THE EFFECT OF TREE MORTALITY ON BIOGEOCHEMICAL RESPONSE AFTER MOUNTAIN PINE BEETLE FOREST INFESTATION

by

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ABSTRACT

Pine species in the American West are experiencing large-scale, bark beetle-induced mortality in association with a changing climate. As resulting terrestrial biogeochemical responses to this ecosystem disturbance are unclear, we hypothesized that a threshold of localized tree mortality must be exceeded before disruption of carbon and nitrogen biogeochemical cycling is observed. In order to isolate the compensatory effects of proximal healthy trees and intertwined hydrologic, energetic and rhizospheric processes, microbiological and geochemical parameters within three near-surface soil horizons were contrasted between healthy and deceased lodgepole pines (Pinus contorta) surrounded by varying extents of tree mortality.

Bark beetle impact altered N cycling, as an increased proportion of the total N pool was inorganic ammonium, as opposed to more stable and less mobile organic N, which dominates in healthy systems. This change in N cycling was dependent on the extent of surrounding tree mortality in the upper soil horizons, but not in the mineral soil. The different response in the upper and lower soil horizons is likely a reflection of how altered inputs after bark beetle infestation are different in each horizon. A threshold response in the upper horizons was found for ammonium, which accumulated only under trees surrounded by at least 40% tree mortality. The elevated ammonium response was associated with increased C recalcitrance and increased relative abundance of N. Concurrently, surrounding tree mortality also affected the soil bacterial community structure with associated increases in alpha diversity and overall community
structure. However, functional processes tended to correlate better with changes in C:N ratio. Collectively, these biogeochemical and microbial indicators suggest that high degrees of beetle-induced mortality shift the terrestrial environment of Colorado Rocky Mountain lodgepole pine forests from an N-limited ecosystem to one where N is in excess.
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LIST OF ACRONYMS

OTU ................................................................. Operational Taxonomic Unit
NUE ................................................................. Nitrogen Use Efficiency
C ................................................................. Carbon
N ................................................................. Nitrogen
DOC ...................................................... Dissolved Organic Carbon
TN ............................................................... Total Dissolved Nitrogen
SUVA ........................................................ Specific Ultraviolet Absorbance
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CHAPTER 1

INTRODUCTION

C and N cycling in forest soils has important implications for water quality, ecosystem resilience, and climate change (Lal, 2005; Jandl et al., 2007). While a critical component in future climate forecasting (Falkowski et al., 2000), knowledge gaps exist due to the highly heterogeneous, interdependent, and biological nature (Schimel & Bennett, 2004). Exploration of nutrient cycle changes after a natural disturbance has multiple benefits. First, we can directly apply new knowledge to infer ecosystem responses after bark beetle infestation. More broadly, utilizing a natural disturbance as a controlled experimental variation also offers the opportunity to investigate C and N cycling mechanisms in a natural system. The resultant understanding of underlying processes is necessary to improve prediction accuracy of climate change outcomes that can contribute to better-informed policy decisions.

Forests are facing many threats that are expected to intensify in the changing climate, including deforestation, drought, disease, insect infestations, and wildfire (Dale et al., 2001). Bark beetle infestations, in particular, have increased in magnitude and severity as a result of warming temperatures (Carroll et al., 2003; Mitton & Ferrenberg, 2012). In North America alone, the recent mountain pine beetle epidemic from 1997-2010 has affected an estimated 10 Mha in the western United States and British Columbia (Meddens et al., 2012). In extreme instances, bark beetle-induced tree
mortality has the potential to change forests from carbon sinks to carbon sources, potentially intensifying climatic feedback loops that will persist after impact (Kurz et al., 2008; Wear & Coulston, 2015). Furthermore, large-scale beetle-induced forest disturbances have been shown to affect water quantity and quality (Mikkelson et al., 2013a; Bearup et al., 2014; Brouillard et al., 2016) while altering terrestrial carbon (C) and nitrogen (N) cycling (Morehouse et al., 2008; Mikkelson et al., 2013b; Norton et al., 2015; Trahan et al., 2015).

Soil biogeochemical parameters such as water content, pH, N and C pools are affected by tree mortality after bark beetle infestation; however, there is significant variability in ecosystem responses post-infestation (Mikkelson et al., 2013b). Many studies have investigated seasonal and inter-annual variability of terrestrial biogeochemical cycling during forest recovery (Štursová et al., 2014; Ferrenberg et al., 2014; Norton et al., 2015; Mikkelson et al., 2016), but few have investigated variability due to the degree of beetle infestation (Cigan et al., 2015).

After bark beetles infest and kill a tree, C and N inputs to the soil system change. Initially, as transpiration ceases, root exudates are no longer deposited, and a source of labile C in the subsurface is lost (Grayston et al., 1996). After several years, beetle-killed trees progress to the grey phase, during which there is a large increase in recalcitrant organic matter input, as needles and coarse woody material fall from dead trees (Edburg et al., 2012). Needle litter from beetle-killed trees is also N enriched relative to naturally senesced litter (Morehouse et al., 2008; Griffin et al., 2011). Overtime, the litter input decays and transformed compounds are transported from the
litter horizon downward through the organic and mineral horizons. These altered inputs may have downstream changes on the fate and storage of C and N.

The decay and the fate of C and N-containing compounds are mediated primarily through soil microorganisms (Booth et al., 2005; Trivedi et al., 2016). C and N cycling is highly interdependent, in part due to their mutual dependence and influence on biological intermediates. As C and N inputs and physicochemical parameters change, soil microorganisms adapt, which in turn can affect C and N cycling (Mooshammer et al., 2014). C and N can be immobilized (i.e. stored) through assimilation by organisms or mobilized through decay processes and biochemical transformations to gaseous or aqueous species (Tate, 1995). Flux of CO\(_2\) and N\(_2\)O, greenhouse gases that intensify climate change, may change as a result of altered soil microbial respiration. Aqueous outputs, such as nitrate and DOC, can be transported to water sources and have downstream effects on water quality. For example, mountain pine beetle-impacted watersheds in the Colorado Rocky Mountains were linked to increased organic carbon and disinfection byproduct formation potential at downstream water treatment plants (Mikkelson et al., 2013a), and bark beetle-impacted forests in the Czech Republic have been linked to increased nitrate runoff, threatening water sources with eutrophication (Zimmermann et al., 2000; Huber, 2005).

In this study, we investigate the effect of tree mortality on biogeochemical cycling after bark beetle infestation. Precedent from other disturbances, such as logging, suggests that some geochemical responses are only observed after a threshold of surrounding tree mortality is surpassed (Parsons et al., 1994; Prescott et al., 2003),
likely because live trees exert a compensatory effect on ecosystem disruption. While
tree harvesting has notable differences from that of mortality caused by bark beetles, we
hypothesized that an analogous threshold of localized tree mortality must be exceeded
before disruption of carbon and nitrogen cycling is observed. To isolate the effects of
the degree of tree mortality on carbon and nitrogen cycling, we studied lodgepole pines
(Pinus contorta) infested by bark beetles in the Colorado Rocky Mountains. Soil
biogeochemical parameters were compared between healthy and deceased trees
surrounded by varying degrees of tree mortality. C and N fractions were quantified and
related to soil bacterial community structure and function using phylogenetic analysis
and enzyme activity assays to integrate the intertwined geochemical and biological
processes. Our findings provide a foundation for better understanding how and to what
degree biogeochemical processes may change in association with this type of large-
scale ecosystem disruption.
The study was performed in the White River National Forest approximately 5 km southwest of Frisco, Colorado, in the summer of 2015. This site was chosen for accessibility, relative homogeneity of vegetation, and uniform slope and aspect. The majority of trees located at the site were lodgepole pine (Pinus contorta), which experienced mountain pine beetle infestation beginning between 2007 and 2008. As bark beetle-induced mortality progresses, trees transition through 3 phases associated with different biogeochemical and hydrologic changes. This study focused on comparisons between the initial ‘green’ phase when a tree is alive, healthy, and transpiring, and the terminal ‘grey’ phase, which starts 3-5 years after a successful attack when the tree has stopped transpiring and has dropped its needles (Mikkelson et al., 2013b). No recent anthropogenic or natural forest disturbance outside of the recent bark beetle infestation has occurred at the site.

2.1 Site Characterization

To investigate the biogeochemical threshold response, we sampled 7 healthy green phase control trees and 31 grey phase trees surrounded by varying levels of tree mortality ranging from 9 to 91%. For this study, we defined surrounding tree mortality as the percent of dead trees relative to the number of trees within an 8-meter radius of the
sample tree. Lodgepole pine roots typically extend 4 meters from the trunk (Parsons et al., 1994). An 8-meter radius was selected to include any trees that may partially overlap with the root system of the sample tree. All surrounding trees were counted if their diameter at 4 feet above ground surface exceeded 3 inches. Surveyed neighboring trees were primarily lodgepole pine with an occasional aspen. A diagram depicting the sampling procedure is shown in Fig. 2.1a. All trees were located on the same hill slope and care was taken to reduce heterogeneity in slope, aspect, soil type, land cover, tree circumference, and sun exposure while capturing a range of surrounding tree mortality (Table 2.1). The aspect at each sample tree was East-Southeast with slopes ranging from 8 to 27%.

2.2. Sample Collection

Sampling was conducted during 3 consecutive days in mid-July of 2015. Three 8” PVC rings were hammered through the litter and organic horizon on the downslope side of each sample tree, approximately 1.5 feet from the trunk. Samples were collected from the litter, organic, and mineral horizon within each PVC ring and hand homogenized (Fig. 2.1b). Due to the variable litter depth, mineral horizon samples were collected 15 cm below the interface between the mineral and organic horizon. 2 g of soil was immediately collected from each bulk sample in the field and preserved in LifeGuard Soil Preservation Solution (MoBio Laboratories) for DNA extraction.

25 trees were sampled in all three horizons (5 green trees and 20 grey phase trees with varying levels of surrounding tree mortality). An additional 13 trees were sampled in the mineral horizon only. Bulk samples stored at 4°C in the dark. Enzyme
Fig. 2.1: Diagram of (a) surrounding tree mortality survey methods and (b) soil sampling methods.
assays were performed in less than 24 hours, and geochemical analyses were performed in less than 1 week. Preserved DNA samples were stored in the dark at -80°C for less than 3 weeks before extracting.

Table 2.1 Table of site descriptive characteristics. Grey phase trees are binned by percent tree mortality.

<table>
<thead>
<tr>
<th></th>
<th>Green Phase Mean (SD)</th>
<th>Grey Phase 0 – 25% Mean (SD)</th>
<th>Grey Phase 25 – 40% Mean (SD)</th>
<th>Grey Phase 40 – 59% Mean (SD)</th>
<th>Grey Phase &gt;59% Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Diameter (inches)</td>
<td>8.68 (1.03)</td>
<td>10.0 (1.06)</td>
<td>9.55 (1.41)</td>
<td>10.23 (1.33)</td>
<td>11.18 (1.34)</td>
</tr>
<tr>
<td>Elevation (feet)</td>
<td>9709 (166)</td>
<td>9789 (164)</td>
<td>9631 (247)</td>
<td>10030 (147)</td>
<td>9894 (182)</td>
</tr>
<tr>
<td>Slope (°)</td>
<td>17.8 (5.2)</td>
<td>17.7 (3.2)</td>
<td>14.1 (4.5)</td>
<td>19.9 (2.3)</td>
<td>19.0 (5.5)</td>
</tr>
<tr>
<td>Aspect (°)</td>
<td>116.6 (10.9)</td>
<td>111.3 (10.6)</td>
<td>114.0 (6.5)</td>
<td>113.4 (11.8)</td>
<td>117.9 (4.9)</td>
</tr>
<tr>
<td>Density (trees/m²)</td>
<td>0.19 (0.05)</td>
<td>0.16 (0.03)</td>
<td>0.18 (0.06)</td>
<td>0.11 (0.04)</td>
<td>0.09 (0.03)</td>
</tr>
</tbody>
</table>

Notes:
SD: Standard deviation
n: Number of trees in bin

2.3 Geochemical Analysis

Moisture, organic content, dissolved organic carbon (DOC), ultraviolet absorbance at 254 nm (UV₂₅₄), total nitrogen (TN), nitrate, ammonium, and pH was measured for each sample. Mineral and organic horizon samples were sieved through
2mm mesh prior to analysis while litter samples were hand homogenized. Moisture and organic content was measured using gravimetric and loss on ignition methods. Samples were dried at 105°C for 24 hours and then placed in a 375°C oven for 16 hours. Measures of DOC, UV$_{254}$, TN, and ammonium were determined by extracting samples in 0.5 M K$_2$SO$_4$ solution at a 1:5 ratio for mineral and organic horizon samples and 1:8 ratio for litter samples for 1 hour (Ferrenberg et al., 2013). Extractions were filtered through 0.45 um polyethersulfone filters prior to analysis. DOC and TN were determined using a Shimadzu TOC-550A Total Organic Carbon Analyzer. UV$_{254}$ was measured using a DU 800 spectrophotometer. Specific UV absorbance (SUVA) was calculated by dividing UV$_{254}$ by DOC concentration to evaluate differences in aromatic structure between extractions (Drewes & Croue, 2002). Ammonium concentrations were measured using the sodium salicylate method and absorbance at 650 nm using a Hach Spectrometer (Mulvaney et al., 1996). Measures of nitrate and pH were determined from 1:5 extractions for mineral and organic horizon samples and 1:8 extractions for litter horizon samples using deionized water (Ferrenberg et al., 2013). Nitrate concentrations were determined using the dimethylphenol method and absorbance at 345 nm with a Hach Spectrometer. The concentration of dissolved organic nitrogen was found by subtracting the sum of nitrate and ammonium from the TN concentration (Kaňa et al., 2012). C:N ratios were calculated as molar ratios of DOC:TN.

### 2.4 DNA Extractions and Sequencing

Preserved samples were extracted using PowerSoil® Total RNA Isolation Kit and DNA Elution Accessory Kit, as specified by the manufacturer (MoBio Laboratories).
Sequencing methods were adapted from those previously described by our laboratory (Weathers et al., 2016). Briefly, Phusion® High-Fidelity DNA Polymerase Master Mix (New England Bioscience) and nearly universal bacterial and archaeal dual indexed primers (Kozich et al., 2013) were used to amplify the hypervariable V4 region. Amplicons were purified and pooled in equimolar concentration using the SequalPrep plate normalization kit (Life Technologies). Pooled samples were concentrated using 30K ultra centrifugal filter devices (Amicon), and quantified using a Qubit 2.0 Fluorometric Quantitation (Life Technologies). The library was sequenced at Biofrontiers, University of Colorado with an Illumina MiSeq using a 2x250 V2 kit.

Dual indexed sequencing outputs were demultiplexed by the Biofrontiers MiSeq Platform. QIIME toolkit (v1.9.0) was used to process demultiplexed libraries (Caporaso et al., 2010). Forward and reverse Illumina reads were joined and quality filtered using the multiple_join_paired_ends.py script. Reads were truncated after a stretch of three low-quality bases. Pick_open_reference_otus.py workflow script was used to assign operational taxonomic unit (OTU) to joined reads. Reads were assigned to OTUs using USEARCH(Edgar, 2010)and taxonomy was assigned using the RDP classifier v2.2 and the Greengenes v13.8 database filtered at 97% identity (DeSantis et al., 2006).

After processing and quality filtering the Illumina 16S rRNA gene reads, a total of 1,998,245 paired-end sequences were obtained from 86 samples. Three samples with fewer than 4000 sequences were dropped from analysis. Samples ranged from 8951 to 59,847 sequences per sample, averaging 23,235. After removing low quality samples, litter samples from under 5 green phase trees and 18 grey phase trees were analyzed.
Organic horizon samples from under 5 green phase trees and 20 grey phase trees were analyzed, and from the mineral horizon, 6 samples from under green phase trees and 29 samples from under grey phase trees were analyzed for phylogenetic structure.

### 2.5 Enzyme Activity Assays

Four enzymes were selected for activity assays to represent metabolic activity over a wide range of substrate recalcitrance. From least recalcitrant to most, enzyme activity potential was measured for α-glucosidase, N-acetylglucosaminidase (NAGase), Endo-1,4-β-glucanase (endocellulase), and laccase. Samples were kept on ice as much as possible during processing. Enzyme activity assays were performed in accordance with prior work with some modifications (Štursová & Baldrian, 2010). For α-glucosidase, NAGase, and endocellulase assays, samples were prepared by homogenizing 1g of soil in 50 mL of 50mM sodium acetate buffer, pH 5.0 by vortexing for 30s. For laccase assays, samples were prepared by adding 1.5g of soil to 10mL of 100 mM sodium phosphate buffer, pH 7.0. Laccase samples were homogenized by vortexing and then extracted for 3 hours on an orbital shaker (100 rpm) at 4°C. Samples were centrifuged for 3 min at 3000xg at 4°C. The supernatant was collected and filtered through 0.45 µm syringe filters and then desalted using PD-10 desalting columns (GE Healthcare) according to the manufacturer’s instructions. Samples were eluted from columns in 100mM citrate, 200mM phosphate buffer, pH 5.0.

To assay α-glucosidase and NAGase, fluorescent reporter linked substrates (4-methylumbelliferyl-α-D-glucopyranoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively) were added to 100 µl of soil homogenate at a final
concentration of 500µM in a 96-well plate with 3 technical replicates. Blanks were used to subtract background fluorescence and standard curves of 4-methylumbelliferyl were used to quantify enzyme activity. Plates were incubated at 37°C for 90 minutes on a plate shaker. Fluorescence was measured using a microplate reader (Permin Elmer, Victor X5), with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Endocellulase activity was assayed using the Cellafluor Kit, according to manufacturer’s instructions (Megazme, Ireland). 20 µl of enzyme sample and 20 µl of Cellafluor reagent, pre-equilibrated to 40°C were added to a 96-well plate in triplicate. The reactions were incubated at 40°C for 10 min and then the reaction was stopped with 60ul of 2% Trizma. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Laccase activity was assayed by quantifying the oxidation of ABTS. A reaction containing 500 µM ABTS and 100µl of extracted enzyme sample was incubated for 90 minutes at 30°C. Absorbance was measured at 405nm and the concentration of oxidized ABTS was calculated using ε = 3.68 x 10^4 L/mol-cm. Enzyme activity is expressed per g dry soil mass per time elapsed.

2.6 Statistical Analyses

Mann-Whitney rank abundance tests were used to compare biogeochemical parameters between green and grey phase trees. Spearman test were used to assess nonlinear correlations and segmented linear regression was used to test for threshold relationships (Muggeo, 2003) between biogeochemical parameters and surrounding tree mortality surrounding grey phase trees. In QIIME, ADONIS tests were used to
compare microbial community composition and identify parameters that may account for clustering patterns.

Microbial sequencing data was analyzed using a rarefied operational taxonomic unit (OTU) table with 8950 randomly selected sequences per sample for all subsequent analyses except differential abundance tests, for which a Negative Binomial model was used to normalize counts (McMurdie & Holmes, 2014). Alpha diversity was assessed using Shannon diversity index and OTU richness in QIIME. Shannon diversity index accounts for species richness and evenness, whereas OTU richness is the number of unique OTUs in each sample. Beta diversity was analyzed using weighted UniFrac distance matrices generated in QIIME and visualized using the Phyloseq package (McMurdie & Holmes, 2013). Differential abundance analyses were used to identify clades that were significantly different between green phase trees and grey phase trees surrounded by high levels of tree mortality (>40%). OTUs with <1% relative abundance were not considered. For differential abundance tests, the unrarefied OTU table was normalized and filtered with the DESeq2 package in R (Love et al., 2014). R was used for all statistical analyses unless otherwise noted (R Core Team, 2015). P-values less than 0.05 were considered significant.
When comparing soil under green and grey phase trees in our study, we found distinct biogeochemical signatures relating to surrounding tree mortality and C:N ratio shifts (Table A.1, A.2, A.3). Some biogeochemical signatures were linked to surrounding tree mortality, while others were disrupted under beetle-impacted trees regardless of the extent of surrounding impact. Supporting our threshold hypothesis, elevated ammonium was only observed under grey phase trees surrounded by a threshold percent of surrounding tree mortality; however this threshold trend was not present for the other variables analyzed. Key results relating to N and C pools, and microbial community shifts are discussed in detail below.

3.1 Proportional Shifts in N Species

The TN pool in soil is comprised of organic N, and inorganic ammonium and nitrate. TN was not significantly different between tree phases, and only associated with surrounding tree mortality in the litter horizon, where it increased modestly in conjunction with surrounding tree mortality (rho = 0.46, p < 0.05). However, there was a marked change in the N species composition of the TN pool as surrounding tree mortality increased (Fig. 3.1).
Fig. 3.1: Changing composition of total nitrogen pool relative to surrounding tree mortality. LOESS smoothing was conducted on N species under grey phase trees. Green phase trees are excluded from smoothing regression but are included for comparison. Shaded area represents the 95% confidence interval.
As more of the forest stand was affected by beetle-induced tree mortality, the proportion of organic N in the soil diminished. Organic N dominated under green phase trees, constituting 70±6%, 65±13%, and 55±7% of the TN pool in the litter, organic, and mineral horizons, respectively. The relative proportion of organic N under grey phase trees decreased as surrounding tree mortality increased (litter: rho= -0.43, p < 0.1; organic: rho= -0.50, p < 0.05; mineral: rho= -0.41, p < 0.05). Concurrently, ammonium generally increased under grey phase trees (Fig. 3.2a). The proportion of ammonium in the TN pool increased in the litter and organic horizon but only when surrounding tree mortality surpassed a threshold of approximately 40%. For grey phase trees surrounded by less than 40% tree mortality, the proportion of ammonium was low and comparable to that observed under green phase trees. In the mineral horizon, on the other hand, the proportion of ammonium increased even at low surrounding tree mortality. In contrast, nitrate was not significantly affected by tree mortality (Table A.1, A.2, A.3). Specifically, the concentration of nitrate was not different between grey and green phase trees in any horizon and was unaffected by surrounding tree mortality.

3.2 Size and Quality of DOC Pool

In addition to N pool speciation, we also investigated the amount and character of the DOC pool. In contrast to soil N trends, the amount of DOC was significantly different between green and grey phase trees only in the mineral horizon. The concentration of DOC was 35% lower under grey phase trees than green phase (p = 0.03), but was unrelated to surrounding tree mortality. Despite DOC levels remaining generally consistent, carbon character was significantly more refractory under grey phase trees
compared to green phase trees in all soil horizons as SUVA increased by 25%, 38%, and 21% in the litter, organic, and mineral horizon, respectively (p < 0.003 for all horizons). Carbon recalcitrance also co-varied with surrounding tree mortality in the mineral horizon (rho = 0.38, p < 0.05).

### 3.3 C:N Ratio Shifts Linked to Surrounding Tree Mortality and Altered N Cycling

The relative abundance of C and N (C:N ratio) was used to examine the relationship between altered N and C pools after bark beetle impact. While N speciation shifted and C recalcitrance increased, the relative amount of C and N also changed under beetle-killed trees. C:N ratios were reduced under grey phase trees when contrasted to healthy, green phase trees. The effect of surrounding tree mortality on C:N ratio shifts differed between horizons. In the upper horizons (litter and organic), C:N ratios decreased as surrounding tree mortality increased (litter: p < 0.05, rho= -0.61; organic: p < 0.05, rho = - 0.78, Fig. 3.2b). In the deepest horizon (mineral), C:N ratios were 40% lower under grey versus green phase trees, regardless of surrounding tree mortality, mirroring ammonium trends in each horizon (p = 0.002, Fig. 3.2a). Not only did C recalcitrance increase under these trees but C abundance relative to N decreased, suggesting C may be less accessible under beetle-killed trees.

In all soil profiles, ammonium concentrations were also coupled to C:N ratios. Ammonium concentrations spiked only under trees below a quantifiable C:N ratio (Fig. 3.2b). The C:N ratio at which this transition from baseline to elevated ammonium concentrations occurred decreased with soil depth, from 29:1 (± 4.2) in the litter, to 21:1 in the organic (± 2.2), to 18:1 in the (± 2.6) mineral horizon (p < 0.001 (all); litter, adj. R =
Thus, the accumulation of ammonium occurred when the abundance of C relative to N decreased, altering N cycling.

Fig 3.2: (a) The proportion of ammonium and (b) C:N ratio under green (▲) and grey (●) phase trees surrounded by varying levels of tree mortality in each horizon. Dotted black line shows the approximate threshold of tree mortality (40%) for an ammonium response in the litter and organic horizon.
Fig. 3.3: The relationship between the concentration of ammonium and C:N ratio under green (▲) and grey (○) phase trees in the (a) litter, (b) organic, and (c) mineral horizon. The inflection point was 28.7 ± 4.2, 20.8 ± 2.2, and 18.1 ± 2.6 in the litter, organic, and mineral horizon, respectively (for all horizons: p < 0.001). (Note: One data point with C:N = 79 omitted from organic horizon to improve data visualization, follows trend).
CHAPTER 4

SOIL MICROBIAL COMMUNITY RESULTS

Phylogenetic analysis and exoenzyme activity assays were used to assess changes to the soil bacterial community structure and function in relation to changing physical and biogeochemical parameters under beetle-impacted trees. Results demonstrated that the soil bacterial community structure changed under grey phase trees compared to green trees and was affected by surrounding tree mortality. Functional bioindicators, such as enzyme activity and functionally conserved clades, were related to C:N ratio changes but not to surrounding tree mortality.

4.1 Soil Microbial Community Shifts following Tree Mortality

The effect of tree mortality on alpha diversity varied in each soil horizon. Using the 40% tree mortality threshold, approximated from the accumulation of ammonium (Fig. 3.2a), samples were categorized as green phase, low impact grey phase (<40% tree mortality), or high impact grey phase (>40% tree mortality). Diversity in the litter horizon did not change between tree phases, or when binned by extent of tree mortality (Fig. 4.1). In the organic horizon, alpha diversity was significantly higher under grey compared to green phase trees, according to both OTU richness ($p = 0.009$) and Shannon ($p = 0.001$) diversity indices. Diversity in the organic horizon also increased in concert with surrounding tree mortality, according to both OTU richness ($\rho = 0.54$, $p =$ 0.001) and Shannon ($p = 0.001$) diversity indices. Diversity in the organic horizon also increased in concert with surrounding tree mortality, according to both OTU richness ($\rho = 0.54$, $p =$ 0.001).
0.02) and Shannon diversity (rho = 0.45, p = 0.05) metrics. When samples are binned based on the ammonium threshold of 40% tree mortality, OTU richness under low impact grey phase trees was not significantly different (Fig. 4.1). In the mineral horizon, only grey phase trees surrounded by >40% tree mortality had significantly higher diversity than green phase trees (Fig. 4.1).

Beta diversity analysis using weighted UniFrac distance matrices demonstrated that bacterial communities clustered separately into green, low impact, and high impact groups in all horizons (litter: r = 0.39, p = 0.04; organic: r = 0.42, p = 0.003; mineral: r = 0.39, p = 0.004). Clustering behavior also changed with depth as demonstrated by principal coordinate analysis of bacterial communities using weighted UniFrac distance matrices (Fig. 4.2). Community structure trends with surrounding tree mortality mirrored ammonium and C:N ratio trends in each horizon—whereby in the upper soil horizons (litter and organic), parameters trended with the extent of surrounding tree mortality, but in the mineral horizon, parameters under grey phase trees differed from under green trees, regardless of surrounding tree mortality. For microbial community distances, green and low impact grey phase clusters overlapped, in the litter horizon, suggesting similar microbial community structure. In the organic horizon, low impact grey phase trees clustered at an intermediate distance between green phase and high impact grey phase, suggesting a transitional microbial community state. In the mineral horizon, high and low impact grey phase tree clusters overlapped, suggesting grey phase trees have similar community structures that differed from communities under green phase trees.
Fig. 4.1: Estimates of soil bacterial community diversity according to (a) Shannon and (b) OTU richness under sample trees binned by green phase, low impact grey phase (<40% tree mortality), and high impact grey phase (>40% tree mortality). Points are outliers and whiskers represent the highest or lowest value within 1.5 x Interquartile Range. P-values are given for bins significantly different from green phase.
Fig. 4.2: Principal coordinate analysis of weighted Unifrac community distance matrices in each horizon. Samples are grouped as green phase trees (▲), grey phase trees surrounded by low levels of tree mortality (■), and grey phase trees surrounded by high levels of tree mortality (●).
4.2 Clade Specific Changes associated with Tree Mortality

In order to investigate which bacterial clades were changing most with respect to surrounding tree mortality, differential abundance analyses were used to identify clades that were significantly different between green phase trees and grey phase trees surrounded by high levels of tree mortality.

In the litter horizon, only 17 OTUs were significantly different; 2 OTUs decreased in abundance and 15 OTUs increased in abundance under high impact grey phase trees compared to green phase trees. In contrast, 167 OTUs changed in abundance in the organic horizon. Consistent with the litter horizon, the majority of OTUs increased in abundance under high impact grey phase trees compared to green phase trees (133) and a minority decreased (34). A similar number of OTUs changed in abundance in the mineral horizon; however, nearly an equal proportion of OTUs increased and decreased. 86 OTUs increased and 70 decreased under high impact grey phase trees compared to green phase trees (Fig. 4.3). Collectively, these OTUs belonged to 9 phyla: Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Gemmatimonadetes, Cholorflexi, Cyanobacteria, Elusimicrobia, and Fibrobacteres. Members of Gemmatimonadetes, Cholorflexi, Elusimicrobia, and Fibrobacteres were always more abundant under high impact trees than green phase trees.

Since C:N ratio was a strong indicator of ammonium accumulation, and therefore changes to N cycling, we also compared cladal abundance in association with C:N ratio variability. Using the C:N ratio where ammonium increased non-linearly in each horizon (Fig. 3.3), we binned samples as high or low C:N. While ten to hundreds of OTUs were
Fig. 4.3: The number of OTUs, categorized by phylum, that either increased or decreased under grey phase trees surrounded by high levels of impact compared to green phase trees. NA represents OTUs that were unclassified at the Phylum level.
different between green phase and high impact grey phase, there were minimal differences in OTU abundance between C:N regimes. There were no OTUs that differed in abundance in the litter horizon, seven OTUs in the organic horizon, and four OTUs in the mineral horizon. In the organic horizon, six OTUs increased in abundance under low C:N conditions and belonged to the phyla Verrucomicrobia, Proteobacteria, Actinobacteria, and Acidobacteria. One OTU from the phyla Verrucomicrobia increased in abundance under low C:N conditions. In the mineral horizon, three OTUs increased in abundance under low C:N conditions and belonged to the phyla Verrucomicrobia, Actinobacteria, and Proteobacteria. One OTU from the phyla Acidobacteria increased in abundance under low C:N conditions.

Of particular interest, the class Nitrospira— a functionally conserved group of aerobic nitrifiers that were predominantly found in the mineral horizon— were more abundant at low C:N ratios. The relative abundance of Nitrospira increased non-linearly at C:N ratios below 13:1 ± 1.2 (Fig. 4.4). In the upper horizons, Nitrospira was low abundance with base mean counts per sample of 1.02 in the litter and 6.62 in the organic horizon, compared to 135.25 counts in the mineral horizon.

4.3 Microbial Functional Response to Tree Mortality

Enzyme activity responses to tree mortality were limited and not consistent across all soil horizons. NAGase activity in the litter horizon decreased as surrounding tree mortality increased (rho = - 0.54, p = 0.01), and α-glucosidase activity decreased in the mineral horizon of grey phase trees compared to green trees (p = 0.03, Table 4.1).
NAGase and laccase activities were also associated with C:N ratios. NAGase activity was significantly elevated at higher C:N ratios in the organic horizon (\(\rho = 0.58, \ p = 0.007\)), while laccase activity was significantly higher in both the organic and mineral horizon at higher C:N ratios (organic: \(\rho = 0.46, \ p = 0.04\); mineral: \(\rho = 0.54, \ p = 0.001\)).

Fig. 4.4: The relative abundance of Nitrospira in the mineral horizon increased under green (▲) and grey (●) phase trees with low C:N ratios. C:N ratio threshold for Nitrospira abundance was 13 ± 1.2 (\(p< 0.001\), adjusted \(R^2=0.42\)).
Table 4.1: Enzyme activity and extent of surrounding tree mortality relationships.

<table>
<thead>
<tr>
<th>Litter</th>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Spearman rho&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-glucosidase Activity (umols/g dry weight-90m)</td>
<td>0.28 (±0.18)</td>
<td>0.26 (±0.22)</td>
<td>43</td>
<td>-0.179</td>
</tr>
<tr>
<td></td>
<td>NAGase Activity (umols/g dry weight-90m)</td>
<td>1.89 (±1.22)</td>
<td>1.76 (±0.95)</td>
<td>48</td>
<td>-0.545</td>
</tr>
<tr>
<td></td>
<td>Cellulase Activity (umols/g dry weight-10m)</td>
<td>0.07 (±0.04)</td>
<td>0.06 (±0.05)</td>
<td>40</td>
<td>-0.175</td>
</tr>
<tr>
<td></td>
<td>Laccase Activity (mmols/g dry weight-90m)</td>
<td>2.16 (±1.11)</td>
<td>3.48 (±3.09)</td>
<td>43</td>
<td>-0.331</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organic</th>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Spearman rho&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-glucosidase Activity (umols/g dry weight-90m)</td>
<td>0.11 (±0.06)</td>
<td>0.1 (±0.06)</td>
<td>42</td>
<td>0.191</td>
</tr>
<tr>
<td></td>
<td>NAGase Activity (umols/g dry weight-90m)</td>
<td>0.61 (±0.2)</td>
<td>0.58 (±0.41)</td>
<td>32</td>
<td>-0.331</td>
</tr>
<tr>
<td></td>
<td>Cellulase Activity (umols/g dry weight-10m)</td>
<td>0.02 (±0)</td>
<td>0.02 (±0.01)</td>
<td>44</td>
<td>-0.259</td>
</tr>
<tr>
<td></td>
<td>Laccase Activity (mmols/g dry weight-90m)</td>
<td>0.62 (±0.41)</td>
<td>0.79 (±0.44)</td>
<td>38</td>
<td>-0.352</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Spearman rho&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-glucosidase Activity (umols/g dry weight-90m)</td>
<td>2.93 (±0.8)</td>
<td>0.07 (±0.04)</td>
<td>70</td>
<td>0.240</td>
</tr>
<tr>
<td></td>
<td>NAGase Activity (umols/g dry weight-90m)</td>
<td>0.12 (±0.09)</td>
<td>0.24 (±0.13)</td>
<td>97</td>
<td>-0.026</td>
</tr>
<tr>
<td></td>
<td>Cellulase Activity (umols/g dry weight-10m)</td>
<td>0.25 (±0.16)</td>
<td>0.03 (±0.01)</td>
<td>78</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>Laccase Activity (mmols/g dry weight-90m)</td>
<td>0.02 (±0.01)</td>
<td>0.24 (±0.3)</td>
<td>98</td>
<td>-0.128</td>
</tr>
</tbody>
</table>

<sup>1</sup>Differences between green and grey phase trees. **Bold** if p < 0.05.

<sup>2</sup>Correlation between parameter under grey phase trees and surrounding tree mortality. **Bold** if p < 0.05.
By studying changes to soil biogeochemical parameters on a continuum of tree mortality, we can also tease out mechanisms that may be driving C and N cycling. In the case of bark beetle infestation, inputs to the soil system are altered as a pulse of needles enriched with N and recalcitrant C is deposited in the litter horizon, while subsurface rhizodeposition ceases (Morehouse et al., 2008).

5.1 Altered N Cycling Under Highly Impacted Grey Phase Trees

Lodgepole pine forests in the Rocky Mountains are typically N-limited (Stump & Binkley, 1993); however, accumulation of ammonium above baseline in forest soils signifies a system is N-saturated (Aber et al., 1989). Hence, the elevated ammonium observed in this study indicates that there is excess N after beetle-induced mortality. Importantly, the transition from N-limited to N-saturated conditions only occurred under dead trees that were surrounded by at least 40% tree mortality in both the litter and organic horizon.

This pattern suggests that the upper soils are buffered until a threshold of surrounding tree mortality is surpassed. In contrast the switch to N-saturated conditions appears to occur even in low impact areas, within the mineral horizon of bark beetle-killed trees. The cessation of root exudates largely occurs in the mineral horizon, based
on the localization of fine root hair structures in lodgepole pines (Walker et al., 2003); therefore, loss of rhizodeposition can explain lower C:N ratios in the deeper subsurface of grey phase trees contrasted with green phase trees. The significant decrease in DOC in the mineral horizon of grey phase trees supports this interpretation. The litter and organic horizon may be more impacted by the top down inputs of increased recalcitrant C- and N-enriched litter, which is mobilized through litter decay and leaching processes.

The disproportionate accumulation of ammonium associated with tree mortality could be the result of multiple mechanisms: (1) the soil microbial community adapts to excess N by decreasing nitrogen use efficiency (NUE) and wasting N through mineralization (Mooshammer et al., 2014a); (2) increased water content, which is typical under beetle-killed grey phase trees (Mikkelson et al., 2013b), may increase micro-anoxic zones inhibiting nitrification, resulting in a buildup of ammonium; and/or (3) N uptake by alive and overlapping roots diminishes as tree mortality increases. It is unlikely that the accumulation of ammonium is due to the second proposed mechanism— increased WC under grey phase trees making the soil system more anaerobic and inhibiting aerobic nitrification. Aerobic nitrifiers increased in abundance in the mineral horizon with both increasing levels of tree mortality and decreasing C:N ratios (Fig. 4.4), and WC did not correlate with any other biogeochemical parameter changes. The third mechanism, decreased ammonium uptake due to tree mortality, is undoubtedly occurring; however, the accumulation of ammonium indicates that growth of soil biota is limited by another growth nutrient or condition. This also does not explain
the accumulation of ammonium in the litter and organic horizon, where the root system has little influence.

Based on the strong correlation between C:N ratios and ammonium concentration along with the changes to the C and N pools in our system, ammonium accumulation is most likely occurring by the first mechanism—decreasing NUE as microbes waste excess N in the form of ammonium. According to the theory of ecological stoichiometry, nutrient limitations occur due to an imbalance between the ratio of nutrients supplied by the substrate and the ratio required for growth (Mooshammer et al., 2014a). Decreased C:N ratios indicate that N becomes more abundant relative to C in the mineral horizon of grey phase trees and at higher levels of tree mortality in the litter and organic horizon. Moreover, the TN pool also transitions from predominantly organic N, which consists of decaying plant detritus, rhizodeposits and microbial biomass, to predominately inorganic ammonium produced by N mineralization. As surrounding tree mortality increases, the pool of organic nitrogen decreases, suggesting less of the total nitrogen pool is assimilated and more is wasted as ammonium. Moreover, the dissolved organic carbon fraction is more recalcitrant under grey phase trees and exacerbated by surrounding tree mortality. This would further limit the availability of organic carbon beyond that captured in a simple C:N analysis. Together, these results suggest that bioavailable carbon is scarce relative to nitrogen under beetle-killed trees.

It was also anticipated that the increase in ammonium would lead to increased nitrate concentrations since there is an abundance of substrate (ammonium), and
Nitrospira was enriched at low C:N ratios. However, this was not evident in our study. The lack of nitrate buildup could be due to: (1) leaching from the system, (2) denitrification, and (3) inhibition of nitrification, which as discussed above, is unlikely. With the largest solubility and repulsive interactions with positively charged soil particles (Tate, 1995), nitrate may exit the soil system rapidly in the aqueous form. Unlike systemically N-saturated European forests, nitrate leaching after bark beetle infestation has not been documented in the Colorado Rocky Mountains (Rhoades et al., 2013; Biederman et al., 2016). Alternatively, nitrate may be transformed through denitrification and exit the system as N$_2$, N$_2$O, and/or NO as was observed in Swiss forests when nitrous oxide flux from soils increased in response to tree girdling experiments (Krause et al., 2013). While we did not measure for these gaseous species, our results suggest that conditions under beetle-killed lodgepole pines favor denitrification; it is possible that widespread beetle infestation may lead to increased flux of these potent greenhouse gases from the soil.

5.2 Terrestrial Bacterial Community Adaptation
Terrestrial bacterial and fungal communities are interdependent and integral components of the C and N cycle. Several reports have shown that fungal but not bacterial communities are affected by bark beetle disturbance (Štursová et al., 2014; Ferrenberg et al., 2014); however, more recent studies (Mikkelsen et al., 2016a) and these results suggest this is not the case. Instead, our results show the soil microbiotic community adapting to tree mortality and changing nutrient regimes through multiple mechanisms. Microorganisms adapt to nutrient availability shifts primarily through three
mechanisms: (1) community structure changes as competitive advantages shift, (2) nutrient use efficiency changes as nutrients become more or less abundant, and/or (3) exoenzyme optimization to increase access to limited nutrients (Mooshammer et al., 2014). We can evaluate community structure changes using phylogenetic analyses; nutrient use changes using geochemical observations; and optimization of enzyme production and microbial functionality using exoenzyme activity assays.

Microorganism adaptation may help to explain intertwined microbial processes and nutrient availability. The accumulation of ammonium is likely a result of changes in NUE, but the soil microbial community is also adapting through the other two mechanisms. Phylogenetic analysis of the bacterial community revealed that alpha diversity increased under grey phase trees in the organic and mineral horizon, both by species richness and evenness (Fig. 4.1). Although, in the mineral horizon, this diversity increase was only significant under grey phase trees surrounded by high levels of tree mortality. These results corroborate similar findings where bacterial diversity was elevated under beetle-killed trees at a site with high percent tree mortality, but did not at an analogous site with low percent tree mortality (Mikkelson et. al., 2016).

Moreover, overall community analysis revealed that samples clustered by extent of surrounding tree mortality, although different clustering patterns were evident in each soil horizon (Fig. 4.2). Near the surface, in the litter and organic horizons, grey phase trees surrounded by low levels of tree mortality had community distances that were a transitional state between green and high impact trees. In the mineral horizon, grey phase trees, regardless of the level of impact, clustered together, separate from green
phase trees. This pattern is consistent with the geochemical trends observed (Fig. 3.2a,b), wherein the litter and organic horizon, C:N ratio and ammonium concentration trend with surrounding tree mortality, but in the mineral horizon, grey phase trees were different from green phase trees, regardless of the surrounding tree mortality.

The limited differences in cladal abundance when contrasting N-limited and N-saturated environments suggests that overall community structure changes were comparatively modest despite the shift in nutrient availability. However, Nitrospira, which utilizes N as an energy source, was enriched in low C:N environments (Fig. 4.4). Interestingly, the relative abundance of Nitrospira increased only below 13:1 C:N ratio. As an autotroph, Nitrospira are typically outcompeted by heterotrophs (Tate, 1995). The enrichment of Nitrospira in these more N-rich environments likely reflects a restructuring in the competitive advantage, as their energy source becomes more abundant, and bioavailable carbon becomes scarcer.

As microbial communities are composed of both bacteria and fungi it is important to take into consideration both components. Subsurface soil fungi have an important role in response to bark beetle disturbances and C/N cycling in general (Štursové et al., 2014, Treu et al., 2014; Karst et al., 2015). Our exoenzyme activity assay results suggest that fungal activity responded to altered C and N inputs, and thus play an important role as the community adapts to a new nutrient regime. Counter intuitively perhaps, NAGase and laccase activity declined in low C:N ratio regimes. It might be expected that exoenzymes that break down recalcitrant carbon molecules would be beneficial when C is less abundant relative to N; however, this was not the case in this
study or in others that looked a enzyme activity after beetle infestation (Štursová et al., 2014). While we cannot yet interpret the underlying mechanisms, one explanation for the decrease in NAGase activity in low C:N environments may be the relative abundance of N. NAGase releases N from chitin, its substrate (Hamid et al., 2013), and the abundance of ammonium in low C:N environments may reduce the value of NAGase as a N-scavenging enzyme compared to others.
Seven to eight years after the initial bark beetle infestation, we observed a threshold ammonium response when approximately 40% or more of trees in the proximal stand are killed. Many lines of evidence support the concept that beetle-infested lodgepole ecosystems are switching from N-limited to N-saturated. When the system has transitioned to N-saturated synchronously ammonium is elevated, N is relatively more abundant than C, and C is less accessible due to increased recalcitrance. This transition is not tied to the extent of beetle-induced tree mortality in the mineral horizon, and these indicators are observable under grey phase trees at any surrounding tree mortality. However, the transition to N-saturation in the litter and organic horizon was only observed in trees with high surrounding tree mortality.

These findings generate more questions of what processes are driving the biogeochemical changes after bark beetle infestation and in terrestrial C and N cycling overall. First, controlled laboratory experiments are necessary to support these observations and establish causal relationships to confirm our interpretations. In particular, conclusive experimental evidence that the system is N-limited or N-saturated under the respective conditions described above would strongly bolster these conclusions. One interesting future direction would be to manipulate C:N ratio in a
column study and track the subsequent alterations in N cycling to confirm that inorganic N species are favored at low C:N ratio.

Another knowledge gap that remains is a deeper understanding of the soil microbial community that modulates C and N cycles and how they are affected by changing nutrient regimes. The above findings are exploratory and not comprehensive. While this work shows that the community responded to tree mortality through both community structure and functional activity shifts, an in depth analysis of the environmental selective pressures is needed. These results demonstrate that the microbial community adapts to tree mortality through community structure, nutrient use efficiency changes, and exoenzyme activity adaptations; each of these adaptation mechanisms could be explored directly in more detail.

The experimental design of this study using a continuum of surrounding tree mortality and, inadvertently, C:N ratios generated an ideal matrix for studying the intertwined C and N cycle in situ as inputs are incrementally changed. The microbial community data is still ripe for data mining, in particular to understand how microbes, both as a community and as individual species, adapt to changing nutrient regimes and to what extent they influence the fate N and C cycling.

Moreover, fungal community structure has not been addressed in this work and will certainly have an interesting role in C and N cycling responses to forest disturbances. The enzyme activity reported here also shows interesting trends; however, meaningful interpretation of these results is dependent on focused, controlled
studies that specifically explore environmental pressures affecting these enzymes and the microbes that produce them.

Finally, and perhaps most impactful, is an investigation into the evolution of greenhouse gases after beetle infestation. Beetle-killed trees appear to create conditions that would favor nitrous oxide evolution. Denitrification may be enhanced due to increased inorganic N availability, higher soil water content, and selective pressures favoring saprotrophic fungi. This continuum of surrounding tree mortality is an ideal experimental matrix for moving beyond purely observations of greenhouse gas evolution to also understanding why nitrous oxide evolution may be increasing under beetle-killed trees.
REFERENCES


Dale VH, Joyce LA, McNulty S (2001) Climate Change and Forest Disturbances Climate change can affect forests by altering the frequency, intensity, duration, and timing of fire, drought, introduced .... ....


### Table A.1 Litter horizon biogeochemical parameters and trends with extent of surrounding tree mortality (n = 20 grey, 5 green).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U¹</th>
<th>Spearman rho²</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.77 (±0.23)</td>
<td>5.19 (±0.42)</td>
<td>18</td>
<td>-0.180</td>
</tr>
<tr>
<td>Water Content (% dry weight)</td>
<td>73.64 (±32.7)</td>
<td>75.78 (±26.14)</td>
<td>40</td>
<td>-0.347</td>
</tr>
<tr>
<td>DOC</td>
<td>58.41 (±13.21)</td>
<td>74.48 (±30.41)</td>
<td>40</td>
<td>-0.054</td>
</tr>
<tr>
<td>UV</td>
<td>1.6 (±0.37)</td>
<td>2.65 (±1.45)</td>
<td>21</td>
<td>0.111</td>
</tr>
<tr>
<td>SUVA</td>
<td>2.75 (±0.21)</td>
<td>3.45 (±0.44)</td>
<td>4</td>
<td>0.218</td>
</tr>
<tr>
<td>Organic Matter (%)</td>
<td>47.59 (±17.69)</td>
<td>56.78 (±16.32)</td>
<td>37</td>
<td>0.205</td>
</tr>
<tr>
<td>Total Nitrogen (mg/L as N)</td>
<td>2.52 (±0.57)</td>
<td>3.34 (±1.36)</td>
<td>29</td>
<td>0.460</td>
</tr>
<tr>
<td>NO3- (mg/L as N)</td>
<td>0.622 (±0.171)</td>
<td>0.818 (±0.397)</td>
<td>38</td>
<td>0.066</td>
</tr>
<tr>
<td>NH4+ (mg/L as N)</td>
<td>0.187 (±0.129)</td>
<td>0.525 (±0.661)</td>
<td>31</td>
<td>0.733</td>
</tr>
<tr>
<td>Organic Nitrogen (mg/L as N)</td>
<td>1.75 (±0.429)</td>
<td>1.996 (±0.801)</td>
<td>42</td>
<td>0.005</td>
</tr>
<tr>
<td>NO3-/TN</td>
<td>0.245 (±0.042)</td>
<td>0.258 (±0.099)</td>
<td>45</td>
<td>-0.281</td>
</tr>
<tr>
<td>NH4+ /TN</td>
<td>0.074 (±0.042)</td>
<td>0.131 (±0.134)</td>
<td>32</td>
<td>0.750</td>
</tr>
<tr>
<td>Organic N/TN</td>
<td>0.696 (±0.058)</td>
<td>0.61 (±0.134)</td>
<td>25</td>
<td>-0.433</td>
</tr>
<tr>
<td>C:N (molar ratio)</td>
<td>27.33 (±5.2)</td>
<td>28.04 (±9.81)</td>
<td>49</td>
<td>-0.607</td>
</tr>
<tr>
<td>ATP (pmols/g dry weight)</td>
<td>58.92 (±34.88)</td>
<td>52.98 (±15.96)</td>
<td>41</td>
<td>-0.007</td>
</tr>
<tr>
<td>Shannon Diversity Index³</td>
<td>9.59 (±0.24)</td>
<td>9.64 (±0.42)</td>
<td>40</td>
<td>0.445</td>
</tr>
<tr>
<td>Observed OTUs³</td>
<td>2385.4 (±166.5)</td>
<td>2420.8 (±308.1)</td>
<td>38</td>
<td>0.305</td>
</tr>
</tbody>
</table>

¹ Differences between green and grey phase trees. Bold if p < 0.05.
² Correlation between parameter under grey phase trees and surrounding tree mortality. Bold if p < 0.05.
³ n_{green} = 5, n_{grey} = 18
Table A.2 Organic horizon biogeochemical parameters and trends with extent of surrounding tree mortality (n = 20 grey, 5 green).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U¹</th>
<th>Spearman ( \rho^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.97 (±0.11)</td>
<td>5.52 (±0.34)</td>
<td>3</td>
<td>-0.014</td>
</tr>
<tr>
<td>Water Content (% dry weight)</td>
<td>12.19 (±4.91)</td>
<td>21.53 (±5.65)</td>
<td>11</td>
<td>-0.104</td>
</tr>
<tr>
<td>DOC</td>
<td>43.81 (±27.08)</td>
<td>27.3 (±11.87)</td>
<td>32</td>
<td>-0.395</td>
</tr>
<tr>
<td>UV</td>
<td>0.96 (±0.31)</td>
<td>0.97 (±0.48)</td>
<td>43</td>
<td>-0.226</td>
</tr>
<tr>
<td>SUVA</td>
<td>2.54 (±0.64)</td>
<td>3.52 (±0.42)</td>
<td>11</td>
<td>0.393</td>
</tr>
<tr>
<td>Organic Matter (%)</td>
<td>5.31 (±1.01)</td>
<td>7.28 (±3.11)</td>
<td>29</td>
<td>0.522</td>
</tr>
<tr>
<td>Total Nitrogen (mg/L)</td>
<td>1.24 (±0.31)</td>
<td>1.48 (±0.54)</td>
<td>35</td>
<td>0.275</td>
</tr>
<tr>
<td>NO3- (mg/L as N)</td>
<td>0.35 (±0.14)</td>
<td>0.41 (±0.14)</td>
<td>37</td>
<td>-0.189</td>
</tr>
<tr>
<td>NH4+ (mg/L as N)</td>
<td>0.075 (±0.018)</td>
<td>0.337 (±0.55)</td>
<td>33</td>
<td>0.758</td>
</tr>
<tr>
<td>Organic Nitrogen (mg/L as N)</td>
<td>0.823 (±0.311)</td>
<td>0.735 (±0.249)</td>
<td>42</td>
<td>-0.256</td>
</tr>
<tr>
<td>NO3-/TN</td>
<td>0.292 (±0.128)</td>
<td>0.298 (±0.107)</td>
<td>45</td>
<td>-0.421</td>
</tr>
<tr>
<td>NH4+/TN</td>
<td>0.063 (±0.008)</td>
<td>0.177 (±0.203)</td>
<td>31</td>
<td>0.795</td>
</tr>
<tr>
<td>Organic N/TN</td>
<td>0.647 (±0.128)</td>
<td>0.525 (±0.154)</td>
<td>27</td>
<td>-0.504</td>
</tr>
<tr>
<td>C:N (molar ratio)</td>
<td>29.667 (±4.43)</td>
<td>22.135 (±7.177)</td>
<td>16</td>
<td>-0.780</td>
</tr>
<tr>
<td>ATP (pmols/g dry weight)</td>
<td>15.79 (±4.58)</td>
<td>20.09 (±9.07)</td>
<td>40</td>
<td>0.308</td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td>9.32 (±0.265)</td>
<td>9.906 (±0.283)</td>
<td>6</td>
<td>0.454</td>
</tr>
<tr>
<td>Observed OTUs</td>
<td>2196.2 (±212.2)</td>
<td>2605.1 (±289.2)</td>
<td>15</td>
<td>0.535</td>
</tr>
</tbody>
</table>

¹ Differences between green and grey phase trees. Bold if \( p < 0.05 \).
² Correlation between parameter under grey phase trees and surrounding tree mortality. Bold if \( p < 0.05 \).
Table A.3 Mineral horizon biogeochemical parameters and trends with extent of surrounding tree mortality (n = 31 grey, 7 green).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Green Average (SD)</th>
<th>Grey Average (SD)</th>
<th>Mann-Whitney U(^1)</th>
<th>Spearman rho(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.04 (±0.17)</td>
<td>5.52 (±0.31)</td>
<td>16.5</td>
<td>-0.045</td>
</tr>
<tr>
<td>Water Content (% dry weight)</td>
<td>8.61 (±3.18)</td>
<td>14.48 (±5.05)</td>
<td>32</td>
<td>-0.193</td>
</tr>
<tr>
<td>DOC</td>
<td>25.14 (±11.96)</td>
<td>16.46 (±7.9)</td>
<td>56.5</td>
<td>-0.106</td>
</tr>
<tr>
<td>UV</td>
<td>0.67 (±0.39)</td>
<td>0.54 (±0.26)</td>
<td>84</td>
<td>0.034</td>
</tr>
<tr>
<td>SUVA</td>
<td>2.7 (±0.57)</td>
<td>3.26 (±0.36)</td>
<td>57</td>
<td>0.379</td>
</tr>
<tr>
<td>Organic Matter (%)</td>
<td>3.39 (±1.57)</td>
<td>3.27 (±1.55)</td>
<td>92</td>
<td>-0.038</td>
</tr>
<tr>
<td>Total Nitrogen (mg/L)</td>
<td>0.95 (±0.17)</td>
<td>1.1 (±0.31)</td>
<td>71</td>
<td>0.156</td>
</tr>
<tr>
<td>NO3- (mg/L as N)</td>
<td>0.19 (±0.11)</td>
<td>0.24 (±0.08)</td>
<td>66.5</td>
<td>-0.022</td>
</tr>
<tr>
<td>NH4+ (mg/L as N)</td>
<td>0.057 (±0.015)</td>
<td>0.209 (±0.186)</td>
<td>45</td>
<td>0.436</td>
</tr>
<tr>
<td>Organic Nitrogen (mg/L as N)</td>
<td>0.696 (±0.096)</td>
<td>0.655 (±0.219)</td>
<td>74</td>
<td>-0.119</td>
</tr>
<tr>
<td>NO3-/TN</td>
<td>0.24 (±0.051)</td>
<td>0.223 (±0.082)</td>
<td>79</td>
<td>-0.167</td>
</tr>
<tr>
<td>NH4+/TN</td>
<td>0.177 (±0.093)</td>
<td>0.181 (±0.139)</td>
<td>55</td>
<td>0.406</td>
</tr>
<tr>
<td>Organic N/TN</td>
<td>0.545 (±0.07)</td>
<td>0.596 (±0.11)</td>
<td>27</td>
<td>-0.408</td>
</tr>
<tr>
<td>C:N (molar ratio)</td>
<td>29.7 (±9)</td>
<td>17.7 (±6.4)</td>
<td>32</td>
<td>-0.232</td>
</tr>
<tr>
<td>ATP (pmols/g dry weight)</td>
<td>0.19 (±0.12)</td>
<td>9.65 (±3.1)</td>
<td>101</td>
<td>-0.100</td>
</tr>
<tr>
<td>Shannon Diversity Index3</td>
<td>9.85 (±1.8)</td>
<td>9.527 (±0.679)</td>
<td>41</td>
<td>0.280</td>
</tr>
<tr>
<td>Observed OTUs3</td>
<td>9.2 (±0.388)</td>
<td>2407.9 (±502.9)</td>
<td>46</td>
<td>0.163</td>
</tr>
</tbody>
</table>

\(^1\)Differences between green and grey phase trees. Bold if p < 0.05.
\(^2\)Correlation between parameter under grey phase trees and surrounding tree mortality. Bold if p < 0.05.
\(^3\)n\(_{\text{green}}\) = 6, n\(_{\text{grey}}\) = 29