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Rapid response of soil GHG emissions and microbial parameters to the addition of biochar and the freeze-thaw cycle**

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Abstract. A reduction in snow cover makes soil more exposed to freezing-thawing processes. We tested the rapid response of greenhouse gas (GHG) emissions in fertilized soil to biochar addition under the freeze-thaw cycle. The soil (at a moisture of 55% water holding capacity) was enriched with biochar at a rate of 20 t ha^{-1}, frozen at -20°C for 12 h and thawed at 20°C. The control was soil without biochar and with biochar incubated at temperature of 20°C. Unfrozen soil was a CO_2 and N_2O source and a weak sink for CH_4. The GHG fluxes were not significantly altered by biochar, although biochar-treated soil emitted CH_4. The frozen soil emitted all the tested GHGs during the thawing period. A pulse of CO_2 and CH_4 emissions (and N_2O in soil with biochar) occurred in the period of rapid temperature growth, while GHG fluxes reached levels similar to unfrozen soil after temperature stabilization at 20°C. In frozen soil, the addition of biochar significantly increased the CH_4 and N_2O peak only in the first phase of thawing with a dynamic temperature growth. Biochar changed microbial parameters, therefore we assume that both physical and biological mechanisms could be responsible for GHG emissions in frozen soils.

Keywords: soil, freezing, thawing, greenhouse gases, biochar, GHG pulse

INTRODUCTION

The soil ecosystem contributes to global warming by emitting and absorbing greenhouse gases (GHGs). Carbon dioxide (CO_2) is emitted as a result of microbial decomposition of soil organic matter, root respiration, rhizomicrobial respiration and the priming effect, and from human activity including land use changes, deforestation and forest fires (Kuzyakov, 2006; Li *et al.*, 2015; Rahman, 2013). Methane (CH_4) is a gas with a 27-fold higher global warming potential (GWP) than CO_2 (IPCC, 2021). It can be produced in soil under anaerobic conditions (methanogenesis) or can be absorbed through oxidation by methanotrophic bacteria (methanotrophy) (Wnuk *et al.*, 2020). Nitrous oxide (N_2O) is a gas with 273-fold greater contribution to global warming than CO_2 (IPCC, 2021). Soil N_2O formation involves biotic and abiotic processes, but is not clearly understood (Butterbach-Bahl *et al.*, 2013). The agricultural sector is a major source of N_2O from soil fertilization and manure management.

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Global climate change alters the frequency and intensity of extreme weather events, causing droughts, floods and freezing-thawing from a reduction of snow cover during winter (Kim *et al.*, 2012; Nabizada *et al.*, 2023). A decrease in snow cover occurs in most regions and has been confirmed in Poland (Tomczyk *et al.*, 2021), although the opposite phenomenon has been observed in some regions (Choi *et al.*, 2010). During the winter, the cold periods vary in length and are interrupted by periods of thaw, therefore soil freeze-thaw cycles are common in the temperate zone (Miranda-Vélez *et al.*, 2023). Such changes in soil temperature are stressful for soil microorganisms and alter the intensity of various biochemical processes (Rosinger *et al.*, 2022). The influence of air temperature on soil dynamics is also determined by land use and management. In 2016 in the European Union (EU), 23% of arable land was left as bare soil without any vegetation cover during winter (Eurostat, 2022). Bare soils are more exposed to erosion, leaching of nutrients and freezing than land covered with vegetation (Bo *et al.*, 2021; Eurostat, 2022). Larger temperature fluctuations during freezing and thawing have been seen for the surface layer (up to 10 cm deep) (Bo *et al.*, 2021), which simultaneously contributes significantly to gas exchange with the atmosphere. In addition to the frozen state of the soil itself, dynamic changes in soil temperature, hydrology and release of nutrients during freeze-thaw events particularly affect GHGs. Studies on agricultural soils have shown stimulation of respiration and N_2O emission under freezing-thawing because of the release of decomposable organic C and N which increases denitrification (DeLuca *et al.*, 1992; Mørkved *et al.*, 2006). Moreover, thawing causes the gases accumulated in the soil profile during winter to be rapidly released in spring (Maljanen *et al.*, 2007).

The significant contribution of agriculture to global warming means that it is important to identify alternatives to traditional fertilization that reduce emissions and increase C sequestration (Lehmann *et al.*, 2011; Radawiec *et al.*, 2023). Among various potential solutions, an ongoing focus of research is the application of biochar, which can improve soil conditions and crop yields (Ding *et al.*, 2016). Enrichment of soils with biochar changes the physical and chemical properties of soil, *e.g.*, it increases soil C content, pH, water and nutrient storage, improves soil aeration and thus affects soil microbiota (as summarized, *e.g.*, by Ding *et al.* (2016) and Lehmann *et al.* (2011)). Beside practical benefits, it is important to recognize the environmental consequences of biochar application, which are not explained by freeze-thaw cycles (Li *et al.*, 2022). Biochar may reduce GHG emissions in agricultural soil, although the mitigation potential is determined by different factors such as soil type, biochar properties, ageing and pyrolysis temperature (Feng *et al.*, 2022; Méndez *et al.*, 2013; Sha *et al.*, 2019). As GHG pulse emissions occur during soil thawing (Kim *et al.*, 2012), it is useful to recognize how biochar in soil may modify GHG fluxes during freeze-thaw cycles. The few studies in this field suggest that the GHG pulse can contribute significantly to their annual balance. Usage of a higher dose of biochar can increase CO_2 emissions more than thawing, but biochar increases the capacity of soil to absorb CH_4 by improving aeration (Liu *et al.*, 2017). The presence of biochar in cultivated soil during the freezing-thawing cycle has been shown to reduce N_2O emissions through decreased soil nitrification (Liu *et al.*, 2016a), but this effect has not been confirmed in all studies (Zhou *et al.*, 2017b).

The aim of the present study was to assess the rapid response of GHG (CO_2, CH_4, N_2O) emissions and microbial parameters of arable soil with and without biochar and under the controlled simulation of the freeze-thaw event.

MATERIALS AND METHODS

A laboratory experiment was conducted on soil samples collected in the Lublin Upland, Poland (coordinates 51°14’69”N, 22°89’39”E). In recent years before sampling, data from the weather station located next to the Institute of Agrophysics, PAS in Lublin (51°13’11”N, 22°37’32”E, 207 m above sea level) confirmed that the average annual temperature in this region was between 8.5 and 10°C and the annual rainfall was between ~500 and 700 mm. During the winter period preceding soil sampling, the average temperature ranged from -1.08°C in December 2022 to 0.82°C in February 2023. Soil samples were collected in April 2023 from a fertilized field under winter wheat. The soil was classified as a fertile arable land, the management of which included a multi-year fertilization regime with annually cultivated winter wheat or oilseed rape. Five representative soil samples (surface layer, *i.e.*, 0-20 cm depth) were collected at 1 m intervals, thoroughly homogenized to provide a representative sample and sieved to <2 mm. The soil was classified as a Luvisol soil, with a pH of 7.61, had silt loam texture, 3.44% of clay, 54.4% of silt, 42.2% of sand, and 22.08 g kg^{-1} of organic C concentrations.

The physico-chemical soil parameters were determined under controlled laboratory conditions (n = 3). Particle size distribution (PSD: clay (diameter < 2 μm), silt (diameter 50-2 μm) and sand (diameter 2000-50 μm)) was determined using a Mastersizer 2000 laser diffractometer with a Hydro G dispersion unit (Malvern Ltd., Malvern, UK) (Polakowski *et al.*, 2023). Soil organic carbon was determined with a TOC-VCPH analyser (Shimadzu, Kyoto, Japan). Soil pH was measured potentiometrically at room temperature in a soil and water slurry, with soil:water ratio of 1:2.5 w/w. Nitrate (NO_3^-) concentrations were determined potentiometrically in the soil solution using an ion-selective electrode and reagents for nitrate contents (HQ40D Portable Multi Meter analyser). Water holding capacity (WHC) of the soil and of the soil with biochar was measured based on the difference in weight of soil samples wetted in water and then dried (Priha and Smolander, 1999).

The biochar was produced in 2018 at 650°C from fir sawdust, with a pH of 7.14, concentrations of 78.46% C and 0.19% of N, and a density of 220 kg m^{-3}, as described previously by Walkiewicz *et al.* (2020). The structure and surface morphology of the biochar were detected by scanning electron microscopy (SEM). The biochar has been stored in the dark at room temperature.

Fresh soil samples of 450 g dry weight were placed in 5.2 dm^{-3} airtight dark vessels (n=3). Biochar was added to half of the soil samples at an amount corresponding to a rate of 20 t ha^{-1}. The soil was enriched with biochar two weeks before the main experiment and preincubated at 20°C in darkness to adapt the microorganisms. Next, the samples were moistened with distilled water to a level corresponding to 55% WHC. After a three-day preincubation period, half of the samples were incubated at temperature of 20°C and the other half placed under frozen conditions (-20°C in a freezer) for 12 h and thawed at 20°C. A similar temperature gradient was used by Müller *et al.* (2002). After sealing the vessels, measurements of changes in gas concentrations (CO_2, CH_4 and N_2O) in the headspace were taken for 10 h at 20°C with a frequency of half or one hour. In frozen samples, GHG measurements started once the vessels were removed from the freezer. Changes in headspace temperature inside the vessels were monitored at each GHG measurement. At the end of the incubation period, soil samples were taken from each variant to measure pH, concentrations of C and NO_3^-, and microbial parameters (basal respiration (BR) and soil microbial biomass (C_{mic})). A visual overview of the experimental design is shown in Fig.1.

GHG concentrations (CO_2, CH_4, and N_2O) in the headspace were measured using a portable Gasmet DX-4040 Fourier-Transform Infrared Gas Analyzer (FTIR-GA) (Gasmet Technologie Oy, Helsinki, Finland). Prior to the measurements, the analyser was zeroed with helium. Microbial parameters included BR, C_{mic} and metabolic quotient qCO_2. Soils in a moisture content corresponding to 55% WHC were weighed (3 g dry mass, n=3) into 60 cm^3 glass vessels. Soil BR was determined based on CO_2 emission after 2 h incubation at 25 °C. C_{mic} was measured using the substrate-induced respiration (SIR) method recommending enrichment of the soil with a glucose solution (10 mg per gram of soil) and CO_2 emission analysis after 2 h incubation at 25°C (Anderson and Domsch, 1978). The CO_2 concentrations were measured chromatographically using a Shimadzu GC-14A equipped with a thermal conductivity detector (TCD) and with the use of a 2 m column (3.2 mm diameter) packed with Porapak Q (Shimadzu Corp., Kyoto, Japan).

GHG emission rates were calculated from the slope of the change in concentration with time, according to the commonly used equations taking into account air temperature, area and volume of vessel (Flessa *et al.*, 1998; Cowan *et al.*, 2014). The rates were calculated for each variant separately and were expressed in mg CO_2-C m^{-2} h^{-1}, mg CH_4-C m^{-2} day^{-1} and µg N_2O-N m^{-2} h^{-1}. In the variant with soil freezing, the GHG emission rates were calculated by dividing the incubation time into two stages depending on the headspace temperature in the vessel: the initial stage of thawing (with a dynamic temperature growth) and the final stage of thawing (after temperature stabilization). Microbial biomass content was expressed in CO_2 g^{-1} h^{-1} and calculated according to Šimek and Kalčík (1998). The qCO_2 was calculated based on the BR:C_{mic} ratio, and expressed as µg CO_2-C mg^{-1} C_{mic} h^{-1}.

The results were statistically analysed with Statistica 13 software (StatSoft Inc., Tulsa, OK, USA). The analysis of variance (ANOVA) test was used to evaluate the significance (at the 5% level) of the differences in soil

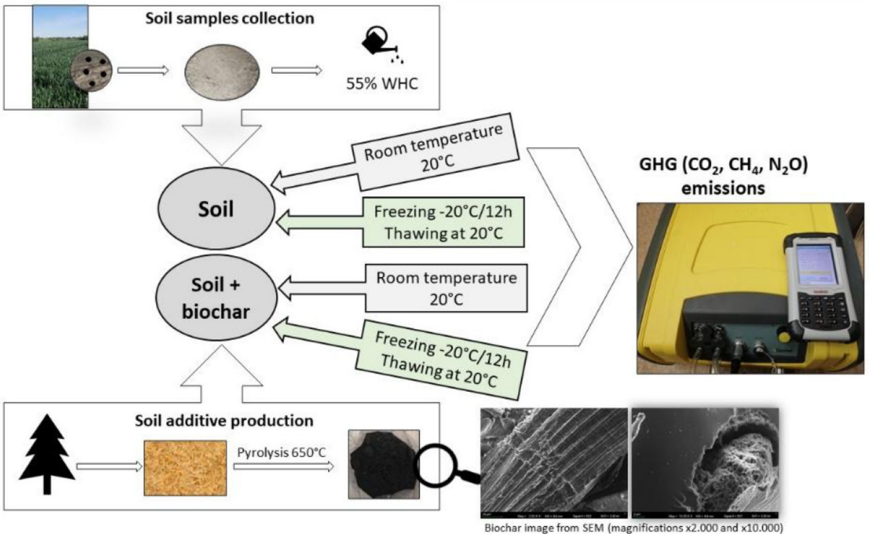

Fig. 1. Design of the experiment.

parameters between variants and in GHG emission rates, separately for each gas. Multifactorial analysis of variance (MANOVA) was used to test which of the factors (biochar, freezing-thawing or their interaction) significantly affected the GHG fluxes. Correlation analyses were conducted (at the 5% level) to recognize significant relationships between different parameters.

RESULTS

Both biochar enrichment and the freeze-thaw cycle changed soil parameters (Table 1). The addition of biochar significantly increased the water storage capacity of soil and doubled the organic C concentration. Soil pH was significantly higher in soil with biochar, which was not altered by freezing. The concentrations of NO_3^- were the lowest in unfrozen soil without biochar, significantly higher in unfrozen soil with biochar and non-enriched frozen soil, and the highest in frozen soil with biochar. Among microbiological parameters, the combination of biochar and freezing-thawing significantly increased BR; the lowest BR was in unfrozen soil with biochar and the highest in thawing soil. C_{mic} increased significantly after the addition of biochar, both in unfrozen and frozen soil. qCO_2 was significantly decreased by biochar and insignificantly increased by the freezing-thawing cycle.

The headspace air temperature in the vessel was maintained at 20°C during GHG measurements in the unfrozen soil which was a source of CO_2 and N_2O, a weak sink for atmospheric CH_4. The GHG fluxes were not significantly altered by biochar, although soil without biochar consumed CH_4, while with biochar emitted CH_4 (Fig. 2).

In the frozen soil variant, the vessel placed at room temperature showed an initial dynamic from 11°C to 20°C which lasted about 2 h. Thereafter, the air temperature in the vessel remained constant at 20°C during soil thawing.

Regardless the biochar presence, the CO_2 emission rates were significantly highest in the first period of soil thawing, with a dynamic increase of ambient temperature. In this time, we observed a significant and positive correlation between CO_2 level and ambient temperature (Fig. 3). However, after temperature stabilization, the CO_2 emission in soils without and with biochar reached a level close to unfrozen soil (Fig. 2). Biochar enrichment resulted in a shift from CH_4 uptake into soil CH_4 emission, although the differences in the rates were not statistically significant. Thawing soil initially showed the highest CH_4 emission peak and soil with biochar emitting 3.5 times more CH_4 than the control. During the dynamic growth of temperature, a positive relationship ($p < 0.05$) was detected between temperature and CH_4 concentration, regardless of biochar presence (Fig. 3). After stabilization of the temperature in the headspace, the soil reabsorbed atmospheric CH_4, regardless of the presence of biochar. The unfrozen soil emitted N_2O at similar levels without and with biochar. During the initial thawing stage, a much higher N_2O emission pulse occurred in the variant with biochar and we observed a significant and positive correlation between air temperature in the vessel and N_2O concentration, similar to CO_2 and CH_4 (Fig. 3). During stabilized temperature in the headspace, the amount of N_2O emitted was similar to the unfrozen control and slight N_2O uptake occurred after the addition of biochar.

MANOVA confirmed that all the tested factors and their interactions significantly ($p < 0.0001$) affected GHG emission rates (considering the rate in unfrozen soil and in the initial thawing stage) in the following order: freezing-thawing ($F = 397.3$) > biochar × freezing-thawing ($F = 200.8$) > biochar ($F = 192.5$).

DISCUSSION

Given the effects of ongoing climate change, it is important to recognize the soil processes occurring during the non-growing season. In recent years, there have been around 95 freezing days (with a daily minimum temperature below 0°C) per year in Poland, which shows a decreasing trend (IOŚ-BIP, 2020). Although the lowest temperatures occur in the winter season (December-February), a long-term analysis for Lublin showed that between 1966 and

Table 1. Parameters of unfrozen and thawed soil without and with biochar

Parameter	Unit	Unfrozen soil		Freezing/thawing soil	
		without biochar	with biochar	without biochar	with biochar
Water holding capacity	($g\ H_2O\ g$ dry soil^{-1})	0.73 ± 0.042 a	0.92 ± 0.016 b	–	–
pH	–	6.56 ± 0.209 a	7.10 ± 0.095 b	6.62 ± 0.286 a	7.11 ± 0.012 b
Organic C concentration	($g\ kg^{-1}$)	24.2 ± 2.19 a	55.3 ± 0.99 c	27.9 ± 6.32 a	46.3 ± 0.38 b
NO_3^--N concentration	($mg\ kg^{-1}$)	43.5 ± 0.57 a	53.3 ± 1.95 b	53.4 ± 1.17 b	77.9 ± 1.44 c
Basal respiration	($\mu g\ C\text{-}CO_2\ g^{-1}\ h^{-1}$)	3.95 ± 0.94 ab	2.54 ± 0.75a	5.07 ± 1.22 b	4.76 ± 0.89 ab
Soil microbial biomass	($CO_2\ g^{-1}\ h^{-1}$)	0.770 ± 0.060 a	1.24 ± 0.243 b	0.858 ± 0.024 a	1.22 ± 0.103 b
Metabolic quotient qCO_2	($\mu g\ CO_2\text{-}C\ mg^{-1}\ C_{mic}\ h^{-1}$)	10.41 ± 3.23 b	4.06 ± 0.70 a	11.85 ± 2.91 b	7.89 ± 1.95 ab

The same letters indicate no statistically significant differences between variants (one-way ANOVA, Tukey post-hoc test, $p < 0.05$, calculated separately for each parameter).

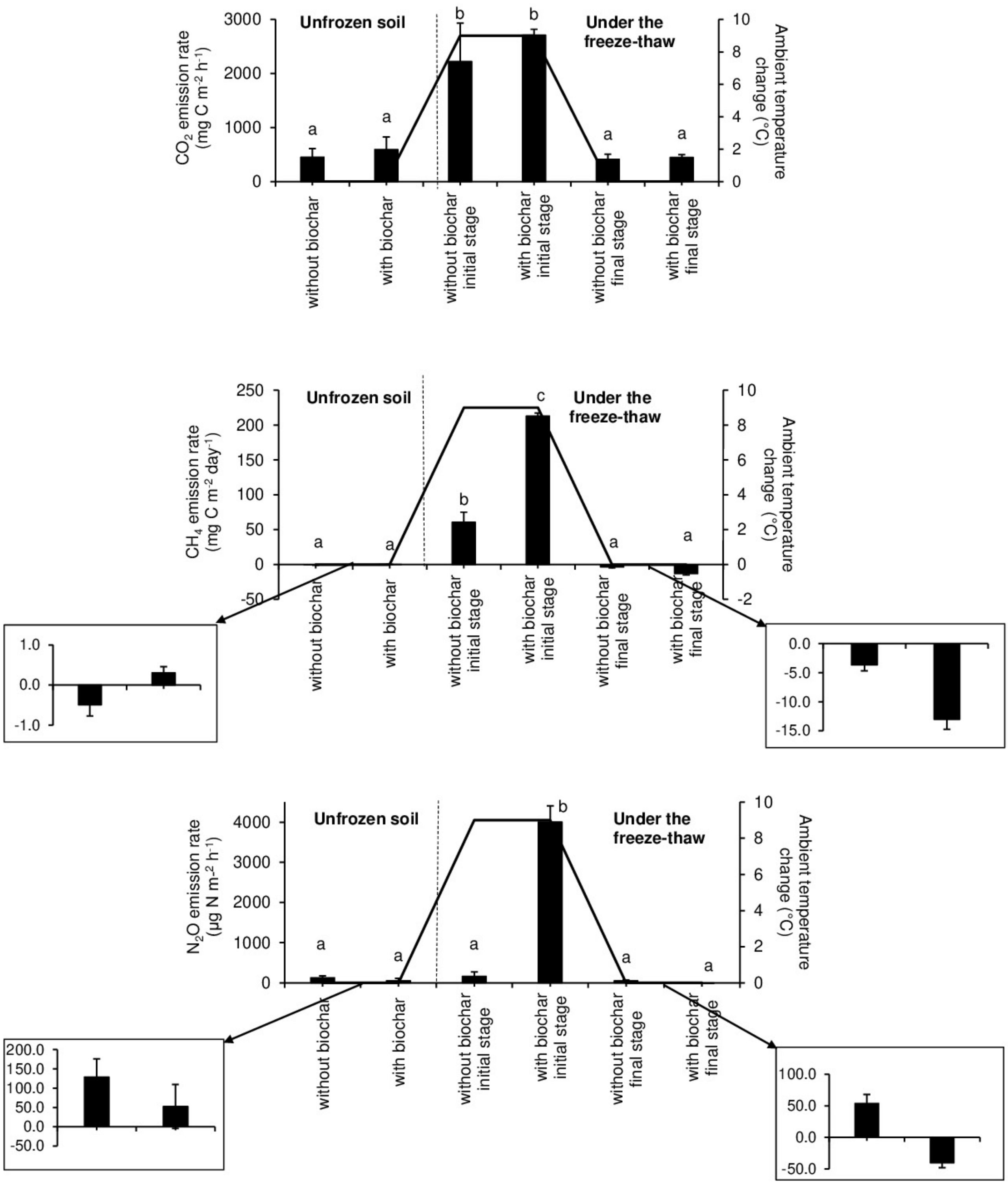

Fig. 2. GHG emission rates (columns) and ambient temperature change (line) in soils without and with biochar including variants: (i) incubation at 20°C; (ii) freezing at -20°C and thawing at 20°C. The initial stage of thawing indicates the GHG emission rate during dynamic temperature growth; the final stage of thawing indicates the rate in constant temperature in the vessel. The same letters above the columns indicate no statistically significant differences between experiment variants, separately for each gas (one-way ANOVA, Tukey post-hoc test, $p < 0.05$).

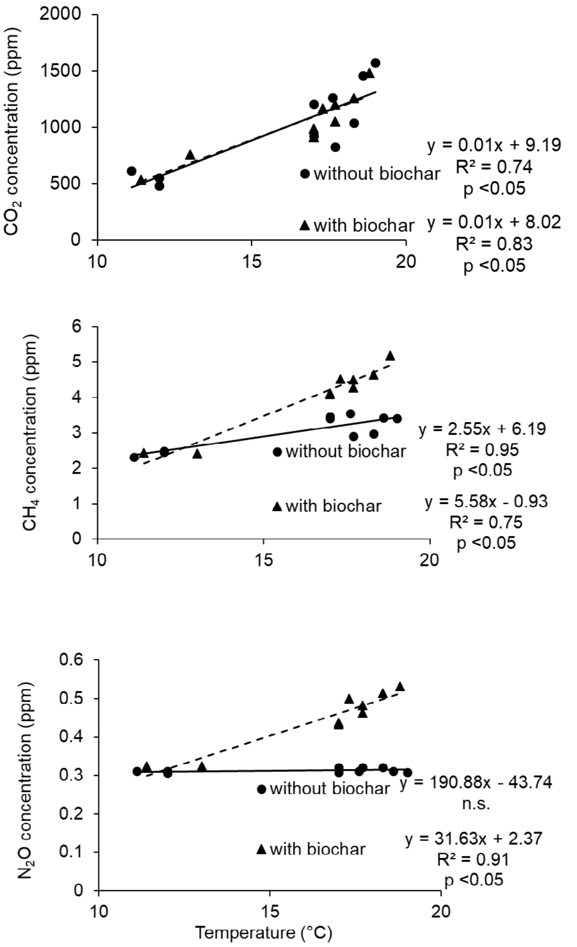

Fig. 3. Relationships between air temperature in the vessel and CO_2, CH_4 and N_2O concentrations during the first stage of soil thawing (significant differences at $p < 0.05$).

2005, the number of frost days (with air temperature T_{min} < 0°C and T_{max} >0°C) ranged from 11 days in March to three or fewer in May and September (IMGW-PIB, 2023). Temperature fluctuations justify conducting tests under freezing-thawing soil conditions in soils from this region.

Biochar changes soil parameters creating different conditions for soil microorganisms (Lehmann *et al.*, 2011). The increased water storage capacity (as a result of porous structure of biochar and water storage in small pores) and pH (since biochars are often alkaline) of unfrozen soil (not altered by freezing-thawing cycle) reported previously (Karhu *et al.*, 2011) was confirmed in our study. The addition of biochar, carbon-rich material, doubled the organic C content, as in other studies (Paetsch *et al.*, 2018). Combination of the addition of biochar and soil thawing significantly in our study increased extractable NO_3^- concentrations. This N form is a product of nitrification but it may have also originated from other sources. The promotion of nitrification or the slowing down of denitrification caused by the presence of biochar has been reported in other studies (Wang *et al.*, 2011; Yang *et al.*, 2020). Biochar increased soil aeration and pH approaching the optimum level for autotrophic nitrifiers from 7.5 to 8 (Szarlip *et al.*, 2010), which creates the proper conditions for nitrification. The moisture content of 55% WHC in our experiment is suitable for this process (Case *et al.*, 2012). Therefore, based on higher NO_3^- concentrations and altered soil conditions after the addition of biochar, we may suppose that NO_3^- originated partly from nitrification during thawing. However, other studies showed that biochar decreased extractable NO_3^- concentrations because of chemical adsorption on the biochar surface (Kameyama *et al.*, 2012), immobilization within microbial biomass (Andersen and Petersen, 2009) or decreased soil nitrification (Liu *et al.*, 2016a), which was not confirmed by our results. In the freezing-thawing cycle, studies on fertilized soils have shown that different processes can be involved in N transformations depending on the freeze-thaw stage, regulated by soil temperature (Müller *et al.*, 2002). The decrease in soil aeration and the increase in moisture content in frozen-thawed soil may have created conditions for the denitrification process in the frozen soil variant (Li *et al.*, 2023; Mørkved *et al.*, 2006). Higher NO_3^- concentrations promoted by thawing-freezing have also been revealed in other soils, suggesting N mineralization as its source (Li *et al.*, 2023). Moreover, the meta-analysis revealed that the freeze-thaw effect significantly promotes ammonification (Song *et al.*, 2017), which also may have occurred in our tests as ammonia emissions were detected (data not shown).

Among soil microbial parameters, the addition of biochar decreased basal respiration and increased soil microbial biomass C. Lower BR has been promoted by thawing as the effect of increased decomposition of thawed C (Natali *et al.*, 2015), although most of the studies show higher BR in soil with biochar (Steiner *et al.*, 2008). C_{mic} was not been changed after freezing which may result from adaptation of microorganisms inhabiting the soil to temperature fluctuations due to seasonal changes and the increasingly rare snow cover in recent years. However, other studies also showed a decline of C_{mic} after freezing-thawing, especially in C-rich soils, as an effect of dry state of cells from altered osmotic potentials and the killing effect (Koponen and Bååth, 2016; Rosinger *et al.*, 2022; Yanai *et al.*, 2004). Similar to our results, the meta-analysis by Zhou *et al.* (2017a) revealed higher soil microbial biomass C in soil with biochar and the meta-analysis by Song *et al.* (2017) revealed no significant response of microbial biomass to freeze-thaw treatments. We observed a positive relationship between soil microbial biomass C and soil pH (Fig. 4). This may suggest a different composition of the microbial community, since soil pH has been reported as a strong regulator of bacterial taxonomic diversity, composition, richness and biomass (Bahram *et al.*, 2018; Gangwar *et al.*, 2022; Szafranek-Nakonieczna *et al.*, 2018).

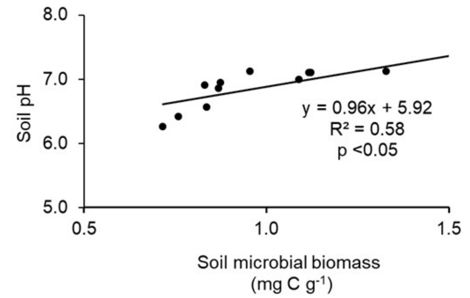

Fig. 4. Relationships between C_{mic} and pH in soil without and with biochar, including unfrozen and frozen variants (statistically significant at $p<0.05$).

An important indicator of microbial health in soils is its metabolic quotient, which relates to the microbial use of carbon for their energy consumption (Anderson and Domsch, 1978) and which is sensitive to short-term soil changes (Zhou *et al.*, 2017a). Lower qCO_2 values after the addition of biochar indicate improved soil biophysical conditions (Zhou *et al.*, 2017a), which was not significantly changed in thawed soil.

The meta-analysis by Shakoor *et al.* (2021) summarized that the biochar effect on soil GHG emissions across global croplands is GHG-specific and determined by many factors such as feedstock, temperature pyrolysis, experimental method, soil texture, biochar dose and the soil and biochar pH and C:N ratio. The authors concluded that biochar shows the mitigation potential of GHGs and may decrease emissions from agriculture. In our study, we tested the rapid response of GHG emissions after the freezing-thawing event, which is important part of the annual GHG output.

Agricultural soils are a source of CO_2, although its emission is regulated by practices used and nitrogen fertilization results in stimulation of soil respiration and an enhancement in soil C mineralization (Lu *et al.*, 2011). Soil parameters may be also modified by the application of biochar, being a commonly investigated practice with the potential of C sequestration, improving soil conditions and crop yield (Lehmann 2011; Liu *et al.*, 2016b). In our study, biochar enrichment did not significantly change soil CO_2 emission, which concurs with other studies on agricultural soils (Castaldi *et al.*, 2011; Huang *et al.*, 2023; Liu *et al.*, 2016b). However, a reduction (Sarkhot *et al.,* 2012) or an increase in CO_2 emission after the addition of biochar (Sagrilo *et al.*, 2014; Walkiewicz *et al.*, 2020) have also been reported. The meta-analysis by Sagrilo *et al.* (2014), which showed a positive effect of biochar on soil respiration, included doses of biochar that were many times higher than those used in our study, which may explain the different effect. The authors found no effect of biochar on soil respiration when the pyrolysis temperature was above 350°C. Our biochar was produced at 650°C, which may also explain the lack of effect on soil respiration. A previous study on different soils, but with the same biochar, revealed a decrease in CO_2 emission in saturated forest soil and an increase in CO_2 emission in saturated orchard soil (Walkiewicz *et al.*, 2020). No effect was observed under 55% WHC of fertilized orchard soil, which was confirmed in our experiment as the same soil moisture condition was used.

The tested soil showed weak methanotrophic activity since low CH_4 uptake was observed in the control sample without biochar. The reduction of methanotrophy in fertilized soils has been reported previously as an effect of N fertilization, often explained by the competitive inhibition of monooxygenase by NH_4^+ (Bodelier and Steenbergh, 2014). Interactions between C and N are still not fully recognized and there are also contrary studies showing stimulation of methanotrophy by N fertilization (Bodelier and Steenbergh, 2014). The biochar addition in our experiment resulted in a shift of soils from weak sink into weak source of CH_4, but the differences were not statistically significant. An experiment on different soils fertilized but enriched with the same biochar showed a stimulation of methanotrophy, especially under saturated conditions, which may have resulted from improved soil aeration and increased O_2 availability for methanotrophs (Walkiewicz *et al.*, 2020). In our experiment, the soil was unsaturated and microorganisms were exposed to O_2 which may lead to an insignificant impact of biochar on soil methanotrophy. However, the current experiment investigated ambient CH_4 uptake over a short incubation period, while the previous study was conducted over a longer time period and under conditions of elevated (2% v/v) CH_4 concentration. Different types of methanotrophs, referred to as low- and high-affinity methanotrophs, are responsible for the methanotrophic process under such different CH_4 concentrations, and their populations may respond differently to environmental conditions (Tate *et al.*, 2012). In fertilized soils, both methanotrophs and nitrifiers may be responsible for CH_4 oxidation as an effect of activity of a similar enzyme, particulate methane monooxygenase (MMO) *vs*. ammonia monooxygenase (AMO) (Hyman and Wood, 1983). The long-term nitrogen fertilization of the soil investigated may have favoured the nitrifier community and a microbial test would be needed to identify the microbes inhabiting it. It is difficult to assess the longer lasting effect of biochar on methanotrophy in a rapid soil response experiment.

The tested soil was a source of N_2O which is typical for fertilized soils. Similarly to CO_2 and CH_4, the rapid response of soil N_2O emission to the application of biochar was not significantly changed over the short incubation period. A longer term incubation period of another soil fertilized with the same biochar showed a reduction in N_2O emissions as an effect of suppression of N_2O production and stimulation of reduction of N_2O to N_2 (Dong *et al.*, 2020). This N_2O mitigation has also been reported in various meta-analyses (e.g. Huang *et al.*, 2023; Kaur *et al.*, 2022; Shakoor *et al.*, 2021). Different results can be found in the literature which underlines that the response of soil to biochar is determined by N fertilizer dose, cation exchange capacity, pH and biochar application rate (Huang *et al.*, 2023; Sun *et al.*, 2017). The mitigation potential of biochar has been reported for pH 8-9, while our biochar has lower pH. The highest reduction of N_2O emission has been reported for soil organic C content in the range from 10-20 mg g^{-1} (Kaur *et al.*, 2022), while our soil has a higher C content. These parameters may explain the lack of effect of biochar on rapid N_2O emission in our study. Another explanation may be the amount applied, since 10-20 t ha^{-1} of biochar did not significantly change N_2O emissions (Kaur *et al.*, 2022).

When interpreting our results, it is important to consider the procedure used of frequent measurements over a short period of time, which is dictated by the course of the soil thawing process, discussed below. Measurements over a longer time period would provide more accurate information on the impact of biochar on GHG fluxes.

Biochemical soil processes in the non-growing season are notably affected by soil thawing because of an increase in the availability of soil water and mobilization of nutrients which results in rehydrate cells, increased microbial metabolism and improved gas diffusion (Kim *et al.*, 2012). Our study found that the frozen soil emitted all the key GHGs during the thawing period, but the magnitude of emissions was dependent on the ambient temperature dynamic. The rapid ambient temperature growth typically lasted up to 2 h after the soil vessels were moved from freezing to a controlled temperature of 20°C. There then occurred a rapid pulse of emissions of CO_2 and CH_4 (and N_2O in soil with biochar) which reached the unfrozen level after the ambient temperature was stabilized at 20°C. Similar results were obtained in thawing forest soils but the N_2O and CH_4 fluxes reached the maximum after 6 h, while the CO_2 flux maximum was observed after 12 h and emissions stabilized within about 48 to 96 h (Wu *et al.*, 2020). The study on alpine meadow and alpine peatland also showed analogous trends, although of longer duration and with GHG fluxes specific to the soils studied (Gao *et al.*, 2015). A similar trend of changes in GHG fluxes during soil thawing was confirmed in our experiment, but the differences in moment and duration of the GHG peaks and next fluxes stabilization may have been due to various lab procedures and soil parameters, particularly the moisture content. Both physical and biological mechanisms could be responsible for GHG emissions in frozen soils (Kim *et al.,* 2012). When interpreting our results, it should be considered that laboratory studies may show a different effect to the practical application of biochar in the field (Huang *et al.*, 2023; Kaur *et al.*, 2022). An important element justifying the different responses of GHG emissions to the addition of biochar is the incubation time of the soils, since the procedure we used was driven by the rate of soil thawing. Therefore, our experiment included the rapid response of the soil by frequent measurements over a short time period.

In our study, in the first stage of thawing, the CO_2 peak was almost five times higher than in unfrozen soil, regardless of biochar presence. A respiration pulse in freezing-thawing events has been reported previously in soils of different ecosystems, but studies have highlighted the dependence of CO_2 pulse on soil type, freeze-thaw cycle frequency, temperature and duration of freezing (Gao *et al.*, 2015; Kim *et al.*, 2012; Koponen and Bååth, 2016). Our results are in agreement with the study on different agricultural soils frozen at -18°C, where CO_2 peak occurred within 1-5 h and the CO_2 rate then returned to the same level as unfrozen soil (Koponen and Bååth, 2016). The soil respiration pulse has been explained by an increase in temperature, moisture content, amount of available C and enhanced microbial metabolism of microorganisms that survived in soil, and improved gas diffusions (Gao *et al.*, 2015; Goldberg *et al.*, 2008; Hou *et al.*, 2020; Kim *et al.*, 2012). The biological mechanism in our soil without biochar was confirmed by the positive and significant correlation between cumulative CO_2 and C_{mic} (Fig. 5), although physical CO_2 release during thawing cannot be excluded either.

The application of biochar did not significantly affect CO_2 emissions in thawing soil, both in the initial and final stages. Similarly to non-amendment soil, a CO_2 pulse occurred during dynamic temperature increase, while CO_2 emission was at similar level to unfrozen soil after ambient temperature stabilization. In soil with biochar, we observed a significant and positive correlation of CO_2 flux with temperature, which also was reported by Hou *et al.* (2020). Research on the effect of biochar on soil CO_2 emissions during thawing is scarce. Although biochar may act as a C

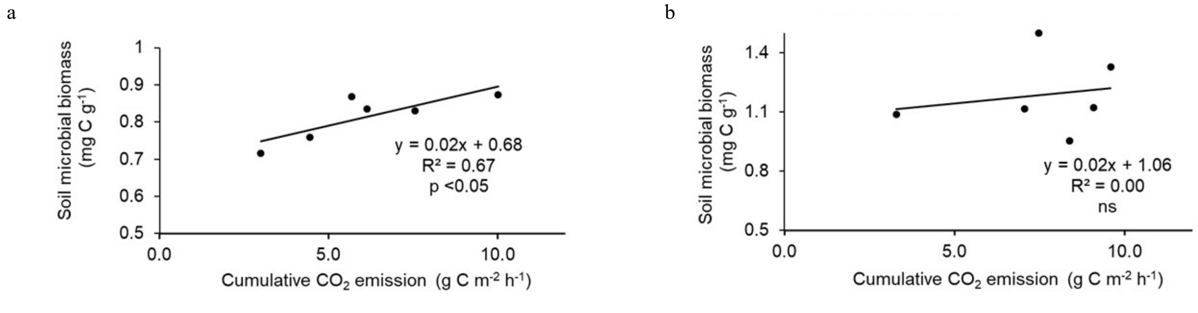

Fig. 5. Relationships between C_{mic} and cumulative CO_2 emissions in soil: a – without and b – with biochar (statistically significant at $p<0.05$).

source for soil microorganisms, a non-significant CO_2 pulse in soil without and with biochar in our study may suggest C stability during freeze-thaw cycles. The lack of a significant correlation between C_{mic} and soil CO_2 emissions in biochar-amended soil may suggest a non-biological mechanism for CO_2 release (Fig. 5). Other authors have shown stimulation of CO_2 emissions in biochar-enriched soil under thawing, but in a longer experiment with a variable number of freeze-thaw cycles (Hou *et al.*, 2020; Liu *et al.*, 2017).

Frozen soil was a significant CH_4 source during the first stage of thawing, while CH_4 uptake was detected after temperature stabilization. This shift from a source to a sink may be an effect of reduced gas diffusion by freezing, lower redox potential and creation of anaerobic conditions in frozen soils that are favourable for CH_4 production by methanogens (Dutaur and Verchot, 2007; Wu and Mu, 2019; Yu *et al*., 2007). The study on alpine peatland soils (which act a CH_4 source) and alpine meadow soils (which act a CH_4 sink) revealed that freezing significantly increased the release of CH_4 from the peatland but reduced the CH_4 uptake in the meadow (Gao *et al.*, 2015). The authors suggest that freezing and thawing accelerated the decomposition of soil organic matter and the C mineralization, providing substrates for methanogenesis. However, the study involved a longer duration of experiment, which may have allowed the methanogens to become active. Considering the short measurement time in our study, we assumed that CH_4 stored in soil could be released during the first stage of thawing as an effect of changes in pressure during freezing (Mastepanov *et al.*, 2008) rather than methanogenesis. The presence of biochar in soil led to a four-fold increase in the CH_4 peak at the initial soil thawing, but the soil started to consume the gas once the ambient temperature was stabilized, regardless of biochar presence. The mechanism responsible for the observed trend may be connected with the porous structure of biochar. We suppose that the frozen soil with biochar may have stored more CH_4 than the soil without biochar addition, because of the sorption capacity of biochar and CH_4 storage in small pores (Memetova *et al.*, 2022). Therefore a higher amount of stored CH_4 was released into the atmosphere at the first thawing stage of soil with biochar. Subsequently, the soil water thaws, moves deeper into the soil profile, the porosity and temperature of the soil increases, enabling methanotrophic bacteria activity (Hou *et al.*, 2020; Fest *et al.*, 2017) whose presence has been confirmed in control soil. We did not detected a significant effect of biochar on CH_4 uptake after ambient temperature stabilization, although a slight increasing trend occurred, which could be verified by a longer soil incubation time. In summary, a physical mechanism (gas diffusion) may have been involved in CH_4 emissions during the first thawing stage and a biological mechanism (CH_4 uptake by methanotrophs) after temperature stabilization. To date, CH_4 has received very little attention in terms of thawing, hence the uncertainties and the need for continued research in this area (Kim *et al.*, 2012).

Nitrification and denitrification are the main processes responsible for soil N_2O emission, which are sensitive to O_2 availability (Szarlip *et al.*, 2010). In our study, biochar did not significantly change the rapid response of N_2O emission in unfrozen soil, but an N_2O pulse occurred during the first stage of thawing of amended soil. Other studies show a significant N_2O pulse, a small increase in emissions or no N_2O response following thawing. A high N_2O peak may be an effect of enhanced microbial metabolism (because of higher temperature and improved substrate supply) and a physical mechanism increasing gas diffusion (summarized by Kim *et al.*, 2012). The soil N_2O mitigation potential of biochar has been often described, *e.g*., Liu *et al.* (2016a) reported that biochar application reduced soil N_2O from decreased soil nitrification which was confirmed by lower NO_3^- concentrations. In our study, higher NO_3^- concentrations in soil with biochar may suggest lowering of denitrification through improving aeration. N_2O peaks many magnitudes higher than in unfrozen soil occurred only in the first thawing stage in the biochar amended soil, similar to a field study on agricultural soil (Zhou *et al.*, 2017b). The authors suggested that porous biochar improves microbial growth by increasing the supply of C and N after freezing and therefore stimulating N_2O production. Frozen conditions in soil create anoxia from enhanced O_2 consumption and therefore promote denitrification (Mørkved *et al.*, 2006, Yang *et al.*, 2022). The application of biochar may improve soil aeration and enhance nitrification during thawing which has been confirmed by the highest extractable NO_3^- concentrations. We may thus suppose that nitrification and gas diffusion could be a sources of N_2O pulse in the frozen variant with biochar.

CONCLUSIONS

1. Biochar addition did not significantly change the rapid response of GHG emissions in unfrozen soil.
2. In thawing soil, the GHG emission rate is determined by the dynamic of growth in ambient temperature. GHG pulses occurred in the period of the fast temperature growth and then reached the level as in the sample without freezing.
3. Biochar application showed negative effect on climate since significantly increased CH_4 and N_2O peak in thawing soil, although did not affect CO_2 emission.
4. Biochar enrichment improve soil conditions as indicated by microbial parameters.
5. Both physical (stored gas diffusion) and biological mechanisms could be responsible for GHG emissions in frozen soils.

Conflict of interest. The Authors declare they have no conflict of interest.

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