ECOLOGICAL PROCESSES

Decoupling of microbial carbon, nitrogen, and phosphorus cycling in response to extreme temperature events

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Predicted changes in the intensity and frequency of climate extremes urge a better mechanistic understanding of the stress response of microbially mediated carbon (C) and nutrient cycling processes. We analyzed the resistance and resilience of microbial C, nitrogen (N), and phosphorus (P) cycling processes and microbial community composition in decomposing plant litter to transient, but severe, temperature disturbances, namely, freeze-thaw and heat. Disturbances led temporarily to a more rapid cycling of C and N but caused a down-regulation of P cycling. In contrast to the fast recovery of the initially stimulated C and N processes, we found a slow recovery of P mineralization rates, which was not accompanied by significant changes in community composition. The functional and structural responses to the two distinct temperature disturbances were markedly similar, suggesting that direct negative physical effects and costs associated with the stress response were comparable. Moreover, the stress response of extracellular enzyme activities, but not that of intracellular microbial processes (for example, respiration or N mineralization), was dependent on the nutrient content of the resource through its effect on microbial physiology and community composition. Our laboratory study provides novel insights into the mechanisms of microbial functional stress responses that can serve as a basis for field studies and, in particular, illustrates the need for a closer integration of microbial C-N-P interactions into climate extremes research.

INTRODUCTION

Given the strong control of microbial communities over critical biogeochemical processes, there is growing consensus that accurate predictions of future biogeochemical cycles require a more mechanistic understanding of perturbations of microbial carbon (C) and nutrient cycling (*1*–*3*). Most microbial communities are sensitive to disturbance either in their activity, or composition, or both (*4*, *5*). Their degree of resistance and resilience to disturbance depends on many factors, such as nutrient availability, substrate quality, microbial community composition, microbial stress tolerance, or adaptation (*6*). Despite major efforts to understand the factors governing microbial functional and structural stability, as well as their interrelationships (*7*), we still lack a thorough mechanistic understanding that can be used to develop a predictive framework of functional responses of microbial communities to disturbances and their consequences for ecosystem functioning and stability.

Environmental disturbances affect microbial process rates through changes in nutrient availability and direct physicochemical effects, which lead to disruption of microorganisms' activities that alter the rate at which they perform a process. However, disturbance-induced changes in microbial community activity are complex because they can occur via nonmutually exclusive mechanisms, including physiological stress responses, changes in growth rate and turnover, or shifts in microbial community composition (*8*, *9*). The response of microbial process rates to persistent disturbances may be predicted on the basis of the change in the physical environment, although only before microbial communities acclimate or adapt, which potentially changes the response itself (*9*, *10*). By contrast, transient but severe disturbances often result in high microbial mortality, impose high physiological costs associated with acclimation and survival-related metabolism (*8*), or induce dormancy states, after which microbes can regain activity when conditions improve (*11*). When these disturbances cease, and with them the direct environmental effect, microbial processes depend solely on the remaining active microbial populations. Understanding the consequence of these transient disturbances on microbial processes is particularly important in the context of extreme weather events. Climate change does not only lead to a gradual increase in mean global temperatures but may also increase the frequency and intensity of extreme weather events, such as heat waves and drought events (*12*). Moreover, the frequency and intensity of soil freezing events increase with reductions of snow cover (*13*), and it has already been shown that the snow cover extent in the northern hemisphere has decreased since the mid-20th century, and this trend is predicted to continue (*12*).

In the case of transient disturbances, numerous studies have reported disturbance-induced bursts of microbial C and nitrogen (N) mineralization, which can be attributed to (i) increased levels of labile substrates resulting from cell lysis (for example, after freeze-thaw or drying-rewetting events) (*14*, *15*), (ii) positive priming effects caused by enhanced turnover of microbial biomass or enhanced mineralization of nonbiomass organic matter (*16*), or (iii) mineralization of accumulated protective molecules (for example, osmolytes, after a combined drought and rewetting event) (*17*). In turn, the effect of disturbances on microbial phosphorus (P) fluxes has received much less attention

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than that of C and N, although microorganisms play a pivotal role in the soil P cycle, including the regulation of P availability to plants (*18*). Moreover, the flow of different nutrients can also be affected by disturbance-induced changes in the catalytic capacity of microbial communities. For example, the production of specific extracellular enzymes may be suppressed or induced by changes in nutrient availability or by shifts in microbial community composition following disturbances (*19*, *20*). The resistance of extracellular enzyme activities also depends on the direct physical effect of disturbances, whereas their resilience will depend on the survival and proliferation of microbial populations capable of producing new enzymes.

To predict the response of C and nutrient cycle processes to environmental changes, we require conceptual and empirical approaches that integrate a wide range of microbial processes involved in the cycling of C, N, and P. However, this integrated knowledge has been hindered by the fact that most of the available information on microbial functional stability is derived from studies investigating only one or few indicator processes of microbial activity (*4*). It has been shown that microbial stability strongly depends on the specific environmental context (for example, soil physicochemical properties and disturbance history) (*6*, *21*), and therefore, combining information on microbial processes from different studies to analyze the sensitivity and similarity of the direction of responses of C, N, and P process rates may be ambiguous.

The objective of this study is to assess the integrated response of microbial C, N, and P cycling processes to transient, but severe, disturbances to improve our mechanistic understanding of the microbial multifunctional response to environmental changes and climate extremes. We hypothesized that transient disturbances, due to disturbance-induced increases in availability of labile compounds derived from microbial cell lysis (*15*), (i) temporarily lead to a more rapid cycling of C, N, and P but (ii) negatively influence the production of extracellular enzymes through end-product inhibition (*20*) and, in addition, by microbial acclimatization to stress when microorganisms shift resources from growth (including enzyme production) to survival pathways (*8*). Immediate disturbance-induced increases in process rates are primarily fueled by the flush of nutrients, whereas negative disturbance effects are more likely to be caused by physiological and structural changes of the microbial communities and by direct physical damage, which will recover slower. Therefore, we hypothesized that (iii) initially stimulated microbial processes recover faster from disturbance than negatively affected processes.

We tested the effect of two different temperature disturbances (freeze-thaw and heat) in a well-controlled laboratory model system, in which beech litter (*Fagus sylvatica* L.) collected from three different sites in Austria [Schottenwald (S), Klausenleopoldsdorf (K), and Ossiach (O)] with similar organic C chemistry but varying N and P content (table S1) was sterilized and inoculated with the same microbial community to eliminate the influence of past disturbance history (that is, selection of a more resistant microbial community). After 3 months of incubation at a constant temperature of 15°C, the plant litter was exposed to either one of two different temperature cycles lasting for 9 days (freeze-thaw, 15°/4°/−15°/4°/15°C; heat disturbance, 15°/23°/30°/23°/15°C) or to no disturbance (control at 15°C). The resistance of microbial processes and community composition were determined 3 days after the temperature cycles were finished, and their resilience was determined 3 months after the disturbance. To accurately assess the response of microbial C and nutrient cycling processes to environmental changes, we systematically tested the effect of the two temperature disturbances on 17 microbial processes involved in major C, N, and P processes during organic matter decomposition, including gross production and consumption rates of five major nutrients (namely, glucose, amino acids, ammonium, nitrate, and phosphate) determined by isotope pool dilution technique, and potential activities of extracellular enzymes (Fig. 1). Moreover, to compare microbial functional stability to microbial structural stability and to normalize activities to the size of the microbial community (*19*, *22*), we assessed viable microbial biomass and community composition via phospholipid fatty acid (PLFA) analysis.

RESULTS

Resistance of microbial processes and community composition to temperature disturbances

Three days after exposure to the disturbances (heat or freeze-thaw), most microbial process rates and potential extracellular enzyme activities (expressed on a litter dry mass basis) were substantially altered in comparison to the controls, although both the magnitude and direction of the disturbance effects varied between processes (Fig. 2 and Table 1). The few unaffected microbial processes were mainly N transformation processes, that is, protein depolymerization, N mineralization, ammonium consumption, and peptidase activity (Table 1). However, effects on protein depolymerization and peptidase activity depended on litter type, as indicated by a significant interaction between disturbance and litter type. Respiration, nitrification, and nitrate consumption rates significantly increased in both treatments, whereas amino acid consumption rates increased only after freeze-thaw disturbance (Table 1). By contrast, rates of glucan depolymerization, glucose consumption, P mineralization, and phosphate consumption declined significantly in both treatments. Among measured gross transformation rates, P mineralization and phosphate consumption were notably the most affected by disturbances,

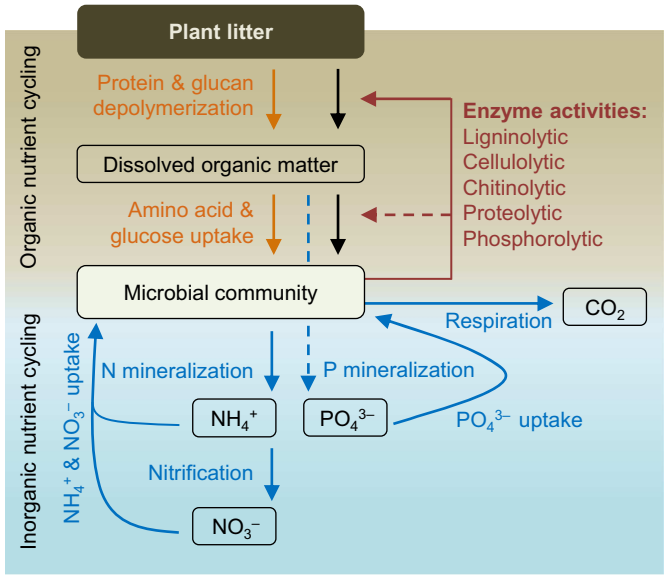

Fig. 1. Schematic representation of the microbial processes studied. Microbial respiration and gross rates of glucan depolymerization, glucose consumption, protein depolymerization, and amino acid consumption were determined, as well as gross rates of inorganic N and P fluxes, namely, N mineralization, ammonium consumption, nitrification, nitrate consumption, P mineralization, and phosphate consumption. As further proxies of responses of microbial functions involved in the breakdown of high–molecular weight organic compounds, potential activities of four extracellular hydrolytic enzymes (cellobiosidase, chitinase, phosphatase, and peptidase) and two oxidative enzymes (peroxidase and phenol oxidase) were determined.

showing a reduction by approximately 90%. The potential activities of cellobiosidase, chitinase, and phosphatase were also particularly sensitive to disturbance, showing a strong reduction after both disturbances. By comparison to hydrolytic enzyme activities, peroxidase and phenol oxidase activities were only reduced after heat disturbance. ANOVA results also showed that, in particular, enzyme activities (except phenol oxidase activity) had a significant disturbance × litter type interaction term, indicating that their response depended on the litter type. Temperature disturbance also resulted in minor changes of the dissolved nutrient pools (Fig. 2 and Table 1): Concentrations of DOC and DON were significantly higher after heat but not after freeze-thaw disturbancer, and concentrations of DOP were significantly lower after both disturbances.

Viable microbial biomass, measured as total PLFA concentration, was sensitive to both temperature disturbances, showing a decline of approximately 20 to 50% after both treatments (Fig. 2). Because process rates can also be controlled by the size of the microbial community, we normalized microbial processes to total PLFA concentration to account for differences in the size of the microbial biomass between litter types and between the disturbance treatments and the control (Fig. 3 and table S2). Overall, the response directions of processes on a biomass basis

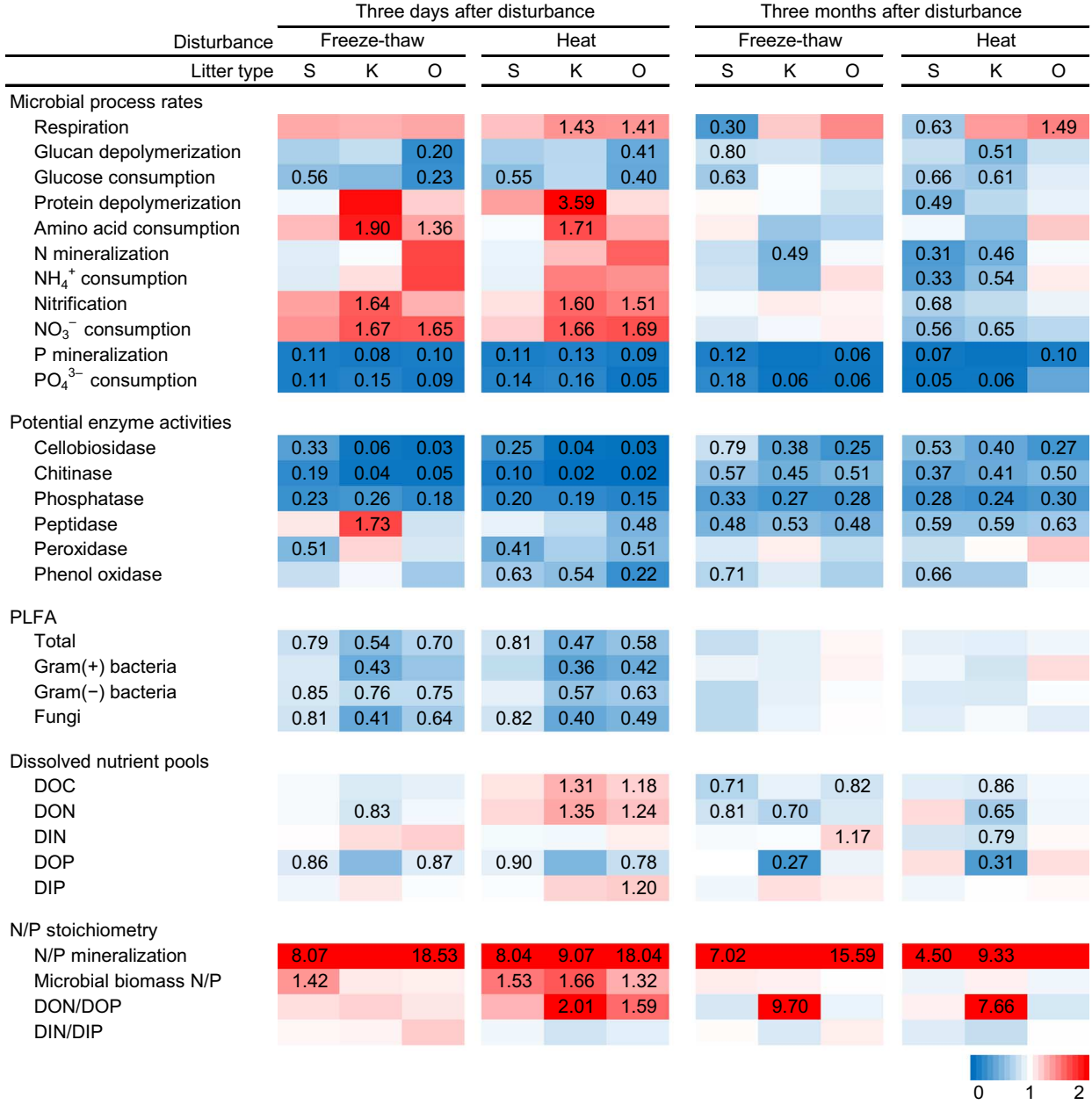

Fig. 2. Mean response ratios of gross microbial process rates, potential enzyme activities, PLFAs, dissolved nutrient pools on a dry mass basis, and N/P stoichiometry. Values are for 3 days (resistance) and 3 months (resilience) after disturbance by freeze-thaw or heat. Response ratios were calculated as the treatment replicate over the mean of the respective control. Shown are only response ratios where the disturbed samples were significantly different from the undisturbed control (*t* test on raw data, $P < 0.05$). DOC, dissolved organic carbon; DON, dissolved organic nitrogen; DIN, dissolved inorganic nitrogen; DOP, dissolved organic phosphorus; DIP, dissolved inorganic phosphorus. Litter type: S, Schottenwald; K, Klausenleopoldsdorf: O, Ossiach.

Table 1. Effects of temperature disturbance and litter type on gross microbial process rates, potential extracellular enzyme activities, and microbial community composition (PLFA profile) 3 days after disturbance. Analyses were performed on process rates and PLFA expressed per gram litter dry weight. Shown are the results of mixed-effect analysis of variance (ANOVA) using litter type as random effect and post hoc pairwise comparisons of the treatments (C, control; FT, freeze-thaw; H, heat). $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

	Three days after disturbance											
	Litter			Disturbance			Litter × disturbance			Pairwise comparison		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	C-FT	C-H	FT-H
Microbial process rates												
Respiration	2,33	8.51	†	2,33	21.17	†	4,33	0.39		*	**	
Glucan depolymerization	2,29	30.80	†	2,29	11.79	†	4,29	1.01		*	*	
Glucose consumption	2,29	26.67	†	2,29	25.59	†	4,29	0.80		**	*	
Protein depolymerization	2,36	0.83		2,36	2.66		4,36	5.17	*			
Amino acid consumption	2,36	21.62	†	2,36	8.59	†	4,36	0.63		*		
N mineralization	2,33	15.69	†	2,33	0.46		4,33	1.22				
NH_4^+ consumption	2,33	19.29	†	2,33	0.39		4,33	1.14				
Nitrification	2,35	14.20	†	2,35	8.67	†	4,35	0.96		*	†	
NO_3^- consumption	2,35	16.98	†	2,35	29.44	†	4,35	0.55		**	**	
P mineralization	2,33	73.58	†	2,33	246.2	***	4,33	0.87		***	**	
PO_4^{3-} consumption	2,33	5.39	†	2,33	44.5	**	4,33	1.33		**	**	
Potential enzyme activities												
Cellobiosidase	2,36	8.69	†	2,36	13.65	†	4,36	56.67	***	*	*	
Chitinase	2,36	12.35	†	2,36	35.47	†	4,36	33.48	***	**	**	
Phosphatase	2,36	5.69	†	2,36	248.3	***	4,36	3.53	†	**	***	
Peptidase	2,36	5.82	†	2,36	3.65		4,36	4.31	*			
Peroxidase	2,36	3.04		2,36	5.26	†	4,36	3.01	†		†	
Phenol oxidase	2,35	20.6	†	2,35	15.9	†	4,35	1.73			*	*
PLFA (phospholipid fatty acid)												
Total	2,36	9.96	†	2,36	15.43	†	4,36	4.62	**	*	*	
Gram-positive bacteria	2,36	10.2	†	2,36	15.55	†	4,36	1.22		*	*	
Gram-negative bacteria	2,36	72.68	***	2,36	11.73	†	4,36	2.32	†	*	*	
Fungi	2,36	0.47		2,36	8.26	†	4,36	16.3	***	†	*	
Dissolved nutrient pools												
DOC	2,36	132.5	***	2,36	10.21	†	4,36	1.65			†	*
DON	2,36	109.1	***	2,36	12.81	†	4,36	1.36			*	*
DIN	2,36	164.7	***	2,36	5.73	†	4,36	0.66				†
DOP	2,36	241.3	***	2,36	16.45	†	4,36	0.76		*	*	
DIP	2,36	711.1	***	2,36	3.52		4,36	0.63				
N/P stoichiometry												
N/P mineralization	2,34	21.56	**	2,34	120.8	***	4,34	0.75		**	**	

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	Three days after disturbance											
	Litter			Disturbance			Litter × disturbance			Pairwise comparison		
	df	F	P	df	F	P	df	F	P	C-FT	C-H	FT-H
Microbial biomass N/P	2,36	10.68	†	2,36	7.22	†	4,36	3.28	*		*	
DON/DOP	2,36	41.43	**	2,36	5.7	†	4,36	2.22			†	
DIN/DIP	2,36	226.4	***	2,36	3.04		4,36	1.06				

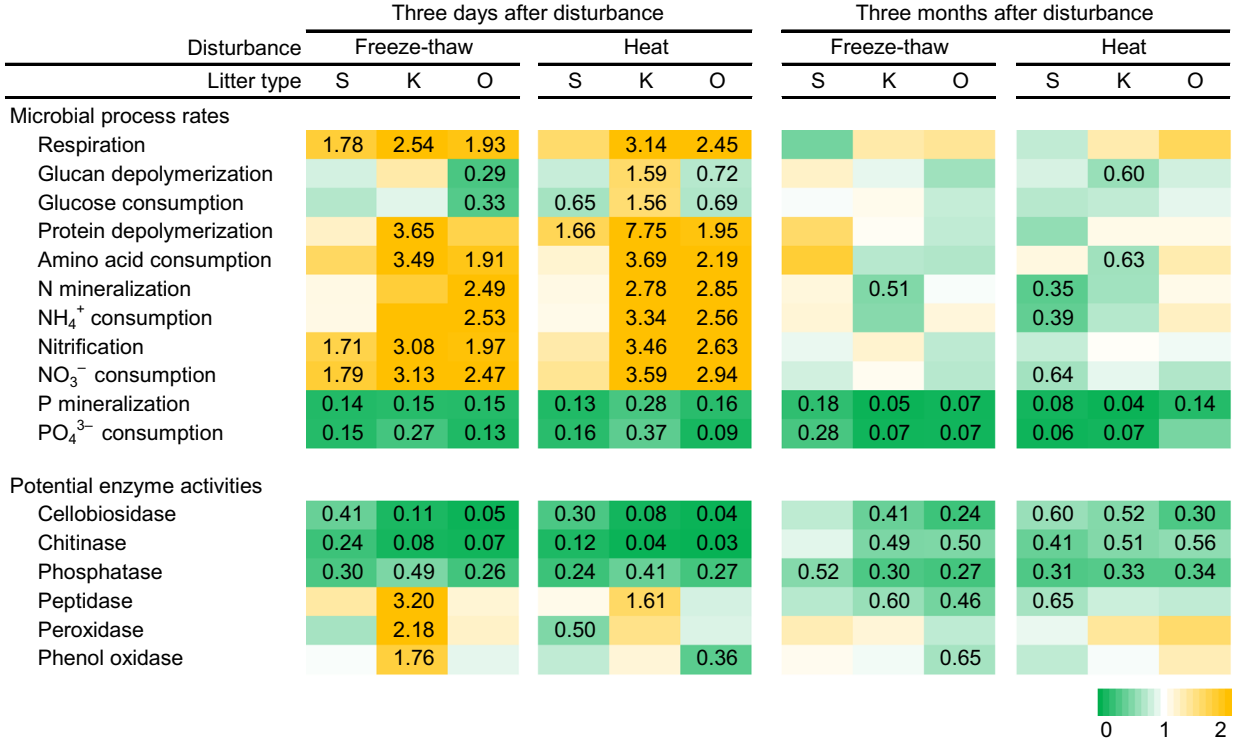

Fig. 3. Mean response ratios of gross microbial process rates and potential enzyme activities normalized to microbial biomass (that is, total PLFA concentration). Values are for 3 days (resistance) and 3 months (resilience) after disturbance by freeze-thaw or heat. Response ratios were calculated as the mean of the treatment replicate over the mean of the respective control. Given are only response ratios where the disturbed samples were significantly different from the undisturbed control (*t* test on raw data, $P < 0.05$).

were similar to those of processes expressed on a dry mass basis because most of the changes in process rates were stronger than those in microbial biomass (Fig. 3). The only processes where normalization by biomass removed the disturbance effect in the two-way ANOVA were the two oxidative enzymes, as well as glucan depolymerization and glucose consumption (table S2). Generally, the normalization of microbial processes to biomass resulted in reduced significance levels for the main litter and treatment effects but in more significant disturbance × litter type interaction terms in the two-way ANOVA (table S2).

To analyze the structural stability of microbial communities, we used PLFA profiling, which has been reliably used to detect treatment effects on microbial community composition (*23*). Principal coordinates analysis (PCO), an unconstrained ordination method, of PLFA profiles showed that different litter types inoculated with the same microbial community at the beginning of the incubations developed distinct microbial communities within the first 3 months of the experiment (Fig. 4A). Furthermore, PCO results indicated a shift in PLFA composition after both disturbances with the exception of litter type S. A significant disturbance effect on the PLFA profiles was confirmed by permutational multivariate ANOVA (PERMANOVA), where we used litter C/N as a covariate to constrain the ordination for the factor "litter type" (Table 2). To further visualize this effect on microbial community composition, we performed a canonical analysis of principal coordinates (CAP), which is a constrained ordination method. By using CAP, we maximized the discrimination among the disturbance types, largely eliminating variation introduced by the litter types (Fig. 4B). CAP analysis showed a clear separation between the two temperature disturbances and the undisturbed control (control, classification rate of 100%) on axis 1, indicating disturbance-induced shifts in microbial community structure. However, there was no clear separation of freeze-thaw and heat disturbances on axis 2 (low classification rate of 53% for freeze-thaw and 60% for heat).

Resilience of microbial processes and community composition to temperature disturbances

Given that microbial community composition and process rates in the undisturbed controls also changed over the 3 months following disturbance (*24*), we compared the temperature-disturbed samples to the

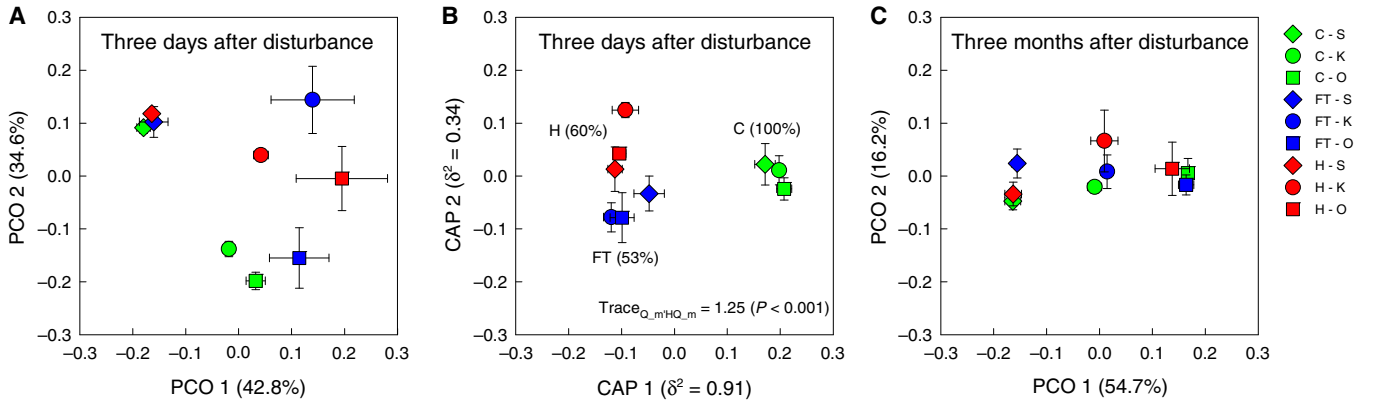

Fig. 4. Microbial community composition based on PLFA profiles of three beech litter types (S, K, and O) 3 days and 3 months after disturbance. (**A** and **C**) The ordination of PCO for 3 days and 3 months after disturbance, respectively. The variance explained by each PCO axis is given in parentheses. (**B**) The results of CAP for 3 days after disturbance. CAP is a constrained ordination that maximizes the differences among a priori defined groups. The canonical correlation (δ^2) of each CAP axis, indicating the association strength between the multivariate data cloud and the hypothesis of differences between disturbances, is given in parentheses. The CAP classification rates (in percent) for each disturbance (C, control; FT, freeze-thaw; H, heat) are given in parentheses next to each cluster. The $trace_{Q\_m'HQ\_m}$ statistic (sum of canonical eigenvalues) tests the null hypothesis of no significant differences in multivariate location among disturbances.

undisturbed controls, both analyzed at the same time (Fig. 2). We found that several microbial process rates expressed on a dry mass basis did not recover to the state of the undisturbed controls (Table 3). The only processes showing full recovery in all litter types (that is, exhibiting no significant main treatment effect and no significant disturbance × litter type interaction term in the two-way ANOVA) were glucan depolymerization, glucose consumption rates, protein depolymerization, amino acid consumption rates, and peroxidase and phenol oxidase activities (Table 3). Compared to this high resilience of oxidative enzyme activities, we found that no hydrolytic enzyme activity had fully recovered. Rates of respiration, N mineralization, and ammonium consumption showed no main disturbance effect but still showed a significant interaction between disturbance and litter type (Table 3), which indicate a disturbance- or litter type–specific recovery. Notably, P mineralization and phosphate consumption were still considerably lower in both disturbances compared to the undisturbed control. There was no significant disturbance effect on DOP or DIP concentrations nor on microbial biomass N/P, DON/DOP, and DIN/DIP, indicating no difference in P availability between control and disturbance treatments (Table 3).

The abundance of indicator lipids for fungi, Gram-positive and Gram-negative bacteria, as well as microbial community composition profiles recovered completely 3 months after disturbances (Figs. 2 and 4C). Because of the recovery of total PLFA concentrations (Fig. 2 and Table 3), microbial process rates normalized to microbial biomass showed similar patterns compared to rates expressed on a dry mass basis (Fig. 3 and table S2).

Similarity in functional responses of microbial communities subjected to different disturbances

We observed only minor differences in microbial functional responses between freeze-thaw and heat disturbance. To further assess the similarity of functional responses of the microbial communities subjected to the two different transient temperature disturbances, we correlated the response ratios of all microbial processes of the two different disturbance types (Fig. 5). The slopes of the linear regressions were 0.94 (±0.04 SE) and 0.98 (±0.05 SE) for the sampling 3 days and 3 months after disturbances, respectively. These slope values, which are close to 1, indicated a high degree of functional similarity between the differently disturbed microbial communities.

DISCUSSION

Microbial processes and community composition were substantially altered by both heat and freeze-thaw disturbances with both the magnitude and direction of the disturbance effects and the recovery from disturbance varying between processes. We hypothesized that transient disturbances lead temporarily to a more rapid cycling of C, N, and P because there is ample evidence of immediate microbial C and N mineralization bursts following drying-rewetting or freeze-thaw events (*14*, *15*, *25*). We found increased microbial respiration after the transient temperature disturbances (Fig. 2 and Table 1), but microbial N processes in our study appeared to be relatively stable, particularly gross N mineralization and ammonium consumption. However, this apparent resistance of gross ammonium fluxes in two of three litter types represented a zero net change in N mineralization, a so-called "portfolio effect" (*26*), which was based on a strong drop in microbial biomass, with the smaller surviving communities being much more active than the initial ones (Fig. 3). In the case of severe heat wave and drought events, other studies reported increased inorganic N availability after the release from the stress, which can support plant growth and, thus, can have positive effects on the recovery of ecosystem C uptake (*27*, *28*). Regarding the N cycle, the disturbance-induced increase in nitrification rates is also of particular interest, showing a temporary higher potential for soil N losses through nitrate leaching and through losses of gaseous N forms (that is, N_2O and N_2) produced by nitrification and denitrification. It has been shown that freeze-thaw events enhance gaseous and solute losses of soil N (*29*, *30*). Therefore, an increase in nitrification alongside with reduced plant nitrate uptake could promote soil N losses following freeze-thaw events.

Depolymerization of soil organic matter mediated by extracellular enzymes controls the rate at which assimilable dissolved organic matter is produced (*31*) and has also been hypothesized to be the rate-limiting step in organic matter decomposition (*32*). We postulated that transient disturbances negatively influence the production, stability, and activity

Table 2. Effect of temperature disturbances on microbial community composition (PLFA profile). Shown are the results of PERMANOVA with litter C/N as covariate and pairwise comparison for the treatments (C, control; FT, freeze-thaw; H, heat).

		Three days after disturbance		Three months after disturbance	
	df	**Pseudo-*F***	***P* (perm)**	**Pseudo-*F***	***P* (perm)**
Covariate	1,41	25.95	0.0001	39.68	0.0001
Disturbance	2,42	4.83	0.0004	1.17	0.2885
Pairwise comparison		*t*	*P* (perm)		
C-FT		2.56	0.0002		
C-H		3.24	0.0001		
FT-H		0.75	0.5841		

Table 3. Effects of temperature disturbance and litter type on gross microbial process rates, potential extracellular enzyme activities, and microbial community composition (PLFA profile) 3 months after disturbance. Analyses were performed on process rates and PLFA expressed per gram litter dry weight. Given are results of mixed-effect ANOVA using litter type as random effect and post hoc pairwise comparison of the treatments (C, control; FT, freeze-thaw; H, heat). $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

	Three months after disturbance											
	Litter			Disturbance			Litter × disturbance			Pairwise comparison		
	df	***F***	***P***	**df**	***F***	***P***	**df**	***F***	***P***	**C-FT**	**C-H**	**FT-H**
Microbial process rates												
Respiration	2,34	1.34		2,34	0.38		4,34	13.43	***			
Glucan depolymerization	2,35	1.31		2,35	3.23		4,35	1.54				
Glucose consumption	2,35	8.12	†	2,35	3.59		4,35	1.36				
Protein depolymerization	2,36	4.16		2,36	2.62		4,36	2.27				
Amino acid consumption	2,36	10.35	*	2,36	0.61		4,36	2.11				
N mineralization	2,33	12.3	*	2,33	2.61		4,33	9.11	***			
NH_4^+ consumption	2,33	12.9	*	2,33	1.22		4,33	7.12	**			
Nitrification	2,33	59.22	**	2,33	7.14		4,33	0.81			†	†
NO_3^- consumption	2,33	148.6	**	2,33	39.83	**	4,33	0.19			**	**
P mineralization	2,31	45.09	**	2,31	153.5	***	4,31	1.19		**	**	
PO_4^{3-} consumption	2,31	2.89		2,31	5.48		4,31	4.58	*	†	†	
Potential enzyme activities												
Cellobiosidase	2,36	47.6	**	2,36	14.9	*	4,36	4.17	*	*	*	
Chitinase	2,35	97.73	**	2,35	63.74	**	4,35	0.50		**	**	
Phosphatase	2,36	6.79	†	2,36	226.5	***	4,36	1.23		**	**	
Peptidase	2,36	1.64		2,36	250.3	***	4,36	0.19		***	**	†
Peroxidase	2,35	32.99	**	2,35	0.47		4,35	2.03				
Phenol oxidase	2,35	37.78	**	2,35	3.49		4,35	1.78				

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	Three months after disturbance											
	Litter			Disturbance			Litter × disturbance			Pairwise comparison		
	df	F	P	df	F	P	df	F	P	C-FT	C-H	FT-H
PLFA												
Total	2,36	12.34	*	2,36	1.56		4,36	0.55				
Gram-positive bacteria	2,36	6.25	†	2,36	0.23		4,36	0.48				
Gram-negative bacteria	2,36	28.75	**	2,36	1.92		4,36	0.85				
Fungi	2,36	2.62		2,36	1.41		4,36	0.68				
Dissolved nutrient pools												
DOC	2,36	7.74	*	2,36	0.13		4,36	33.71	***			
DON	2,36	9.86	*	2,36	0.68		4,36	18.08	***			
DIN	2,36	110.4	***	2,36	4.46	†	4,36	1.7				
DOP	2,36	3.01		2,36	0.96		4,36	39.29	***			
DIP	2,36	938.8	***	2,36	1.56		4,36	0.67				
N/P stoichiometry												
N/P mineralization	2,28	16.5	*	2,28	27.28	**	4,28	3.32	*	**	**	
Microbial biomass N/P	2,36	11.3	*	2,36	1.64		4,36	0.66				
DON/DOP	2,36	1.16		2,36	0.74		4,36	89.6	***			
DIN/DIP	2,36	188.2	***	2,36	1.85		4,36	2.25	†			

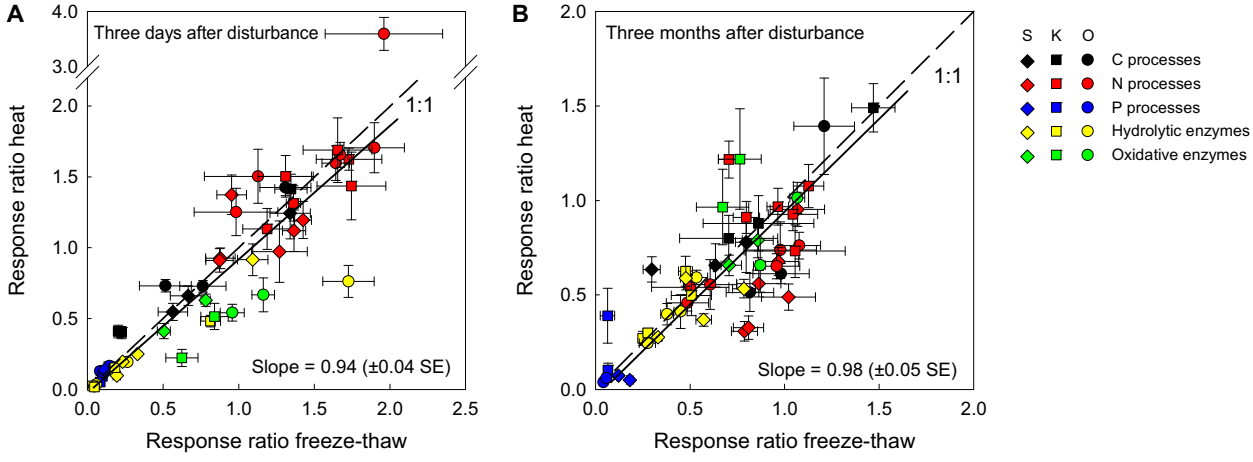

Fig. 5. Similarity of the functional response of microbial communities perturbed by heat and freeze-thaw treatments. Given are reduced major axis (RMA) regressions (solid line) between response ratios of the heat and freeze-thaw treatment of the three litter types (S, K, and O) (**A**) 3 days after disturbance and (**B**) 3 months after disturbance. The closer the slope of the regression line is to the 1:1 line (dashed line), the higher is the similarity in the functional response of the differently perturbed microbial communities. Response ratios of protein depolymerization of litter type K were excluded from linear regression analysis because the high response ratio of 3.59 in the heat disturbance greatly influenced the regression equation (slope of 1.11 when included in the linear regression). Microbial processes were grouped as follows: C processes: respiration, glucan depolymerization, and glucose consumption rates; N processes: protein depolymerization, amino acid consumption, N mineralization, ammonium consumption, nitrification, and nitrate consumption rates; P processes: P mineralization and phosphate consumption rates; hydrolytic enzymes: potential cellobiosidase, chitinase, phosphatase, and peptidase activities; and oxidative enzymes: potential peroxidase and phenol oxidase activities.

of extracellular enzymes. Primarily, enzyme production is energetically and nutritionally expensive, requiring microbial investment of C and nutrients (*33*). Therefore, microbial enzyme production may decrease when microbes acclimate to stress by altering their allocation of resources from growth to survival pathways (*8*), although this different allocation of resources may quickly reverse when disturbances cease. In addition, disturbance-induced releases of labile compounds possibly cause end-product inhibition of enzyme production or, in turn, induce enzyme activity by increased substrate availability (*20*). Extracellular enzymes can also be negatively affected by direct physical damage

triggered by disturbances. Adaptations of extracellular enzymes to specific temperature regimes (here, constant at 15°C) may also lead to a limited range of thermal stability around their temperature optima, resulting in loss of function of the active site and/or of enzyme conformation outside their temperature tolerance range (*34*). We found reduced potential activities, that is, amounts of enzymes, of cellobiosidase, chitinase, and phosphatase after both heat and freeze-thaw disturbances (Fig. 2 and Table 1). These hydrolytic enzyme activities expressed per biomass unit were also strongly reduced at both sampling times (Fig. 3 and table S2), indicating that their reduced activity was not controlled by the size of the microbial community but by a down-regulation of microbial enzyme production per unit of microbial biomass. Moreover, we observed reduced P mineralization and glucan depolymerization rates concomitant with the decline of phosphatase and cellobiosidase activities, which suggested that the reduction in hydrolysis of organic P compounds and glucans was due to the loss of enzyme activity. In soils, the binding of extracellular enzymes to mineral phases causes a decline in the activity of the enzyme pool, but this also greatly enhances its stability (*35*). Here, the lack of mineral binding in litter might therefore also have decreased the resistance of extracellular enzymes to temperature disturbances. However, stress responses of extracellular enzyme activities were not consistent—oxidative enzyme activities were more resistant and resilient than hydrolytic enzyme activities. It has been suggested that the controls on microbial expression and environmental turnover differ between these two broad classes of enzymes (*36*). In general, these different disturbance effects on enzyme activities (that is, prolonged reduction in hydrolytic but complete recovery of oxidative enzyme activities) indicate differential allocation of resources to extracellular enzymes and possibly differential stability of these enzyme classes. Because hydrolytic enzymes target labile organic compounds (for example, cellulose and protein) and because oxidative enzymes are important for the cycling of recalcitrant organic matter, such as lignin and humic substances (and are therefore closely linked to C sequestration), our results show that extreme temperature events can trigger relative changes in the pools and activities of extracellular enzymes targeting labile or recalcitrant C (as well as N and P) compounds.

Immediate disturbance-induced increases in process rates are primarily fueled by the availability and release of nutrients from lysed microbial cells, whereas negative disturbance effects are more likely to be caused by direct physical damage and physiological and structural changes of the microbial communities, which will likely recover slower. We thus postulated that microbial processes that were stimulated immediately after disturbance recover faster than negatively affected processes. We observed a full recovery of almost all processes after 3 months, except of microbial P dynamics and hydrolytic enzyme activities, which remained substantially lower 3 months after disturbance and, hence, showed the slowest recovery of all processes. The strong reduction in P mineralization and hydrolytic enzyme activities were not accompanied by changes in microbial community structure because we found a complete recovery of microbial community composition (at least at the resolution of PLFA). These results suggest that microbial functional resilience is not necessarily tightly coupled to structural resilience and that extreme weather events can cause prolonged changes in nutrient cycling through physiological responses of microbial communities that are not accompanied by significant changes in community composition. However, for a more thorough testing of the relationship between functional and structural resilience, it may require high-resolution microbial community profiling techniques using next-generation sequencing combined with "meta-omics" approaches (metatranscriptomics or metaproteomics) with which specific enzymes can be assigned to their producer (*35*).

The slow recovery of extracellular phosphatase activities and consequently of P mineralization and microbial phosphate consumption rates over 3 months could have been caused by several mechanisms. First, the production of extracellular phosphatases could have been down-regulated due to enhanced P availability derived from disturbance-induced release of P from cell lysis, which would have persisted over 3 months. However, we found no disturbance effect on proxies of P availability, such as litter DOP and DIP concentrations, microbial biomass N/P, and DON/DOP and DIN/DIP ratios, 3 months after disturbance ceased. Second, a lower microbial P demand due to slower growth or decreased microbial biomass in the stressed microbial communities could have led to a lower production of phosphatases relative to the undisturbed microbial communities. To investigate microbial activity based on substrate utilization, we also performed 24-hour incubations with ^{13}C-labeled amino acids, which showed no significant difference between the quantities of ^{13}C incorporated into PLFAs between disturbed and undisturbed microbial communities after the 3-month recovery period (*37*). These results [that is, lack of difference in microbial growth (^{13}C incorporation into PLFA)] and the fact that the microbial biomass fully recovered suggest that microbial growth and biomass and, for that reason, most likely microbial P demand were similar in disturbed and undisturbed microbial communities. Third, disturbance could have selected for microbial communities with lower potential to produce extracellular phosphatases or with lower P demand. However, the full recovery of microbial biomass and of microbial community composition does not indicate that a shift in microbial community composition (at least at the resolution of PLFA) is responsible for the sustained suppression of microbial P cycling processes. Fourth, the P use of the stressed microbial communities may have shifted from phosphate to other P forms, such as organic P compounds or polyphosphates, which is intrinsically difficult to determine and was not tested here. Fifth, sufficient available P due to the lack of (i) plant competition for available P and (ii) loss of P through litter fragmentation or leaching could have caused suppressed phosphatase production. This removal of excess available P could accelerate the recovery of microbial P processes. However, this can be neglected in our controlled laboratory study because plants, mesofauna, and leaching were excluded in both control and disturbance treatments, but they may be important in natural plant-soil systems. Overall, the disturbance response and recovery of microbial P cycling appear to be more complex than initially realized and cannot be clearly explained by our data. This certainly warrants future work, especially because the disturbance response of microbial cycling of P was so distinct from that of N.

Microbial function is strongly tied to soil resources, and, in turn, it has been suggested that soil resources interact with the stability of microbial communities and their function (*4*, *20*). Nevertheless, it remains uncertain how particular nutrient availability influences microbial functional stress responses. To more closely assess this interaction and, thus, to gain insight into the underlying mechanisms of the environmental dependency of microbial stability, we used three litter types with similar organic C chemistry and content but different N and P content. We found that extracellular enzyme activities were particularly affected by interactions between litter type and disturbances. Although all plant litter types were inoculated with the same microbial community, they were colonized by different microbial communities over time (Fig. 4, A and C), most likely as a result of different litter nutrient contents. It has been suggested that extracellular enzyme activities are potentially more sensitive to shifts in microbial community composition, because certain extracellular enzymes can

only be synthesized by a limited range of soil microorganisms (*19*, *38*), in contrast to broad processes that are carried out by a greater diversity of soil microorganisms or that are measured as a single process but are actually the sum of multiple distinct physiological processes (for example, respiration or N mineralization) (*39*). According to this, we would expect that the disturbance responses of extracellular enzymes differ between microbial communities because the enzyme activities depend on the community composition of the microbial population remaining after disturbance. In contrast, broad processes would show similar disturbance responses across different microbial communities because we expect them to be independent from community structure. Our results support this and, thus, show that the short-term stress response of extracellular enzyme activities, but not that of intracellular processes, was dependent on microbial community structure, which differed between litter types.

It is well established that the frequency and severity of disturbances are critical determinants of microbial community responses through the selection of resistant taxa (*40*–*42*). For example, microbial communities that have been previously exposed to disturbances are typically more resistant to following disturbances than those that have not (*8*, *43*). To assess the effect of disturbance on microbial function without the confounding effect of disturbance history, we inoculated different sterilized plant litter provenances with the same microbial community and incubated them for a relatively long time (that is, 3 months) at a constant temperature to favor the establishment of taxa that are not particularly resistant, neither to heat nor to cold stress. Notably, microbial communities showed considerably similar functional responses to the contrasting temperature disturbances (Fig. 5), and the two temperature disturbances did not select for structurally distinct microbial communities (Fig. 4B and Table 2). This is interesting because the two temperature disturbances have distinct physical effects on microbial cells: Subzero temperatures can induce intracellular ice crystal formation and consequently rupture cells, whereas high temperatures can increase membrane permeability and have harmful effects on the internal cell organization (*44*). However, freeze-thaw and heat both trigger temperature stress (low- versus high-temperature stress) combined with moisture stress. High temperatures come with drought stress through increased water evaporation (*45*). Here, the heat-disturbed samples had about four times lower water content (approximately 7% water content) compared to the freeze-disturbed and control samples after the disturbance because we did not adjust the water content during the temperature treatments. During freezing, ice formation causes a decline in water availability (*46*) and thereby negatively affects microbial activity beyond the effect of low temperature alone (*47*). The similar functional and structural responses observed therefore suggest that direct physical stress effects and costs associated with the stress response were comparable (*8*), possibly because, in both cases, microorganisms experience osmotic stress, and they produce protective molecules and induce repair mechanisms. Freezing, heating, and desiccation can lead to osmotic stress, enhanced free radical production causing oxidative damage, nucleic acid damage, adverse changes in membrane functionality causing cytosolic leakage, and enzyme dysfunction in the intracellular as well as the extracellular compartment (*48*). The different stressors therefore hold several generalities in the microbial community stress response, such as increased reactive oxygen species scavenging, diversion of metabolic flux from acquisition (extracellular enzyme production) toward repair and maintenance, the production of osmoprotectants and protective proteins (chaperones and heat and cold shock proteins), reformation of biomembranes to optimize to current conditions, production of extracellular polymeric substances, and induction of dormancy (*8*, *45*, *49*). Distinct transient temperature disturbances as used in the present study therefore can induce common physiological stress responses in unadapted microbial communities, resulting in functionally similar microbial communities. This is particularly important because different extreme weather events, such as heat waves, freeze-thaw, or drought events, are likely to become more frequent and more severe toward the end of the century (*12*).

From molecular to global scales, biogeochemical cycles are biologically coupled, owing to the relatively conserved elemental stoichiometry of plants and microbes that drive the cycling of C, N, and P (*50*). Understanding the particular role of soil microbes in controlling these element fluxes has become an area of great interest because we strive to understand and predict how global change will influence ecosystem functioning. Despite the fact that cycling of C, N, and P is tightly coupled through microbial immobilization and mineralization (*50*, *51*), we demonstrated here that, following extreme temperature events, microbial P cycling can decouple from those of C and N as a consequence of differential stress responses of these processes. The faster cycling of N but slower cycling of P resulted in higher N/P mineralization ratios. Altered mineralization stoichiometry indicates (i) a different fate of N and P, that is loss of N and retention of P, and (ii) altered nutrient availability to plants, that is, higher N and lower P availability, after extreme weather events. Climate change affects the nutrient stoichiometry of terrestrial plants (*52*, *53*), as well as soils (*54*). This reduced P mineralization after extreme weather events may not only influence the nutrient stoichiometry of plants but may also negatively affect plant productivity, especially when plants become increasingly limited by P under enhanced atmospheric N deposition (*55*). The slow recovery of P mineralization rates was a result of physiological responses by microbial communities that were not accompanied by significant changes in community composition. In this case, predictive models of stress responses of ecosystem processes would not be improved by the incorporation of microbial community data. However, it needs to be tested whether these strong reductions in P mineralization rates hold true under field conditions when stress responses of both soil microbes and vegetation are included. We also showed that the sensitivity of extracellular enzyme activities, but not that of intracellular microbial processes, was dependent on the resource nutrient content through its effect on microbial physiology and community composition, suggesting that microbial community composition data (and resource quality) have the potential to strengthen predictions of certain, but not all, microbial processes. Our study thus provides novel insights into the mechanisms of the microbial functional stress response to disturbance in a well-controlled model system. Although these approaches are invaluable for the understanding of ecosystem functioning, they may not exactly represent the disturbance-related responses in natural ecosystems. Therefore, in-depth knowledge and prediction of ecosystem responses to extreme climate events will necessarily require the integrated knowledge of microbial multifunctional stress responses from mechanistic studies and field observations. In particular, our results call for a closer examination of the P cycle and C-N-P interactions from a microbial ecophysiological perspective under extreme climate conditions.

MATERIALS AND METHODS

Experimental design

Three beech litter provenances (*F. sylvatica* L.) similar in their organic C chemistry and content but varying in N and P content were collected at

different locations in Austria: sites S, K, and O (*24*). Site descriptions and litter nutrient contents are given by Wanek *et al.* (*56*) and in table S1. Litter C, N, and P content and concentrations of dissolved nutrient pools (DOC, total dissolved N and P, ammonium, nitrate, and phosphate) were analyzed by standard protocols described in the Supplementary Materials. The collected litter was dried at 40°C for 48 hours, finely chopped (1 to 20 mm), and sterilized by gamma-ray treatment. To exclude the effect of disturbance history on microbial stability, we inoculated all litter types with 1.5% (based on the dry weight of the respective litter) of a litter/soil mixture [1:1 (w/w)] from one of the sites (site K). Of each inoculated litter type, 60 g was placed in mesocosms constructed from polyvinyl chloride tubes (height, 10 cm; diameter, 12.5 cm) and kept at constant temperature (15°C). Litter water content was maintained at 60% fresh weight by adding autoclaved tap water weekly. For each treatment and sampling, separate mesocosms of each litter type ($n = 5$) were established.

Three months after inoculation of the plant litter, the mesocosms were subjected to either a freeze-thaw treatment, a heat treatment, or no disturbance (control). Starting from the standard incubation temperature of 15°C, the mesocosms were submitted to the following temperature cycles: 3 days at 4°C, 5 days at −15°C, and 1 day at 4°C for the freeze-thaw treatment and 3 days at 23°C, 5 days at 30°C, and 1 day at 23°C for the heat treatment. The temperature cycles of both treatments were completed within 9 days. After the last temperature step, all mesocosms were incubated at the standard incubation temperature of 15°C for another 3 days (i) to reduce the interference of increased levels of labile substrates and liberated intracellular enzymes derived from cell lysis on the determination of microbial processes and (ii) to determine the composition of the resistant microbial community composition by allowing the turnover of the PLFA content of cells killed by the disturbances. Litter water content was readjusted 2 days before sampling to avoid any differences in the water content caused by the two different treatments. In addition to the sampling shortly after the treatment application, a second set of samples was subjected to the same temperature cycle and control treatments and was sampled 3 months later to determine the resilience of microbial processes and community composition.

Microbial processes

Net rates represent the sum of two opposing processes: gross production and gross consumption rates. Gross rates are not only more informative than net rates but also the interpretation of disturbance responses of net transformation rates can lead to erroneous conclusions about microbial functional stability. For that reason, we analyzed gross rates of glucan depolymerization, glucose consumption (*57*), protein depolymerization, amino acid consumption, N mineralization, ammonium consumption, nitrification, nitrate consumption (*56*), P mineralization, and phosphate consumption (*24*) using isotope pool dilution assays. This technique is based on labeling the target pool (glucose, amino acid, ammonium, nitrate, or phosphate) by adding the respective ^{13}C-, ^{15}N-, or ^{33}P-labeled compound. The quantification of the decrease in the isotopic label and the change in concentrations over time allows calculation of the respective gross production and consumption rates (*58*). Heterotrophic respiration was measured using an infrared CO_2 gas analyzer (EGM-4, PP systems). Potential activities of extracellular hydrolytic and oxidative enzyme were determined fluorimetrically and photometrically, respectively, according to standard assays (*59*, *60*), as previously described by Keiblinger *et al.* (*61*). A detailed description of the procedures is given in the Supplementary Materials.

PLFA analysis

PLFA profiles were used to characterize the microbial community composition and to quantify viable microbial biomass. Phospholipids were extracted from plant litter according to Frostegård *et al.* (*62*). The procedure is described in the Supplementary Materials. Twenty-five PLFAs were extracted and quantified. We used the following PLFAs as indicators of specific microbial groups: 18:1ω9c, 18:1ω9t, 18:2ω6c, 18:2ω6t, and 18:3ω3c for fungi (*63*, *64*); 16:1ω7c, 18:1ω7c, cy17:0, and cy19:0 for Gram-negative bacteria; and i15:0, a15:0, i17:0, and a17:0 for Gram-positive bacteria (*65*, *66*). For Gram-positive bacteria, we only included PLFAs where the pairs of anteiso/iso were present. Because physiological changes in Gram-positive bacteria may change the anteiso/iso ratio, the inclusion of only one of them might bias the structural interpretation of the disturbance effect on this microbial group (*67*). We used the sum of all PLFAs described above together with the unspecific PLFAs (14:0, i14:0, 15:0, 16:0, i16:0, 16:1ω5t, 17:0, i17:1ω8c, 18:0, 18:3ω6c, 20:0, and 20:4ω6c) to define microbial community composition and as a measure of viable microbial biomass (*68*).

Data and statistical analyses

The difference between undisturbed controls and disturbance treatments is given as response ratio, which was calculated as the treatment replicate over the mean of the respective controls. Statistical significance of the difference between control and disturbance treatment was analyzed by *t* test on raw data. The effects of disturbance treatment and litter type were tested by two-way ANOVA. We used a mixed-effect model with treatment as fixed and litter type as random effect. For the 17 studied microbial processes, we controlled the false discovery rate by using the approach developed by Benjamini and Hochberg (*69*). ANOVA was followed by Tukey honest significant difference post hoc test. We used RMA regressions to describe the similarity of the functional response of microbial communities to disturbances.

All multivariate tests of the PLFA profiles were based on a χ^2 distance matrix calculated from raw data. χ^2 distance computes relative abundance and thus tends to emphasize compositional changes more than changes in abundance, for example, compared to the Bray-Curtis measure. To determine structural dissimilarities among microbial communities, we conducted a PCO. Because the a priori hypothesis concerned differences among groups, we also performed CAP using disturbance treatment as the constraining variable. In this case, CAP uses PCO followed by canonical discriminant analysis to provide a constrained ordination that maximizes the differences among a priori defined groups and may reveal patterns that are masked in unconstrained ordinations (*70*, *71*). The multivariate null hypothesis of no difference among a priori defined groups was also examined using PERMANOVA (*72*, *73*). Significance levels calculated in CAP and PERMANOVA were determined with 9999 permutations. ANOVA was performed in Statgraphics Centurion XVI (Statistical Graphics Inc.; www.statgraphics.com). For PCO, CAP, and PERMANOVA, we used the free FORTRAN program provided by M. J. Anderson.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1602781/DC1

Supplementary Materials and Methods

table S1. Description of sites of beech litter collection and nutrient content of collected litter types.
table S2. Effects of temperature disturbance and litter type on gross microbial process rates and potential extracellular enzyme activities normalized to microbial biomass (total PLFA concentration).
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