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The relative importance of the bacterial pathway and soil inorganic nitrogen increase across an extreme wood-ash application gradient

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Abstract

Ash from combustion of biofuels, for example wood chips, is often deposited as waste, but due to its high content of essential plant nutrients and alkalinity, it has been proposed to recycle ash as a fertilizer and liming agent in biofuel production forest. However, current legislation sets rather strict limitations for wood-ash application in biofuel production systems. The soil microfood web, that is microorganisms and their microfaunal grazers, protozoa and nematodes, is pivotal for essential ecosystem processes such as decomposition and plant nutrient release. Therefore, a thorough assessment of the impacts on microfood web structure and functioning must precede actions towards raising the currently allowed application rates. In a Danish Norway spruce plantation, we evaluate the impact of wood ash applied at dosages from 0 to the extreme case of 90 t ash ha^{-1} on the microfood web, the bacterial community structure, soil content of inorganic nitrogen, organic matter, dissolved organic carbon and nitrogen. Using structural equation modelling (SEM), we disentangled the direct effect of the disturbance imposed by ash per se, the associated pH increase and changes in prey abundance on individual organism groups in the microfood web. The SEM showed that the pH rise was the main driver of increasing abundances of culturable heterotrophic bacteria with increasing ash doses, and via trophical transfer, this also manifested as higher abundances of bacterial grazers. Fungal-feeding nematodes were unaffected by ash, whereas carnivorous/omnivorous nematodes decreased due to the direct effect of ash. Increasing ash doses enhanced the difference between bacterial communities of control plots and ash-amended plots. The ash-induced stimulation of culturable heterotrophic bacteria and bacterial grazers increased inorganic nitrogen availability at ash doses of 9 t ha^{-1} and above. Hence, raised limits for ash application may potentially benefit tree growth via enhanced N mineralization activity of the soil food web.

Keywords: bacterial community structure, high-resolution melt curve (HRM) analysis, inorganic nitrogen, nematodes, Norway spruce, protozoa, structural equation model (SEM)

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Introduction

Wood ash – waste product or a valuable fertilizer?

Renewable energy sources such as wood chips play an increasing role in the global energy production. For instance, the European Council has set a mandatory target of 20% renewable energy of the total energy consumption by 2020 (COM, 2006). Long-term sustainable use of wood biomass for energy production calls for optimized forest management, where the nutrient export at harvest is balanced by similar nutrient inputs.

As wood ash contains high amounts of plant nutrients, an obvious strategy is to apply the wood ash as a biofertilizer in biofuel production plantations (Jacobson *et al.*, 2014). The major components of wood ash are calcium, potassium, magnesium and phosphorus, while it contains virtually no nitrogen (N) (Demeyer *et al.*, 2001). Therefore, the efficiency of ash as a fertilizer depends on the existing N in the system and/or the rate of N deposition. In spite of the negligible N content in wood ash, the increased pH can indirectly increase microbial activity and thus N mineralization and availability

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(Genenger *et al*., 2003; Jäggi *et al*., 2004). Positive effects of wood-ash application on tree production have been demonstrated at sites, which receive N deposition or are rich in organic matter, like peat soils, whereas ash does not enhance tree production on, for example, mineral soils where N is the limiting nutrient (Huotari *et al*.; 2015; Karltun *et al*., 2008). In N-limited systems, the combined application of ash and N may be a solution to the unbalanced nutrient content in wood ash (Saarsalmi *et al*., 2006, 2012).

However, the extremely high pH of wood ash as well as the potentially high heavy metal content may challenge the sustainability of wood-ash application as fertilizers. The application of ash typically raises forest floor pH 0.5–3 units depending on the amount of ash applied (Huotari *et al*., 2015). Wood ash consists of a range of trace elements that originate from the incinerated wood such as arsenic, silver, molybdenum, mercury, nickel, vanadium, zinc and cadmium (Cd) (Etiégni & Campbell, 1991; Demeyer *et al*., 2001; Karltun *et al*., 2008). Some of these are micronutrients; some are nonessential heavy metals (Huotari *et al*., 2015). The biggest concern is the heavy metal content, particularly Cd (Saarsalmi *et al*., 2001). The Cd concentration in wood ash typically varies between 1 and 20 mg kg^{-1} (Korpilahti *et al*., 1998). Presently, the Cd content defines the maximum limit for wood-ash application to 3 t ha^{-1} three times during a 75 years period according to Danish legislation (DEA, 2008), and similar rules are followed in, for example, Sweden and Finland. A substantial proportion of ash produced during biofuel combustion, including valuable plant nutrients, is therefore currently deposited as waste at a considerable cost (Ingerslev *et al*., 2011). Enhanced recirculation of wood ash as fertilizers in biofuel production would therefore benefit the economy of biofuel-based energy production through reduced expenses to fertilizers from alternative sources and through reduced expenses to ash deposition. Increasing doses of 6–9 t ash ha^{-1} would be feasible and realistic. However, thorough evaluation of potential negative side effects of enhanced wood-ash recirculation on the environment must be conducted before actions are taken towards changing the currently allowed application rates.

The high alkalinity of ash often reduces moss and lichen coverage resulting in the replacement of the original moss species with other colonizing species. Likewise, herbs and grasses often increase at the expense of shrubs (Huotari *et al*., 2015). Generally, ash application does not change plant Cd concentrations (Huotari *et al*., 2015) or Cd concentrations in soil or aboveground invertebrates (Lodenius *et al*., 2009), although earthworms may accumulate Cd to nontoxic concentrations (M. Vestergård, R. Rønn and M.F. Hovmand, unpublished; Lodenius, 2003). Studies on the Cd effect on soil invertebrates after ash application are scarce, but no toxic effects have been observed (Lundkvist, 1998; Lodenius *et al*., 2009).

Thorough assessments of wood-ash application impacts on the forest ecosystem must also include detailed evaluation of effects on microorganisms and higher trophic levels of the soil food web as well as key microbial functioning.

The importance of the soil microfood web for decomposition and nutrient mineralization

The soil microfood web, including microorganisms and their microfaunal grazers, protozoa and nematodes (Dam *et al*., 2017), plays an extremely important role for key ecosystem processes, for example, decomposition and mineralization of nitrogen and other plant nutrients and hence plant growth (Rønn *et al*., 2012). Protozoa and nematodes are the primary microbial grazers in terrestrial ecosystems. They regulate the size and functioning of fungal and bacterial populations (Ingham *et al*., 1985; Rønn *et al*., 2012) in the soil, and nematode and protozoan grazing enhance microbial activity and nutrient mineralization (Rønn *et al*., 2012). Nematodes are represented at several trophic levels of the soil decomposer food web; that is bacteriovorous, fungivorous, omnivorous and carnivorous taxa (Yeates *et al*., 1993), and the abundance of these individual feeding groups often mirrors growth responses of their respective food sources (Ferris, 2010; Christensen *et al*., 2012). Transfer of energy and elements in the soil food web is thought to be compartmentalized into two separate pathways that are either based on bacterial or fungal production (Moore & Hunt, 1988; Moore *et al*., 1996). Especially lower order consumers in the soil food web such as protozoa and nematodes reflect the carbon flow through the bacterial or fungal channel (Rooney *et al*., 2006).

Ash-induced effects on the soil microfood web

The pH increase caused by ash can have profound effects on microbial composition and productivity. In general, bacterial growth peaks at higher pH than fungal growth (Rousk *et al*., 2010a; Rousk & Bååth, 2011; Cruz-Paredes *et al*., 2017), and often increasing pH benefits bacterial abundance and productivity, whereas fungal productivity usually declines or remains unaffected (Majdi *et al*., 2008; Rousk *et al*., 2009, 2011). Shifts towards bacterial relative to fungal decomposition activity are likely to enhance mineralization rates and diminish soil C storage capacity (Waring *et al*., 2013; Malik *et al*., 2016). In general, soil pH is a principal driver of soil bacterial community composition (Fierer & Jackson, 2006; Rousk *et al*., 2010b); hence, also the composition of

bacterial communities may respond to wood-ash application (Perkiömäki & Fritze, 2002; Noyce *et al.*, 2016; Bang-Andreasen *et al.*, 2017; Cruz-Paredes *et al.*, 2017).

We know much less about the effects of increased pH on organisms at higher trophic levels of the soil food web. Further, there is a striking paucity of data on the effects of ash application and pH increase on protozoa, and results on the effects of wood-ash and lime application on nematodes in acidic forest soils are somewhat contradictory. Ash and lime application enhanced populations of bacterial-feeding nematodes in Finnish pine forest soils (Hyvönen & Huhta, 1989; Nieminen, 2009), whereas ash had a transient negative effect on fungal-feeding nematodes, but no effect on bacterial-feeding nematodes in a laboratory experiment with similar soil (Liiri *et al.*, 2007). Further, defaunated soil that was buried in acidic pine forest plots attained higher abundances of microbial-feeding nematodes, but lower abundances of omnivorous nematodes after colonization from the surrounding forest soil that had been treated with wood ash than from unamended soil (Liiri *et al.*, 2002). The variable responses may reflect that nematode responses were assessed under variable experimental conditions and may also reflect variable responses of prey organisms or co-occurring microbivores under the different experimental conditions.

Soil organisms will not only experience pH changes, but also the general system disturbances imposed by ash application, for example reduced moss coverage and nutrient and heavy metal addition (Huotari *et al.*, 2015). Especially long-lived nematode taxa with long life cycles and slow reproduction, most of which are higher-order consumers such as omnivores and predators, are sensitive to environmental disturbance (Bongers, 1990; de Goede & Dekker, 1993).

Testing ash dose effects in a spruce forest

Our aim is to assess the effects of ash application across a wide range of doses on the bacterial community structure and the microfood web that is based on microbial production in a spruce plantation, which represents a system where enhanced application of wood ash as fertilizer is most relevant. Further, we will evaluate if ash-driven changes of the bacterial community structure and the microfood web have implications for the inorganic nitrogen availability. We also relate responses of the organisms and inorganic nitrogen availability to ash effects on soil organic matter content and labile organic substrates, that is dissolved organic carbon and nitrogen. To our knowledge, no studies report the concerted response of the major groups of the soil microfood web; that is microorganisms, protozoa and nematode trophic groups.

Effects of the application of potentially environmentally harmful products are best evaluated in dose–response trials that also include very high or extreme treatment doses. Extreme treatments increase the likelihood of detecting potential negative effects of the application of a given substance or product; vice versa, lack of effects even at extreme doses allows us to conclude with confidence that a product does not elicit harmful effects on investigated variables. Such trials are rarely conducted at field scale, but here we designed a field experiment with a gradient of ash application doses including currently allowed and extremely high doses; that is, doses that far exceed the doses that would realistically be applied in forestry.

Within recent years, the use of structural equation modelling (SEM) has remarkably advanced our causal understanding of the shifts in belowground food webs and ecological processes associated with environmental changes (Eisenhauer *et al.*, 2013; Vestergård *et al.*, 2015). With SEM, we will analyse whether treatment effects on abundances of culturable heterotrophic bacteria, protozoa and nematode feeding groups can primarily be ascribed to the ash application per se, to the direct effect of the associated pH raise or to ash- or pH-induced changes in the abundance of prey organisms.

For SEM, the analyst constructs a hypothesized network of variables including hypothesized causal relationships between variables. SEM is a tool that statistically evaluates the probability of the hypothesized network including the implied causal relationships between variables (McCoach *et al.*, 2007). Therefore, significant correlation between variables does not as such prove causality (Kazantis *et al.*, 2001). One limitation for the inference of causality between variables is that it is rarely, if ever, possible to identify and incorporate all relevant causes for a given variable (McCoach *et al.*, 2007), and causality inference should always be interpreted with some caution.

We hypothesized that the liming effect of wood ash was the principal driver of soil organism responses to ash application, and that the pH raise would enhance the abundance of culturable heterotrophic bacteria with cascading positive effects on bacterial consumers and possibly carnivorous/omnivorous nematodes. In contrast, we expected no or a modest negative response of the fungal-based compartments of the soil food web to the pH increase. Alternatively, we hypothesized that differential sensitivity to environmental disturbances at higher trophic levels could decouple the responses of microbial grazers or higher-order consumers of the microfood web from the anticipated bacterial productivity increase at wood-ash application. Further, we hypothesized that the bacterial community composition would respond to the pH increase in a dose-dependent

manner, and we expected enhanced soil inorganic nitrogen content with enhanced microbial activity at increasing ash application doses.

Materials and methods

Field experiment and sampling

Our field site is a Norway spruce (*Picea abies* (L.) Karst.) plantation located in Mid Jutland, Denmark (N 56°16.633′, E 9°05.200′), 51 m above sea level. The climate is temperate with a mean annual temperature of 8.4 °C and a mean annual precipitation of 850 mm. The plantation is a second-generation plantation established in 1957 on former heathland with a well-developed podzolization formed on a well-drained, sandy glacial till. The ground vegetation is heavily dominated by mosses with very limited contribution of vascular plants, mainly *Deschampsia flexuosa* and *Vaccinium vitis-idaea*.

The experiment was laid out as a randomized block design with six levels of wood-ash application in five replicate rows, that is in total 30 2 × 2 m plots, within a 20 × 15 m area. In April 2014, we applied the equivalent of 0, 3, 9, 15, 30 or 90 t wood ash ha^{-1} to the individual plots, where 3 t ha^{-1} represents the currently maximum allowed dose. The wood ash was a mixture of bottom- and fly ash originating from the burning of spruce bark chips at the nearby Brande heating plant. The ash had not been exposed to any stabilization treatment. Details of wood-ash contents and alkalinity are shown in Table 1.

In April 2015, we sampled all plots. In each 2 × 2 m plot, three samples randomly positioned at least 30 cm from the edge of the plot were taken with a 5 cm auger to a depth of 5 cm including the moss (when present), O horizon and the top of the A horizon (3 × 98 cm^{-3} per plot). The samples were transported to the laboratory under cooled conditions and kept at 4 °C until processed the following day.

Sample processing and determination of physicochemical parameters

The three subsamples from each plot were pooled into one composite sample per plot.

We sieved (5 mm) and homogenized the individual composite samples. All variables were measured on fractions of the homogenized composite samples.

pH was measured on 10 g (fw) soil suspended in 25 mL distilled H_2O on a Phm 240 pH/ION meter. For soil water determination, we measured the weight loss of 5 g (fw) soil after 24 h drying at 110°C, and subsequently we determined soil organic matter (SOM) content as mass loss on ignition at 550°C for 6 h.

For dissolved organic carbon (DOC), dissolved organic nitrogen (DON), ammonium (NH_4^+) and nitrate (NO_3^-) determination, we extracted 5 g (fw) soil in 50 mL distilled H_2O for 60 min on a shaker at 200 rpm. The extracts were filtered and immediately frozen at −18°C. We measured the DOC content in the filtrates on a Shimadzu TOC5000A analyser (Shimadzu, Kyoto, Japan). DON contents in digested filtrates and NH_4^+ and NO_3^- contents in the filtrates were measured with a flow injection analyser (5000 FIASTAR, Höganäs, Sweden).

Enumeration of soil organisms

For the enumeration of culturable heterotrophic bacteria and protozoa, we suspended 3 g of each composite sample (fw) in 100 mL sterile distilled H_2O and blended for 1 min in a Waring laboratory blender at full speed at 20 °C. We determined the density of culturable heterotrophic bacteria as colony forming units (CFUs) after plate spreading. Duplicates of 0.1 mL of 10^{-3}, 10^{-4} and 10^{-5} dilutions of the soil suspension were spread on tryptic soy broth (TSB) (0.3 g L^{-1}) (Becton, Dickinson and Company, Sparks MD) agar plates, and the number of CFUs was counted after 14 days incubation at 12 °C. Like any other method for bacterial quantification (Blagodatskaya & Kuzyakov, 2013), this culture-dependent method is biased; generally, it underestimates the total cell number by approximately a factor of 10–100 (Vestergård *et al*., 2011). Nevertheless, it gives a cost-efficient and reproducible estimate of between-sample variation in bacterial abundances that correlates with total cell counts (Rønn *et al*., 2002) and most likely represents the most metabolically active fraction of the bacterial community (Olsen & Bakken, 1987).

Numbers of protozoa (amoebae/flagellates and ciliates) were estimated with the most probable number (MPN) method. For amoebae/flagellates, eight replicated threefold dilution series of each soil suspension were prepared in 96 wells microtitre plates with 0.3 g L^{-1} TSB in amoeba saline (Page, 1988) as medium. After 7 and 21 days of incubation at 12 °C, we inspected the wells for the presence of protozoa (amoebae/flagellates) using an inverted microscope at 200-400× magnification (Rønn *et al*., 1995). For ciliates, eight replicates of 1 mL of 10^{-0}, 10^{-1} and 10^{-2} dilutions were incubated with a sterile barley grain in wells of 12 wells titre plates. After 7 days of incubation at 12 °C, we inspected the wells for the presence of ciliates to determine the MPN of ciliates (Ekelund *et al*., 2002).

We extracted nematodes from 30 g of soil from each composite sample by a combination of the Baermann pan and the Whitehead tray (Whitehead & Hemming, 1965) extraction

Table 1 Elemental content (ppm dw) and pH of the wood ash applied in the field experiment

Al	As	Ba	Ca	Cd	Co	Cu	Cr	Fe	K	Mg	
6183	1.8	986	116 024	7.1	4	88	14	5289	29 115	15 341	
Mn	Mo	Na	Ni	P	Pb	Si	U	V	Zn	Zr	pH
9689	1	4963	18	14 682	29	666	0.4	13	639	9	12.95

methods. Samples were extracted for 72 h, and individuals of nematode trophic groups were counted while live based on morphological characteristics of mainly the buccal cavity and oesophagus (Yeates *et al.*, 1993; Dam *et al.*, 2017) using an inverted microscope at 200× to 400× magnification. We focus on abundances of nematodes that feed on bacteria and fungi or are carnivores or omnivores, as the quantitative responses of these trophic groups often reflect growth responses of their respective food sources (Rooney *et al.*, 2006; Ferris, 2010; Christensen *et al.*, 2012).

Fingerprinting of the bacterial community structure

DNA was extracted from 3 g of frozen soil with the PowerMax Soil® kit (MoBIO, Carlsbad, USA). Differences in bacterial community structure between samples were examined using real-time PCR combined with high-resolution melt curve analysis of the amplified DNA, as described by (Hjelmsø *et al.*, 2014). PCRs were run on a real-time Bio-Rad T100 Thermal Cycler in technical triplicates using a master mix consisting of 4 μL HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Riia, Estonia), 2 μL of bovine serum albumin (BSA) (20 mg mL^{-1}; BIORON, Ludwigshafen, Germany), 0.4 μL of 10 μM forward and reverse primers (354f: 5′-CCTAYGGGRBGCASCAG-3′ and 806r: 5′-GGACTACNNGGGTATCTAAT-3′) (Hansen *et al.*, 2012), 1 μL DNA template and 12.2 μL sterile water to a final volume of 20 μL. Sterile water and genomic DNA from Escherichia coli K-12 (Blattner *et al.*, 1997) were used as negative and positive controls, respectively. Thermal conditions were an initial denaturation at 95 °C for 12 m followed by 35 amplification cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s and ending with 72 °C for 3 m. High-resolution melt curves were generated by melting the resulting 16S rRNA gene amplicons using 0.1 °C increments from a starting temperature of 72 °C and ending at 95 °C with fluorescence measurements at every temperature increment.

Melting curves were normalized to relative fluorescence units (RFU) in the melt region of the 16S rRNA gene amplicons (83.8–90.9 °C) using the Precision Melt Analysis™ Software (Bio-Rad Laboratories, Richmond, CA, USA). Based on the normalized curves, we calculated the difference in RFU for each temperature step. These values represent the amount of DNA melting at a given temperature, and the data provide a fingerprint of the bacterial community (Hjelmsø *et al.*, 2014).

Data analysis

Our aim was to quantify the treatment impacts in the top five cm of the forest floor, where the organic matter content and hence soil density vary considerably. We therefore present all variables on a volume basis, that is cm^{-3}, to accommodate comparison on a comparable basis. We analysed the relationship between the independent variables, that is ash application dose, and the response variables, that is soil pH, abundance of culturable heterotrophic bacteria, protozoa, nematode trophic groups, SOM, DOC, DON, NH_4^+–N and NO_3^-–N with linear and nonlinear regression models in SigmaPlot version 13.0. Where necessary, data were log-transformed to obtain normality and homoscedasticity. For variables that responded significantly to ash application, we subsequently tested the significance of pairwise differences between ash doses with Tukey's test.

We used SEM to analyse the relationships between ash application level, pH and abundances of organisms at different trophic positions in the soil microfood web to examine whether treatment effects on abundances could primarily be ascribed to the ash application per se, to the associated pH raise or to ash- or pH-induced changes in the abundance of prey organisms. We arranged the measured variables in a causal network according to our prior knowledge on soil-ecological cause–effect relationships (Fig. 6). Some of the variables were log-transformed to achieve approximate normal distribution and linear relationships between variables, and some of the variables were rescaled to achieve approximately equal variances for all variables. We fitted the variables to the SEM with the software package lavaan (Rosseel, 2012) in R (R Core Team, 2014).

We analysed overall differences in bacterial community structure with a principal component analysis (PCA), where we treated the difference in RFU for each temperature step of the HRM analysis as proxy operational taxonomic units (OTUs). We ran the PCA in PC-ORD ver. 5 (McCune & Mefford, 1999).

Results

Physicochemical parameters

pH increased dramatically ($P < 0.0001$) from 3.8 in the control treatment to 7.7 at the highest ash application dose at 90 t ha^{-1} (Fig. 1).

The organic matter content in the upper 5 cm was quite variable within individual ash doses, but overall it decreased with increasing ash doses ($P = 0.0012$), although only two treatments, that is 3 t ash ha^{-1} with a mean organic matter content of 1.4 g cm^{-3} and 90 t ash ha^{-1} with a mean of 0.93 g cm^{-3}, were significantly different (Tukey, $P < 0.05$) (Fig. 2a). Likewise, DOC and DON varied considerably within individual ash application levels, but both variables increased with ash doses up to 15–30 t ash ha^{-1}. At 90 t ash ha^{-1}, the variation within both labile organic pools increased even further, but there was an overall decline in DOC and DON compared to the maximum values found at lower ash doses (Fig. 2a,b).

Soil ammonium concentration increased dramatically from 1.5 to 1.7 μg NH_4^+–N at 0 to 3 t ash ha^{-1} to 11 μg NH_4^+–N at 9 t ash ha^{-1}, and remained at a high level between 14 and 19 μg NH_4^+-N at 15–90 t ash ha^{-1} (Fig. 3). At 0 and 3 t ash ha^{-1}, nitrate concentrations were very low (0.1 μg NO_3^-–N cm^{-3}), but it increased linearly ($P < 0.0001$) with ash doses to 6 μg NO_3^-–N cm^{-3} at 90 t ash ha^{-1} (Fig. 3).

Soil organism abundances

The density of culturable heterotrophic bacteria increased ($P < 0.0001$) more than twentyfold from the

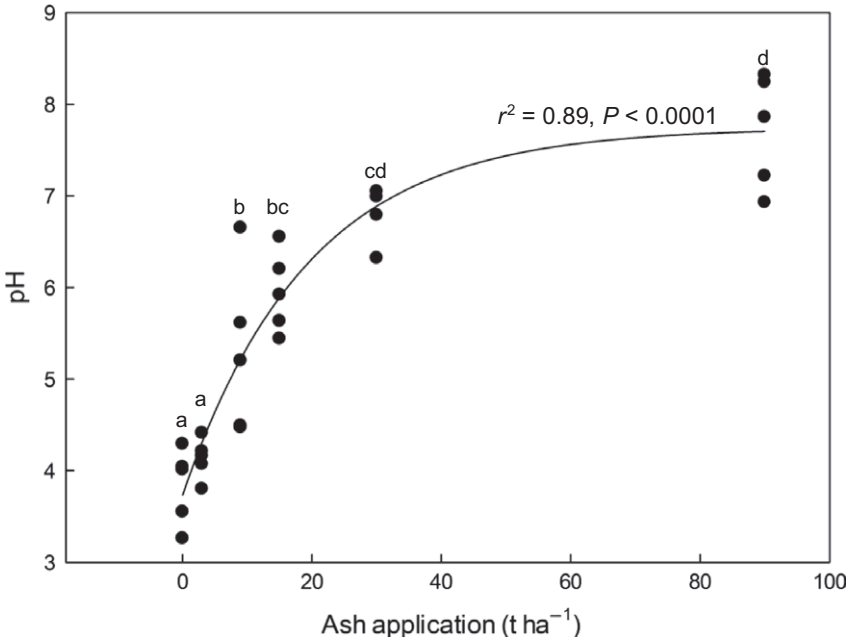


Fig. 1 pH in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). The curve represents the fit to an exponential rise to maximum regression model, where pH = 3.7340 + 4.0103 × (1−e$^{-0.0515\times t\ ash/ha}$) with r^2 and P values for the regression model. Ash doses with different letters are significantly different (Tukey $P < 0.05$).

control plots to 15 t ash ha^{-1}, whereas there was no further increase in densities from 15 to 90 t ash ha^{-1} (Fig. 4).

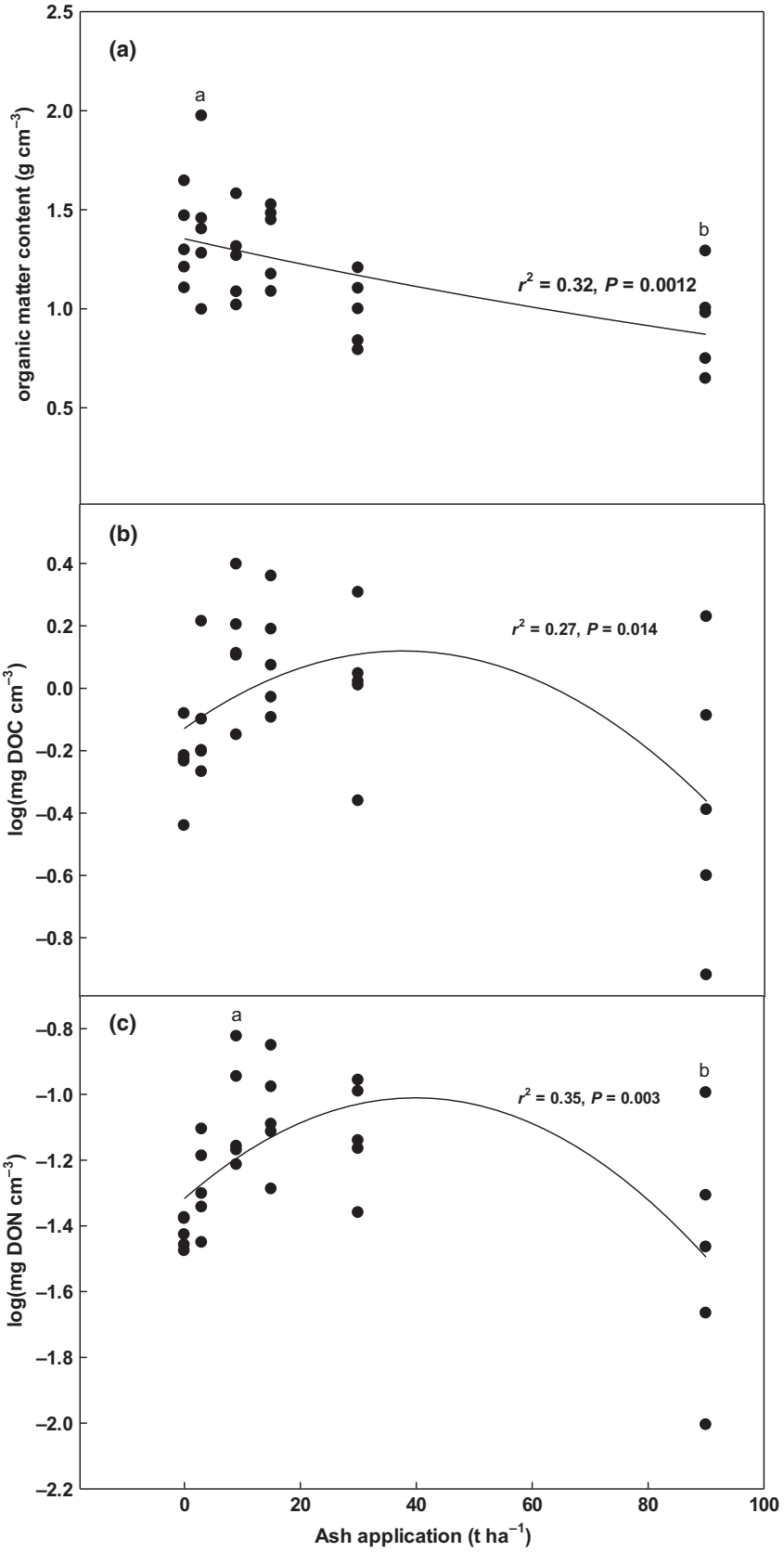
Similarly, densities of amoebae/flagellates ($P = 0.0006$) and ciliates ($P = 0.006$) increased 15 and 25 times, respectively, from 0 to 15 t ash ha^{-1} and remained at this high level from 15 to 90 t ash ha^{-1} (Fig. 4).

The abundances of bacterial-feeding nematodes were variable, but numbers increased modestly ($P = 0.015$) and roughly doubled from the control to the highest ash dose (Fig. 5a). However, there were no significant pairwise differences in bacterial-feeding nematode abundance between individual ash application doses (Tukey $P > 0.05$). Fungal-feeding nematodes, on the other hand, were not affected by ash application ($P = 0.21$; Fig. 5a), and the density of carnivorous/omnivorous nematodes decreased ($P = 0.0002$) with increasing ash application doses; their mean densities were already reduced by 50% at 3 t ash ha^{-1} and were close to zero at 30 and 90 t ha^{-1} (Fig. 5b).

Mechanisms of ash effects

The SEM adequately fit the data ($\chi^2 = 5.474$, df = 6, $P = 0.485$). The significant positive regression coefficient between pH and culturable heterotrophic bacteria revealed that the pH raise was driving the bacterial density increase at increasing ash doses, whereas the residual effect of ash dose was insignificant (Fig. 6). The positive pH effect on abundances of culturable heterotrophic bacteria transferred to amoebae/flagellates, as the relationship between culturable heterotrophic bacteria and amoebae/flagellates was significantly positive and further to ciliates that were positively related to amoebae/flagellates. In addition to the bacterial-mediated association between pH and amoebae/flagellates, there was also a direct positive relationship between the pH rise and abundances of amoebae/flagellates. There was no direct influence of ash or pH on bacterial-feeding nematodes, but a negative relationship between abundances of culturable heterotrophic bacteria and bacterial-feeding nematodes.

Fig. 2 (a) Soil organic matter (SOM), dissolved organic (b) C (DOC) and (c) N (DON) content in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). The curves represent fits to (a) an exponential decay model, where SOM = 1.3535 × e$^{-0.0049\times t\ ash/ha}$, and (b,c) second-order polynomial models, where log DOC = −0.1289 + 0.0132 × t ash ha^{-1}–0.0002 × (t ash ha^{-1})^2 and log DON = −1.3171 + 0.0154 × t ash ha^{-1}–0.0002 × (t ash ha^{-1})^2, with r^2 and P values for the regression models. Ash doses with different letters are significantly different (Tukey $P < 0.05$).


(a)
a
b
r^2 = 0.32, P = 0.0012
organic matter content (g cm^{-3})
2.5
2.0
1.5
1.0
0.5
(b)
r^2 = 0.27, P = 0.014
log(mg DOC cm^{-3})
0.4
0.2
0.0
–0.2
–0.4
–0.6
–0.8
(c)
a
b
r^2 = 0.35, P = 0.003
log(mg DON cm^{-3})
–0.8
–1.0
–1.2
–1.4
–1.6
–1.8
–2.0
–2.2
0
20
40
60
80
100
Ash application (t ha^{-1})

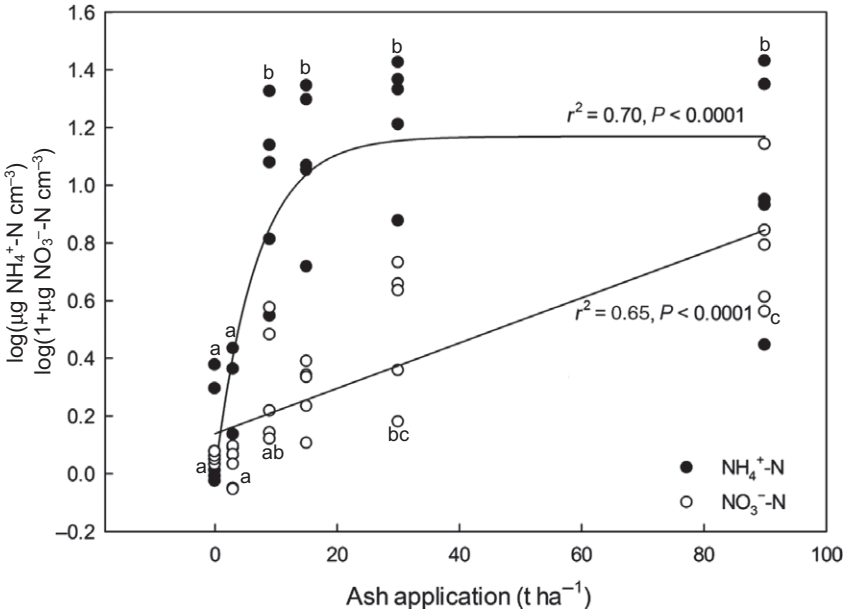


Fig. 3 Concentrations of NH_4^+–N (log(μg NH_4^+–N cm^{-3})) and NO_3^-–N (log(1 + μg NO_3^-–N cm^{-3})) in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). Curves represent fits to an exponential rise to maximum regression model, where log(μg NH_4^+–N cm^{-3}) = 1.169 × (1−$e^{-0.1461\times t\ ash/ha}$) and a linear regression model, where log(1 + μg NO_3^-–N cm^{-3}) = 0.1389 + 0.0078 × t ash ha^{-1}, respectively, with r^2 and P values for the regression models. Ash doses with different letters are significantly different (Tukey $P < 0.05$).

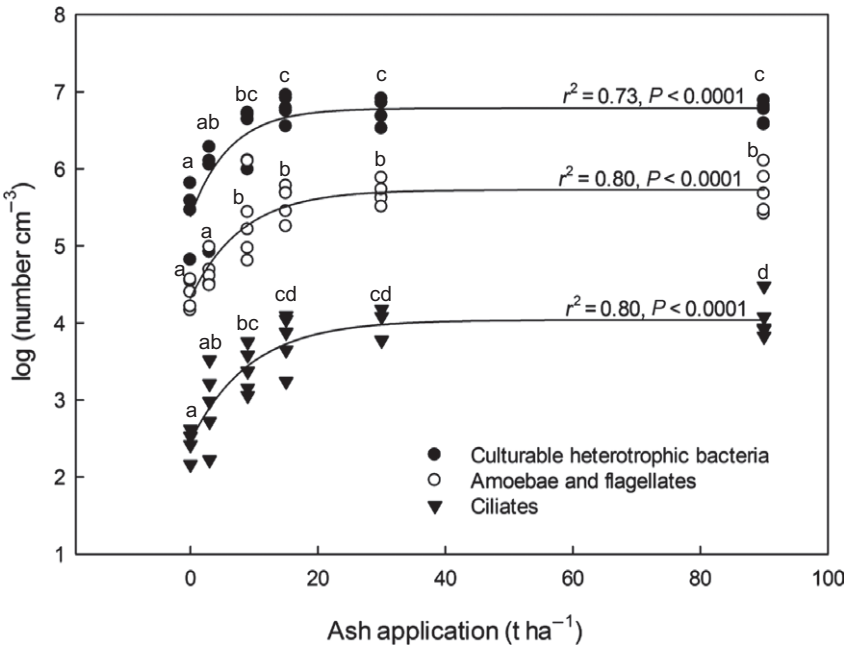


Fig. 4 Abundance (number cm^{-3}) of culturable, heterotrophic bacteria, amoebae/flagellates and ciliates in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). The curves represent the fits to exponential rise to maximum regression models, where log(culturable heterotrophic bacteria) = 5.3853 + 1.4001 × (1−$e^{-0.1642\times t\ ash/ha}$), log(amoebae/flagellates) = 4.3214 + 1.407 × (1−$e^{-0.1246\times t\ ash/ha}$) and log(ciliates) = 2.4606 + 1.5799 × (1−$e^{-0.1084\times t\ ash/ha}$) with r^2 and P values for the regression models. Ash doses with different letters are significantly different (Tukey $P < 0.05$).

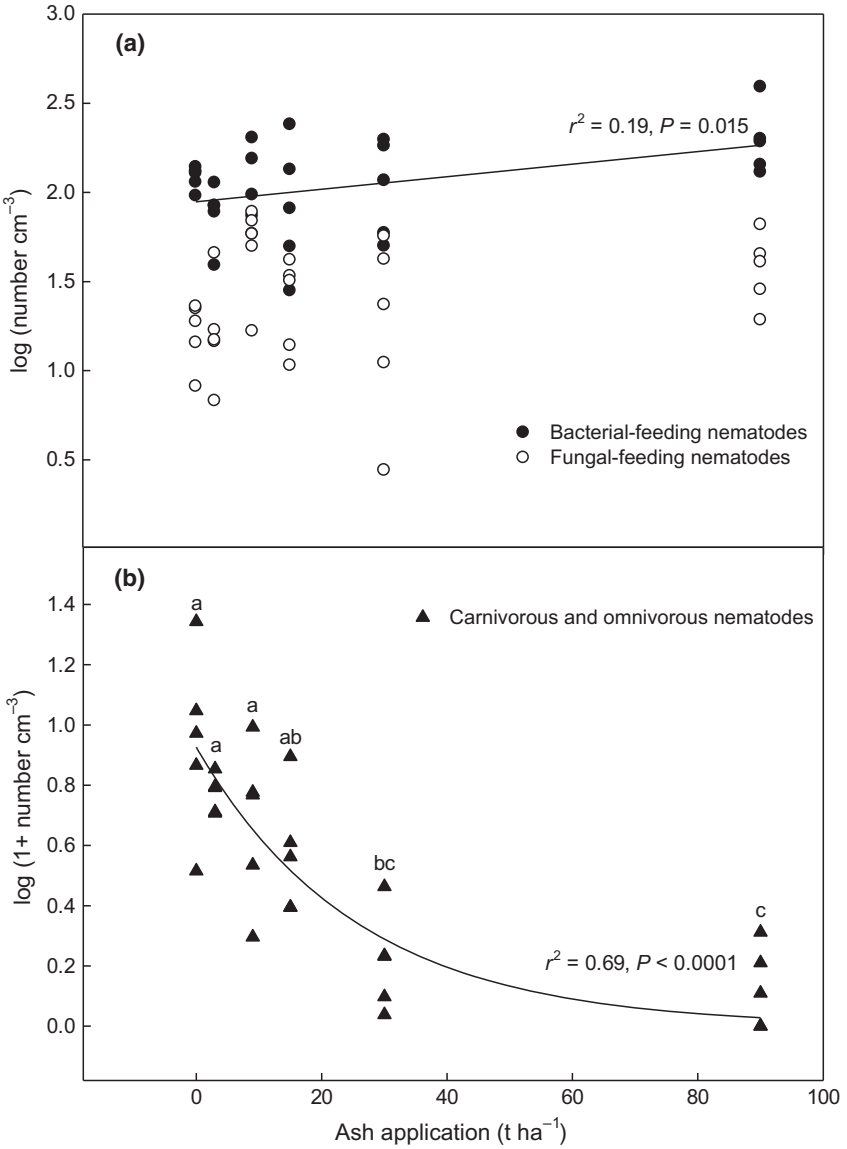


Fig. 5 Abundance (log number cm^{-3}) of (a) bacterial-feeding, fungal-feeding and (b) carnivorous/omnivorous nematodes in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). In (a), the solid line represents the fit to a linear regression model, where log(bacterial-feeding nematodes) = 1.9473 + 0.0035 × t ash ha^{-1}, in (b) the curve represents the fit to an exponential decay regression model, where log(1+carnivorous/omnivorous nematodes) = 0.9261 × $e^{-0.0388 \times t\ ash/ha}$ with r^2 and P values for the regression models. Ash doses with different letters are significantly different (Tukey $P < 0.05$).

Besides pH, the only variable that responded positively to ash application per se was the ciliates, and only carnivorous/omnivorous nematodes responded negatively to the direct effect of increasing ash doses.

Bacterial community structure

The first and second axes of the PCA explained 53.9% and 25.3% of the variance of the total variation in the high-resolution melting analysis, respectively (Fig. 7). The first axis was highly correlated with soil pH and clearly separated bacterial communities in control plots from bacterial communities in ash-treated plots and communities in plots exposed to 3 t ash ha^{-1} from communities in plots exposed to higher ash application doses (Fig. 7). Despite a tenfold increased ash application dose from 9 to 90 t ha^{-1}, there was no clear separation of bacterial communities subjected to ash levels exceeding 3 t ha^{-1}. The variation between bacterial communities within individual treatments was large for

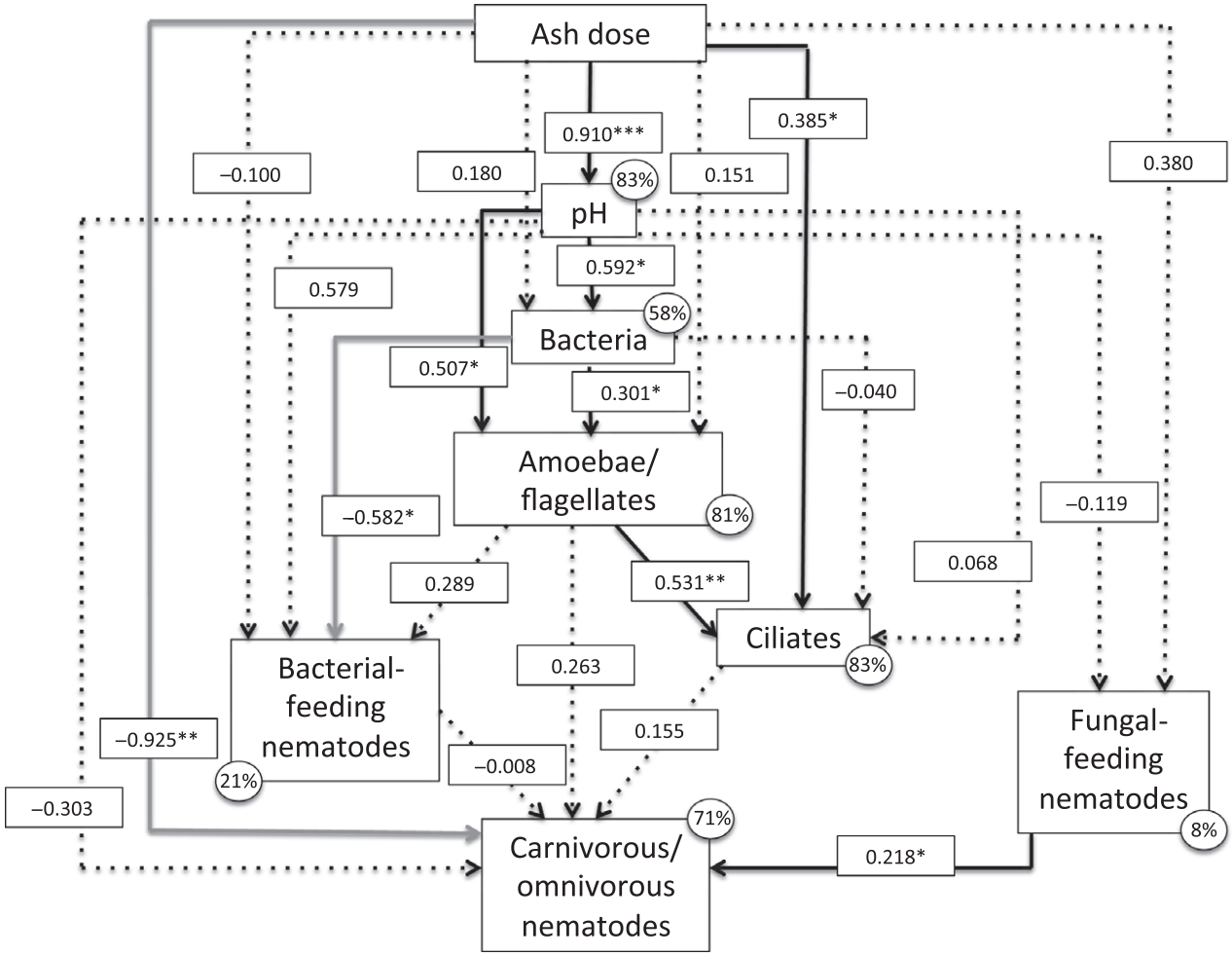


Fig. 6 Structural equation model of effects of ash application doses on soil pH, abundances of culturable heterotrophic bacteria, amoebae/flagellates, ciliates, bacterial-feeding, fungal-feeding and carnivorous/omnivorous nematodes in the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ash ha^{-1}). Numbers on arrows are standardized regression coefficients. Regression coefficients marked with asterisks are significant (***$P < 0.001$, **$0.001 \leq P < 0.01$, *$0.01 \leq P < 0.05$). Black, solid arrows indicate positive relationships, and grey arrows indicate negative relationships, dashed arrows indicate nonsignificant relationships. Percentages depict the variance explained by the model (R^2).

treatments above 3 t ha^{-1} compared with the control and 3 t ha^{-1} treatments.

Discussion

pH increase is the principal driver of microfood web responses to wood-ash addition

As hypothesized, the pH increase caused by ash application (Fig. 1) increased abundances of culturable heterotrophic bacteria and bacterivorous protozoa (Fig. 4). Even at the extremely high ash doses, the densities of culturable heterotrophic bacteria and their protozoan grazers remained at levels that were ca. 20 times higher than the densities in control plots. The increase is consistent with the increase in labile substrate (DOC and DON) for microbial growth from 0 to 30 t ash ha^{-1} (Fig. 2b,c) and confirms the correspondence between the DOC and microbial biomass response to ash addition seen in mesocosms (Pugliese *et al.*, 2014). In a microcosm experiment, the addition of high ash doses (40 t ash ha^{-1}) also enhanced the DOC and DON contents in the soil (Ludwig *et al.*, 2000). We note that microbial metabolites associated with increased microbial activity may also contribute to the increase of labile organic fractions (Hansen *et al.*, 2016). At 90 t ash ha^{-1}, we measured low DOC and DON contents in some of the plots. This reflects that the mineral soil contributed relatively more to the top 5 cm of the forest floor as a consequence of the reduced SOM content at 90 t ha^{-1} (Fig. 2a).

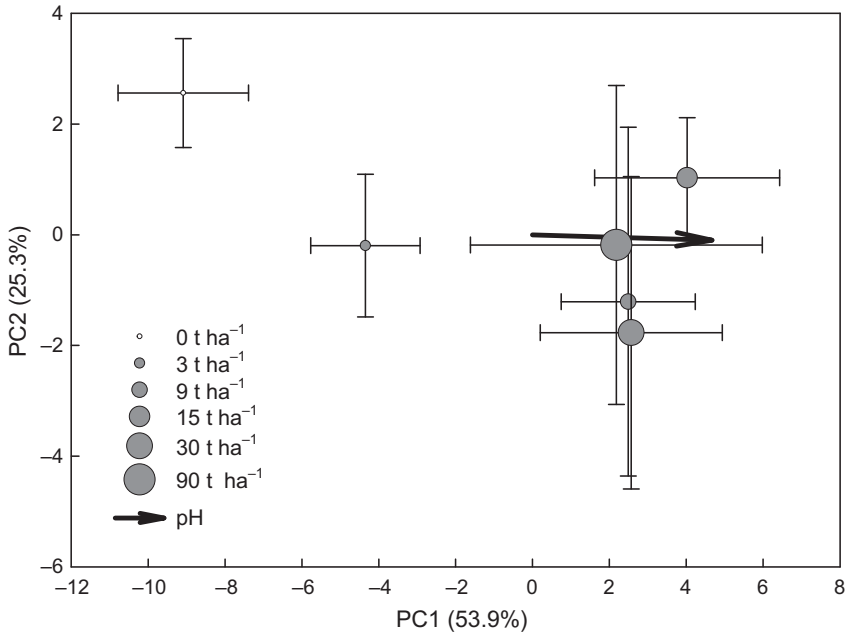


Fig. 7 Principal component analysis of bacterial community composition based on high-resolution melt curve analysis of bacterial DNA extracted from the top 5 cm of forest floor plots 1 year after the application of no wood ash (0) or increasing doses of wood ash (3, 9, 15, 30 or 90 t ha^{-1}). Plotted values are group mean ± SE. The first and second principal component axes represent 53.9% and 25.3% of the total variation, respectively.

Similarly to our findings, wood-ash application enhanced microbial activity and abundance in mesocosms with coniferous forest soil (Fritze *et al*., 2000) as well as bacterial-feeding nematode abundances (Nieminen, 2009). It is well-known that liming of acidic environments like the forest floor investigated here stimulates bacterial activity and production (Rousk *et al*., 2009, 2011; Cruz-Paredes *et al*., 2017), and Räty & Huhta (2003) found that a pH increase from 4.9 to 6.2 enhanced bacterial-feeding nematodes. The SEM analysis supported that the pH rise, and not the ash as such, was the main driver of the increased density of culturable heterotrophic bacteria (Fig. 6). The significant relationship between culturable heterotrophic bacteria and amoebae/flagellates and further between amoeba/flagellates and ciliates demonstrates that the positive effect of ash application on protozoa could be partly ascribed to the underlying pH driven increase of their prey.

Previously, it was assumed that declining densities of naked amoebae and flagellates at increased soil acidification were solely driven by the concomitant reduction of microbial prey availability (Ekelund & Rønn, 1994), but the SEM suggested that the reduced acidity in itself adds to the positive effect on amoebae/flagellates. Further, the SEM revealed a direct positive coupling between ash application doses and ciliate abundance. Ciliates are known to display much lower activity than expected considering the availability of food items ('ciliotstasis') (Foissner, 1987). This is probably caused by hitherto unknown chemical substances in the soil (Ekelund *et al*., 2002). Certain types of disturbances break ciliotstasis; for example drying-rewetting, and components in the ash may work similarly. We also note that the general food quality of available food items probably improved with the nutrients applied with the ash.

The abundance of bacterial-feeding nematodes varied considerably within the individual ash application levels, and even though there was an overall positive relationship between ash dose and bacterial-feeding nematode abundance (Fig. 5a), this was a modest increase compared to the protozoan response.

The enhanced energy flow through the bacterial-based decomposer channel of the microfood web was not transferred to the next trophic level, that is carnivorous/omnivorous nematodes. Their densities decreased with increasing ash application doses (Fig. 5b), and the SEM showed that the negative ash effect was not related to the liming effect, rather there was a direct negative relationship between ash doses and carnivorous/omnivorous nematodes (Fig. 6). Thus apparently, differential sensitivity to wood-ash application at different trophic levels decoupled higher-order trophic levels from the enhanced productivity at the first- and second-order level of the decomposer food web. As previous investigations did not detect any Cd effects on natural

nematode communities or typically disturbance-sensitive nematode taxa (Korthals *et al*., 1996; Bakonyi *et al*., 2003; Nagy *et al*., 2004), Cd added with the ash probably did not cause the decline of carnivorous/omnivorous nematodes. In our field experiment, ash application reduced and changed the species composition of the moss cover (Ethelberg-Findsen *et al*., in prep), and this overall habitat disturbance may explain the negative response of the most sensitive nematodes. Further, at reduced organic matter content (Fig. 2a), the average soil pore diameter declines, which can restrict the large-sized carnivorous/omnivorous nematodes from accessing prey residing in small pores spaces (Mikola & Setälä, 1998a). We speculate that the modest increase in bacterial-feeding nematode abundances with increasing ash doses may reflect reduced predatory control from the declining populations of carnivorous/omnivorous nematodes (Mikola & Setälä, 1998b). In general, populations of carnivorous/omnivorous nematodes increase slower than populations of typically more r-selected bacterial feeders (Bongers & Bongers, 1998). Here, we study responses to ash 1 year after application; the positive ash effect on abundances of lower trophic organisms may thus later manifest as enhanced carnivorous/omnivorous nematode densities or to some degree counteract the immediate negative effect on carnivorous/omnivorous nematodes.

Contrary to the bacterial-based compartment of the soil food web, ash application did not affect fungal-feeding nematode abundances (Fig. 5a). The abundance of bacterial- and fungal-feeding nematodes has been shown to correlate with the activity of their food sources, that is bacteria and fungi, respectively (Christensen *et al*., 2012). Our data thus support previous findings that fungal production responds very little, if at all, to rising pH (Rousk *et al*., 2009, 2011). Rousk *et al*. (2010b) stated that fungal diversity is largely unresponsive to soil pH changes, but there is also evidence that fungal diversity is highest in acidic soils, and soil pH is an important driver of fungal and particularly ectomycorrhizal fungal community composition (Tedersoo *et al*., 2014; Kjøller *et al*., 2017).

pH-related impacts on bacterial community structure

There is strong evidence that pH is the main driver of bacterial community composition at both continental scales (Fierer & Jackson, 2006) and local scales (Lanzén *et al*., 2015; Zhalnina *et al*., 2015), and our analysis confirms that the wood ash-induced community change strongly correlates with pH (Fig. 7). In our system, the largest bacterial community changes were associated with pH increases from 3.8 to 5.3, that is from 0 to 9 t ash ha^{-1}.

Within this range of ash doses and pH increase, our analyses thus confirm previous findings that modest ash application doses and the associated pH increase significantly alter the composition of microbial, especially bacterial, communities (Frostegård *et al*., 1993; Perkiömäki & Fritze, 2002; Noyce *et al*., 2016) resulting in community-wide tolerance to higher pH values (Cruz-Paredes *et al*., 2017). However, contrary to our expectation ash doses above 9 t ash ha^{-1} did not separate bacterial communities in a dose-dependent manner. The larger variation of bacterial community composition within treatments exposed to 9–90 t ash ha^{-1} suggests that stochastic processes to a larger extent governed the development of spatially separated bacterial communities exposed to extreme ash application doses.

The importance of pH as the driver of microbial community composition probably also explains why low wood-ash application rates of maximum 3.3 t ha^{-1} did not affect the microbial community composition in drained peatlands, where wood ash did not raise the pH (Björk *et al*., 2010).

Given that wood-ash application did not induce enhanced Cd tolerance in bacterial communities sampled at our field site (Cruz-Paredes *et al*., 2017), we consider it highly unlikely that the Cd content in the ash applied in our experiment (7.1 mg kg^{-1} ash) contributed to the changes in microbial community structure. Further, in another study, spiking wood ash with very high Cd levels (up to 1,000 mg kg^{-1} ash) did not in any way affect the microbial community composition (Fritze *et al*., 2000).

Implications of wood-ash application on inorganic nitrogen availability and soil C storage

As wood ash contains practically no nitrogen, its applicability as a fertilizer critically depends on its impact on inorganic nitrogen availability, especially in N-limited systems.

We found that application of 9 t ash ha^{-1} and above increased ammonium concentrations more than 10 times, and at 30 and 90 t ash ha^{-1}, mean nitrate concentrations were 18 and 41 times higher than in the control, respectively. This is in accordance with the concurrent increase of labile organic N (DON), readily available for microbial turnover (Kielland *et al*., 2007) and the pronounced stimulation of culturable heterotrophic bacteria and bacterial grazers that are responsible for soil nitrogen mineralization (Rønn *et al*., 2012). At our study site, vascular plants contribute very little to the forest floor vegetation, which is totally dominated by mosses. Ash application reduced the moss cover, and the increased inorganic N content with ash application could thus partly reflect reduced N uptake by mosses. However,

the 10-fold increase in ammonium was already apparent at 9 t ash ha^{-1}, whereas the moss cover did not decline before 15–90 t ash ha^{-1} (Ethelberg-Findsen *et al.*, in prep.), and we therefore consider increased mineralization the most likely cause of the increased ammonium concentration.

In Finnish and Swedish studies, 3–4 t ash ha^{-1} either had no or low, transient positive impact on soil ammonium concentrations (Jacobson *et al.*, 2004; Nieminen *et al.*, 2012). At one site, ammonium concentrations in the humus layer increased ca. 2 and 4 times at 6 and 9 t ash ha^{-1}, respectively, whereas at another site, ash did not affect ammonium concentrations (Jacobson *et al.*, 2004). We found no increase in the inorganic N availability at 3 t ash ha^{-1}. Taken together, these results suggest that consistent and lasting positive effects on ammonium availability require ash application doses above the currently allowed limits.

Whereas the NH_4^+-N pool increased dramatically already at 9 t ash ha^{-1}, the NO_3^--N pool increased more moderately and gradually. Although the traditional notion that nitrification is limited in acidic soils has been challenged (Booth *et al.*, 2005; Nicol *et al.*, 2008), our study shows that significant nitrate production is not evident before near-neutral pH is reached.

Our plots of 2 × 2 m are too small to relate the increased inorganic N availability to tree N uptake and growth, which is key to assessing the economic and environmental sustainability of ash application, but positive effects of ash application on tree production have been reported for sites on organic soils (Karltun *et al.*, 2008; Huotari *et al.*, 2015).

The main rationale for the use of wood as an energy source is the replacement of fossil fuel and the conversion of energy production towards systems where CO_2 uptake during biofuel production compensate for CO_2 emissions. However, if ash-induced mineralization of soil C exceeds the CO_2–C fixed during tree growth the climate change mitigating rationale may prove untenable. Therefore, on the one hand, the ash-induced increased activity of the bacterial-based decomposer food web jeopardizes the sustainability of biofuel production by increasing soil organic matter decomposition, but on the other hand, the concomitant N mineralization may enhance tree growth and thus CO_2 fixation. Further research is pertinent to clarify, if the ash-induced enhanced inorganic N content will positively impact tree growth and adequately compensate for ash-induced mineralization of soil organic C.

In conclusion, wood-ash application increased labile organic pools and the production of culturable heterotrophic bacteria in the top 5 cm of the forest floor with cascading effects on the bacterial-feeding trophic level. This response was governed by the liming effect of wood ash rather than by ash per se. In contrast, ash application per se, rather than the associated pH change, reduced abundances of carnivorous/omnivorous nematodes, confirming that nematode taxa at this trophic level are sensitive to environmental disturbances.

Further, wood-ash application and the associated pH increase changed the bacterial community structure, but the changes induced by the most extreme ash dose of 90 t ha^{-1} did not exceed the changes induced at 9 t ash ha^{-1}.

As expected, the stimulation of bacterial-based microfood web enhanced inorganic nitrogen availability. At extreme ash doses, soil organic matter decreased, but at 0–15 t ash ha^{-1} the soil organic matter pool was unaffected. Overall, the negative effects of ash application on abundances of soil biota were very limited, and the microfood web functioning proved robust to dosages exceeding currently allowed doses up to 30 times. Hence, we do not foresee negative implications for the ecosystem parameters investigated here, if legislative limits for ash application doses are raised to, for example, 6–9 t ash ha^{-1}.

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