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RESEARCH ARTICLE

No evidence that conifer biochar impacts soil functioning by serving as microbial refugia in boreal soils

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Abstract

It is well established that application of biochar to soils can promote soil fertility, which ultimately may enhance plant growth. While many mechanisms have been proposed to explain this, one specific mechanism, the "microbial refugia hypothesis," suggests that biochar may provide physical protection for soil microbe from soil microfauna that otherwise exert top-down control on microbial biomass and activity. We tested the microbial refugia hypothesis by incubating two boreal soils with and without biochar derived from a wood mixture of boreal tree species (*Picea abies* and *Pinus sylvestris*), and with and without soil nematodes. We measured phospholipid fatty acids (PLFA) as a relative measure of microbial biomass, and several variables indicative of microbial activity, including extractable nutrient concentrations (NH_4^+, NO_3^-, and PO_4^-), heterotrophic N_2-fixation, and soil respiration. Contrary to our expectations, we found that biochar by itself did not stimulate microbial biomass or activity. Furthermore, we found that nematode addition to soil stimulated rather than depressed the biomass of several bacterial PLFA groups. Finally, interactive effects between the nematode treatment and biochar never worked in a way that supported the microbial refugia hypothesis. Our findings suggest that a typical boreal biochar applied to boreal soils may not have the same stimulatory effect on microbial biomass and activity that has been shown in some other ecosystems, and that enhanced plant growth in response to biochar addition sometimes observed in boreal environments is likely due to other mechanisms, such as direct nutrient supply from biochar or amelioration of soil pH.

KEYWORDS

biochar, boreal forest, carbon sequestration, microbial activity, microbial biomass, microbial refugia, soil microfauna, top-down control

1 | INTRODUCTION

Human-caused carbon (C) emissions from fossil fuel combustion and land-use change are responsible for rising atmospheric CO_2 concentrations and global warming that are expected to have pronounced negative effects on global ecosystems (IPCC, 2021). Intentionally pyrolyzed organic matter added to soil as an amendment, hereafter "biochar," has been proposed for offsetting atmospheric gaseous C by converting labile biogenic C into recalcitrant soil C (Lehmann & Joseph, 2015). The pyrolysis and combustion of organic matter produces biochar with high C content (often >80%) and includes fractions that are highly resistant to microbial degradation and that can remain stable in soils for hundreds to thousands of years (Barrow, 2012; Gavin et al., 2003; Laird et al., 2008; Liang et al., 2008; Meyer et al., 1995). In addition to its contribution to the stable soil C pool, biochar has been shown to enhance soil fertility and plant productivity, leading to even further C uptake and storage (Barrow, 2012; Jeffery et al., 2011). While there are many mechanisms through which biochar is known to enhance soil fertility and plant growth, one poorly explored mechanism is its physical role as a habitat for soil microorganisms (Pietikäinen et al., 2000; Saito & Marumoto, 2002; Wardle et al., 2008; Zackrisson et al., 1996). Both internal pore and external surfaces of biochar particles have been shown to harbor high densities of soil microbes (Li et al., 2020; Warnock et al., 2007). It has been suggested that the unique habitat characteristics of biochar such as the physical pore structure or its adsorption of organic compounds may promote a high level of microbial activity (Wardle et al., 2008; Zackrisson et al., 1996), which could subsequently enhance native organic matter decomposition, nutrient turnover, and availability.

Direct evidence for microbial habitats occurring within biochar particles emerges from studies that report physical association of fungi, bacteria spores, and plant roots on internal and external surfaces of biochar, as assessed with microscopy (de la Rosa et al., 2018; Pietikäinen et al., 2000; Quilliam et al., 2013; Zackrisson et al., 1996). For example, in agricultural soils, biochar incubated in-situ for 3 years showed 25%–60% microbial colonization of biochar surfaces (Quilliam et al., 2013). In a mesocosm study, Pietikäinen et al. (2000) showed that the ability of charcoal (i.e., char that is produced during wildfire) to adsorb organic compounds from boreal plant species led to increased microbial growth and activity. Furthermore, not only did microbial colonization of biochar occur in particles incubated in the field, but biochar surfaces showed higher CO_2 evolution and microbial C use efficiency when provided with a labile C source relative to native soil (Quilliam et al., 2013), suggesting a highly active microbial community on biochar surfaces. Recently, De la Rosa et al. (2018) provided evidence of microbial decomposition of biochar C made from a variety of feedstocks in Spanish Mediterranean environments, which also contains a small labile C fraction, after a 120-day incubation. This resulted in a reduction in total C and a shift in the ^{13}C-NMR spectral signature, indicative of a change in the aromaticity of the biochar. The intimate physical association between biochar and microbes may be a mechanism driving patterns of higher microbial activity (Lehmann et al., 2011).

One mechanism through which biochar has been hypothesized to support a high level of microbial colonization and activity is by providing physical protection from soil animals that graze upon microbes (Pingree & DeLuca, 2017; Zackrisson et al., 1996). Soil food web interactions can potentially serve as a strong control on soil microbes, and the key soil processes and functions they control such as C and nutrient cycling (de Vries & Caruso, 2016). Biochar surfaces provide unique physical habitats in the soil environment by adding microporosity and macroporosity, which varies according to biochar feedstock and pyrolysis conditions (Chia et al., 2015; Schnee et al., 2016). Generally, the pore size in wood biochar can be on average smaller compared to its precursor feedstock (Chia et al., 2015), which can contribute substantially to soil porosity (Li et al., 2018), and potentially influence soil communities (Pingree & DeLuca, 2017). Keech et al. (2005) showed a high proportion of micropores of <50 μm in biochar produced at 450°C from common boreal tree species. Soil organisms, on the other hand, range in size from bacteria that are less than 2 μm, to grazers of microbes such as protozoa and nematodes that can be over 100 μm in diameter, as well as larger arthropods (Brackin et al., 2017; Paul, 2007). It has been proposed that biochar microporosity may act as a natural refuge for microorganisms from predation from grazers in cases where pore sizes limit the access to larger soil animals that would otherwise exert top-down control (i.e., the "microbial refugia hypothesis", Hockaday et al., 2007; Warnock et al., 2007; Zackrisson et al., 1996). The physical protection from microfaunal grazers that biochar pore spaces provide could allow microbes within those pores to carry out key soil processes differently, such as higher C utilization and associated nitrogen mineralization and immobilization, or specific nitrogen transformations carried out by specialized microbes such as heterotrophic N_2-fixation and nitrification. Despite speculation that biochar may alter soil functioning by serving as a microbial refugia, this mechanism has never been explicitly tested in boreal forests.

In this study, we tested the microbial refugia hypothesis by creating experimental mesocosms that were treated

with or without biochar, and with or without nematodes, which are common microbial grazers in boreal soils (Fanin et al., 2019). We first hypothesized that biochar amendment to forest soils would enhance soil microbial biomass and activity, both because it can contain a labile fraction that could serve as a source of bio-available C and because it may serve as a physical habitat for microbes that enhances microbial biomass. We further anticipated that the higher microbial biomass and activity associated with biochar would lead to higher concentrations of extractable soil nutrient pools, which has been shown in numerous other studies (DeLuca et al., 2015; Xu et al., 2021). Second, we hypothesized that amending soils with nematodes would exert a top-down control on soil microbes, which would reduce microbial biomass, and cause an associated change in nutrient availability and other soil processes. Third, we hypothesized that biochar amendment would interact with soil nematode addition such that top-down effects on microbes and microbial processes would be minimized, as predicted by the microbial refugia hypothesis. We tested these hypotheses using two soil horizons characteristic of the boreal forest, that is, organic surface soil and underlying mineral soil; both of these are mixed with biochar when biochar management is implemented in boreal forests (Gundale et al., 2016). Testing these hypotheses will help to provide insights into a relatively unexplored mechanism through which biochar may potentially enhance nutrient availability and supply to plants.

2 | METHODS

We performed a laboratory incubation experiment using boreal soils to evaluate whether biochar serves as a refuge habitat for soil microbes, which may, in turn, impact microbial community composition and nutrient cycling. We conducted a full factorial incubation experiment consisting of two soil types (mineral and organic soil), three substrate amendment treatments (control, biochar, and pumice addition), and two food web treatments (control and nematode addition), with 12 replicates for each treatment combination, resulting in 144 experimental units. The pumice addition treatment served as a structural control for biochar (hereafter referred to as "pumice-control treatment"), because it has similar density and particle size, but differs in porosity (Pietikäinen et al., 2000; Zackrisson et al., 1996). Soils were collected in November of 2018 at Svartberget Experimental Forest (64°140 N, 19°460 E, 175 m above sea level) in northern Sweden, from 10 randomly chosen locations. The forest at the site consisted of a ca. 120-year-old dominant *Pinus sylvestris* overstory, and sub-dominant *Picea abies*, with *Calluna vulgaris* and *Vaccinium vitis-idaea* as dominant ground layer vegetation. Soil at the site is a fine sandy loam Typic Haplocryod (FAO, Cambic Podzol) formed from glacial outwash sediment (Gundale et al., 2016). The %C, %N, and C:N ratio of the organic horizon soil was 9.47, 0.21, and 45.1, and for mineral soil was 1.66, 0.05, and 33.2, respectively, and the soil pH in the organic and mineral soils was 3.3 and 4.0, respectively (Forsmark et al., 2020). The organic horizon (ca. 10 cm depth) was collected using a spade, and mineral soils were collected to a depth of 20 cm using a 100 mm soil probe. Soils were kept at 5°C, and then sieved. Mineral and organic soils were sieved (4 and 8 mm, respectively), and then bulk samples were split in two halves, where one half was sterilized (γ-irradiation, see below) to eliminate the entire soil food web, and the other was reserved for use as microbial inoculum and nematode inoculum.

Before the experiment was established, both soils and substrate amendments (i.e., biochar and pumice) were sterilized using γ-irradiation (minimum dose 30 kGy, maximum dose 36 kGy, by STERIS, Ede, The Netherlands), which is an effective and established technique to kill the soil microbes and fauna (Gundale et al., 2019; McNamara et al., 2003). We then added either 121 g of sterilized field-moist mineral soil or 61 g of sterilized field-most organic soil to 250 ml glass jars (21 and 208% moisture content, respectively), which for both soil types filled the jars to a ca. 100 ml volume. For jars assigned with biochar or the pumice-control treatment, we added 3.12 g of these substrates. Soil mesocosms were divided into three blocks (i.e., 48 jars per block), with equal representation of treatment combinations within each block. Both charcoal and pumice substrates had a particle size range of between 1 and 5 mm and a bulk density of 0.1 $g\,m^{-3}$. The relatively small particle size of biochar we added, meant that each jar with biochar had a relatively uniform concentration of biochar per volume (ca. 10% of total volume) and per cross-sectional area of the jar. This application rate equated to 10 t biochar or pumice ha^{-1}. This amount also equates to the amount of biochar used in a field experiment adjacent to where soils were collected for the current study (Gundale et al., 2016) and also represents the higher range estimates of natural charcoal resulting from wildfires that has been measured across several sites in Northern Swedish forests (Ohlson et al., 2009). Pumice was supplied from VWR International AB and biochar from Vindelkol AB, Sweden. Biochar supplied by this private company was made from *P. sylvestris* and *P. abies* wood and bark, which are the two most common tree species in northern European boreal forests. The biochar had a pH of 8.04, C content of 74%, and extractable concentrations of NH_4^+, NO_3^-, and PO_4^- of 1.26, 0.14, and 1.38 $mg\,kg^{-1}$, respectively (Gundale et al., 2016). We characterized the pore

size distribution of the charcoal using scanning electron microscopy. We randomly selected 20 charcoal samples, which we mounted on electron micrograph plates. From each plate, we selected the most clear and visible transversal image, where pore surfaces were visible. We then zoomed in on an area of 205 × 143 μm, which were then each digitized as a tiff image. Images were then imported into Image J software, where two perpendicular diameters were measured from every pore on every image (Figure 1; Figure S1), using the measurement tool, which allowed us to estimate the pore size distribution. We further calculated the transversal porosity of each image by creating a black and white contrast image, and then using the "analyze particles" tool. This function was repeated on each image (Figure S1), which provided a mean and standard error transversal porosity for the biochar we used in the study.

A microbial community was established in each jar by extracting live microbial inoculum from either mineral or organic soil (i.e., soil not subjected to γ-irradiation). This was done for each soil type by first adding 1 L of tap water to 1 kg of soil, and stirring for 30 s. We then allowed the heavy particles to settle after 4 h, and passed the liquid through two successive sieves of different pore size: 70 μm followed by 38 μm. The majority of soil microorganisms are <10 μm, whereas nematodes and other soil grazers are typically >38 μm, which allowed us to exclude a majority of micro-arthropods, nematodes, and protists from the inoculum (Ames et al., 1987; Kardol et al., 2007). The resulting live inoculum was then added (20 ml) to sterilized soils in all mesocosms, using a pipette, whereby each mesocosm was inoculated with an equivalent volume of extracted soil. This sterilization and inoculation approach followed experimental design type A as described by Gundale et al. (2017, 2019), whereby both soils and inoculum were derived from composite samples, which was appropriate because our research question was not focused on describing spatial variation in responses. After inoculation with the microbial community, mesocosms were homogenized thoroughly, sealed with perforated Parafilm™

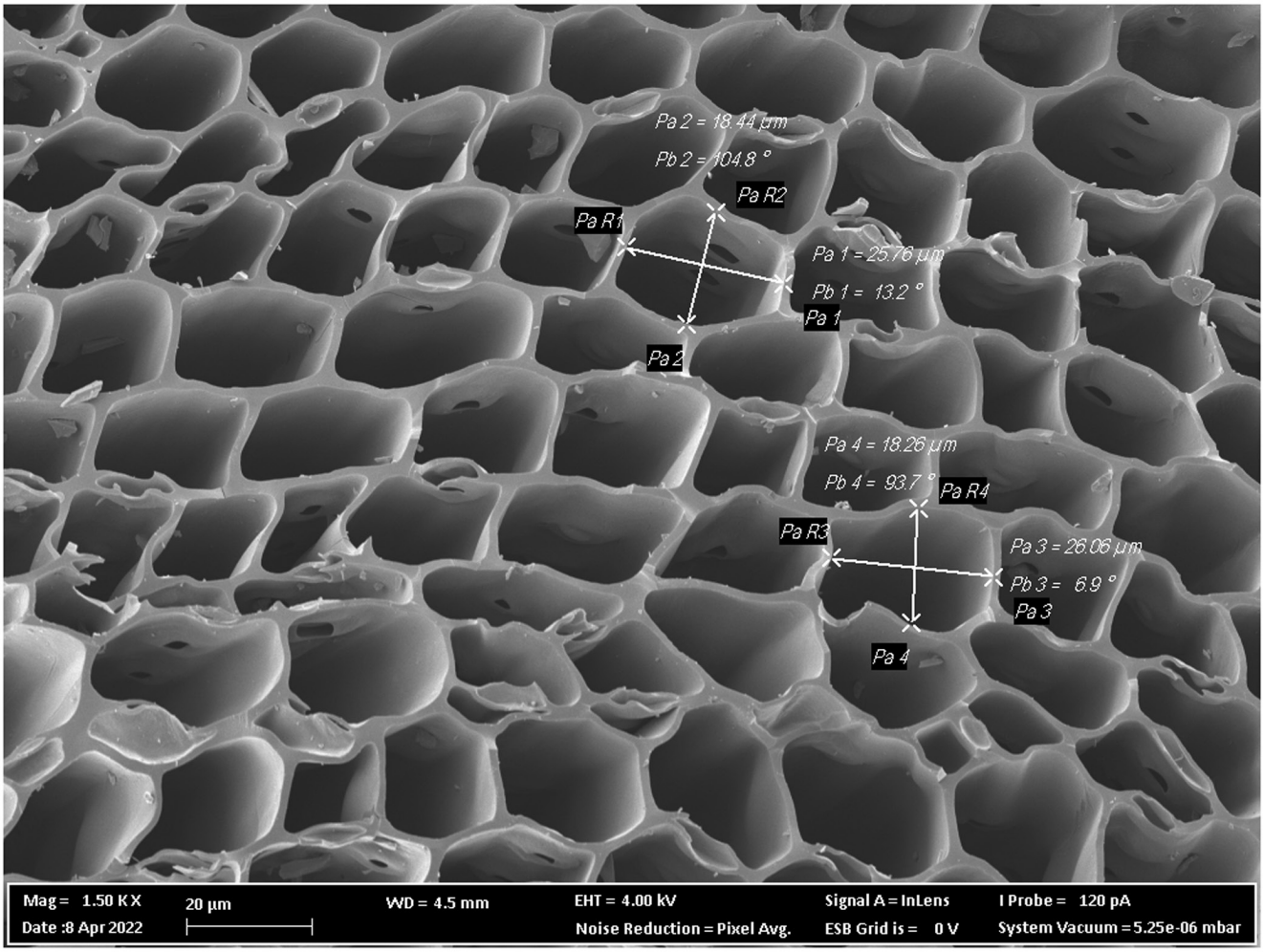

FIGURE 1 A scanning electron micrograph image of a biochar particle surface. The pore size distribution from 20 such images was extracted (see Figure S1).

to allow for air exchange, and incubated in the dark at 25°C and 50% relative humidity for 4 months to provide ample time for the microbial community to develop and colonize each mesocosm.

After 4 months of incubation, half of each block was then treated with nematodes. Soil nematodes were extracted in bulk from the originally collected unsterilized organic and mineral soils, which was kept at 5°C for the duration of the incubation time. We extracted nematodes using a sugar flotation method (Jenkins, 1964) from an equivalent soil mass as contained in each jar (i.e., 121 g of mineral soil or 61 g of organic soil for each jar). Nematode density was quantified on a subset of extracts for each soil type ($n = 6$), and the nematode supernatant was fixed with formaldehyde to quantify nematode abundance in soil samples. On average, approximately 6 and 159 nematodes were present per gram dry mineral and organic soil respectively, which equates to 630 and 3217 total nematodes added to each mineral or organic soil mesocosm, respectively. The proportion of the nematode community consisting of bacterivores and fungivores in similar forest types has previously been shown to be approximately 28 and 12%, respectively (Maaroufi et al., 2018). Once nematodes were added, mesocosms were incubated for 8 days after which the experiment was harvested. After that time, soil subsamples were removed from each jar for measurements of moisture content, KCl-extractable N and P, phospholipid fatty acid (PLFA) analysis, and biological N-fixation rates. The remaining soil in the mesocosm jar was then used to measure CO_2 basal respiration per gram of soil C, as described below.

Moisture content was determined on a dry mass basis after drying 10 g mineral or 5 g organic soil subsamples for 48 h at 60°C (Gardner, 1986). Extractable ammonium (NH_4^+-N), nitrate (NO_3^--N), and phosphate (PO_4^{-3}-P) were extracted from soil subsamples by adding 35 ml of 1 M KCl to 5 g organic soil or 20 g mineral soil, then shaken for 1 h and filtered with Whatman #42 paper (Mulvaney 1996). Extracts were analyzed using standard spectrophotometric methods, using an Auto Analyzer 3 (Omniprocess), as done in previous studies (Ibanez et al., 2021; Pluchon et al., 2014).

Mesocosm subsamples were analyzed for PLFA markers to quantify soil microbial community structure (Bligh & Dyer, 1959). A 1 g subsample was finely ground, freeze-dried, and extracted using a modification of the Bligh and Dyer liquid extraction method (Bligh & Dyer, 1959; Frostegard & Baath, 1996; Maaroufi et al., 2019; Pluchon et al., 2016), which was analyzed on a Perkin Elmer Claris 500 Gas Chromatograph (GC). The abundance of identified PLFAs was reported as micromoles per gram soil using conventional nomenclature and subsequently converted to relative abundance. Different types of PLFA markers represent different components of the soil microflora. The PLFAs 18:1w9 and 18:2w6 were used to estimate the contribution of fungi, while the branched fatty acids 10me16:0, 10me17:0, and 10me18:0 were used to estimate actinobacteria. The PLFAs i-15:0, a-15:0, 15:0, i-16:0, 16:1×9, 16:1×7t, i-17:0, cy-17:0, a-17:0, 18:1×7, and cy-19:0 were summed to represent total bacteria. Gram-positive bacteria were represented by branched fatty acids i-15:0, a-15:0, i-16:0, i-17:0, and a-17:0, whereas cy-17:0, cy-19:0, and 18:1×7 were used to represent gram-negative bacteria. We note that some biochars have been reported to cause a lower extraction efficiency for PLFAs from soils, and in this light, a comparison of PLFAs with both biochar amended and pumice amended soils enabled us to detect if this was an issue.

Heterotrophic nitrogen fixation was determined by acetylene reduction on a sub-sample of soil from each jar, after a 24-h incubation period, and gas samples were analyzed on a headspace GC (Clarus 580, PerkinElmer) and headspace sampler (TurboMatrix 110, PerkinElmer) with a flame ionization detector (FID) (Gundale et al., 2010; Kardol et al., 2016). Acetylene-free control soils and acetylene-only samples were used to calibrate ethylene values (Gundale et al., 2013; Stuiver et al., 2015). Soil basal respiration was determined by sealing mesocosms with a septum, and measuring CO_2 produced at time zero, and after 30 minutes, and calculated in terms of dry soil mass (Grau-Andres et al., 2021; Gundale et al., 2011, 2016). Headspace samples were collected directly from the mesocosm headspace into 22 ml vacuumed vials and analyzed on the same GC as above. We suffered an instrument malfunction when measuring basal respiration of organic soils, which meant that only 8, 5, 9, 9, 5, and 9 replicates were measured for control, biochar-only, pumice-only, nematode-only, biochar plus nematodes, and pumice- and nematode-treated mesocosms, respectively. This led to lower replication and an unbalanced design for this variable, but there were sufficient numbers of replicates in all treatment combinations for data analysis to remain viable.

2.1 | Statistical analyses

All statistical analyses were conducted in the R environment (R Core Team, 2020). Response variables in this study consisted of available nutrient concentrations (mg NO_3^--N, NH_4^+-N, or PO_4-$^-$P g dry soil^{-1}), N-fixation rates (μg N_2 fixed g dry soil$^-$1 day^{-1}), basal respiration (CO_2 ppm min^{-1} g dry soil^{-1}), and individual PLFA groups (nmol g dry soil^{-1}). Each response variable was considered separately for mineral and organic soils in a two-way ANOVA with amendment type (control, pumice, or biochar), nematode treatment (nematodes added

or not added), and the interaction between amendment type and treatment as fixed factors, and block as a random error term. Soil types (mineral and organic soils) were tested separately because it was difficult to satisfy parametric assumptions when they were included in a single analysis. Logarithmic transformations were employed when necessary to meet assumptions of normality and homoscedasticity required for ANOVA. Where the interaction term was significant, we used a post-hoc comparison for all factor combinations to explore differences among means, using Tukey's pairwise contrasts with least squares means in the *emmeans* package (Lenth, 2020). Extractable soil phosphate in mineral soils was excluded from analysis because the concentration of phosphate in the majority of replicates fell below detection limits. Ammonium extracted from both mineral and organic soils, and mineral soil N-fixation, could not be transformed to meet normality assumptions, and these variables were instead analyzed with a Kruskal–Wallis nonparametric rank sum test testing for the effects of amendment type, nematode treatment, interaction between these factors, and block; this was followed by Dunn's contrast tests (Ogle et al., 2021).

3 | RESULTS

3.1 | Biochar porosity

The biochar used in our study had a mean (±SE) transversal porosity of 66.3% (±2.7%). The mean and median pore diameter were 19.7 and 18.9 μm, and the most abundant pore diameter class was 15–20 μm, which consisted of 27% of all pores (Figure 2).

3.2 | Microbial community responses

Total PLFAs and PLFAs of different microbial functional groups showed many significant responses to the amendment types for both organic and mineral soils, and responses to the nematode treatment were frequently found in mineral soil. However, significant interactive effects of amendment type and nematode treatments were never found ($p < 0.05$, Tables 1 and 2). For both mineral and organic soils, biochar amendment never significantly altered PLFA functional groups relative to control soils, whereas pumice amendment often reduced PLFA concentrations in both soil types. In mineral soil, the pumice-control treatment reduced total PLFAs (Figure 3) and all PLFA functional groups except for fungi, while it increased the fungal to bacterial ratio (Figure 4). In organic soils, the pumice-control treatment reduced total PLFAs (Figure 3) and all PLFA functional groups, and it increased the fungal to bacterial ratio. The finding that PLFA groups were never altered in response to biochar amendment, but were altered by pumice amendment, indicates that biochar did not result in altered extraction efficiency of PLFAs. Total bacteria, actinomycetes, Gram-positive, and Gram-negative bacteria positively responded to nematode addition in mineral soils, whereas the fungi to bacteria ratio declined (Table 1; Figure 4). In organic soils, nematode addition had few significant effects on PLFA functional groups, except for the fungal to bacterial ratio, which increased (Table 2; Figure 5).

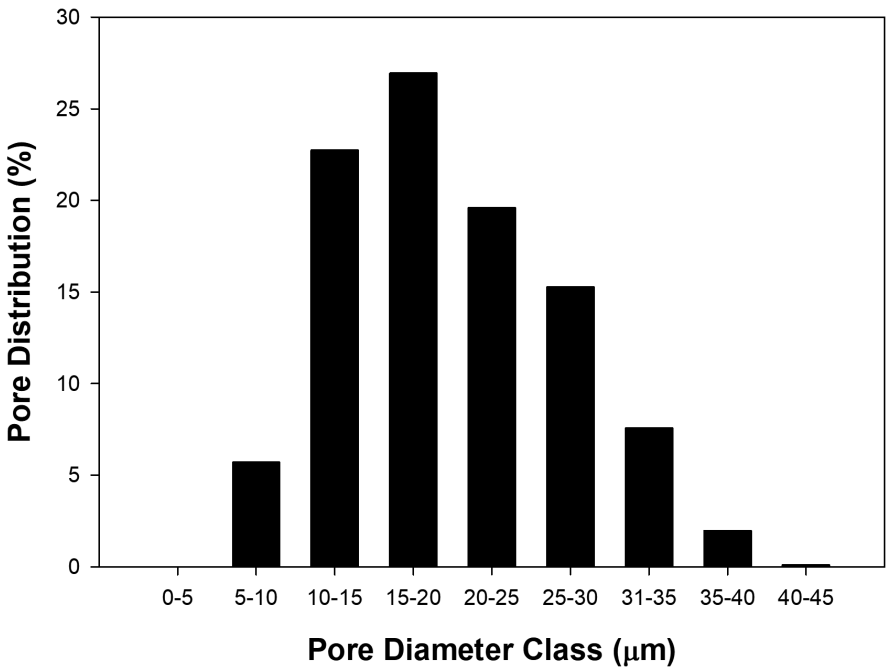

FIGURE 2 The pore size distribution obtained from 20 scanning electron micrograph images of biochar surfaces. Pores are categorized into 5 μm diameter classes, and are described as percent of total pores.

TABLE 1 Analysis of variance results performed for an incubation experiment using mineral soils, using a two-way factorial model with amendment (i.e., biochar, pumice, and control) and nematode treatment (with or without nematodes added) serving as fixed factors, and blocking used as an error term

Microbial biomass (nmol g dry soil^{-1})	Factor	df	*F*-value	*p*-value
Total PLFA	Amendment (A)	2	13.84	**<0.001**
	Nematodes (N)	1	48.08	**<0.001**
	A×N	2	1.58	0.21
	Residuals	63		
Fungi	Amendment (A)	2	0.84	0.44
	Nematodes (N)	1	3.95	0.51
	A×N	2	0.92	0.41
	Residuals	63		
Bacteria	Amendment (A)	2	16.72	**<0.001**
	Nematodes (N)	1	68.53	**<0.001**
	A×N	2	1.98	0.1468
	Residuals	63		
Actinomycetes	Amendment	2	5.57	**<0.01**
	Nematodes	1	4.79	**<0.05**
	A×N	2	0.53	0.59
	Residuals	63		
Gram-positive	Amendment (A)	2	15.57	**<0.0001**
	Nematodes (N)	1	14.99	**<0.0001**
	A×N	2	2.91	0.06
	Residuals	63		
Gram-negative	Amendment (A)	2	15.52	**<0.0001**
	Nematodes (N)	1	119.23	**<0.0001**
	A×N	2	1.48	0.24
	Residuals	63		
Fungi: bacteria	Amendment (A)	2	78.73	**<0.0001**
	Nematodes (N)	1	147.78	**<0.0001**
	A×N	2	1.87	0.16
	Residuals	63		

Data were log transformed.

Values in bold are signficant

3.3 | Soil nutrient pools and fluxes

Soil amendment and nematode treatments, as well as interactions between these two factors, sometimes altered soil nutrient pools and fluxes (Tables 3 and 4). The soil amendment treatment had few effects in mineral soils, except for heterotrophic N_2-fixation which was reduced in biochar amended soils relative to control and pumice-control soils (Figure 6). In organic soils, amendments affected extractable NO_3^-, PO_4^-, and N_2-fixation rates (Table 4). For extractable NO_3^-, all amendment treatments significantly differed from each other, with the pumice-control treatment resulting in the lowest concentrations, and the control having the highest (Figure 7). For extractable PO_4^- and N_2-fixation, pumice-control treatment resulted in lower values compared to the other amendments (Figure 5).

Nematode addition significantly reduced extractable NH_4^+ and NO_3^- in mineral soils, and had a weak positive effect on extractable NH_4^+ concentrations in organic soils (Tables 3 and 4; Figures 6 and 7). Several variables also responded to an interactive effect of soil amendment and nematode addition (Tables 3 and 4). In mineral soils, the negative effect of nematode addition on extractable NH_4^+ was more pronounced for pumice-control treated soils, and on extractable NO_3^- was most pronounced for biochar-amended soils and least for unamended (control) soils (Figure 6). For N_2-fixation in mineral soils, the negative effect of biochar appeared to be stronger when nematodes were not added. In organic soils, the weak positive effect of nematode addition on extractable NH_4^+ occurred only in the control treatment (Figure 7). We further found that basal respiration was unresponsive to both soil

TABLE 2 Analysis of variance results performed for an incubation experiment using organic soils, using a two-way factorial model with amendment (i.e. biochar, pumice, and control) and nematode treatment (with or without nematodes added) serving as fixed factors, and blocking used as an error term

Microbial biomass (nmol g dry soil^{-1})	Factor	df	*F*-value	*p*-value
Total PLFA	Amendment (A)	2	43.31	**<0.001**
	Nematodes (N)	1	0.24	0.6
	A×N	2	1.52	0.2
	Residuals	60		
Bacteria	Amendment (A)	2	49.85	**<0.001**
	Nematodes (N)	1	0.02	0.90
	A×N	2	1.96	0.15
	Residuals	60		
Fungi	Amendment (A)	2	20.57	**<0.001**
	Nematodes (N)	1	2.21	0.14
	A×N	2	1.20	0.31
	Residuals	60		
Actinomycetes	Amendment (A)	2	48.78	**<0.0001**
	Nematodes (N)	1	0.03	0.86
	A×N	2	1.65	0.20
	Residuals	60		
Gram positive	Amendment (A)	2	42.76	**<0.0001**
	Nematodes (N)	1	2.28	0.14
	A×N	2	2.50	0.09
	Residuals	60		
Gram negative	Amendment (A)	2	54.94	**<0.0001**
	Nematodes (N)	1	2.07	0.16
	A×N	2	1.54	0.22
	Residuals	60		
Fungi:Bacteria	Amendment (A)	2	15.44	**<0.0001**
	Nematodes (N)	1	7.53	**<0.01**
	A×N	2	0.31	0.74
	Residuals	60		

Values in bold are signficant

amendments and nematode treatments in both mineral and organic soils, and that extractable PO_4^- was below detection limit in mineral soils.

4 | DISCUSSION

4.1 | Main effect of biochar on microbial community and soil functioning

Contrary to our first hypothesis, we never observed effects of biochar amendment on microbial properties and rarely on nutrient pools or soil fluxes. The lack of significant differences between biochar-treated and non-amended control soils is inconsistent with several previous studies showing that biochar often increases a variety of metrics describing the microbial biomass (Liu et al., 2016; Palansooriya et al., 2019; Xu et al., 2021). However, a recent meta-analysis by Xu et al. (2021) revealed that microbial biomass responses to biochar can vary substantially depending on both the rate of biochar addition and soil properties. Their analysis also showed that while biochar properties and addition rate served as major determinants of fungal responses to biochar addition, bacterial responses depended more on the properties of the soils to which biochar was added as well as the extent to which fungi were dominant relative to bacteria.

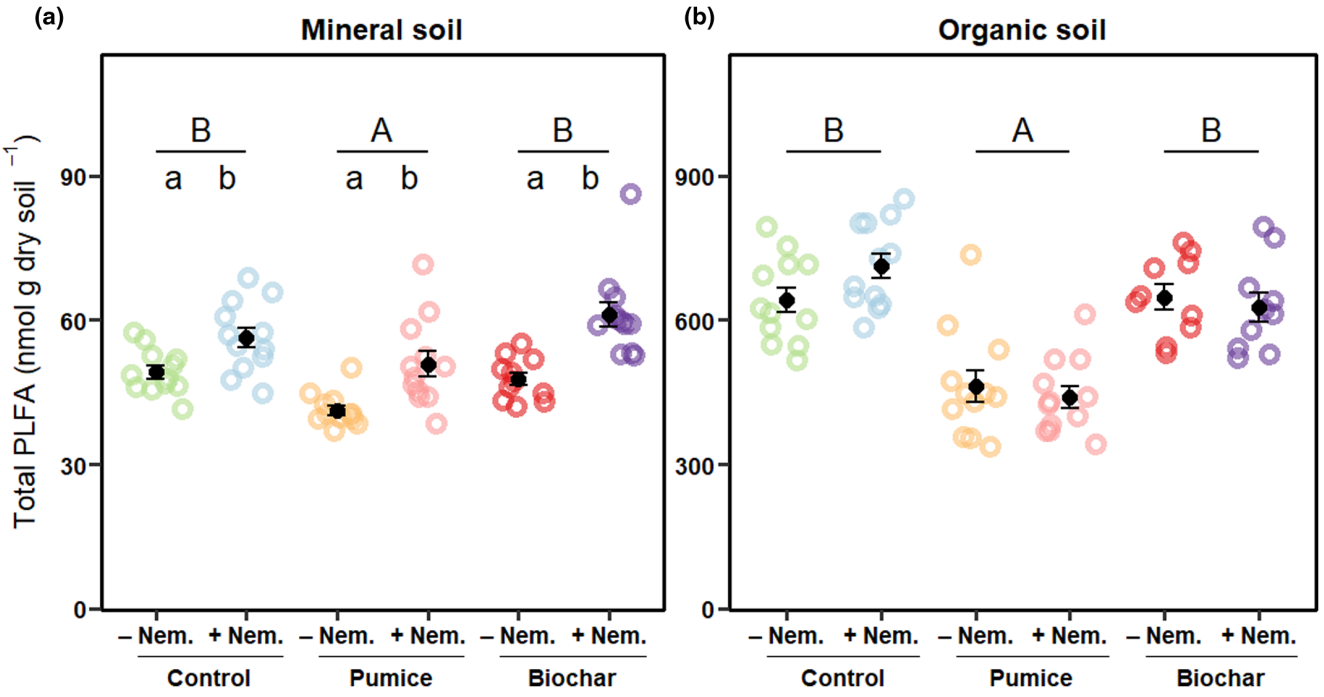

FIGURE 3 Total phospholipid fatty acids (PLFA; nmol g dry soil^{-1}) in mineral and organic soil amended with biochar and pumice, and treated or not treated with nematodes. Capital letters (A or B) indicate significant differences between soil amendments, and lower case letters (a or b) indicate significant responses to nematode treatment ($p < 0.05$).

In our experiment, we applied biochar at a rate equivalent to 10 t ha^{-1} which is the upper limit for naturally occurring wildfire-produced charcoal present in coniferous forests of northern Sweden (Ohlson et al., 2009). This amount of charcoal has previously been shown to exert a stimulatory effect on microbial biomass in similar forests in the region (Pietikäinen et al., 2000). It is possible that the addition rate or properties of the biochar that we used, even though regionally relevant, may have been insufficient to illicit the types of strong microbial responses frequently reported in other ecosystems where a range of other types of primarily angiosperm biochars are used (Jaafar et al., 2015; Palansooriya et al., 2019; Xu et al., 2021). For example, Jaafar et al. (2015) found that fungi colonized the pore spaces of several biochar types made from Australian angiosperm wood, and that hyphal colonization mainly occurred in the largest pore sizes. This is notable because angiosperm wood typically has larger pore sizes associated with their vessel anatomy relative to the tracheid anatomy found in gymnosperms. Furthermore, the soils used in our experiment were derived from intact boreal conifer forest, which is frequently characterized by high fungal to bacterial ratios (Forsmark et al., 2020, 2021 Maaroufi et al., 2019) that may constrain bacterial responses to biochar addition (Xu et al., 2021). However, the lack of microbial community responsiveness to biochar amendment that we observed is consistent with a recent field experiment in northern Sweden, where the same biochar material was applied to 0.1 ha plots containing similar soils, and for which few direct responses of PLFA functional groups were detected after 4 years (Gundale et al., 2016). It is also consistent with some studies that have shown biochar derived from boreal tree species to have little impact on microbial communities and soil functioning (Palviainen et al., 2018; Soinne et al., 2020).

The biochar that we used was derived from *P. sylvestris* and *P. abies*, which represent two gymnosperm genera that are very abundantly distributed in boreal forests. While the mean pore diameter of the charcoal we used appeared to be suitable for providing microbial habitat (19.7 μm) and for providing physical protection from grazers (i.e., ≥38 μm in our study), it is possible that the tracheid structure of gymnosperm biochar may not be as easily colonized by microorganisms, notably fungi, due to the very small pit diameters that connects tracheids. In contrast, angiosperm wood may be more easily colonized by soil microbiota due to the greater connectivity between vessel elements (Pearce, 1996). A glasshouse study by Pluchon et al. (2014) showed that biochar made from boreal gymnosperm species had very little impact on boreal tree seedling growth, whereas biochar made from boreal angiosperm species had much larger impacts, especially for angiosperm

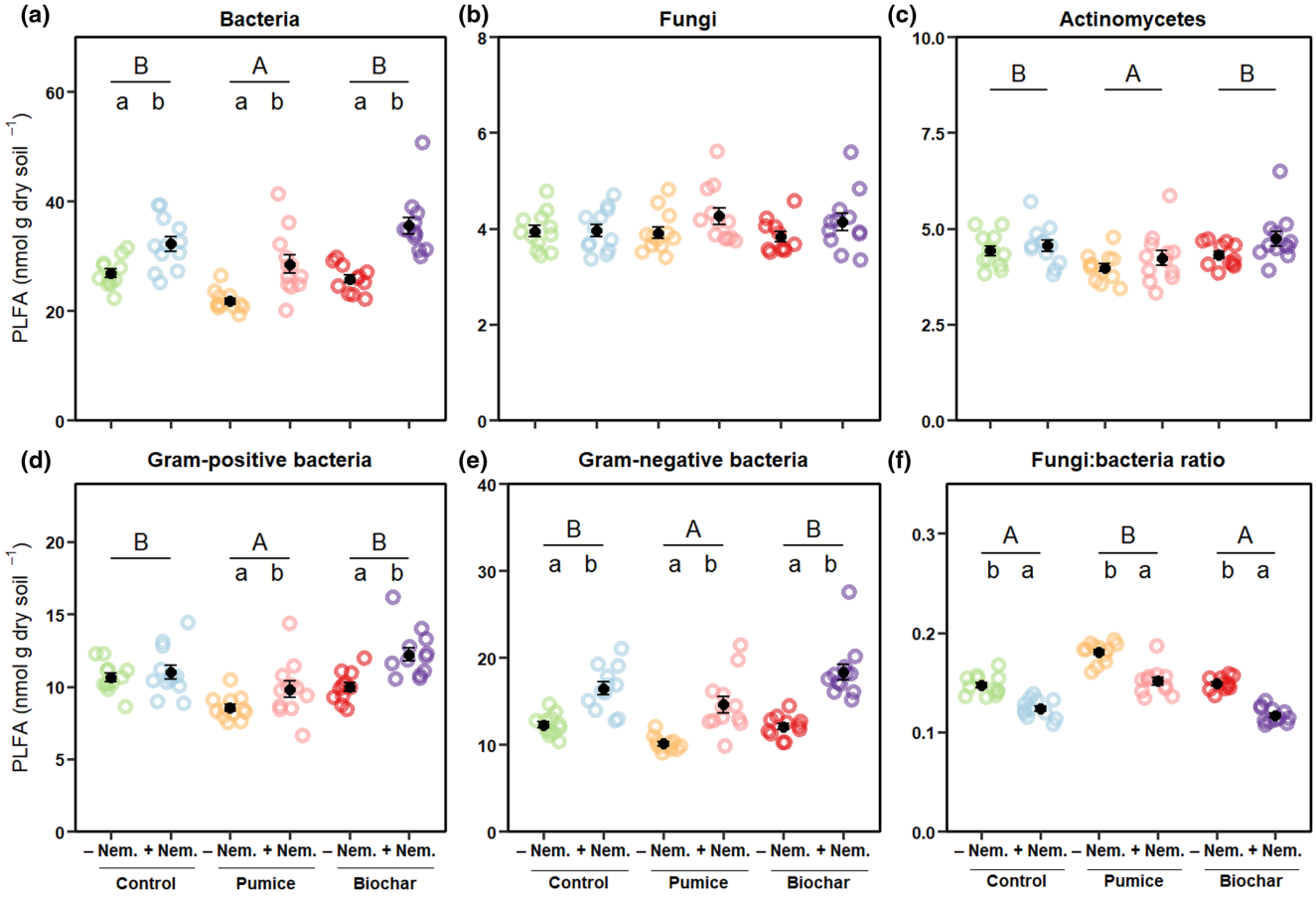

FIGURE 4 Phospholipid fatty acid (PLFA) functional group responses (nmol g dry soil^{-1}) in mineral soil amended with biochar and pumice, and treated or not treated with nematodes. Capital letters (A or B) indicate significant differences between soil amendments, and lower case letters (a or b) indicate significant responses to nematode treatment ($p < 0.05$).

seedling species. They proposed that this difference was due to higher nutrient supply rates associated with angiosperm biochar together with greater nutrient demand by angiosperm seedlings. If these differences between angiosperm and gymnosperm biochars in promoting seedling growth also correspond to differences in biochar effects on the soil microbial community, it could help explain why several studies in the boreal forest that have used biochar derived from gymnosperm species have not observed strong microbial community responses (Palviainen et al., 2018, Soinne et al., 2020).

4.2 | Effect of nematode addition and interaction with biochar amendment

Nematode addition resulted in strong effects on the microbial community, notably in mineral soils, but contrary to our second hypothesis these effects were generally positive, especially for bacterial groups (i.e., total bacteria, Gram-positive, and Gram-negative). A meta-analysis of bacterivore effects on the soil bacterial biomass showed that a majority of studies applying microfaunal treatments show negative impacts on soil bacterial biomass, but also that many studies have also observed positive effects such as we observed (Trap et al., 2016). The stimulation of bacterial biomass by nematode addition that we observed may be caused by nematodes altering the community composition or activity of the bacterial population. A higher turnover of bacteria due to nematode predation may cause a shift from slower to faster growing bacteria which could establish a larger microbial biomass (Ekblad et al., 2013; Mamilov et al., 2000). The stimulation of bacterial biomass by nematode addition also corresponded to a decline in extractable NH_4^+ and NO_3^- in mineral soils, which could be the result of immobilization, in that a higher bacterial biomass would immobilize more N. Our data also showed that the nematode treatment generally only had strong effects in mineral soils, which could be because mineral soils provide a less aggregated

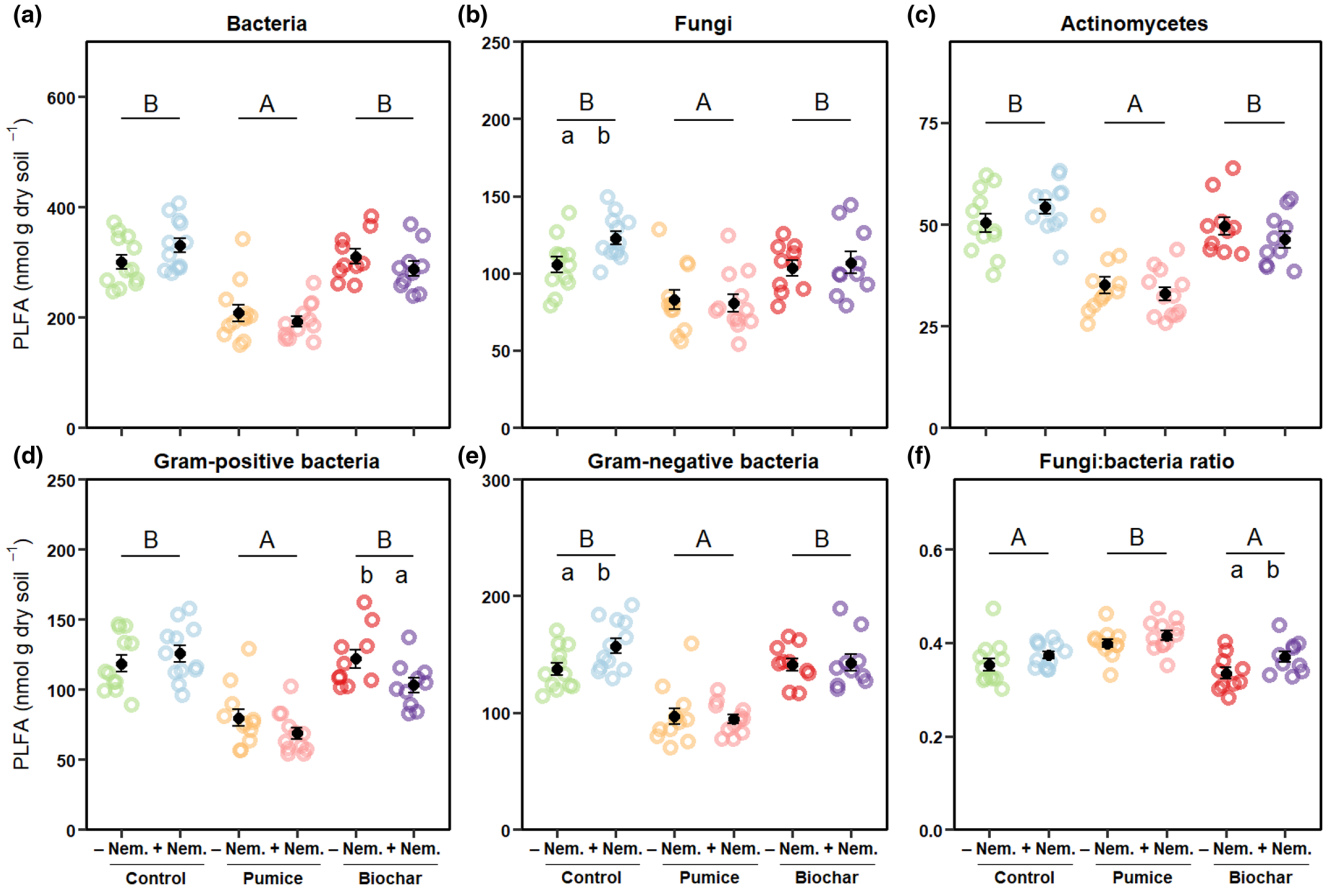

FIGURE 5 Phospholipid fatty acid (PLFA) functional group responses (nmol g dry soil^{-1}) in organic soil amended with biochar and pumice, and treated or not treated with nematodes. Capital letters (A or B) indicate significant differences between soil amendments, and lower case letters (a or b) indicate significant responses to nematode treatment ($p < 0.05$).

physical structure that offer bacteria less physical protection from bacterivores relative to organic soils (Trap et al., 2016). In line with this, Trap et al. (2016) showed that bacterivores have stronger positive effects on soil N and P mineralization in mineral compared to organic soils.

Our data did not provide any support for our third hypothesis that biochar refugia promotes higher rates of soil processes, because there were no significant interactive effects between biochar amendment and nematode addition on any of the microbial groups. Given the strong positive responses of bacteria to nematode addition we observed in mineral soil, it is clear that our treatment duration was adequate to provide the opportunity for interactions between nematodes, biochar, and microbes to emerge. One explanation for the lack of interactive response we observed in mineral soils (where nematode effects on bacteria were strongest) is that biochar contributed relatively little to the total soil volume in these mesocosms (ca. 10%), which could mean that microbial biomass in biochar pore spaces made a relatively minor contribution to the total soil microbial biomass (Razzaghi et al., 2020). This means that we might have observed a significant effect of biochar refugia on soil processes if the proportion of biochar relative to soil volume was higher (i.e., >10%), or if the biochar had been added to soil with an inherently low micropore volume (e.g., such as a course sandy soil; Razzaghi et al., 2020). However, we note that the amount of biochar we added was realistic in the context of natural wildfire-derived charcoal content in boreal forests, as well as in the context of field experiments focused on biochar management in boreal forests (Gundale et al., 2016; Ohlson et al., 2009).

While we did not find evidence that the microbial community was responsive to the interactive effect of biochar and nematode addition, we did find several interactive effects between soil amendment and nematode addition on soil nutrient pools and fluxes (i.e., NH_4^+, NO_3^-, and N_2-fixation in mineral soils, and NH_4^+ in organic soils), and one interactive effect that specifically appeared to be linked to biochar per se (i.e., on extractable NO_3^- in

TABLE 3 Results from analysis of variance or Kruskal–Wallis tests for mineral soils, evaluating the effects of soil amendment (biochar, pumice, or control) and nematode treatment (added or not added), and their interaction on a variety of soil pools or fluxes

Response variable (units)	Factor	df	*F*- or chi-squared-value	*p*-value
Extractable N-NH_4^+ (mg^{-1} g dry soil)	Amendment (A)	2	3.30[a]	0.19
	Nematodes (N)	1	8.61[a]	**<0.01**
	A×N	5	15.29[a]	**<0.01**
	Block	2	0.10[a]	0.95
Extractable N-NO_3^- (mg^{-1} g dry soil)	Amendment (A)	2	0.63	0.54
	Nematodes (N)	1	103.06	**<0.001**
	A×N	2	3.23	**<0.05**
	Residuals	64		
N fixation rate (μg N_2 fixed g^{-1} soil d^{-1})	Amendment (A)	2	17.40[a]	**<0.001**
	Nematodes (N)	1	0.44[a]	0.51
	A×N	5	18.69[a]	**<0.01**
	Block	2	1.30[a]	0.52
Basal respiration[b] (μg CO_2 min^{-1} g dry $soil^{-1}$)	Amendment (A)	2	0.04	0.96
	Nematodes (N)	1	2.77	0.10
	A×N	2	2.30	0.11
	Residuals	64		

[a]Kruskal–Wallis test was performed, and chi-squared values reported instead of *F*-values.

[b]Data were log transformed.

Values in bold are signficant

TABLE 4 Results from analysis of variance or Kruskal–Wallis tests for organic soils, evaluating the effects of soil amendment (biochar, pumice, or control) and nematode treatment (added or not added), and their interaction on a variety of soil pools or fluxes

Response variable (units)	Factor	df	*F*- or chi-squared value	*p*-value
Extractable N-NH_4^+ (mg^{-1} g dry soil)	Amendment (A)	2	2.20[a]	0.33
	Nematode (N)	1	4.22[a]	**<0.05**
	A×N	5	14.47[a]	**<0.05**
	Block	2	3.04[a]	0.22
Extractable N-NO_3^- (mg^{-1} g dry soil)	Amendment (A)	2	31.23	**<0.001**
	Nematode (N)	1	0.95	0.33
	A×N	2	0.66	0.52
	Residuals	60		
Extractable P-PO_4^{-3} (mg^{-1} g dry soil)	Amendment (A)	2	24.34	**<0.001**
	Nematode (N)	1	2.66	0.11
	A×N	2	0.08	0.92
	Residuals	60		
N fixation rate (μg N2 fixed g^{-1} soil d^{-1})	Amendment (A)	2	7.05	**<0.01**
	Nematode (N)	1	0.89	0.35
	A×N	2	1.02	0.37
	Residuals	60		
Basal respiration (μg CO_2 min^{-1} g dry $soil^{-1}$)	Amendment (A)	2	2.75	0.08
	Nematode (N)	1	1.93	0.17
	A×N	2	0.11	0.9
	Residuals	38		

[a]Kruskal–Wallis test was performed, and chi-squared values were reported instead of *F*-values.

Values in bold are signficant

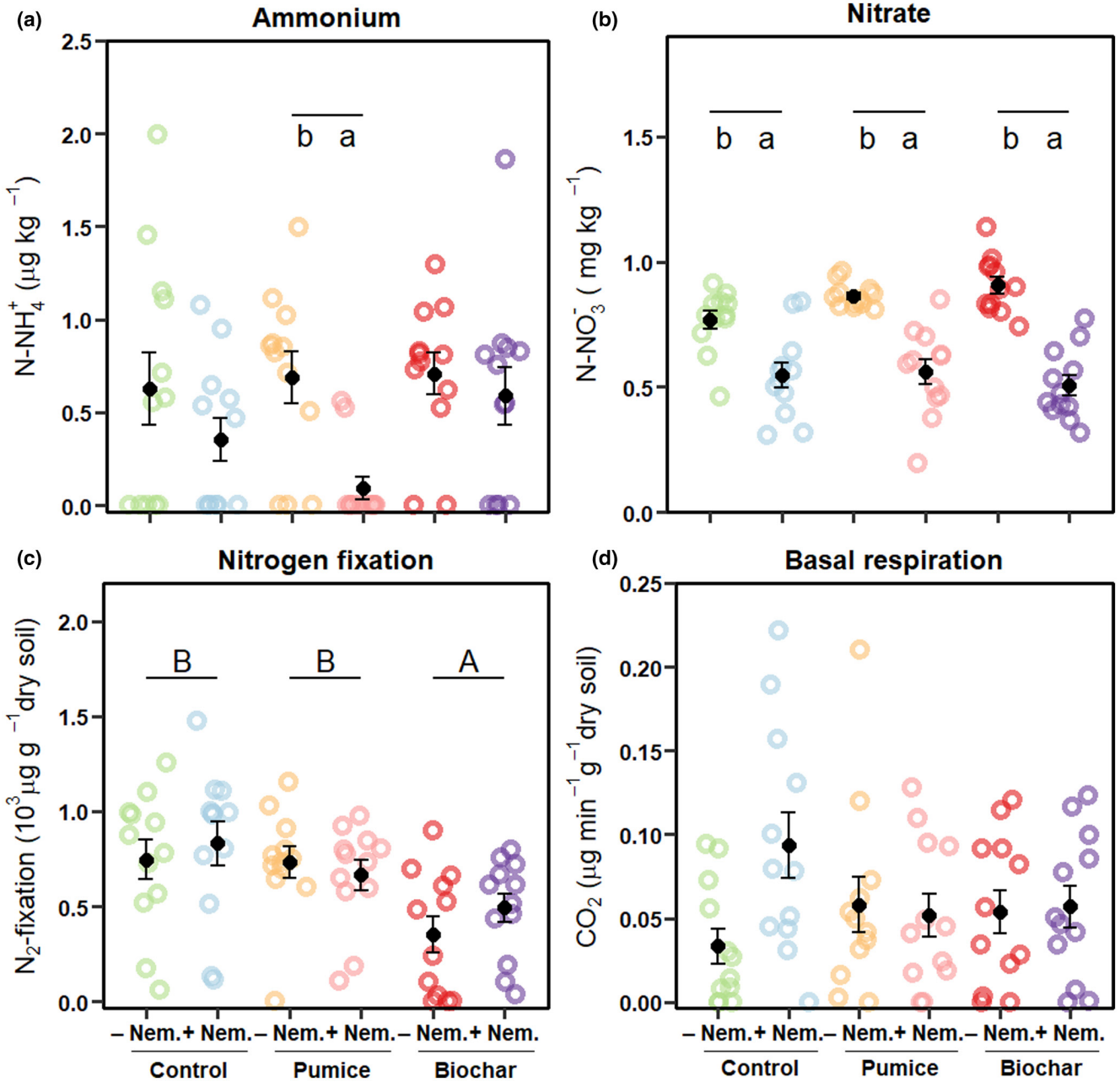

FIGURE 6 Extractable nutrients (NH_4^+, NO_3^-), nitrogen fixation rates (µg N_2 day^{-1} g dry $soil^{-1}$), and basal respiration rates (CO_2 µg min^{-1} g dry $soil^{-1}$) in mineral soils amended with biochar, and pumice reported before and treated or not treated with nematodes. Capital letters (A or B) indicate significant differences between soil amendments, and lower case letters (a or b) indicate significant responses to nematode treatment ($p < 0.05$).

mineral soil). Specifically, we found that the reduction of extractable NO_3^- concentrations in response to nematode addition was strongest in the presence of biochar relative to the other soil amendment treatments. This response is notable, because it has been suggested that nitrifying bacteria may gain particular benefit from protection from grazers in the internal pore spaces of biochar (DeLuca et al., 2006; Prommer et al., 2014), which could lead to higher rates of nitrification and NO_3^- accumulation. However, in contrast to this hypothesis we instead observed a reduction in extractable NO_3^- in the presence of nematodes and biochar. A potential explanation for our observed interactive effect is that the higher bacterial biomass stimulated by nematodes could have resulted in stronger N immobilization when bio-available C associated with biochar was present (Hangs et al., 2016).

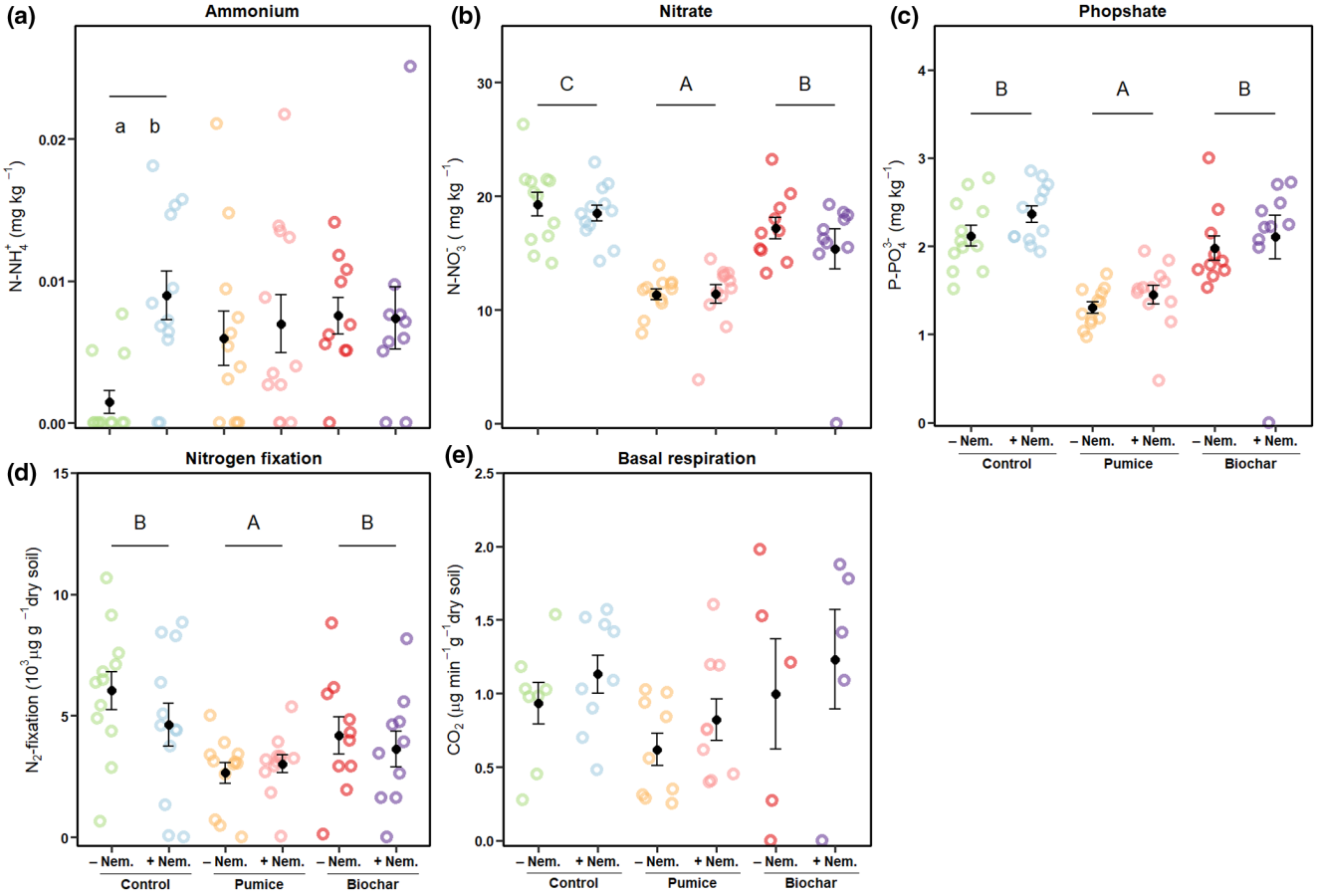

FIGURE 7 Extractable nutrients (NH_4^+, NO_3^-, and PO_4^{-3}), nitrogen fixation rates (μg N_2 day^{-1} g dry $soil^{-1}$), and basal respiration rates (CO_2 μg min^{-1} g dry $soil^{-1}$) from organic soils amended with biochar and pumice, and treated or not treated with nematodes. Capital letters (A or B) indicate significant differences between soil amendments, and lower case letters (a or b) indicate significant responses to nematode treatment ($p < 0.05$).

5 | CONCLUSIONS

We applied biochar made from boreal wood biomass to a boreal soil to evaluate a specific mechanism through which biochar has been proposed to enhance soil functioning, that is, via the microbial refugia hypothesis. In testing this, we found little evidence that biochar by itself altered soil microbial communities or soil functioning, or that it interacted with microfaunal grazers to regulate soil processes. The lack of evidence for the microbial refugia hypothesis in our experiment may be due to a combination of factors. First, the dominant coniferous tree species found in boreal forests, which are members of the Pinaceae, may yield to a different quality of biochar with a different capacity to serve as microbial refugia, relative to angiosperm biochars that are more common at lower latitudes. Furthermore, the type of microbial communities found in boreal soils may not benefit from refugia to the same degrees as lower latitude soil communities where bacteria are relatively more dominant. The greater dominance of fungi in boreal forests may constrain the responses to biochar of key bacterial functional groups that may particularly benefit from refugia, such as nitrifying bacteria. However, despite a lack of support for the microbial refugia hypothesis, we note that several other studies have demonstrated positive impacts of boreal biochar on microbial activity, soil fertility, and forest growth (Gundale et al., 2016; Pietikäinen et al., 2000; Saarnio & Kettunen, 2020; Soinne et al., 2020). These studies suggest that biochar may enhance soil functioning through other types of mechanisms (e.g., source of soil nutrients, sorption of specific C compounds, or by improving soil physical structure). Future research should isolate the relative importance of these alternative mechanisms, which will enable a better understanding and use of biochar as an effective tool in the boreal region to simultaneously increase soil C storage, and enhance soil functioning, which may subsequently promote C uptake through enhanced forest growth.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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