaining the negative relationship between maximum attainable growth rate and cellular C : N or C : P. In our experiment, the relationship between asymptotic growth rate and microbial community C : N was statistically significant, but that was not the case for $(C : P)_m$. As we discussed above, microbial community C : P was affected by P storage compounds more than by the amount of catalytic macromolecules and thus the relationship was not found. There was also some residual variability in the negative linear relationship between asymptotic growth rate and microbial community C : N, possibly caused by different ribosome-specific protein synthesis rate, which is species specific (Vrede *et al.* 2004).

K_M parameter

The half saturation constant (K_M) was the most important parameter of the modified Monod equation, because it was shown to be proportional to the critical P amount, which in turn determines C : P critical ($C : P_{CR}$). In 6 representative soils, whose $C : P_{CR}$ range from 26·6 to 227·2, nearly 80% of the variability in K_M could be explained by the first three principal components of the PLFA profiles (Fig. 5). The first principal component, which accounts for the most explained variability (66%), was negatively correlated with fungal-specific markers (18:2n6,9, 18:1n9) and positively correlated with four G+ or G− bacteria-specific markers (a15:0, 16:1n7, cy17 and 18:1n7). The first principal component mainly separated spruce forest ecosystem soils (Certovo, Plesne and Pop Ivan) with $C : P_{CR}$ around or above 100, from Siberia(Ajj) and Siberia(BCg) soils with $C : P_{CR}$ below 30 (Fig. 5a). In this respect, our findings are partly in agreement with the general theory (eqn 1), which predicts higher $C : P_{CR}$ of fungi compared to bacteria because of higher fungal C : P and therefore lower demand for P (Cleveland & Liptzin 2007;

Mouginot *et al*. 2014). Nevertheless, we have no direct evidence to support a general relationship between cellular C : P and C : P_{CR} (Fig. S5a). This is in disagreement with our initial hypothesis. There was also no relationship between soil P and C : P_{CR} (Fig. S5b). We argue that $(C : P)_m$, soil P and C : P_{CR} are not necessarily connected, because each of them could be independently and differently affected by edaphic conditions.

1. *Microbial community C : P does not reflect demands of growing cells for P and thus it is not related to C : P_{CR}.* As we showed above, the concentration of P in microbial cells is modified by P storage compounds and it is also very likely affected by the microbial physiological status (active/dormant). Both formation of P storage compounds and dormant structures is not directly connected to growth. Therefore, across these widely different soils, the microbial community C : P ratio *per se* cannot be directly related to C : P_{CR}.
2. *C : P_{CR} is not related to available soil P because microbial communities do not adapt to avoid P limitation.* Usually, P availability is not the main driver of microbial community adaptation. One of the most important drivers is depth because temperature, substrate concentration, moisture, texture, etc. change along with increasing depth (e.g. Gittel *et al*. 2014b; Schnecker *et al*. 2014). Very often, P availability decreases with increasing depth (Kalcik & Santruckova 1994), which was also the case for soils from Siberia, Skogaby and Stråsan (Table S1). With decreasing P availability along increasing depth, C : P_{CR} decrease in Siberian and Skogaby soils, in contrast with our expectation. This unexpected trend could be explained by the fact that abundance of fungi as well as fungi to bacteria ratio generally decrease with increasing depth, independent of P availability (e.g. Gittel *et al*. 2014a,b). With increasing abundance of bacteria with low C : P ratio, C : P_{CR} can decrease with depth (eqn 1).

Our data show that no simple predictor of C : P_{CR} exists. C : P_{CR} is very likely driven by many edaphic factors like substrate availability, P availability, pH, temperature, moisture, etc. and their mutual combinations. These factors modify C : P_{CR} via their effects on soil microbial community structure and physiology (CUE and k_I/k_D in eqn 1). The relative abundance of PLFA is a manifestation of effects of edaphic conditions on soil microbial community structure, explaining why PLFA is the only significant predictor of K_M and thus of C : P_{CR}.

C : P CRITICAL IN ECOSYSTEM MODELS

We showed that C : P_{CR} could vary from 26·6 to 465·1. Accounting for 95% confidence interval of K_M parameter estimate, the limits of C : P_{CR} are even wider – from 20·9 to 740·7. Using constant or narrowly fluctuating C : P_{CR} in ecosystem models is therefore inaccurate. However, implementation of variable C : P_{CR} into models can be problematic at present, because no simple predictor of C : P_{CR} could be found. C : P_{CR} does not reflect ecosystem type, biome, land management, microbial community C : P or P availability, but is related to community structure (Fig. 5). More data would be needed in future, especially from deep soil horizons, to develop the process-based understanding of variations in C : P_{CR} required for P cycle models.

Acknowledgements

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Data accessibility

All data used in this manuscript are present in the manuscript and supporting information.

References

Ågren, G.I. & Bosatta, E. (1998) *Theoretical Ecosystem Ecology. Understanding Element Cycles*. Cambridge University Press, Cambridge, UK.

Ågren, G.I., Hyvonen, R., Berglund, S.L. & Hobbie, S.E. (2013) Estimating the critical N: C from litter decomposition data and its relation to soil organic matter stoichiometry. *Soil Biology and Biochemistry*, **67**, 312–318.

Blagodatskaya, E. & Kuzyakov, Y. (2013) Active microorganisms in soil: critical review of estimation criteria and approaches. *Soil Biology & Biochemistry*, **67**, 192–211.

Boer, V.M., Crutchfield, C.A., Bradley, P.H., Botstein, D. & Rabinowitz, J.D. (2009) Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. *Molecular Biology of the Cell*, **21**, 198–211.

Bosatta, E. & Berendse, F. (1984) Energy or nutrient regulation of decomposition – implications for the mineralization immobilization response to perturbations. *Soil Biology & Biochemistry*, **16**, 63–67.

Brookes, P.C., Powlson, D.S. & Jenkinson, D.S. (1982) Measurement of microbial biomass phosphorus in soil. *Soil Biology & Biochemistry*, **14**, 319–329.

Brookes, P.C., Landman, A., Pruden, G. & Jenkinson, D.S. (1985) Chloroform fumigation and the release of soil-nitrogen - a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biology & Biochemistry*, **17**, 837–842.

Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M. & West, G.B. (2004) Toward a metabolic theory of ecology. *Ecology*, **85**, 1771–1789.

Cleveland, C.C. & Liptzin, D. (2007) C : N : P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry*, **85**, 235–252.

Cotner, J.B., Makino, W. & Biddanda, B.A. (2006) Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microbial Ecology*, **52**, 26–33.

Cross, W.F., Hood, J.M., Benstead, J.P., Huryn, A.D. & Nelson, D. (2015) Interactions between temperature and nutrients across levels of ecological organization. *Global Change Biology*, **21**, 1025–1040.

Elser, J.J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T. *et al*. (2003) Growth rate-stoichiometry couplings in diverse biota. *Ecology Letters*, **6**, 936–943.

Elser, J.J., Fagan, W.F., Kerkhoff, A.J., Swenson, N.G. & Enquist, B.J. (2010) Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. *New Phytologist*, **186**, 593–608.

Enriquez, S., Duarte, C.M. & Sandjensen, K. (1993) Patterns in decomposition rates among photosynthetic organisms - the importance of detritus C-N-P content. *Oecologia*, **94**, 457–471.

Frostegard, A., Baath, E. & Tunlid, A. (1993) Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty-acid analysis. *Soil Biology & Biochemistry*, **25**, 723–730.

Gittel, A., Barta, J., Kohoutova, I., Mikutta, R., Owens, S., Gilbert, J. *et al*. (2014a) Distinct microbial communities associated with buried soils in the Siberian tundra. *ISME Journal*, **8**, 841–853.

Gittel, A., Bárta, J., Kohoutová, I., Schnecker, J., Wild, B., Capek, P. *et al*. (2014b) Site- and horizon-specific patterns of microbial community

structure and enzyme activities in permafrost-affected soils of Greenland. *Frontiers in Microbiology*, **5**, 541.
Hall, E.K., Maixner, F., Franklin, O., Daims, H., Richter, A. & Battin, T. (2010) Linking microbial and ecosystem ecology using ecological stoichiometry: a synthesis of conceptual and empirical approaches. *Ecosystems*, **14**, 261–273.
Herbert, D. (1961) The chemical composition of microorganisms as a function of their environment. *Microbial Reaction to Environment: Eleventh Symposium of the Society for General Microbiology* (ed. H. Gooder), pp. 426. Cambridge University Press, Cambridge, UK.
Hill, G.A. & Robinson, C.W. (1974) Measurement of aerobic batch culture maxim specific growth-rate and respiration coefficient using a dissolved-oxygen probeS. *Biotechnology and Bioengineering*, **16**, 531–538.
Kadouri, D., Jurkevitch, E., Okon, Y. & Castro-Sowinski, S. (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Critical Reviews in Microbiology*, **31**, 55–67.
Kahm, M., Hasenbrink, G., Lichtenberg-Frate, H., Ludwig, J. & Kschischo, M. (2010) grofit: Fitting Biological Growth Curves with R. *Journal of Statistical Software*, **33**, 1–21.
Kalcik, J. & Santruckova, H. (1994) Profile distribution of the forms of soil-phosphorus in dependence on the degree of anthropogenic effect. *Rostlinna Vyroba*, **40**, 305–314.
Kieft, T.L., Wilch, E., Oconnor, K., Ringelberg, D.B. & White, D.C. (1997) Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. *Applied and Environmental Microbiology*, **63**, 1531–1542.
Kulaev, I., Vagabov, V. & Kulakovskaya, T. (1999) New aspects of inorganic polyphosphate metabolism and function. *Journal of Bioscience and Bioengineering*, **88**, 111–129.
Lennon, J.T. & Jones, S.E. (2011) Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology*, **9**, 119–130.
Linder, K. & Oliver, J.D. (1989) Membrane fatty-acid and virulence changes in th viable but nonculturable state of vibrio-vulnificus. *Applied and Environmental Microbiology*, **55**, 2837–2842.
Makino, W., Cotner, J.B., Sterner, R.W. & Elser, J.J. (2003) Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C : N : P stoichiometry. *Functional Ecology*, **17**, 121–130.
Manzoni, S. & Porporato, A. (2009) Soil carbon and nitrogen mineralization: theory and models across scales. *Soil Biology and Biochemistry*, **41**, 1355–1379.
Manzoni, S., Trofymow, J.A., Jackson, R.B. & Porporato, A. (2010) Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecological Monographs*, **80**, 89–106.
McGill, W.B. & Cole, C.V. (1981) Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma*, **26**, 267–286.
Moore, T.R., Trofymow, J.A., Prescott, C.E., Fyles, J. & Titus, B.D. (2006) Patterns of carbon, nitrogen and phosphorus dynamics in decomposing foliar litter in Canadian forests. *Ecosystems*, **9**, 46–62.
Mouginot, C., Kawamura, R., Matulich, K.L., Berlemont, R., Allison, S.D., Amend, A.S. *et al.* (2014) Elemental stoichiometry of fungi and bacteria strains from grassland leaf litter. *Soil Biology & Biochemistry*, **76**, 278–285.
Nikel, P.I., Chavarria, M., Martinez-Garcia, E., Taylor, A.C. & de Lorenzo, V. (2013) Accumulation of inorganic polyphosphate enables stress endurance and catalytic vigour in *Pseudomonas putida* KT2440. *Microbial Cell Factories*, **12**, 1–14.
Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H. & Wagner, H. (2015) vegan: Community Ecology Package. R package version 2.3-2, https://CRAN.R-project.org/package = vegan.
Oliver, J.D. (1993) Formation of viable but nonculturable cells. *Starvation in Bacteria* (ed. K. Staffan), pp. 277. Plenum Press, New York, NY.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2015) nlme: Linear and nonlinear mixed effects models. R package version 3.1-122, http://CRAN.R-project.org/package = nlme.
R Core Team (2014) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Schade, J.D., Espeleta, J.F., Klausmeier, C.A., McGroddy, M.E., Thomas, S.A. & Zhang, L.X. (2005) A conceptual framework for ecosystem stoichiometry: balancing resource supply and demand. *Oikos*, **109**, 40–51.
Schmidt, K.S. (1992) Models for studying the population ecology of microorganisms in natural systems. *Modeling the Metabolic and Physiologic Activities of Microorganisms* (ed. J.C. Hurst), pp. 31–59. John Wiley & Sons, Inc., New York, NY, USA.
Schnecker, J., Wild, B., Hofhansl, F., Alves, R.J.E., Barta, J., Capek, P. *et al.* (2014) Effects of soil organic matter properties and microbial community composition on enzyme activities in cryoturbated arctic soils. *PLoS ONE*, **9**, 1–10.
Sinsabaugh, R.L., Shah, J.J.F. & Follstad Shah, J.J. (2012) Ecoenzymatic stoichiometry and ecological theory. *Annual Review of Ecology, Evolution, and Systematics*, **43**, 313–343.
Sinsabaugh, R.L., Manzoni, S., Moorhead, D.L., Richter, A. & Elser, J. (2013) Carbon use efficiency of microbial communities: stoichiometry, methodology and modelling. *Ecology Letters*, **16**, 930–939.
Sterner, R.W. & Elser, J.J. (2002) *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton University Press, Princeton, NJ, USA.
Vance, E.D., Brookes, P.C. & Jenkinson, D.S. (1987) An extraction method for measuring soil microbial biomass-C. *Soil Biology & Biochemistry*, **19**, 703–707.
Veldkamp, H. (1970) Enrichment cultures of prokaryotic organisms. *Methods in Microbiology* (eds J.R. Norris & D.W. Ribbons), pp. 305–355. Academic Press, London & New York, NY, USA.
Vrede, T., Dobberfuhl, D.R., Kooijman, S. & Elser, J.J. (2004) Fundamental connections among organism C : N : P stoichiometry, macromolecular composition, and growth. *Ecology*, **85**, 1217–1229.
Wang, J.G. & Bakken, L.R. (1998) Screening of soil bacteria for poly-beta-hydroxybutyric acid production and its role in the survival of starvation. *Microbial Ecology*, **35**, 94–101.
Xu, X.F., Thornton, P.E. & Post, W.M. (2013) A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. *Global Ecology and Biogeography*, **22**, 737–749.
Zechmeister-Boltenstern, S., Keiblinger, K.M., Mooshammer, M., Penuelas, J., Richter, A., Sardans, J. *et al.* (2015) The application of ecological stoichiometry to plant-microbial-soil organic matter transformations. *Ecological Monographs*, **85**, 133–155.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Oxygen consumption over time.
Fig. S2. Modified Monod kinetic of microbial community growth rate response to amount of available P.
Fig. S3. Relationship between soil microbial community and soil C : N (A) and C : P (B).
Fig. S4. (A) Ordination of 6 representative soil samples [Certovo, Plesne, Pop Ivan, Siberia(Ajj), Siberia(BCg) and Siberia(O)]. Data include all PLFA markers described in Materials and Methods section. Different open symbols represent different soils. Grey symbols represent samples before soil manipulation and incubation and black symbols represent samples after soil manipulation and incubation. (B) Ordination of PLFA markers with highest loadings on principal component 1 and 2. Other PLFA markers were removed from the ordination to avoid over plotting.
Fig. S5. Plots of critical C : P against microbial biomass C : P (A) or soil available P (B).
Table S1. Basic characteristics of soil samples used in this study.
Table S2. Modified Michaelis-Menten equation parameters estimated for 18 soils. Mean values and 95% confidence intervals is given.
Appendix S1. Critical C to P ratio calculations.
Appendix S2. Experimental settings, Materials and methods.

Supplementary information

Appendix S1 - Critical C to P ratio calculations

In this Appendix we show how the critical C:P ratio, above which P limitation starts, can be linked to microbial traits, with a focus on the specific experimental setup presented here.

Let us consider an organic substrate with a given C:P ratio, $(C:P)_s$, a pool of bio-available inorganic P, P_i, and a microbial community with a chemical composition $(C:P)_m$ and a C-use efficiency CUE. Because P mineralization takes place outside the microbial cells thanks to extracellular phosphatases (McGill & Cole 1981), no organic P can be directly assimilated, and all P from organic matter decomposition is transferred to P_i before it is consumed by microbes. Therefore, the stoichiometric demand for inorganic P of a homeostatic microbial community is defined as:

$$\Phi_P = D\frac{CUE}{(C:P)_m}, \qquad [A1]$$

where D is the C-limited organic matter decomposition rate (in C-flux units, e.g., mol C/kg soil/day) and the product $D \times CUE$ represents the microbial growth rate. For simplicity, the flux D is described by a multiplicative model (Manzoni & Porporato 2009),

$$D = k_D C_s C_m, \qquad [A2]$$

where C_s and C_m indicate the sizes of the substrate and microbial C pools, respectively, and k_D is a kinetic constant.

The microbial P demand can be met by immobilization from P_i if this pool is replenished by mineralization (at a rate $D/(C:P)_s$) or other amendments. When enough P can be supplied, immobilization from P_I exactly matches the P demand and the microbial community grows at a C-limited rate $D \times CUE$. In contrast, when the concentration of inorganic P is low, immobilization becomes a function of P_i and the demand cannot be met, resulting in P-limited microbial growth. In this case, in analogy to Equation [A2], immobilization can be described by a multiplicative model of P_i and C_m, with kinetic constant k_I.

These two regimes can be incorporated in a single equation for the P immobilization rate,

$$I_P = \min\left[k_I P_i C_m, k_D C_s C_m \frac{CUE}{(C:P)_m}\right]. \quad [A3]$$

The first term in Equation [A3] represents the P immobilization rate when inorganic P is limiting, whereas the second term represents the stoichiometric P demand that guarantees a C-limited growth.

The microbial growth rate per unit biomass can be obtained by converting the P flux in Equation [A3] to microbial C using $(C:P)_m$, and dividing by C_m,

$$\mu = \min[k_I P_i (C:P)_m, k_D C_s\, CUE]. \quad [A4]$$

According to Equation [A4], the growth rate first increases linearly with P_i (P-limited regime) and then transitions to a constant value that only depends on organic C availability and CUE (C-limited regime). The growth rate normalized by the rate of uptake of C substrates is shown in Figure 1A (main text), as a function of the ratio C_s/P_i. Mathematically, this normalized growth rate is given by the function $\min\left[\frac{k_I}{k_D}\frac{P_i}{C_s}(C:P)_m, CUE\right]$, where the C-limited regime is characterized by the constant CUE and the P-limited regime is an inverse function of C_s/P_i.

The C_s/P_i ratio at which the two terms of Equation [A4] are equal defines the transition point from C- to P-limitation (Equation [1] and dots in Figure 1 in the main text),

$$\frac{C_s}{P_i} = \frac{k_I}{k_D}\frac{(C:P)_m}{CUE}. \quad [A5]$$

Despite the simplifications, Equation [A4] is conceptually comparable with the measured microbial growth-P_i curves (Section "Mathematical Description" in the main text) and can thus be used to interpret them. In the proposed experiment, C_s is set by providing abundant labile C to the medium, whereas P_i is varied (i.e., all inorganic P is supplied externally and not through organic substrates), and microbial growth curves as a function of P_i are obtained. The critical C_s/P_i is estimated at the transition between the near-linear behavior

and the asymptotic growth rate value, and is thus conceptually equivalent to Equation [4]. Therefore, even if the parameters k_D and k_I cannot be estimated with this method, Equation [1] provides a useful conceptual model to generate hypotheses to explain patterns in the measured critical C_s/P_i ratios.

References

Manzoni, S. & Porporato, A. (2009) Soil carbon and nitrogen mineralization: Theory and models across scales. *Soil Biology & Biochemistry,* **41,** 1355-1379.

McGill, W.B. & Cole, C.V. (1981) Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma,* **26,** 267-286.

Appendix S2 - Experimental settings, Materials and Methods

In appendix 2 we include details about experimental design and explain units used in the main text. We also append Supporting figures that illustrate our complete dataset.

Soil samples

Soils samples used in our study represent a wide range of environmental conditions including extreme ones in terms of climate and land management. Here we include detailed description of sampling sites and horizons. Sampling sites Tsomoriri and Marka are located in Ladakh, Jammu & Kashmir State, India. The area is a part of the Trans-Himalaya, being delimited by the Eastern Karakoram Range in the north and by the Great Himalaya Range in the south. The sites are extremely arid due to its position in the rain-shadow of the Himalaya Range (average annual precipitation100 mm). Because of their high elevation (5200 and 5300 m a.s.l. for Marka and Tsomoriri respectively), almost no vegetation is present (Dvorsky *et al.* 2013) and the soil microbial community is only present in biological soil crusts (Bowker *et al.* 2014).

The soil Ajj horizon sample from the Turbic Cryosol (Siberia (Ajj)) is a pocket of organic material subducted by cryogenic process into the mineral soil. Recent data indicate that cryoturbated organic horizons have distinctly

different microbial community composition and enzyme activities from the regular soil horizons (Siberia (O) and (Bcg); Harden *et al.* 1992; Gittel *et al.* 2014; Schnecker *et al.* 2014), which presumably retards decomposition rate, leading to high age of cryoturbated organic horizons (Kaiser *et al.* 2007; Wild *et al.* 2013). Soil samples from Marka, Tsomoriri and the Ajj horizon of Siberian Turbic Cryosol represent the most extreme of our soil samples in terms of climatic and edaphic conditions.

In terms of land management, our dataset includes pristine sampling sites with no intervention regime (Marka, Tsomoriri, Petunia, Suilven, Plesne, Pop Ivan), semi-natural sampling sites with intervention regime (Certovo, Forsinard, Strasan), and sampling sites with extensive (Ohrazeni, Skogaby) and intensive agriculture (Malvirata) or forestry (Tarvisio).

Plesne and Certovo sampling sites represent special case in regards to land management and P cycling. Both sites have similar vegetation, elevation, mean annual temperature and precipitation (Tab. 1), but different land management. Both sites underwent bark beetle outbreaks. The Plesne site is not managed, whereas the Certovo site is managed. Therefore, tree-level dieback occurred on Plesne site, but not on Certovo site, where attacked trees were cut and removed (Kopacek *et al.* 2015). That let to dissimilar changes in soil chemistry and biological activity (Kaňa, Tahovská & Kopáček 2012; Kaňa *et al.* 2015). Both sites are of special importance for this work because recent studies suggest unknown biological controls over P leaching from these sites (Tahovská *et al. in press*; Kopacek *et al.* 2015).

The latitudinal gradient of spruce forest ecosystems Plesne – Certovo – Skogaby – Stråsan represents a trend of decreasing atmospheric N deposition (Rappe-George *et al. in prep.*). While the Strasan site received very low atmospheric N deposition, Plesne and Certovo sites were heavily polluted and their soils are considered to be N saturated. The

Ohrazeni sampling site is a managed oligotrophic meadow, which is mowed annually (Lepš 2014). This site represents the extensive agriculture type of land management. The intensive agriculture type of management is represented by the two sampling sites Malvirata and Tarvisio. The first one is a paddy field with continuous rice plantation (one harvest per year, no winter cover crops, regularly fertilized) and the second one is a managed spruce forest undergoing regular clear cutting.

Experimental set-up

Appendix 1 highlighted the importance of microbial biomass C as driver of both degradation of the organic C substrate and immobilization of inorganic P (C_m, equations [A2] – [A4]). To compare the different soils, it is essential to have the same C_m in all tested samples, but natural variation in C_m is large among soils (Tab. 1). Thus, soils were manipulated to achieve the same C_m in the final samples used for incubation. To this aim, all soils were mixed with sterile sand in the same ratio of 25:1 (w/w; sand:soil), but the amount of the sand-soil mixture that was incubated was chosen in proportion to C_m of the original soil. As a result, each incubated sample (soil-sand mixture of variable amount) contained 35 µmol of C_m at the beginning of incubation. Because of this procedure, we had to adjust amount and concentration of basal medium accordingly. Basal medium was added in volume to achieve 75% WHC of the incubated mixture (to avoid water limitation). The concentration of basal medium had to be appropriately manipulated to add organic C equal to 20 times C_m, which is 700 µmol. In this way, we achieved precisely defined conditions, which are microbial biomass specific. Thus, the growth rate and P amount are microbial biomass-specific. The measured growth rate therefore represents the growth rate of the whole microbial community and the P amount represents all available P supplied in the growth medium, which is evenly distributed in the sample.

The high dilution of soil with sterile sand was done in order to minimize the background effect of soil organic C and nutrients, which naturally differ among soils. For example, soil residual P was on average 150 times lower than P_{CR} across all soil samples. Similarly, residual soil organic C was on average 80 times lower than added amount of organic C in basal medium. We did not account for residual soil C, N or P in our calculations. First of all, it is uncertain whether all K_2SO_4 extractable C, N, and $NaHCO_3$ extractable P are directly available to the microbial community, but even if they were available, their amount in the final sand/soil mixture was negligibly small.

Microbial and soil analysis

Microbial biomass C (C_m) and N (N_m) were estimated as follows: 5g of naturally wet soil was weighted in 100 ml bottles. Half of them were fumigated for 24 hours in the dark. Fumigated and unfumigated soil samples were shaken with 20 ml of 0.5 M K_2SO_4 for 30 min at room temperature and 170 rot./min. Extracts were centrifuged for 10 min at 3000 g and filtered through glass filters

(MN GF-5, Macherey-nagel, Germany). The aliquots were kept frozen until measurement. The concentration of dissolved organic C and dissolved total N in the aliquots was measured with a DOC/DN analyzer (LiquicTOC II, Elemantar, Germany). Microbial biomass C and N were respectively calculated as differences between dissolved organic C and dissolved total N extracted from fumigated and non-fumigated samples. Correction factors of 0.35 and 0.4 were used for microbial biomass C and N calculation, respectively (Brookes *et al.* 1985; Vance, Brookes & Jenkinson 1987).

For microbial biomass P (P_m) determination, 2g of fumigated and unfumigated soil was shaken with 30 ml of $NaHCO_3$ (pH=8.5) for 45 min at room temperature and 170 rot./min. After centrifugation, extracts were acidified and filtered. The aliquots were frozen until measurement. P concentration in the aliquots was measured spectrophotometrically using a method modified after Murphy and Riley (1962). Microbial biomass P was calculated as a difference between P extracted from fumigated and non-fumigated samples with a correction factor of 0.4 (Brookes, Powlson & Jenkinson 1982).

The concentrations of C, N and P in non-fumigated samples represents available soil C, N and P.

Phospholipid fatty acid (PLFA) identification and quantification

Briefly, an appropriate amount of soil or soil/sand mixture containing approx. 100 µmol of microbial C was extracted twice with one-phase extraction mixture consisting of chloroform, methanol and citrate buffer. After phase separation (overnight), the organic phase was purified on silica columns (SPE-SI Supleclean 500mg/3 mL; Supelco, USA) using chloroform, acetone and methanol. The polar (methanol) fraction containing phospholipids was trans-esterified to the fatty acid methyl esters (FAME) by a mild alkaline methanolysis (Bossio & Scow 1998). The following fatty acids were used to describe the microbial community composition: a14:0, i15:0, a15:0, i16:0, i17:0, a17:0, 17:1n9, i18:0 as markers of Gram-positive bacteria (*G+*); 16:1n11, 16:1n9, 16:1n7, 16:1n5, cy17:0, 18:1n7, cy19:0 as markers of Gram-negative bacteria (*G-*) (Kaiser *et al.* 2010); 13Me:16, 10Me16:0, 10Me17:0, 10Me18:0 as markers of *Actinobacteria* (Kroppenstedt 1985) and 18:1n9, 18:2n6,9 as markers of fungi (Frostegard & Baath 1996). Total bacterial biomass was calculated as a sum of general bacterial markers 15:0, 17:0, 18:1n11 and markers for *G+* and *G-* bacteria. The PLFAs 14:0, 16:0, 18:0 and 20:0 were considered as nonspecific markers (Kaiser *et al.* 2010). All PLFAs were

quantified by an internal standard calibration procedure using methyl-nonadecanoate (19:0) as an internal standard. To identify the FAMEs, retention times and mass spectra were compared with those obtained from standards (Bacterial Acid Methyl Esters standard, the 37-component FAME Mix, PUFA-2, and PUFA-3; all standards purchased from Supelco, USA). The ISQ instrument equipped with Focus gas chromatograph (Thermo Fisher Scientific, USA) was used for detection and chromatographic separation.

References

Bossio, D.A. & Scow, K.M. (1998) Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. *Microbial Ecology*, **35**, 265–278.

Bowker, M.A., Maestre, F.T., Eldridge, D., Belnap, J., Castillo-Monroy, A., Escolar, C. & Soliveres, S. (2014) Biological soil crusts (biocrusts) as a model system in community, landscape and ecosystem ecology. *Biodiversity and Conservation*, **23**, 1619–1637.

Brookes, P.C., Landman, A., Pruden, G. & Jenkinson, D.S. (1985) Chloroformfumigation and the release of soil-nitrogen - a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biology & Biochemistry*, **17**, 837–842.

Brookes, P.C., Powlson, D.S. & Jenkinson, D.S. (1982) Measurementof microbial biomass phosphorus in soil. *Soil Biology & Biochemistry*, **14**, 319–329.

Dvorsky, M., Dolezal, J., Kopecky, M., Chlumska, Z., Janatkova, K., Altman, J., de Bello, F. & Rehakova, K. (2013) Testing the Stress-Gradient Hypothesis at the Roof of the World: Effects of the Cushion Plant Thylacospermum caespitosum on Species Assemblages. *Plos One*, **8**.

Frostegard, A. & Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biology and Fertility of Soils*, **22**, 59–65.

Gittel, A., Barta, J., Kohoutova, I., Mikutta, R., Owens, S., Gilbert, J., Schnecker, J., Wild, B., Hannisdal, B., Maerz, J., Lashchinskiy, N., Capek, P., Santruckova, H., Gentsch, N., Shibistova, O., Guggenberger, G., Richter, A., Torsvik, V.L., Schleper, C. & Urich, T. (2014) Distinct microbial communities associated with buried soils in the Siberian tundra. *Isme Journal*, **8**, 841–853.

Harden, J.W., Sundquist, E.T., Stallard, R.F. & Mark, R.K. (1992) DYNAMICS OF SOIL CARBON DURING DEGLACIATION OF THE LAURENTIDE ICE-SHEET. *Science*, **258**, 1921–1924.

Kaiser, C., Koranda, M., Kitzler, B., Fuchslueger, L., Schnecker, J., Schweiger, P., Rasche, F., Zechmeister-Boltenstern, S., Sessitsch, A. & Richter, A. (2010)

Belowground carbon allocation by trees drives seasonal patterns of extracellular enzyme activities by altering microbial community composition in a beech forest soil. *The New phytologist*, **187**, 843–58.

Kaiser, C., Meyer, H., Biasi, C., Rusalimova, O., Barsukov, P. & Richter, A. (2007) Conservation of soil organic matter through cryoturbation in arctic soils in Siberia. *Journal of Geophysical Research*, **112**.

Kaňa, J., Tahovská, K. & Kopáček, J. (2012) Response of soil chemistry to forest dieback after bark beetle infestation. *Biogeochemistry*, **113**, 369–383.

Kaňa, J., Tahovská, K., Kopáček, J. & Šantrůčková, H. (2015) Excess of Organic Carbon in Mountain Spruce Forest Soils after Bark Beetle Outbreak Altered Microbial N Transformations and Mitigated N-Saturation. *PloS one*, **10**, e0134165.

Kopacek, J., Cudlin, P., Fluksova, H., Kana, J., Picek, T., Santruckova, H., Svoboda, M. & Vanek, D. (2015) Dynamics and composition of litterfall in an unmanaged Norway spruce (Picea abies) forest after bark-beetle outbreak. *BOREAL ENVIRONMENT RESEARCH*, **20**, 305–323.

Kroppenstedt, R.M. (1985) Fatty acid and menaquinone analysis of actinomycetes and related organisms. *Chemical methods in bacterial systematics* (eds M. Goodfellow & D. Minikin), pp. 173 – 194. Academic Press, London.

Lepš, J. (2014) Scale- and time-dependent effects of fertilization, mowing and dominant removal on a grassland community during a 15-year experiment (ed S Wan). *Journal of Applied Ecology*, **51**, 978–987.

Murphy, J. & Riley, J.P. (1962) A modified single solution method for determination of phosphate in natural waters. *Analytica Chimica Acta*, **26**, 31–&.

Rappe-George, M.O., Choma, M., Capek, P., Börjesson, G., Kastovska, E., Santruckova, H. & Gärdenäs, A.I. Microbial variables in Norway spruce forest organic horizons as affected by long-term nitrogen addition and deposition.

Schnecker, J., Wild, B., Hofhansl, F., Alves, R.J.E., Barta, J., Capek, P., Fuchslueger, L., Gentsch, N., Gittel, A., Guggenberger, G., Hofer, A., Kienzl, S., Knoltsch, A., Lashchinskiy, N., Mikutta, R., Santruckova, H., Shibistova, O., Takriti, M., Urich, T., Weltin, G. & Richter, A. (2014) Effects of Soil Organic Matter Properties and Microbial Community Composition on Enzyme Activities in Cryoturbated Arctic Soils. *Plos One*, **9**.

Tahovská, K., Čapek, P., Kaňa, J., Kopáček, J. & Šantrůčková, H. Measurement of in situ phosphorus availability in acidified soils using iron infused resin. *Communications in Soil Science and Plant Analysis*.

Vance, E.D., Brookes, P.C. & Jenkinson, D.S. (1987) Anextraction method for measuring soil microbial biomass-C. *Soil Biology & Biochemistry*, **19**, 703–707.

Wild, B., Schnecker, J., Bárta, J., Čapek, P., Guggenberger, G., Hofhansl, F., Kaiser, C., Lashchinsky, N., Mikutta, R., Mooshammer, M., Šantrůčková, H., Shibistova, O., Urich, T., Zimov, S.A. & Richter, A. (2013) Nitrogen dynamics in Turbic Cryosols from Siberia and Greenland. *Soil Biology and Biochemistry*, **67**, 85–93.

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Curriculum Vitae

Personal information

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Date of birth	27 April 1984
University address	Department of Ecosystem Biology, Faculty of Science, University of South Bohemia Branišovská 1760, CZ – 370 05 České Budějovice Czech Republic

Education and Training

2009-present: Ph.D. program, Department of Ecosystem Biology, Faculty of Science, University of South Bohemia in České Budějovice (2009-2010 – "Degradation of MUSK compounds in a constructed wetland"; 2011-present – "Various aspects of soil microbial ecology as revealed by phospholipid fatty acid (PLFA) analysis", supervisor: Mgr. Eva Kaštovská, Ph.D)

2013: defence of a rigorous thesis

2007-2009: Master of Biology (Mgr.), Department of Ecosystem Biology, Faculty of Science, University of South Bohemia in České Budějovice, student of Ecosystem Biology (supervisor: Prof. Ing. Jan Tříska, CSc.)

2003-2007: Bachelor of Biology (Bc.), Department of Ecosystem Biology, Faculty of Science, University of South Bohemia in České Budějovice, student of Environmental Management (supervisor: Doc. Ing. Josef Hejzlar, CSc.)

Scientific Focus

Ecology of soil microorganisms, plant-microbial-soil interactions, PLFA and metabolite profiling.

Work and science experience

2017-present: co-investigator on GAČR project "Linking functional traits of three organism levels as driving mechanisms of ecosystem functions in the Arctic" (project no: 17-20839S)

2016-present: co-investigator on GAČR project "C:N stoichiometry in plant-soil interactions: effects on plant metabolism and processes in the rhizosphere (project no: 31-16-21743S)

2015-present: part-time researcher, University of South Bohemia, faculty of Science, Institute for Chemistry and Biochemistry

2015: 2 weeks internship on US Geological Survey, Denver, Colorado, USA

2013: 2 weeks internship on Centre for Ecological Research and Forestry Application (CREAF), Barcelona, Spain

2013: 6 weeks residency on Department of Terrestrial Ecosystem Research, University of Vienna, Austria

2012: Member of field works conducted by the Centre for Polar ecology on Svalbard, Faculty of Science, University of South Bohemia

2009-2017: part-time researcher, Global Change Research Institute, Laboratory of metabolomics and isotope analyses

Other scientific skills: use of stable isotopes in biological research, extensive experience with gas chromatography coupled to FID, ECD, and various MS detectors, use of MALDI-TOF analysis in proteomics and microbial biotyping

International courses
2013: Soil-Water-Plants Interactions, University of Life Sciences, Tartu, Estonia
2012: Polar Ecology – field course, Svalbard
2011: Stable isotope course (Freising, Germany)
2011: Spectroscopy: Analytical methods and mass spectrometry

Personal skills and competences

Mother tongue:	Czech
Other languages:	English – B2-C1
	German – A2

Other skills and competences
Driving licence holder (B), experienced driver

Publications in peer-reviewed scientific journals
Shaliutina-Kolešová, A., **Kotas, P.**, Štěrba, J., Rodina, M., Dzyuba, B., Cosson, J., Linhart, O. 2016. Protein Profile of Seminal Plasma and Functionality of Spermatozoa During the Reproductive Season in Common Carp Cyprinus

carpio and Rainbow trout Oncorhynchus mykiss. Molecular Reproduction and Development 83, 968–982. (IF = 2.32)

Vesely, M., Vajglová, Z., **Kotas, P.**, Křišťál, J., Ponec, R., Jiřičný, V. 2015. Model for photodegradation of polybrominated diphenyl ethers. Environ Sci Pollut Res 22, 4949–4963. (IF = 2.76)

Šámek, O., Jonáš, A., Pilát, Z., Zemánek, P., Nedbal, L., Tříska, J., **Kotas, P.**, Trtílek, M. 2010. Raman Microspectroscopy of Individual Algal Cells: Sensing Unsaturation of Storage Lipids in vivo. Sensors 10, 8635–8651. (IF = 1.78)

Participation in international conferences

Kotas, P., Kaštovská, E.: The effect of foliar N fertilization on the root exudation pattern of two plant species with different exploitation strategy. Biogeomon 2017. Litomyšl, Czech Republic. (poster)

Kotas, P., Kaštovská, E.: Affects N fertilization intensity and composition of root exudation from two plant species differing in their exploitation strategy? EGU 2017 - European Geosciences Union General Assembly 2017, Vienna, Austria. (poster)

Kotas, P., Kaštovská, E., Šantrůčková, H., Elster, J.: Spatial variation and general patterns of soil microbial community structure across altitudinal gradients in Billefjorden, Central Svalbard, 1st Global Soil Biodiversity Conference 2014, Dijon, France. (poster)

Čapek, P., **Kotas, P**., Manzoni, S., Šantrůčková, H.: What is the optimal carbon to phosphorus resource ratio of soil microbial community demand? Biogeomon 2014, University of Bayereuth, Germany. (poster)

Hehenberger, A., **Kotas, P.**, Tříska, J.: Enolisation-silylation reaction of selected steroids. CECE conference 2014, Brno, Czech Republic. (poster)

Kotas, P., Elster,J., Tříska, J.: The soil microbial community structure along altitudinal gradients in arctic mountains surrounding Petuniabukta, Svalbard. Polar Ecology Conference 2012, České Budějovice, Czech Republic. (poster)

Special Awards

3rd place in "Best poster presentation", Biogeomon 2017. Litomyšl, Czech Republic.

Other professional activities

Member of the Czech Society for Mass Spectrometry
Supervisor specialist - 3 bachelor thesis and 2 master thesis

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