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Ash application enhances decomposition of recalcitrant organic matter

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ABSTRACT

Harvesting whole-tree biomass for biofuel combustion intensifies removal of nutrients from the ecosystem. This can be partly abated by applying ash from the combustion back to the system, as the ash is rich in nutrients. Ash is very alkaline and ash application raises soil pH, which in turn can stimulate microbial activity and thus decomposition and mineralization. Our aim was to test if ash induced decomposition activity was associated with enhanced turnover of recalcitrant, i.e. relatively old, organic pools. Two experiments were conducted in the same coniferous plantation after the application of 0, 3, 4.5 and 6 t ash ha^{-1}, and 0, 3, 9, 15 and 30 t ash ha^{-1}, respectively. We used natural abundance of ^{15}N in mosses, mites and ectomycorrhizal fungi 26 months after ash application, as well as temporal variation in $\delta^{15}N$ values of ectomycorrhizal fungi, as an indicator of decomposition of recalcitrant organic matter in the first experiment. Furthermore, in the second experiment we used measurements of extracellular manganese peroxidase activity almost 4 years after ash application as an indication of potential decomposition of lignin, an important component of recalcitrant organic matter.

The $\delta^{15}N$ signature increased significantly for ectomycorrhizal fungi, dead moss, Nothroid and Gamasida mites, and manganese peroxidase activity tended to increase, with increasing ash doses. This suggests that ash application stimulates turnover of recalcitrant organic matter, which can increase the available pool of nitrogen in the system. This will potentially enhance the fertilizer value of ash. However, the $\delta^{15}N$ in ectomycorrhizal fungi tended to peak at 18 months after ash application, before decreasing, suggesting that the turnover of recalcitrant organic matter is reduced again with time.

1. Introduction

As a part of the European Council's strategy to reduce fossil fuel use, renewable energy sources, like wood chips, play an increasing role in the global energy production (European Commission, 2018). The increasing demand for renewable energy sources has shifted harvesting of biofuel from trunk only, towards whole-tree biomass removal. This has intensified nutrient removal from whole-tree harvested sites, which subsequently can reduce tree growth. Long-term sustainable forest management thus requires a similar input of nutrients (Helmisaari et al., 2014). Wood ash is a significant by-product from biofuel production, generating up to 1% ash per dry weight biomass (Pitman, 2006), and contains the mineral nutrient elements necessary for plant growth, except for nitrogen (N) which evaporates during combustion (Demeyer et al., 2001). Applying ash back to the biofuel plantation could thus close the bioeconomic circle by sustaining the soil nutrient status, as well as minimize combustion waste (Ingerslev et al., 2011). However, ash is very alkaline and significantly raises soil pH after application (Vestergård et al., 2018). Because of the limited N content, ash has only shown a fertilization effect on tree growth at sites receiving relatively high N deposition or sites rich in organic matter (OM) (Karltun et al., 2008; Huotari et al., 2015). Most of total N in boreal and temperate coniferous forests is bound in recalcitrant OM of the soil (Korhonen et al., 2013). The raised soil pH can increase microbial activity, which in turn increases decomposition and N mineralization (Genenger et al., 2003; Vestergård et al., 2018) potentially increasing N availability for plant uptake and growth. However, the question arises: What are the sources of the extra inorganic N flow; does the older, presumably more recalcitrant OM contribute?

Recalcitrant compounds in the soil can be identified by their ^{15}N-to-^{14}N ratio ($\delta^{15}N$). Isotopically heavy ^{15}N-bonds demand more energy to break than ^{14}N-bonds, and during decomposition of organic matter,

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^{14}N-bonds will therefore on average be mineralized slightly faster than ^{15}N-bonds (Robinson, 2001). Consequently, the remaining recalcitrant matter becomes ^{15}N enriched over time (Natelhoffer and Fry, 1988). Hence, decomposition of recalcitrant OM will elevate the δ^{15}N signature of inorganic N. Likewise, organisms that incorporate N derived from recalcitrant OM will attain elevated δ^{15}N signatures in their biomass N (Melody and Schmidt, 2012). Lignin-derived humus compounds and humic acids are a significant part of forest recalcitrant OM (Dungait et al., 2012). Degradation of lignin-derived recalcitrant OM requires oxidative reactions by a complex set of ligninolytic enzymes, of these, manganese peroxidase (MnP) plays a crucial role (Hofrichter, 2002; Steffen et al., 2002; Sinsabaugh, 2010). MnP is suggested to play an initial role in lignin degradation (Perez and Jeffries, 1992; Tuomela et al., 2002). For various litter decomposing basidiomycete fungi, MnP is the most important ligninolytic enzyme (Hofrichter, 2002). Therefore, MnP measurements can be used to indicate the decomposition of recalcitrant organic matter.

We hypothesized that ash application increases mineralization of recalcitrant OM. We tested this by two complementary methods: 1) Assessment of natural abundance of ^{15}N in moss, mites and a group of primary decomposers, ectomycorrhizal fungi; 2) Extracellular MnP activity in soil as a proxy for the decomposition of lignin and lignin-derived compounds.

2. Materials and methods

2.1. Field site description

The field site is a coniferous biofuel plantation, Gedhus plantation, located in central Jutland, Denmark (56 16.3922N; 9 5.672E) 51 m above sea level. The mean precipitation is 850 mm year^{-1} (Wang, 2013). The soil type is Albic Podzol (FAO, 2014) with the horizons O (6–0 cm), A (0–10 cm), E (10–20 cm), Bh (20–30 cm), Bs (30–45 cm) and C (45- cm) developed on glacio-fluvial sand (Hansen et al., 2018). The O horizon consists of 86.9% organic matter (OM), with a C/N ratio of 29.9, and the A horizon consists of 18.4% OM, with a C/N ratio of 37. Further details on texture, density and chemical composition can be found in Hansen et al. (2018).

When the experiment was established, the plantation consisted of a 2nd generation, 57-year-old, Norway spruce (*Picea abies* (L.) Karst.) stand, planted on previous heathland. Density of the stand was 457 trees ha^{-1}. One growing season after the start of the experiment, the average tree height was 18.8 m and the diameter at breast height 25.3 cm. The ground vegetation is dominated by mosses, in particular heath plait-moss (*Hypnum jutlandicum* Holmen & Warncke), broom forkmoss (*Dicranum scoparium,* Hedw.) and red-stemmed feather moss (*Pleurozium schreberi* (Willd. ex Brid.) Mitt), with only very few liverworts and the vascular plants lingonberry (*Vaccinium vitis-idaea* L.) and wavy hair-grass (*Deschampsia flexuosa* (L.) Trin.).

The plantation had not been fertilised before this study commenced and the individual plots were homogeneous with respect to stand data.

2.2. Ash application

The ash used for the experiment originated from incinerated coniferous wood chips. It was a mixed ash of bottom and fly ash from the district heating plant in Brande, Denmark, see Table 1 (Maresca et al., 2017). Further data on the ash can be found in Maresca et al. (2017), where this ash is mentioned as MA-9c ash. The ash had not undergone pre-treatments before spreading, as it already met the requirements set by the Danish authorities for the utilisation of wood ashes for fertilization purposes in forest ecosystems (Danish Ministry of the Environment, 2008). Three composite replicates of 1 g homogenized and sieved ash was transferred to 5 mm × 9 mm tin capsules (E12007, EuroVector s.p.a. Italy) before isotope ratio mass spectrometry with a EuroVector EA (Redevalle, Italy). The mean δ^{15}N of the ash was estimated to −3.5‰ (STD 2.8).

Table 1
Physical and chemical composition of the ash used in the experiment (Maresca et al., 2017).

Ash type	Particle size < 1 mm	pH	Total organic carbon	Carbon content	Nitrogen content	δ^{15}N
Mixed	70%	12.7	5.84%	6.64%	0.7 g kg^{-1}	−3.5‰

At the start of the experiments, the ash was manually spread evenly over the soil surface, in a single application, with ash doses from 3 to 30 t ash ha^{-1}, corresponding to 0.3–3 kg ash m^{-2}.

2.3. Experimental set-ups

In April 2014, we established two field experiments in Gedhus Plantation, hereafter named “Realistic experiment” and “Extreme experiment”. In both experiments, experimental plots were distributed in a randomised block design, with either three (Realistic experiment) or five blocks (Extreme experiment), each including all treatments. The two experiments were set up in close proximity with a distance of 10 m between the two experimental sites.

In the Realistic experiment we applied ash amounts equivalent to or up to twice the dose that is allowed according to current Danish legislation i.e. four different treatments: 0, 3, 4.5 and 6 t ash ha^{-1}. Treatments were replicated three times, adding to 12 plots. Each plot measured 20 m × 25 m and included 24–39 trees.

In the Extreme experiment we applied ash doses far above the allowed threshold, which could provide the basis for predicting a critical limit for ash application, i.e. five different treatments: 0, 3, 9, 15 and 30 t ash ha^{-1}. Treatments were replicated five times, adding to 25 plots. Each plot measured 2 m × 2 m. Due to the small size the extreme plots were positioned between the tree trunks, in order to allow for sufficient material for soil sampling.

2.4. δ^{15}N analysis of moss and mites

In September 2016, 26 months after ash application, we sampled the Realistic experiment to test for ^{15}N enrichment in the system. We chose the most abundant plant and microarthropods at the site, moss and mites, respectively.

Moss samples were collected by hand. The moss layer (depth ranging from 4 cm to > 6 cm) was separated from the soil. Heath plait-moss dominated the moss cover. Moss was divided into two categories: living moss (green) and dead moss (brown, but still recognizable to genus level). Three moss samples taken evenly distributed in each plot were pooled and 1 g randomly selected taken out. The moss was then dried overnight and ground with a mixer mill MM 200 (Retsch, Haan, Germany). Approximately 1 mg ground moss biomass was transferred to 5 mm × 9 mm tin capsules (E12007, EuroVector s.p.a. Italy) for stable isotope ratio mass spectrometry (IRMS).

For mite extractions we collected three soil cores (diameter: 5.5 cm, depth: 0–6 cm including vegetation) from each plot. Mites were extracted with a Tullgren funnel (Macfadyen, 1953) for 8 days and collected in 20% NaCl. Samples were stored in 20% NaCl solution at −18 °C until identification. Samples did not solidify, due to the high salt content. With the aid of a dissection microscope, the mites were separated into four groups: Oribatida, Nothroids, Phthiracaridae and Gamasida. The four groups represent at least two feeding strategies, fungivores/detrivores (Oribatida, Nothroids and Phthiracaridae) and carnivores (Gamasida), covering several trophic levels (Díaz-Aguilar and Quideau, 2013). To rinse off any salt that might interfere with the sample dry weight, the mites were rinsed three times in milliQ water. The mites were then transferred to 8 mm × 5 mm ultra-clean tin

capsules (D1035, Elemental Microanalysis, Devon, UK) for IRMS. If necessary, mites collected within a plot were pooled, in order to achieve a minimum of 10 μg N per sample required for analysis (≈0.07 mg DW mite, depending on the group). The preferred range for conventional IRMS was 50–100 μg N per sample (0.4–1.0 mg DW mite depending on the group). However, some mite groups did not reach this weight criterion.

Moss and mite samples were sent for analysis at KOSI, Center for Stable Isotope Research and Analysis, Göttingen, Germany. In order to analyse the small mite biomass samples, μEA-IRMS measurements of isotope ratios were made by high-temperature dry combustion using a modified elemental analyzer (Eurovector, Milano, Italy) coupled to an isotope ratio mass spectrometer (Thermo Delta Vplus; Thermo Scientific, Bremen, Germany) as described by Langel and Dyckmans (2014).

2.5. Time series $\delta^{15}N$ analysis of ectomycorrhizal fungi

For ectomycorrhizal fungal (EMF) sampling, cylindrical fungal in-growth mesh bags were constructed of polyamide mesh (Sintab, Oxie, Sweden) with a mesh size of 50 μm and filled with 2 g of ion-exchange resin beads (Amberlite MB6113 mixed resin, BDH Laboratory Supplies, Poole, UK) covered with 50 g quartz river sand (0.7–1.2 mm) (Cruz-Paredes et al., 2019). The mesh size of 50 μm allows hyphal in-growth but prevents roots from entering (Wallander et al., 2001). The first set of in-growth mesh bags was installed December 2, 2013, four months before ash addition. A 2-cm soil corer was gently pushed down to 13 cm depth while taking care not to damage the moss layer. The soil was taken out and the mesh bag was installed in the hole: i.e. the mesh bags spanned the entire organic horizon and penetrated into the top mineral horizon with the top of the bags just visible above the moss. Mesh bags were collected and replaced with identical ones every six months for two and a half years (Cruz-Paredes et al., 2019).

To extract the mycelia, the outside of the mesh bags was first cleaned to avoid contamination with organic matter, and then the bags were carefully opened. The colonized sand was mixed with 1 l of water and shaken to separate the mycelia from the sand. The extracted mycelia were freeze dried, weighed, and used to calculate the biomass and to analyse $\delta^{15}N$. The weights were corrected for possible contamination of sand particles setting the C content of EMF to 40% (Clemmensen et al., 2006). From each sample, between 0.5 and 10 mg of dried mycelia were transferred to 5 mm × 9 mm tin capsules (E12007, EuroVector s.p.a. Italy) for measuring $\delta^{15}N$ using isotope ratio mass spectrometry with a EuroVector EA (Redevalle, Italy) coupled to an Isoprime IRMS (Cheadle Hulme, UK).

2.6. Manganese peroxidase activity in soil

In January 2018, almost four years after ash application we sampled soil in the Extreme experiment to test for increased lignin degradation potential by measuring manganese peroxidase (MnP) activity. MnP activity was assessed in the Extreme experiment, in order to study the enzyme response over a pH gradient that included the pH optimum of the enzyme. This was not possible in the Realistic experiment with a maximum ash dose of 6 t ash ha^{-1}.

Three soil cores (diameter: 2 cm, depth: 0–5 cm including vegetation) were collected evenly in each plot, and pooled together to obtain a homogenous composite sample. We stored the samples at −20 °C until analysis, which has very limited influence on results of enzymatic assays (Wallenius et al., 2010; Peoples and Koide, 2012; Hewins et al., 2016).

For analysis, samples were thawed and 0.5 g soil from each sample was homogenized with 5 ml cold 50 mM acetate buffer for 1 min at 10000 rpm with a Sorvall mixer. Further 45 ml acetate buffer was added before centrifugation (10 min, 4300 rpm). A boiled aliquot of the supernatant served as a negative control. Extractable MnP activity was measured via the oxidative coupling of DMAB (3-dimethylaminobenzoic acid) and MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) in the presence of Mn^{2+} and H_2O_2 (Bödeker et al., 2014). Samples of 50 μl extract were mixed with 140 μl reaction solution (100 mM sodium lactate and 100 mM sodium succinate, adjusted to pH 4.5 with glacial acetic acid, 50 mM DMAB, 1 mM MBTH, 1 mM $MnSO_4$–$4H_2O$ solution) in a flat-bottomed, transparent microtiter-plate. We then added 10 μl of 5 mM H_2O_2 and estimated background peroxidase activity by substituting $MnSO_4$ with 2 mM Na_2-EDTA·$2H_2O$. No standard was added and MnP activity results are thus relative.

Absorption at 590 nm was measured every 3 min over 1 h at 26 °C in a micro-plate reader (BIO-TEK ELx 808).

2.7. Data analysis

All $\delta^{15}N$ and MnP activity data were log-transformed prior to statistical analysis to attain homoscedasticity.

For moss and mites, we used ANCOVA to test if ash had an overall effect on $\delta^{15}N$ signatures, and if there was a difference in $\delta^{15}N$ signatures between groups. Here, taxonomic groups were a factor, ash was the predictor co-variable and $\delta^{15}N$ of the individual taxonomic groups were the response variable. In order to test the ash effect on the $\delta^{15}N$ signature of individual taxonomic groups, we used linear regression, where ash was the predictor variable and $\delta^{15}N$ signature of the specific taxonomic group was the response variable.

In order to test the effect of time on EMF $\delta^{15}N$ signature within the individual ash treatments, we used regression analysis, where time was the predictor variable and $\delta^{15}N$ signature of EMF the response variable. To test if ash and/or time had an overall effect on $\delta^{15}N$ signatures in EMF we used ANCOVA. Time was a factor, ash was the predictor co-variable and log transformed $\delta^{15}N$ the response variable. Due to the quadratic equation of the regression, shown by the individual regression analysis, the predictor variable, time, was squared for the ANCOVA.

Relative MnP activity is measured as absorbance increase h^{-1} g^{-1} soil DW, where the ash mass has been subtracted from the soil DW. Based on a biological assumption that enzyme activity decreases at extreme treatments (e.g. very high pH), we used a polynomial, quadratic regression model on the data. Ash dose was the predictor variable and MnP activity the response variable.

We obtained R^2 for model fits and set significance level to $P = 0.05$. Statistical analyses were performed in SigmaPlot 13.0.

3. Results

3.1. $\delta^{15}N$ signatures of moss, mites and ectomycorrhizal fungi 26 months after ash application

Increasing ash doses significantly increased $\delta^{15}N$ in the moss, mites and ectomycorrhizal fungi 26 months after ash application (One-Way ANCOVA, $Covariate_{Ash\ treatment}$ $P < 0.001$) (Fig. 1, Table 2). The taxonomic groups were significantly different from each other (factor $_{Taxonomic\ groups}$ $P < 0.001$), and there was an interaction between ash treatment and taxonomic groups ($interaction_{Ash\ treatment \times\ Taxonomic\ groups}$ $P = 0.01$).

Linear regression analysis of the individual taxonomic groups showed that $\delta^{15}N$ increased significantly with increasing ash doses for four of the groups: Ectomycorrhizal fungi ($P = 0.04$), dead moss ($P = 0.02$), Nothroid mites ($P = 0.002$) and Gamasida mites ($P = 0.02$). Live moss showed a trend ($P = 0.06$) and Phthiracaridae and Oribatida mites did not increase significantly ($P = 0.28$ and $P = 0.11$, respectively). (Fig. 1, Table 2).

3.2. Time series $\delta^{15}N$ signatures of ectomycorrhizal fungi

Increasing ash doses significantly increased $\delta^{15}N$ in EMF (One-Way

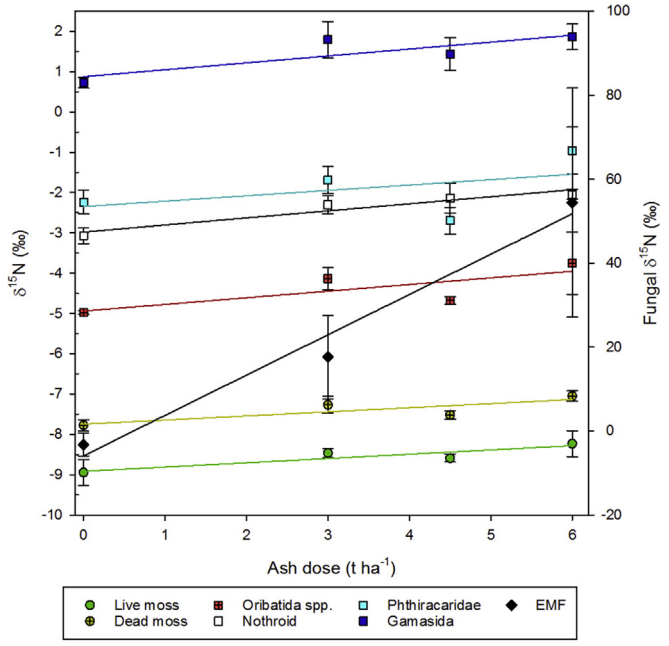

Fig. 1. $\delta^{15}N$ signatures in moss, mites and EMF (ectomycorrhizal fungi) after ash application. Mean values (± SE) of $\delta^{15}N$ (‰) for six soil food web groups of a spruce forest, as a response to increasing ash doses (t ha^{-1}). Circles (•) are plant material: Living moss (green) and dead moss (yellow), squares (■) are animals: Oribatida mites (red), Nothroid mites (white), Phthiracaridae mites (turquoise), Gamasida mites (blue), diamonds (♦) are ectomycorrhizal fungi. See Table 2 for linear regression coefficients, R^2 and P-values. One-Way ANCOVA results: $Covariate_{Ash\ treatment}$ $P < 0.001$, factor $_{Taxonomic\ groups}$ $P < 0.001$, $interaction_{Ash\ treatment \times\ Taxonomic\ groups}$ $P = 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ANCOVA, $Covariate_{Ash\ treatment}$ $P = 0.004$). Time was not significant in the model ($factor_{Time}$ $P = 0.26$), but there was a trend for an interaction between ash treatment and time ($interaction_{Ash\ treatment \times Time}$ $P = 0.06$).

Regression analysis of the individual ash treatments showed different effects of ash over time (Fig. 2). With time, EMF $\delta^{15}N$ declined in 0 t ash ha^{-1} (linear regression, $P = 0.03$, $R^2 = 0.33$) and remained unaltered in 3 t ash ha^{-1} (polynomial, quadratic regression, $P = 0.56$, $R^2 = 0.1$). At 6 t ash ha^{-1}, EMF $\delta^{15}N$ tended to increase during the first 18 months after ash application, and thereafter it started to decrease (polynomial, quadratic regression $P = 0.07$, $R^2 = 0.36$).

3.3. Manganese peroxidase activity in soil

MnP activity tended to increase from 0 to 14 t ash ha^{-1} (polynomial, quadratic regression $P = 0.078$, $R^2 = 0.21$). At higher ash doses, the MnP activity gradually declined (Fig. 3).

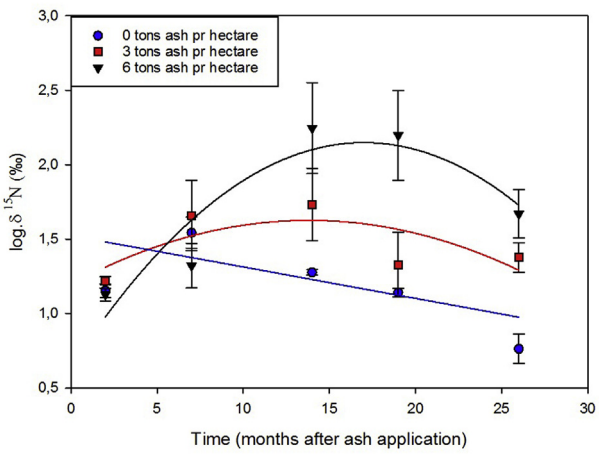

Fig. 2. Ectomycorrhizal fungi $\delta^{15}N$ response over time to ash application. Mean values (± SE) of $\delta^{15}N$ (‰) in ectomycorrhizal fungal hyphae for three ash treatments in a spruce forest, as a response to time (months after ash application). Data is fitted for 0 t ash ha^{-1} with linear regression, log ($\delta^{15}N$) = −0.0206*time + 1.455, $P = 0.03$, $R^2 = 0.33$; for 3 t ash ha^{-1} with polynomial, quadratic regression, log($\delta^{15}N$) = −0.0025 $time^2$ + 0.0703 time + 1.167, $P = 0.56$, $R^2 = 0.1$; for 6 t ash ha^{-1} with polynomial, quadratic regression, log($\delta^{15}N$) = −0.0052 $time^2$ + 0.177 time + 0.644, $P = 0.07$, $R^2 = 0.36$.

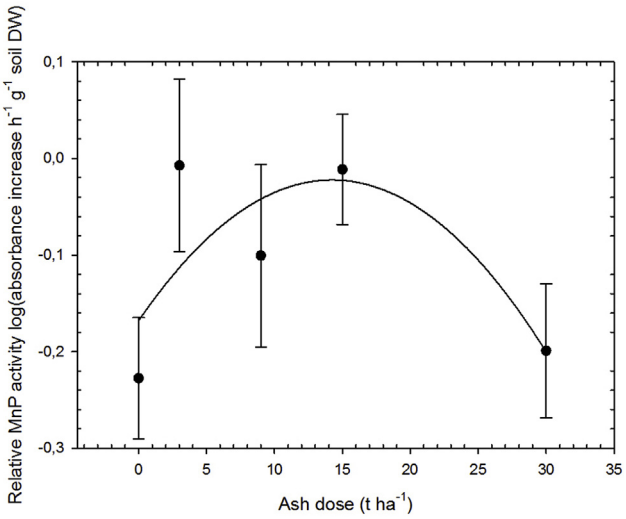

Fig. 3. Relative extracellular manganese peroxidase activity in soil after ash application. Mean log(values) (± SE) for extracellular manganese peroxidase activity in spruce forest soil at increasing ash doses (t ha^{-1}). Data is fitted with polynomial, quadratic regression, log(MnP activity) = −0.0007 t ash per ha ^{2} + 0.0177 t ash per ha - 0.168, $P = 0.078$, $R^2 = 0.21$.

Table 2
Linear regression coefficients, R^2, P-values, degrees of freedom (DF) and F-values for $\delta^{15}N$ as an effect of increasing ash dose.

Food web group	Group	Equation	R^2	P	DF	F
Microorganisms	Ectomycorrhizal fungi	$\delta^{15}N = 9.61$ t ash ha^{-1}-5.91	0.98	0.04	1	6.7
Plants	Live moss	$\delta^{15}N = 0.11$ t ash ha^{-1}-8.92	0.83	0.06	1	4.5
	Dead moss	$\delta^{15}N = 0.10$ t ash ha^{-1}-7.75	0.69	0.02	1	7.5
Animals	Oribatida mites	$\delta^{15}N = 0.17$ t ash ha^{-1}-4.98	0.6	0.11	1	3.1
	Nothroid mites	$\delta^{15}N = 0.18$ t ash ha^{-1}-2.98	0.93	0.002	1	10.4
	Phthiracaridae mites	$\delta^{15}N = 0.13$ t ash ha^{-1}-2.35	0.21	0.28	1	1.3
	Gamasida mites	$\delta^{15}N = 0.17$ t ash ha^{-1} + 0.84	0.71	0.02	1	5.8

4. Discussion

4.1. $\delta^{15}N$ signatures in moss, mites and ectomycorrhizal fungi

We found that mosses, mites and EMF became significantly ^{15}N enriched, with increasing ash doses. This suggests that N derived from recalcitrant OM, which is generally ^{15}N enriched (Natelhoffer and Fry, 1988) contributed relatively more to the biomass in ash amended plots than in unamended plots. In other words, our results indicate that ash enhances the decomposition of recalcitrant OM and the subsequent incorporation of N derived from recalcitrant OM into forest soil organisms.

Furthermore, we found that EMF responded more to ash than the other taxonomic groups. The $\delta^{15}N$ value increased with 9.61‰ per t ash ha^{-1} for EMF, 26 months after ash application, compared to less than 1‰ per t ash ha^{-1} for the other groups. This markedly elevated $\delta^{15}N$ in EMF compared to the other groups may have two explanations. First, EMF are generally ^{15}N enriched, because ^{15}N is retained during transfer of nutrients to the associated host plant (Hobbie and Ouimette, 2009). Second, because EMF may possess the enzyme matrix needed for direct decomposition of organic compounds and recalcitrant OM (Bödeker et al., 2014; Lindahl and Tunlid, 2015), they probably base a larger proportion of their biomass production on these ^{15}N enriched sources than the mosses and soil animals.

Although EMF generally are ^{15}N enriched, the value we observe after ash application (54.4‰, SE ± 15.8 at 26 months, 6 t ash ha^{-1}), is very high compared to other studies (e.g. 2.2–10.0‰ in Hobbie and Ouimette, 2009). An explanation for the high $\delta^{15}N$ level could lie in the type of ecosystem that we studied. The field site was an acidic, nutrient depleted coniferous plantation. Under these conditions, most plant N is probably derived from EMF, rather than direct plant uptake (Hobbie and Ouimette, 2009). Also, soil N limitations have shown to promote decomposition by fungi (Thevenot et al., 2010). As such, the EMF in the system must be capable of mineralizing OM efficiently, thus facilitating a rapid increase in mineralization and ^{15}N enrichment. Another possible explanation that potentially could add to the high ^{15}N levels is the increased competition between bacteria and fungi after ash application. By adding ash to this system the bacterial activity is stimulated, increasing decomposition of labile components (Cruz-Paredes et al., 2017b). Perhaps the increased bacterial activity could stimulate fungi to decompose recalcitrant OM, rather than more labile compounds. However, the high ^{15}N level in EMF could also be a product of nitrification, as ash has shown to stimulate nitrification activity (Vestergård et al., 2018). Ash increases soil pH, and generally, nitrification rates increase with elevated pH. The isotopic fractionation between ammonium and nitrate is 15–35‰ (Robinson, 2001), thus during nitrification the remaining ammonium is enriched by 15–35‰ ^{15}N. The elevated $\delta^{15}N$ in EMF could reflect EMF uptake of ^{15}N-enriched ammonium rather than an increased decomposition of recalcitrant organic matter. At pH 5.5, the highest levels reached in the Realistic experiment (Mortensen et al. in prep), nitrification rates are expected to be low (Ste-Marie and Paré, 1999). However, EMF's also take up nitrate (Nehls and Plassard, 2018); hence, the EMF uptake of ^{15}N-enriched ammonium would to some extent be counteracted by uptake of ^{15}N-depleted nitrate. As nitrification rates have not been investigated in this experiment, further studies are needed to confirm this notion.

Ash can also change fungal community structure (Kjøller et al., 2017; Taylor and Finlay, 2003), and EMF species are known to vary in N acquisition strategies (Hobbie and Agerer, 2010), which in turn could account for some of our observed differences in ^{15}N-enrichment. However, at our Realistic experimental site, ash did not change EMF community structure (Cruz-Paredes et al., 2019), and as such we find this explanation unlikely. Yet, bacterial community composition in soil from our experimental site has shown to change with increasing ash application (Bang-Andreasen et al., 2017). Thus, the influence of microbial community on $\delta^{15}N$ signatures needs further studies to be clarified.

The $\delta^{15}N$ value in EMF significantly increased with ash dose. At 6 t ash ha^{-1}, $\delta^{15}N$ peaked at 18 months and declined at later time points. This bell shaped temporal development of $\delta^{15}N$ suggests that the increased decomposition of recalcitrant OM is a transient phenomenon – an instant response to ash application, which decreases over time. This correlates well with pH over time, which for 6 t ash ha^{-1} peaked at 16 months (Mortensen et al. in prep). Elevated soil pH can increase microbial activity and thus decomposition and N mineralization (Genenger et al., 2003; Vestergård et al., 2018). In turn it is reasonable to assume that decreasing pH will decrease microbial activity. Thus, the increased microbial activity is likely to decrease over time (Cruz-Paredes et al., 2017b).

4.2. Manganese peroxidase activity in soil

Contrary to the $\delta^{15}N$ signatures, the MnP activity was measured in the Extreme experiment to include the pH optimum of the enzyme. On the other hand, due to the small plot size, it was not possible to sample for microarthropods in the Extreme experiment. However, as the two experiments were situated close to each other within the same forest, we are confident that the MnP results are also representative for the MnP response to ash application in the Realistic experiment.

The ash effect was highly variable, but tended to increase MnP activity up 14 t ash ha^{-1}, and reduced it at higher doses.

Because MnP is an important enzyme in degradation of lignin-derived humus compounds and humic acids (Steffen et al., 2002; Sinsabaugh, 2010), a higher MnP activity increases the degradation of recalcitrant OM (Fujii et al., 2013). The response of the MnP activity to ash application is likely explained by the microbial activity response to the pH change. pH in the extreme plots increased from 3.8 in the control treatment to c. 6 at 15 t ash ha^{-1} and 7 at 30 t ash ha^{-1} (Vestergård et al., 2018). MnP is produced by basidiomycete fungi (Hofrichter, 2002), and at our study site fungal growth peaks at 15 t ash ha^{-1} and then declines at higher dosages (Cruz-Paredes et al., 2017b). This fits very well with the course of the MnP response to ash application.

The pH increase with ash application could directly influence extracellular MnP activity in the field. In pure culture, the optimal range for MnP activity was between pH 4.5–5, and the enzyme was active from pH 3.0–6.0 (Rüttimann-Johnson et al., 1994). As mentioned, the pH in the Extreme experiment increased from 3.8 in the control to 7 at 30 t ash ha^{-1} (Vestergård et al., 2018). We assessed MnP activity in a pH buffered assay, hence measured differences in enzyme activity likely reflects differences in enzyme concentration in the soil.

Other factors in the ash, such as the Mn content, may also directly influence extracellular MnP activity (Perez and Jeffries, 1990, 1992). The ash used for the experiment contained 7.43 g Mn kg^{-1} ash (Maresca et al., 2017), a substantial Mn addition, considering that the Mn concentration was 0.005 ppm in the top 15 cm soil of the control treatment (Hansen et al., 2018). However, Mn concentration increases linearly with ash dose, which is not the case for MnP activity. As such, Mn addition probably only stimulate MnP activity, as long as microbial activity is not hampered by soil acidity.

Previously, only certain basidiomycete fungi, i.e. white-rot fungi, were known to produce MnP (Hofrichter, 2002), but several studies have now shown MnP activity in a phylogenetically wide range of EMF (Bödeker et al., 2009, 2014). EMF is especially important for nutrient depleted coniferous forests, where the EM fungi-plant association supports plant nutrient uptake (Courty et al., 2010). Ash amendment increases mycorrhizal biomass (Hagerberg and Wallander, 2002; Majdi et al., 2008; Cruz-Paredes et al., 2017a), which correlates well with our observed MnP activity. However, more studies are needed to confirm the degree of MnP productivity in the EMF species present in the system.

4.3. Ash induction of recalcitrant OM decomposition

Both organismal $\delta^{15}N$ signatures and soil MnP activity increased with increasing ash application, which indicates that ash stimulates the turnover of recalcitrant OM and increases the mineralization or organismal uptake of N otherwise bound in the organic matrix. Because ash contains negligible amounts of N, the full fertilization effect of ash is dependent on available N in the system. Hence, our results suggest that ash induces the mobilization of and plant access to recalcitrant N, potentially increasing tree productivity. Our results align with Vestergård et al. (2018), who, in the same field experiment, found that ash increased soil ammonium concentrations almost 10 times the control at 9 t ash ha^{-1}.

The use of wood ash to abate nutrient depletion in biofuel plantations can close the bioeconomic circle, e.g. removing 200 t ha^{-1} dry weight wood biomass, would generate up to 2 t ash via combustion (Pitman, 2006). The application of 2 t ash ha^{-1} could counteract app. 48–68% of the exported nutrients (Ingerslev et al., 2011). Recycling could thus be a significant route for disposing off wood ash. However, the ash-induced mineralization may also undermine the sustainability of using wood as an energy source. The ash-induced decomposition of recalcitrant soil OM will emit CO_2. The balance between primary production and respiration of decomposer organisms determines whether the forest system acts as a net C sink or net CO_2 emitter to the atmosphere (Dungait et al., 2012). If this balance shifts towards stimulation of decomposition it can result in an increase of atmospheric CO_2. On the other hand, by fertilizing a nutrient depleted forest, ash may shift the C-balance towards primary production and subsequently increase C sequestration in the soil. Ash effects on tree productivity vary considerably with location and time since fertilization (Moilanen et al., 2013; Saarsalmi et al., 2014). However, generally there is a positive effect on tree biomass, when ash is applied together with N or on soil with high organic N content. The impact of ash application on the balance between CO_2 sequestration and emission from the system is still to be determined at our field site, but the CO_2 emission of the soil does not increase up to 4 years after ash application (Christensen et al., in prep). Thus, the increased mineralization seems to be a minor part of the total respiration in the system. Furthermore, it seems to be a temporary response to ash application; hence, for EMF, where we see the strongest ash-induced $\delta^{15}N$ increase, the ^{15}N enrichment peaks and levels off 18 months after ash application.

5. Conclusion

We found that moss and soil food web $\delta^{15}N$ signatures increased with increasing ash application and there was a similar tendency for manganese peroxidase activity. This suggests that ash application stimulates turnover of recalcitrant organic matter, which can contribute to the available pool of N in the system. This will potentially enhance the fertilizer value of ash.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.05.021.

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