



# Soil age and soil organic carbon content shape biochemical responses to multiple freeze–thaw events in soils along a postmining agricultural chronosequence

Christoph Rosinger · Michael Bonkowski

Received: 3 December 2020 / Accepted: 24 May 2021 / Published online: 7 June 2021  
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**Abstract** Freeze–thaw (FT) events exert a great physiological stress on the soil microbial community and thus significantly impact soil biogeochemical processes. Studies often show ambiguous and contradicting results, because a multitude of environmental factors affect biogeochemical responses to FT. Thus, a better understanding of the factors driving and regulating microbial responses to FT events is required. Soil chronosequences allow more focused comparisons among soils with initially similar start conditions. We therefore exposed four soils with contrasting organic carbon contents and opposing soil age (i.e., years after restoration) from a postmining agricultural chronosequence to three consecutive FT events and evaluated soil biogeochemical responses after thawing. The major microbial biomass carbon losses occurred after the first FT event, while microbial biomass N decreased more steadily with subsequent

FT cycles. This led to an immediate and lasting decoupling of microbial biomass carbon:nitrogen stoichiometry. After the first FT event, basal respiration and the metabolic quotient (i.e., respiration per microbial biomass unit) were above pre-freezing values and thereafter decreased with subsequent FT cycles, demonstrating initially high dissimilatory carbon losses and less and less microbial metabolic activity with each iterative FT cycle. As a consequence, dissolved organic carbon and total dissolved nitrogen increased in soil solution after the first FT event, while a substantial part of the liberated nitrogen was likely lost through gaseous emissions. Overall, high-carbon soils were more vulnerable to microbial biomass losses than low-carbon soils. Surprisingly, soil age explained more variation in soil chemical and microbial responses than soil organic carbon content. Further studies are needed to dissect the factors associated with soil age and its influence on soil biochemical responses to FT events.

Responsible Editor: Stuart Grandy.

C. Rosinger (✉) · M. Bonkowski  
Terrestrial Ecology Group, Institute of Zoology, University of Cologne, Zùlpicher StraÙe 47b, 50476 Cologne, Germany  
e-mail: christoph.rosinger@boku.ac.at

## Present Address:

C. Rosinger  
Institute of Soil Research, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences (BOKU), Peter Jordan StraÙe 82, 1190 Vienna, Austria

**Keywords** Agricultural post-mining chronosequence · Basal respiration · Biogeochemistry · Metabolic quotient · Microbial biomass · Multiple freeze–thaw

## Abbreviations

C Carbon  
DOC Dissolved organic carbon  
FT Freeze–thaw

MB-C	Microbial biomass C
MB-N	Microbial biomass N
N	Nitrogen
qCO <sub>2</sub>	Metabolic quotient
SOC	Soil organic carbon
TDN	Total dissolved nitrogen

## Introduction

Freeze–thaw (FT) events have a major impact on soil biogeochemical processes (Bardgett et al. 2008; Congreves et al. 2018). Soil microbial dynamics are significant drivers of these biogeochemical responses (Sharma et al. 2006; Henry 2007), as their ecophysiology is greatly affected by FT (Schimel and Mikan 2005; Schimel et al. 2007; Mooshammer et al. 2017; Miura et al. 2019). Although soil microbes can physiologically adapt to freezing (or rather near-freezing) temperatures, for example by (i) upregulating protein synthesis, (ii) increasing membrane fluidity (through changes in the fatty acid composition), (iii) taking up or producing cryo-protecting osmolytes or (iv) regulating specific metabolic pathways (reviewed e.g. in Shivaji and Prakash 2010; Margesin and Miteva 2011), empirical evidence shows that a considerable portion of the soil microbial biomass can be lost after FT events (Feng et al. 2007; Song et al. 2017; Han et al. 2018). This die-back can be caused by physiological stress imposed by freezing itself or by the subsequent thawing process, when soil microbes must rapidly reverse their freezing adaptations to prevent cell rupture (Schimel et al. 2007). In any case, increased levels of labile organic compounds are commonly recorded after FT in soils (Matzner and Borken 2008). Depending on the prevailing environmental conditions, these nutrients can either be lost through leaching, through gaseous emissions, or metabolized by the remaining viable microbes, thus explaining the commonly observed peaks in CO<sub>2</sub> and N<sub>2</sub>O evolving from soils after FT (Joseph and Henry 2008; Matzner and Borken 2008; Song et al. 2017; Congreves et al. 2018). Soil carbon (C) and nitrogen (N) losses after FT can be substantial (Matzner and Borken 2008; Congreves et al. 2018) and have been inherently linked to soil microbial biomass dynamics (Christensen and Christensen 1991; Hermann & Witter 2002; Matzner and Borken 2008; Risk et al.

2013). For example, Hermann and Witter (2002) could show that around 65% of the C flush after FT was attributed to C derived from soil microbial biomass. Moreover, Sharma et al. (2006) found increased activity and expression of denitrifying genes of the soil microbial community after FT, suggesting that increased N<sub>2</sub>O emissions after FT were a result of increased microbial activity. Therefore, a better understanding of the mechanisms that drive microbial responses to FT events is crucial. This is particularly the case as extreme weather events—such as drought or freezing—are suggested to have a greater impact on microbial-mediated ecosystem responses than overall future changes in temperature and precipitation (Bardgett et al. 2008).

Several factors influence the impact of FT effects on soil biogeochemistry. For example, physical and chemical attributes such as soil organic matter content, soil pH, or soil pore size influence the response of soil microbial biomass to FT (Yanai et al. 2004; Freppaz et al. 2007; Song et al. 2017). Other important aspects governing microbial responses to FT are the intensity, duration and frequency of FT events (Feng et al. 2007; Schmitt et al. 2008; Han et al. 2018). Given this multifactorial complexity, we require a better and comprehensive mechanistic understanding of the drivers of soil chemical and microbial responses to FT events.

We therefore examined the effect of multiple FT events on soils from a 23-year postmining agricultural chronosequence in Western Germany. Due to highly comparable initial soil conditions, chronosequences allow more targeted investigations of microbial responses to FT along gradients of soil organic C (SOC) formation (Walker et al. 2010) and are well suited to disentangle the important driving factors. For example, SOC content may influence responses through potentially increased porosity and water retention capacity in high-SOC soils (Yang et al. 2013). Soil age was recently identified as an important local-scale driver of ecosystem properties such as soil pH, soil N:P and C:P ratios and total soil C stocks (Delgado-Baquerizo et al. 2020). Moreover, a history of soil freezing selects for more frost-tolerant and physiologically adapted microbial communities (Walker et al. 2006; Garcia et al. 2020), suggesting that soil age might be important too. Accordingly, we used four different soils of varying SOC contents (from 0.39 to 1.04% C) and time after soil restoration

(from 4 to 23 years; from now on referred to as ‘soil age’) and exposed these to three severe FT cycles. We analysed dissolved organic C (DOC), total dissolved N (TDN), microbial biomass C (MB-C) and N (MB-N), as well as respiratory responses before freezing and 36 h after each respective FT event. We hypothesized that MB-C and MB-N will decrease with consecutive exposure to FT, with subsequent increases in DOC and TDN. Moreover, we hypothesized that SOC content and soil age shape biochemical and microbial responses to FT.

## Materials and methods

### Study site

The sampling site is located in the open-cast mining area Gartzweiler around 6 km south of Mönchengladbach (6° 15' 0'' E to 6° 21' 0'' E; 50° 50' 5'' N to 50° 53' 0'' N). Mean annual temperature and precipitation is 9.8 °C and 829 mm, respectively. Lignite is extracted in this open-cast mining site by RWE Power AG (Essen, Germany) after removing topsoil and parent material. After lignite extraction, loess material from topsoil (characterized as Luvisol) and parent material (unweathered loess from the Weichselian glaciation period; Lucas et al. 2019) are mixed (in a 1:100 ratio) and used as the basis for subsequent agricultural recultivation, thus creating a 56-year agricultural post-mining chronosequence. The soil texture is a silty clay loam with around 65% silt and 31% clay (Lucas et al. 2019). The bulk density in soil layers not affected by soil tillage is around 1.6 g cm<sup>-3</sup> (Pihlap et al. 2019). Reclaimed soils typically received an initial fertilization of 60, 120 and 180 kg ha<sup>-1</sup> of each, N and P and K fertilizer in the first year, and are cultivated for three subsequent years with alfalfa (*Medicago sativa*) without any further fertilizer or biocide applications. Four to seven years after reclamation, fields typically undergo a wheat and barley crop rotation. Cereals are fertilized with mineral NPK and CAN (Calcium Ammonium Nitrate) fertilizers, and receive in addition in the fourth and seventh year of reclamation 30 t ha<sup>-1</sup> of organic fertilizer (compost), resulting in a total annual fertilization rate of 200, 80, 60 and 40 kg ha<sup>-1</sup> of N, P, K and Mg fertilizer, respectively. After 7 years, fields are returned to the previous owners and

are usually managed with a sugar beet-winter wheat crop rotation (Lucas et al. 2019; Pihlap et al. 2019).

According to the German weather service (DWD), soils in the study area typically undergo up to ten FT cycles in the winter period, when soil temperatures typically cycle around the freezing point. However, differences between daily minimum and maximum air temperatures of more than 21 °C within 72 h have also been recorded, thus showing that soils in this area are exposed to severe but short temperature differences around the freezing point.

### Experimental setup

Soil samples were taken in July/August 2019 on 8 different agricultural sites along the chronosequence. The crop on all agricultural sites was either barley or wheat. The minimum distance between sites was ca. 500 m. Five soil samples were taken randomly at each sampling site with a minimum distance of 20 m between replicates. The soil samples themselves consisted of 5 pooled subsamples taken within a 1 × 1 m square. Samples were taken from the upper A<sub>p</sub> horizon to a depth of 10 cm using a soil sampling auger (5.5 cm diameter). Samples were put in plastic bags and brought to the laboratory the same day, where they were sieved to pass 2 mm and stored at 4 °C until experimental initiation in January 2020.

In order to analyse soil chemical and microbial responses to multiple FT events, we chose four agricultural soils with SOC contents ranging from 0.39 to 1.04% (Table 1). We deliberately chose soils with similar SOC contents but different soil ages (soils 2 and 3) and vice versa (soils 3 and 4) in order to better disentangle SOC content and soil age effects on FT responses. In this study, we chose a harsh freezing treatment at - 21 °C followed by thawing at 10 °C, which resembles a rather strong cold snap. Therefore, and because we wanted to amplify potential effects, we chose this temperature regime.

For the incubation, the five samples taken per agricultural site were pooled, and ca. 30 g of fresh soil were weighed into 50 ml plastic tubes with five technical replicates. The water content was set to field capacity with deionized water. Samples were then pre-incubated in the dark for 21 days at 10 °C. Water content was determined gravimetrically every three days and adjusted as needed. Tubes were loosely covered with a plastic lid to allow gas exchange. At the

**Table 1** Time since restoration (years), soil pH, SOC and total N contents (%) of the 4 different soils used in the experiment

Soil	Time since restoration (years)	Soil pH	SOC (%)	Soil N (%)
1	4	8.59 <sup>c</sup> (0.02)	0.39 <sup>a</sup> (0.003)	0.009 <sup>a</sup> (0.001)
2	12	8.52 <sup>b</sup> (0.01)	0.77 <sup>b</sup> (0.005)	0.031 <sup>b</sup> (0.003)
3	23	8.25 <sup>a</sup> (0.02)	0.76 <sup>b</sup> (0.004)	0.043 <sup>c</sup> (0.002)
4	23	8.23 <sup>a</sup> (0.01)	1.04 <sup>c</sup> (0.007)	0.052 <sup>d</sup> (0.002)

Given is the mean  $\pm$  1SD (in brackets) of three technical replicates that have been analysed for each of the four pooled soil samples. Different letters behind means indicate significant differences ( $p < 0.05$ ; post-hoc Tukey test) between soils

end of the pre-incubation period, a first set of the soil samples were analysed for DOC, TDN, MB-C, MB-N and basal respiration. Immediately after, all soil samples were exposed to severe freezing conditions ( $-21\text{ }^{\circ}\text{C}$ ) for 48 h. After the freezing event, soils were thawed at  $10\text{ }^{\circ}\text{C}$ . Exactly 36 h after the freezing event, the second set of soil samples was analysed for DOC, TDN, MB-C, MB-N and basal respiration. We chose a time period of 36 h to make sure that all the soil in the tubes was properly thawed by the point of analysis. The remaining soil samples were kept at  $10\text{ }^{\circ}\text{C}$  for 5 days until the next FT cycle. These steps were repeated for two more sets of soil samples, i.e. a total of three FT cycles. Therefore, a total of 80 samples was analysed in this experiment (4 soils  $\times$  5 technical replicates  $\times$  4 treatments (i.e. before freezing and three FT events)).

#### Soil analysis

MB-C and MB-N were analysed using the chloroform-fumigation extraction (CFE) method (Vance et al. 1987; Witt et al. 2000). Briefly, two subsamples of 5 g fresh wt soil were taken from each soil sample. One subsample was shaken in 20 ml of 0.5 M  $\text{K}_2\text{SO}_4$  for 30 min and centrifuged at 4500 rpm for 10 min. Thereafter, 3 ml of the supernatant were transferred into new 15 ml plastic tubes and frozen until further determination of DOC and TDN. The other subsample was fumigated under vacuum (using a desiccator) with 50 ml of ethanol-free chloroform for 24 h at room temperature prior to extraction (as above). Extracts were analysed on a Multi N/C analyser (2100S, Analytik Jena, Jena, Germany). MB-C and MB-N were calculated as the difference between fumigated and non-fumigated DOC and TDN, respectively. MB-C and MB-N were not corrected by any extraction

efficiency coefficients. All results are expressed in  $\mu\text{g g dry soil}^{-1}$ .

Basal respiration was measured by an automated electrolytic micro-respiratory apparatus (Scheu 1992). Briefly, ca. 4 g of fresh soil were weighed into glass vessels and connected to the respirometer. The  $\text{O}_2$  consumption rates were measured at  $21\text{ }^{\circ}\text{C}$  every 30 min and subsequently converted to  $\text{CO}_2$  production using the molar gas constant. Basal respiration is expressed in  $\mu\text{g CO}_2\text{-C g dry soil}^{-1}\text{ h}^{-1}$ . The metabolic quotient ( $q\text{CO}_2$ ) was calculated as basal respiration per unit MB-C and is expressed in  $\text{mg CO}_2\text{-C g MB-C}^{-1}\text{ h}^{-1}$ .

#### Statistical analysis

We conducted a one-way multivariate analysis of covariance (MANCOVA) to test for significant differences in DOC, TDN, the DOC:TDN ratio, MB-C, MB-N, the MB-C:N ratio, basal respiration and  $q\text{CO}_2$  between the time points before freezing and after the first, second and third FT event. FT treatment was used as a fixed factor (from now on referred to as ‘FT’ or ‘FT treatment’), and SOC content and soil age were treated as covariates. We used a SS type III model to test for significant treatment effects as well as significant interactions with SOC content and soil age, respectively. To evaluate the statistical significance of the overall model, the Wilks’ lambda distributions ( $\lambda$ ) and derived F-values, p-values and the partial eta squared ( $\eta^2$ ) for main and interaction effects are stated. As the freshly recultivated loess material contains carbonates which disaggregate over time in the upper soil horizon of the chronosequence, soil age and soil pH were negatively correlated ( $R = -0.978$ ,  $p = 0.022$ ). The use of both factors in a single one-way MANCOVA showed that soil age

( $F = 12.584$ ,  $p < 0.001$ , Wilks'  $\lambda = 0.014$ , partial  $\eta^2 = 0.659$ ) appeared to be the stronger explanatory variable as compared to soil pH ( $F = 9.088$ ,  $p < 0.001$ , Wilks'  $\lambda = 0.03$ , partial  $\eta^2 = 0.585$ ). Accordingly, we omitted the colinear soil pH as a covariate. Moreover, SOC and total N were positively correlated ( $R = 0.957$ ,  $p = 0.043$ ). Using the same approach as above, the SOC content ( $F = 9.206$ ,  $p < 0.001$ , Wilks'  $\lambda = 0.03$ , partial  $\eta^2 = 0.585$ ) was kept in the model as it had the slightly higher explanatory power compared to the soil N content ( $F = 9.108$ ,  $p < 0.001$ , Wilks'  $\lambda = 0.03$ , partial  $\eta^2 = 0.583$ ).

Post-hoc Tukey tests using Šidák correction for multiple pairwise comparisons were used to evaluate significant differences across all soils between the respective FT events, while simple post-hoc Tukey tests were applied to evaluate significant differences between FT treatments within soil samples. Moreover, we used linear regression analyses and Pearson correlation coefficients to evaluate the relationship between MB-C and MB-N before freezing and after the first, second and third FT event. We refer to significant differences at the  $p < 0.05$  level (with \* $p < 0.05$ ; \*\* $p < 0.01$ ; and \*\*\* $p < 0.001$ ). All statistical analyses and figures were conducted in SPSS 26.  $p$ -values in the results section refer to both MANCOVA (main and interaction effects) and post-hoc statistics.

## Results

Overall, the one-way MANCOVA revealed a strong main effect of the three FT events ( $F_{1,27} = 19.583$ ,  $p < 0.001$ ) on our tested soil chemical and microbial parameters. However, the response to FT events was dependent on SOC ( $F_{2,27} = 4.918$ ,  $p < 0.001$ ) and soil age ( $F_{2,27} = 9.323$ ,  $p < 0.001$ ), respectively. The detailed test statistics can be found in Table 2.

### Soil chemical responses to FT

DOC contents in all four soils decreased after the first FT event (by 19.8, 24.2, 21.8 and 5.4% in soils 1 to 4, respectively;  $p = 0.001$ ), yet increased with the second and third FT event back to initial DOC levels (Fig. 1A,  $p < 0.01$ ). TDN contents did not change after the first FT event as compared to before FT, but

increased after the second FT in soils 2 and 4, and after the third FT event in soils 1 and 3 (Fig. 1B). After three FT events, TDN contents were increased by 78, 11, 16 and 21% in soils 1 to 4, respectively, as compared to before freezing ( $p < 0.001$ ). Overall, DOC:TDN ratios decreased following the three FT events (Fig. 1C). The strongest decrease ( $-42.7\%$ ) was recorded in soil 1 (Table 1), which also displayed the highest DOC:TDN ratio ( $p < 0.01$ ). In soils 2–4, the DOC:TDN ratio decreased after three FT events by 10–15%. In summary, soil age significantly shaped the response of DOC, TDN and the DOC:TDN ratio to multiple FT, while the SOC content had no significant effect (Table 2, Fig. 1A–C).

### Soil microbial responses to FT

MB-C was strongly affected by the three FT events (Fig. 1D). MB-C dropped sharply already after the first FT (34, 17, 21 and 40% in soils 1 to 4, respectively;  $p < 0.001$ ) and remained rather constant following the second and third FT cycles. The magnitude of the decline increased with soil age ( $p < 0.001$ ) and was less, but significantly dependent on SOC content ( $p < 0.05$ ). After the third FT event, MB-C had declined to 69, 70, 72 and 54% of pre-freezing values in soils 1 to 4, respectively ( $p < 0.001$ ). MB-N instead decreased rather steadily with consecutive FT events (Fig. 1E). Declines in MB-N were rather strong, with decreases of 48, 61, 57 and 66% in soil 1 to 4, respectively, after the third FT event as compared to before FT ( $p < 0.001$ ), and not influenced by soil age or C content. MB-C:N ratios tended to increase with multiple FT, yet overall effects were quite variable and found to be marginal ( $p = 0.067$ ; Fig. 1F). Only between the second and third FT event we noticed a substantial increase of the MB-C:N ratio of 48, 87, 56 and 62% in soils 1 to 4, respectively ( $p < 0.002$ ). Overall, MB-C responses to multiple FT were dependent on soil age and the SOC content, while neither MB-N nor MB-C:N ratios were significantly affected by soil age or soil C content (Table 2, Fig. 1D–F).

Linear regression analyses and Pearson correlation coefficients showed a significant positive relationship between MB-C and MB-N before freezing (Fig. 2A), but the correlation disappeared already after the first FT (Fig. 2B), and was not regained after the second (Fig. 2C) and the third FT event (Fig. 2D).

**Table 2** Test statistics of the MANCOVA analysis to evaluate the effect of FT as well as interactions between FT and SOC content (FT \* soil SOC) and time since restoration (FT \* soil age) on soil and soil biochemical (DOC, dissolved organic C; TDN, total dissolved N) and microbial parameters (MB-C, microbial biomass C; MB-N, microbial biomass N; MB-C:N, microbial biomass C:N ratio; microbial basal respiration; qCO<sub>2</sub>, respiratory quotient)

MANCOVA statistics						
	df	F-value	p-value	Wilks' $\lambda$	partial $\eta^2$	
FT treatment	1,32	20.76	< 0.001	0.004	0.743	
FT * soil SOC	2,32	5.08	< 0.001	0.109	0.426	
FT * soil age	2,32	9.68	< 0.001	0.031	0.582	
	FT treatment		FT * soil SOC		FT * soil age	
	F-value	p-value	F-value	p-value	F-value	p-value
DOC	78.95	< 0.001	0.40	0.805	16.77	< 0.001
TDN	6.58	< 0.001	1.42	0.24	31.55	< 0.001
DOC:TDN ratio	75.36	< 0.001	2.37	0.063	3.99	0.006
MB-C	25.28	< 0.001	3.47	0.013	18.15	< 0.001
MB-N	16.99	< 0.001	1.2	0.321	1.04	0.394
MB-C:N ratio	2.31	0.068	0.63	0.64	1.78	0.145
Basal respiration	54.47	< 0.001	4.16	0.005	3.11	0.022
qCO <sub>2</sub>	54.45	< 0.001	1	0.413	5.93	< 0.001

### Respiratory responses to FT

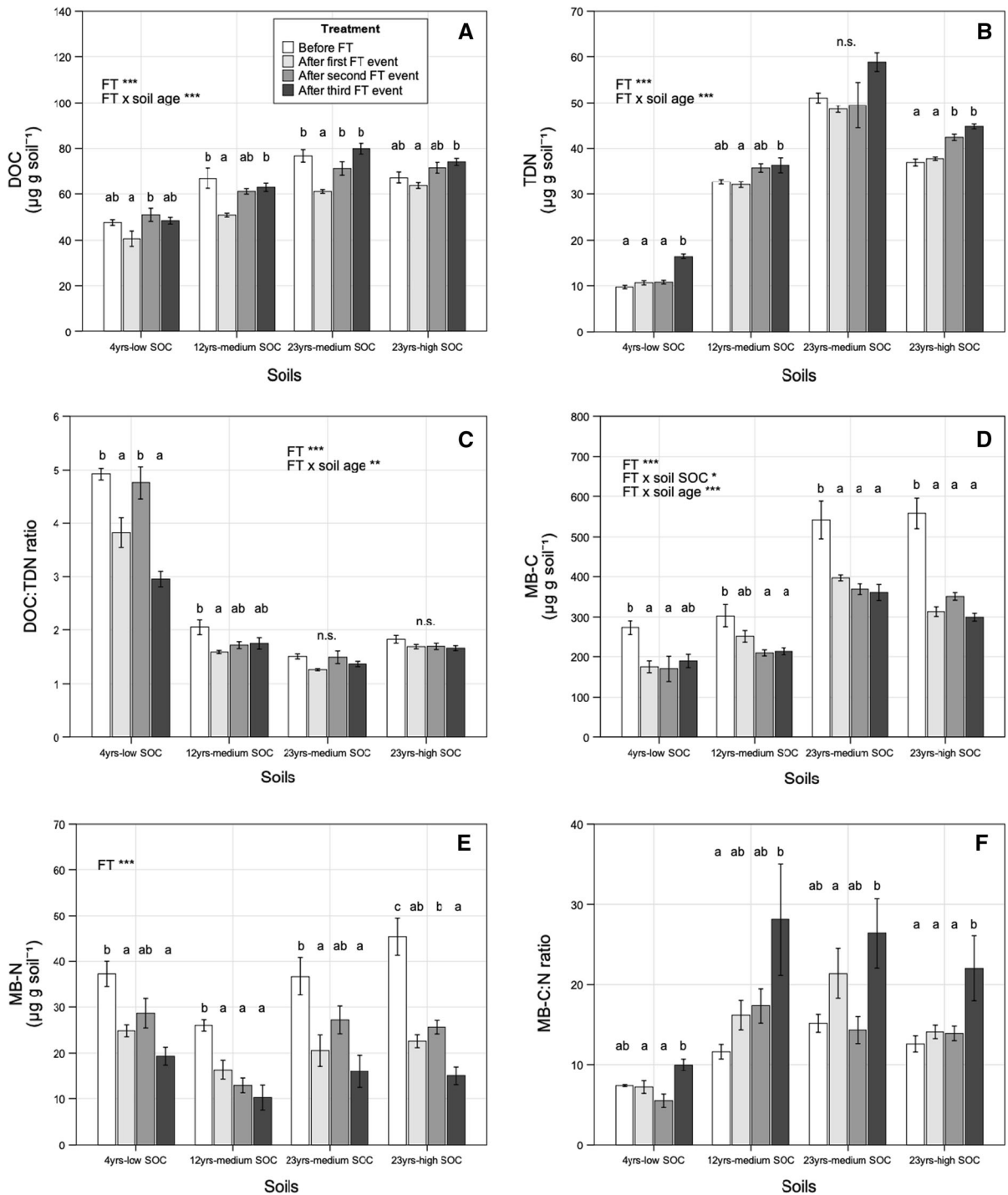
Overall, multiple FT events had a significant impact on basal respiration and the metabolic quotient. Basal respiration initially increased after the first FT event ( $p = 0.004$ ), but then dropped after the second and third FT event (both  $p < 0.001$ ) as compared to before freezing (Fig. 3A). After three FT cycles, basal respiration had decreased by 45, 59, 25 and 44% in soils 1 to 4, respectively, as compared to before freezing. FT responses of qCO<sub>2</sub> mimicked basal respiratory responses (Fig. 3B). After the first FT event, qCO<sub>2</sub> significantly increased as compared to before freezing ( $p < 0.001$ ); by 95, 22, 74 and 84% in soils 1 to 4, respectively, but after the second and third FT event, the qCO<sub>2</sub> decreased to initial levels (in the soils 1, 3 and 4) or below (in soil 2) ( $p < 0.001$ ). The SOC content significantly influenced the response of basal respiration to multiple FT (Table 2, Fig. 3A), while soil age significantly affected the response of both basal respiration and the qCO<sub>2</sub> (Table 2, Fig. 3B).

### Discussion

MB-C losses after multiple FT events were substantial, with ca. 30–40% of the MB-C being lost (Fig. 1D); such losses are in correspondence with

other studies (Yanai et al. 2004; Schimel et al. 2007). The vast majority of this microbial C was lost after the first FT event, which is in line with previous studies (Larsen et al. 2002; Walker et al. 2006). Schimel et al. (2007) assumed that the vulnerable part of the microbial biomass is lost after the first FT event, while the remaining microbes appear to be more resistant to additional FT events. In this study, the soil with the highest SOC content showed the highest C losses from MB-C (Fig. 1D), which corresponds to previous findings from a similar chronosequence study (Rosinger et al., submitted). Because the soil microbial community tends to be more active in high-SOC soils (Barrett and Burke 2000; Allison et al. 2007a), and FT damage was found to be greater when soil microbes were more active and growing (Schimel and Clein 1996), high-SOC soils might be particularly vulnerable to MB-C losses caused by FT. Our data indicate that microbial communities in high-SOC soils could be less resistant to environmental stresses, although a confirmation would require further testing on a larger set of soil samples. Moreover, it needs to be determined whether high-SOC soils on the other hand might be more resilient and thus compensate their losses faster after FT stress (Griffiths and Philippot 2013).

MB-N was also subject to substantial losses after multiple FT events (Song et al. 2017), with ca.



50–65% of MB-N being lost in our soils after three FT cycles (Fig. 1E). Just as MB-C, the strongest losses occurred after the first FT event and in the soils with the highest SOC content, further underlining the

vulnerability of high-SOC soils to FT. While initially MB-C and MB-N of the microbial community were strongly correlated, FT caused an immediate and lasting decoupling of MB-C and MB-N contents

◀ **Fig. 1** The response of **A** dissolved organic C (DOC), **B** total dissolved N (TDN), **C** the DOC:TDN ratio, **D** microbial biomass C (MB-C), **E** microbial biomass N (MB-N) and **F** the microbial (MB-C:N) ratio to three freeze–thaw (FT) events in four soils (Soil 1: 4 years, 0.39% SOC; Soil 2: 12 years, 0.77% SOC; Soil 3: 23 years, 0.76% SOC; Soil 4: 23 years, 1.04% SOC) of a postmining agricultural chronosequence. Bars represent the mean  $\pm$  1SE (n = 5). Significant effects of the FT treatment and significant interaction effects between FT treatment and soil SOC content as well as soil age (as derived from a one-way MANCOVA), respectively, are indicated with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Different letters above bars indicate significant differences ( $p < 0.05$ ; post-hoc Tukey test) between FT treatments within each tested soil sample

(Mooshammer et al. 2017; Fig. 2). Such a stoichiometric decoupling can lead to an intensified or a shifted nutrient limitation of soil microorganisms (Sterner and Elser 2002); in chronosequence soils of similar loess substrate of the Inden mine 50 km south of Garzweiler, Rosinger et al. (submitted) found evidence for a short-term oversaturation of MB-N relative to -C after a first FT cycle, which would well explain FT-driven N losses (Matzner and Borken 2008).

It is evident that with soil FT, substantial amounts of N can be released, and subsequently lost through leaching (Joseph and Henry 2008; Matzner and Borken 2008) or gaseous emissions (Congreves et al. 2018). The magnitude of N losses that can be directly attributed to FT may amount to 35–65% of total N<sub>2</sub>O emissions from croplands (Wagner-Riddle et al. 2017). We recorded significant increases in TDN with an increasing number of FT events (Fig. 1B). TDN increases were particularly marked after the second and third FT cycle and in the youngest soil with the lowest SOC content. In natural soil systems, these dissolved N substrates could be easily lost through leaching. Overall, TDN increases in all four soils after three FT events could only explain 35–50% of the N reductions in MB-N, suggesting that the remaining 50–65% of N must have been lost via gaseous emissions. This could explain the commonly observed high N<sub>2</sub>O fluxes in agricultural fields with spring thaw (Furon et al. 2008).

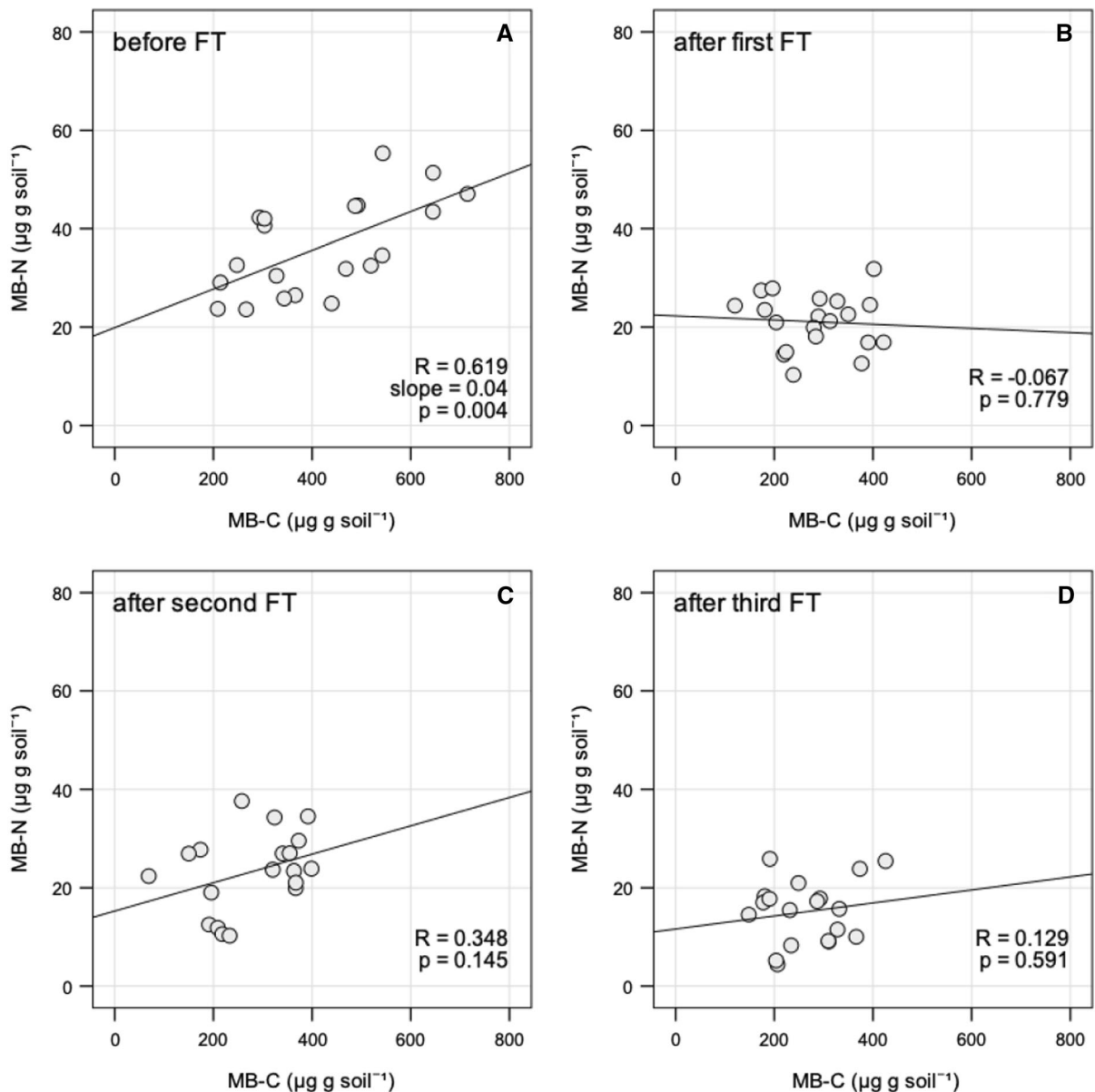
FT of soils commonly causes increases in DOC (Song et al. 2017; Matzner and Borken 2008). The released DOC (as well as TDN) after FT is thought to originate from the disruption of plant cells in litter and roots (Su et al. 2010; Campbell et al. 2014), soil

macro-aggregates (Kværnø and Øygarden 2006; Chai et al. 2014), and microbial cells (Larsen et al. 2002). Contrary to this commonly observed pattern, our soils showed 5–25% reduced DOC contents after the first FT event (Fig. 1A). Only with the second and third FT cycle, DOC contents increased again and reached similar or slightly higher than initial pre-freezing levels at the end of our experiment, suggesting that C losses via DOC were of minor importance as compared to losses via CO<sub>2</sub> emission in our soils (Matzner and Borken 2008).

Along with declining DOC and MB-C after FT, we recorded a significantly increased basal respiration after the first FT event (Sharma et al. 2006; Fig. 3A). Such a CO<sub>2</sub> burst is common in soils after FT and assumed to result from the dissimilation of easily accessible organic compounds released by FT (Schimel and Clein 1996; Schimel et al. 2007). Hermann and Witter (2002) showed that the killed microbial biomass was a major contributor to the pool of accessible organic compounds and their subsequent release as CO<sub>2</sub> burst after FT. Overall, the increased basal respiration and qCO<sub>2</sub> after the first FT together with decreasing DOC and MB-C indicate that a substantial part of the DOC released by FT could not be assimilated, but led to respiratory C losses by the soil microbial community. This further underlines that substrate availability controls to a substantial degree the respiratory responses to FT (Feng et al. 2007). Basal respiration decreased below initial levels after the second and third FT cycle, which is in agreement to several other studies (Larsen et al. 2002; Koponen et al. 2006; Han et al. 2018). The observed trends in qCO<sub>2</sub> resembled the trends of basal respiration rather well, suggesting that the reduced MB-C was responsible for this decline. The metabolic quotient is very responsive to FT (Mooshammer et al. 2017; Fig. 3B). Its sharp increase after the first FT event reflects disproportional dissimilatory (i.e., respiratory) C losses relative to microbial biomass (Fig. 3B), thus being a sensitive measure of the metabolic state of the soil microbial community (Wardle and Ghani 1995).

We deliberately chose soils that allowed us to disentangle the effects of SOC content and soil age on chemical and microbial responses to multiple FT events, because these factors often auto-correlate in chronosequences, and disentangling their effect is often difficult (Walker et al. 2010). A further important assumption is that sites of different ages follow the



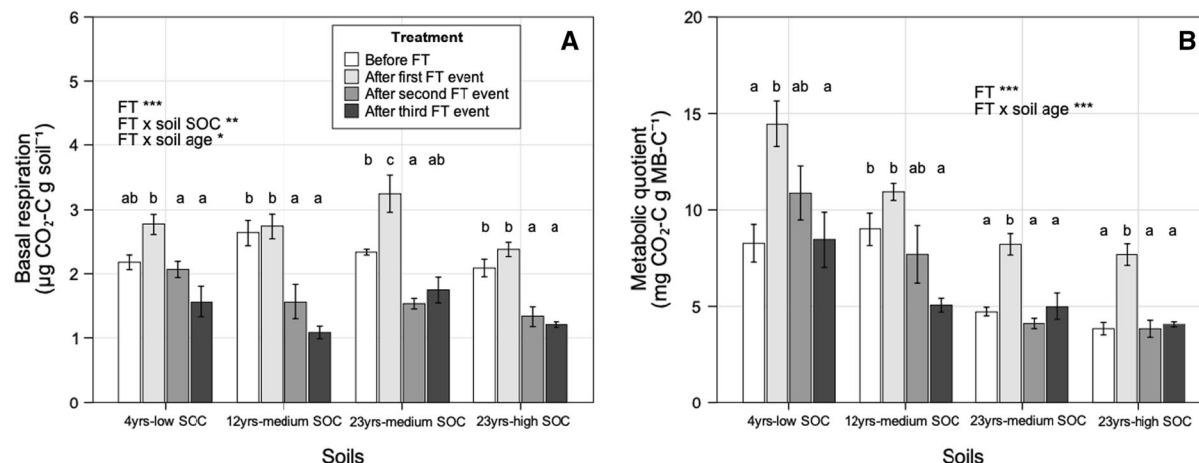


**Fig. 2** The relationship between MB-C and MB-N before freezing (A) and after the first (B), second (C) and third (D) freeze–thaw (FT) event. Linear regression analysis and

Pearson correlation coefficient ( $R$ ) was used to test for significant relationships at  $p < 0.05$  ( $n = 20$ ). The dashed lines represent the 95% confidence interval

same trajectory in soil development. Although farmers do follow area-typical management practices, factors such as fertilizer inputs and/or crop rotations might slightly deviate from field to field. Our analyses showed that soil age was the more important determinant compared to SOC content. While soil age had a significant and strong influence on DOC, TDN, MB-C and both respiratory responses to FT, SOC content

explained much less variation and had only a significant influence on MB-C and basal respiration (Table 2). High SOC content has been associated with higher microbial activity (Barrett and Burke 2000; Allison et al. 2007a), suggesting that a higher portion of active microbial assemblages is vulnerable to FT events (Schimel and Clein 1996). Studies on soil chronosequences have shown that progressing soil age



**Fig. 3** The response of **A** basal respiration and **B** the metabolic quotient ( $qCO_2$ ) to three freeze–thaw (FT) events in four soils (Soil 1: 4 years, 0.39% SOC; Soil 2: 12 years, 0.77% SOC; Soil 3: 23 years, 0.76% SOC; Soil 4: 23 years, 1.04% SOC) of a postmining agricultural chronosequence. Bars represent the mean  $\pm$  1 SE ( $n = 5$ ). Significant effects of the FT treatment and

significant interaction effects between FT treatment and soil SOC content as well as soil age (as derived from a one-way MANCOVA), respectively, are indicated with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Different letters above bars indicate significant differences ( $p < 0.05$ ; post-hoc Tukey test) between FT treatments within each tested soil sample

not only implies increasing SOC contents, but also changes in soil organic matter quality (Shi et al. 2006; Egli et al. 2010; Clark et al. 2012; Chaudhuri et al. 2015; Kumar et al. 2018; Khedim et al. 2020), which in turn dictates biogeochemical processes (Shi et al. 2006; Banning et al. 2008). Recently, Delgado-Baquerizo et al. (2020) showed that soil age indeed drives local-scale ecosystem properties associated with biological activity, such as soil N:P and C:P ratios, microbial biomass, fungal:bacterial dominance or SOC stocks. Thus, microbial responses to FT events may be indirectly linked to soil age via temporal changes in soil biogeochemical characteristics (Yanai et al. 2004; Allison et al. 2005, 2007b; Moore et al. 2010; Delgado-Baquerizo et al. 2020). In line with this, the climatic history of soils can lead to a functional reorganization of the soil microbial community towards more tolerant species (Bardgett and Caruso 2020). Accordingly, a longer history of soil freezing (i.e. older soils) may select for frost-tolerant microbial traits and communities that are physiologically better adapted to FT cycles (Walker et al. 2006; Garcia et al. 2020). Indeed, a recent metagenomic study by Schmidt et al. (2020) showed highly distinct clusters of bacterial communities with soil age in restored loess soils of a comparable soil chronosequence in Inden. Because the restored loess material contains carbonates which disintegrate over time, soil

age and soil pH were negatively correlated along the chronosequence. Although pH differences were not strong (8.59 and 8.23 in the youngest and oldest soil, respectively), it is important to mention that soil pH can have significant effects on nutrient availability (Amelung et al. 2018), microbial community composition (Rousk et al. 2010) and their functions (Kemmitt et al. 2006; Fuentes et al. 2006). Thus, there are likely other parameters covarying with soil age and important in shaping soil microbial responses to FT that we have not captured in our study.

To conclude, this study demonstrated that a single FT event caused a thorough decoupling of MB-C:N stoichiometry. Soils lost a significant proportion of both MB-C and MB-N after the first FT cycle; but while MB-C remained rather unchanged, MB-N continued to decrease with subsequent FT cycles. Our four tested soils lost between 28 and 43% of MB-C and, even stronger, 47–66% MB-N when exposed to three severe FT events. The MB-C released during the first FT event was mainly lost through respiration and could not be reassimilated into biomass, leading to initially lower DOC values. Increasing DOC with subsequent FT cycles, despite constant levels of MB-C and decreasing MB-N suggest that liberated DOC was less and less metabolized in the subsequent FT cycles. This is corroborated by strongly decreasing rates of  $qCO_2$  with subsequent FT cycles, indicating that the

surviving microbial community showed less and less metabolic activity. The discrepancy of FT-driven MB-N losses to TDN gains was most likely due to gaseous N losses, potentially driven by a short-term oversaturation of MB-N relative to -C. Our study partly allowed to dissect the importance of soil age and SOC content when explaining variation in soil biochemical responses to multiple FT events, and our results suggest that different mechanisms regulate soil chemical and microbial responses to multiple FT events. Especially soil age turned out to be a significant factor and explained substantial variation in chemical and microbial responses to multiple FT, while SOC content explained much less variation and only affected MB-C and basal respiration. There is an urgent need to better understand these soil processes under future climate change projections. Although conducted on a limited set of samples, our study shows that soil chronosequences can be valuable tools to identify important drivers of microbial responses, such as soil age and SOC content to re-occurring temperature extremes. Future studies should aim to further dissect the processes associated with soil age, which was a major driver of FT-driven changes in soil biochemistry.

**Acknowledgements** The project was funded by the German Federal Ministry of Education and Research (BMBF) as part of the BonaRes initiative, in the project INPLAMINT: ‘Increasing agricultural nutrient-use efficiency by optimizing plant-soil-microorganism interactions’ (Grant No. BMBF-FKZ 031B0508F). The authors thank the INPLAMINT project team, especially Jessica Clayton and Rüdiger Reichel for planning and organizing the soil sampling campaign, Nicolas Brüggemann for leading the consortium. We further thank Gregor Esser and Ernst-Michael Ortmann of the ‘Forschungsstelle Rekultivierung’ for their logistic support in selecting the chronosequence sites. Special thanks to Irene Brockhaus, Anna Herzog and Conny Thielen for help in soil analyses.

**Funding** Open Access funding enabled and organized by Projekt DEAL. The project was funded by the German Federal Ministry of Education and Research (BMBF) as part of the BonaRes initiative, in the project INPLAMINT: ‘Increasing agricultural nutrient-use efficiency by optimizing plant-soil-microorganism interactions’ (BMBF-FKZ 031B0508F).

**Data availability** The presented data will be submitted to the BonaRes data repository (<https://datenzentrum.bonares.de/data-portal.php>).

## Declarations

**Conflict of interest** The authors declare no conflicts of interest.

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