**Altered edaphic parameters couple to shifts in terrestrial bacterial community structure associated
with insect-induced tree mortality**

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Abstract

Unprecedented insect-induced tree mortality has been observed globally and while hydrologic and biogeochemical changes have been recorded, alterations to terrestrial microbial communities, which influence as well as respond to these shifts, are not well understood. The objective of this work was to better understand how bacterial communities are coupled to perturbations in biogeochemically-relevant soil physicochemical parameters resulting from beetle-induced tree death. To this end, soils beneath trees across a beetle-kill spectrum were contrasted in the central Rocky Mountains at both heavily impacted (Chimney, 85% mortality) and moderately impacted (Niwot, 18% mortality) field sites. Soil organic matter (OM), dissolved organic carbon (DOC) and dissolved organic nitrogen were all significantly altered when contrasting soils under healthy versus beetle-attacked trees at Chimney. Bacterial alpha diversity measurements were found to increase with tree death and beta diversity measures showed significant clustering with relation to tree phase. The site was characterized by a significantly higher relative abundance of bacterial clades under healthy trees that were correlated to OM and DOC concentrations. In contrast, compositional changes in soil bacterial communities and edaphic parameters associated with tree phase were not observed at the less impacted Niwot site. Our findings reveal a coupled response between shifts in organic carbon cycling and the bacterial assemblage as a result of large-scale, beetle-induced tree mortality with implications for heterotrophic respiration in near-surface soils and suggests a possible dependence on the level of forest mortality before manifestation.

Keywords: bark beetle, bacterial community, soil biogeochemistry, tree mortality, 16S rRNA gene sequencing

1. Introduction

Climate change is altering terrestrial ecosystems throughout the world. As a result of increased temperatures and drought, stressed forests are more susceptible to insect attack as evidenced by recent unprecedented tree mortality in North America, Europe, and Asia (Kurz *et al.*, 2008; Williams *et al.*, 2012). In extreme cases such as the Colorado region of the Rocky Mountains, forests are reaching close to 100% tree mortality which has resulted in significant alterations to hydrological and biogeochemical cycles (Morehouse *et al.*, 2008; Mikkelsen *et al.*, 2013a; Bearup *et al.*, 2014a). Decreased forest canopy and transpiration due to widespread tree die off (Mikkelsen *et al.*, 2013c) reduces soil water uptake and potentially increases groundwater recharge (MacDonald and Stednick, 2003; Bearup *et al.*, 2014a) and soil moisture (Morehouse *et al.*, 2008). Cumulative changes in canopy cover, litter fall, and tree respiration also alter incident radiation possibly increasing soil temperatures (Hais and Kučera, 2008). In concert with these biological responses, these processes have resulted in shifts to the terrestrial biogeochemical cycling of carbon (Kurz *et al.*, 2008; Mikkelsen *et al.*, 2013a; Mikkelsen *et al.*, 2013b), nitrogen (Clow *et al.*, 2011; Griffin *et al.*, 2011) and metal/metalloids (Bearup *et al.*, 2014b; Mikkelsen *et al.*, 2014). As recent studies have observed changes to microbial communities after forest disturbance (Hartmann *et al.*, 2012; Štursová *et al.*, 2014) and terrestrial microbial communities have been known to change when soil properties (Hackl *et al.*, 2004; Lauber *et al.*, 2008; King *et al.*, 2010) and tree-specific carbon inputs change (Urbanová *et al.*, 2015), the bark beetle infestation is a relevant scenario in which to investigate the coupled response of terrestrial bacterial communities and the associated edaphic parameters.

Near surface microbial communities mediate essential ecosystem functions such as carbon mineralization and subsurface cycling with their soil-surface CO₂ efflux exceeding emissions from fossil fuels by a factor of seven (IPCC, 2007). It has been demonstrated that historical land disturbances (fire, clear-cutting, fertilization, etc.) and the resultant terrestrial alterations are coupled to shifts in the prominent microbial communities within the perturbed ecosystem (Hart *et al.*, 2005; Hannam *et al.*, 2006; Chatterjee *et al.*, 2008; Hartmann *et al.*, 2012; Wertz *et al.*, 2012). However, the bark beetle presents a

unique disruption whose correlation to these other disturbances is not fully understood (Adams *et al.*, 2012). After death, the tree remains standing while the roots stop excreting carbon into the soil, which is a primary source of dissolved organic carbon (DOC) for subsurface microbial communities (Grayston *et al.*, 1997). Annual litterfall is also reduced followed by a pulsed drop of needles to the forest floor several years later that provides the heterotrophic microbial community with a different quantity and form of organic carbon (Kaňá *et al.*, 2012). Eventually the degradation of the tree roots and other woody material that has fallen to the ground provides another source of terrestrial carbon to below ground microorganisms. To this end, it is possible that alterations in bioavailable carbon, along with changes in biogeochemical and hydrologic inputs associated with beetle-induced tree mortality have the potential to shift microbial community composition and its associated function as seen in other systems (Hackl *et al.*, 2004; Lauber *et al.*, 2008; Harris, 2009).

Previous research has hypothesized that with the changing soil moisture and temperature regime, coupled to increased substrate availability after needle drop during a bark beetle infestation, microbial decomposition rates will increase (Edburg *et al.*, 2012; Hicke *et al.*, 2012). Surprisingly though, multiple studies have measured above ground carbon fluxes or soil respiration after an insect infestation and in the short term (~years after infestation) have observed either no change or a decrease in soil carbon efflux (Hancock *et al.*, 2008; Morehouse *et al.*, 2008; Nuckolls *et al.*, 2009). Few studies have quantified the number of heterotrophic bacteria after an insect infestation and to our knowledge it has only been reported after a phytophagous insect outbreak in a pine forest (in which the needles are consumed but the tree is not necessarily killed) (Streminska *et al.*, 2006) where they observed an increase in Actinobacteria and fungi due to a larger supply of easily degradable organic matter in litterfall.

In this study we hypothesized that shifts in both edaphic parameters and the terrestrial bacterial community could be correlated to beetle infestation; in essence, that the terrestrial ecosystem under a beetle-impacted tree would be distinct from one under a healthy tree. As recent investigation has shown increases in soil respiration and terrestrial CO₂ release with additional soil carbon inputs in a warming climate (Giardina *et al.*, 2014), it is imperative that we fully understand the response of soil microbial

communities in analogous scenarios such as climate-induced forest disturbances where terrestrial carbon inputs can be altered. A recent study in the Bohemian Forest mountain range in Central Europe observed significant changes in fungal biomass after spruce beetle infestation but saw smaller changes in bacterial biomass (Štursová et al., 2014). While that study performed a detailed analysis of fungal communities, it did not focus on structural changes in bacterial ecology. Research in North America found that despite increases in soil nitrogen pools after beetle-induced tree death of limber pines (*Pinus flexilis*), the bacterial community remained unaffected (Ferrenberg et al., 2014). Our study complements these recent investigations by comparing soil physicochemical parameters and bacterial community structure (using next-generation sequencing of the 16S rRNA gene) under lodgepole pines (*Pinus contorta*) across multiple time points in two North American bark beetle infested sites. Importantly, the two sites encompass varying levels of forest mortality with one site more severely impacted by beetle-induced tree death than the other (85% mortality compared to 18% mortality) with field sampling spanning the snow-free period of 2013. We ensured adequate sampling depth by temporally sampling composite soils under a carefully selected matrix involving the same green phase (healthy, transpiring trees), red phase (beetle-killed, no longer transpiring but retain needles) and grey phase (beetle-killed, dropped needles) trees at both sites.

2. Material and Methods

2.1 Field Sites and Sample Collection

Two montane, beetle-impacted sites were chosen for sample collection due to similar climates, geology and prior comparison studies (Biederman et al., 2014), but varying levels of bark beetle infestation. Though residing in different states, both sites are in the Central Rocky Mountains around 3000m in elevation with mean annual air temperatures of 1-3°C and precipitation around 800mm/yr. Both sites contained Inceptisol soils with weakly developed soil horizons. Additional soil characteristics for Niwot can be found in Xiong et al. (2011) and for Chimney in Mikkelsen et al. (2014) or Knight et al. (1985). To determine the local level of tree mortality (including all mature tree species), sampling areas

within the sites consisted of three 65'x20' rectangles. The first site, Niwot Ridge Long-Term Ecological Research Site (Niwot) in Colorado (40°1'58"N, 105°32'47"W) has been moderately impacted by bark beetles with a local site mortality of $18.2 \pm 4.5\%$ and a local tree density of 34 ± 12 trees per sampling rectangle. This led to many of the sampled dead trees being completely surrounded by alive and transpiring trees. The second, heavily infested site is located at Chimney Park (Chimney), Wyoming (41°40'82"N, 106°7'93"W) and had experienced over 75% tree mortality by 2011 (Biederman et al., 2014) with a local level of mortality within the sampling plots of $84.6 \pm 6.3\%$ and a local tree density of 44 ± 3 trees per sampling rectangle.

Samples were taken in the summer of 2013 from under lodgepole pine (*Pinus contorta*) trees at both sites; this was two years after the analogous 2011 Ferrenberg et al. (2014) sampling event of *Pinus flexilis* in a nearby section of Niwot. As trees begin to die from bark beetle attack, they undergo a temporal succession in which various hydrological and biogeochemical changes occur. This succession has been divided up into phases, which will be referenced throughout the study. The 'green' phase is considered to be when the tree is still alive, healthy and transpiring. The 'red' phase marks mortality, occurring within a year after a successful attack; at this point, the tree has ceased transpiration but still retains the majority of its needles (now red in color). The 'grey' phase occurs approximately 3-5 years after infestation at which point the tree has dropped its needles. Soil was sampled downslope within 1m of the trunk and from the top 5cm of the mineral soil layer with litter and visible organic material excluded (Ferrenberg et al., 2013; Ferrenberg et al., 2014). Soil samples were taken using a sterilized stainless steel spoon and 50ml conical tubes to sample horizontally from three soil core pits per tree (Li et al., 2012). To ensure a representative soil sample from under each tree, three boreholes were dug under each tree and ten samples collected per borehole, creating a homogenized 30 grab-sample composite per tree. Unlike Ferrenberg et al. (2014) where they sampled 50 random trees once during March of 2011, our soil samples for biogeochemical and microbial analyses were taken at both sites from under the same 3 green phase, 3 red phase and 3 grey phase trees at each sampling event with 1 green tree, 1 red tree and 1

grey tree per sampling rectangle as described above. This enabled us to explore seasonal variation in addition to beetle-impact with three distinct sampling events taking place at Niwot (June, July and August) and with two events at Chimney (June and August). Overall, each sampling event at each site contained $n = 9$ composite samples (3 green, 3 red and 3 grey) for a season total of $n = 27$ (9 green, 9 red and 9 grey) at Niwot and $n = 18$ (6 green, 6 red and 6 grey) at Chimney. Samples were then transported on ice back to the laboratory where approximately 25g of the soil was stored at -20°C until DNA extraction with the remaining soil being stored at 4°C prior to physicochemical analyses.

2.2 Soil Analysis

Archived soil was sieved to 2mm before all edaphic analyses. Soil samples were analyzed for pH, organic matter content (OM), moisture/water content (WC), dissolved organic nitrogen (DON) and DOC. pH was measured in a 0.01 M CaCl_2 solution (1:10). OM was determined by loss on ignition and gravimetric WC was measured immediately after field sampling by drying overnight at 105°C . DOC/DON were measured using a Shimadzu TOC-550A Total Organic Carbon analyzer after equilibrating 10g of soil in 40ml of 0.5M K_2SO_4 for an hour (Ferrenberg et al., 2014), filtering through a $0.45\mu\text{m}$ polyethersulfone filter and acidifying with phosphoric acid down to a $\text{pH}=2$.

2.3 DNA extraction and preparation

Individual soil samples were homogenized after removing larger sized pebbles and plant materials and DNA was extracted from 1g of homogenized soil using the PowerSoil DNA Isolation Kit (MO BIO laboratories) as specified by manufacturer. Quantitative PCR using Perfecta iQ SYBR Green Supermix and universal 16S primers (515F/806R) (Bergmann et al., 2011) were applied to quantify total Bacteria. The v4 hypervariable region of the 16S rRNA gene was amplified from the DNA extracts using Phusion® High-Fidelity DNA Polymerase Master Mix with a nearly universal bacterial and archaeal primer set (Caporaso et al., 2011) coupled to unique 12-bp error-correcting Golay barcodes on the reverse primer (Fierer et al., 2008). Triplicate amplifications were conducted for each sample and pooled before

bead purification using Agencourt® AMPure XP beads. Purified PCR products were quantified using a Qubit 2.0 fluorometer (Life Technologies), and approximately equal amounts of PCR products for each sample were pooled and sent to Biofrontiers at the University of Colorado for Illumina MiSeq 16S rRNA gene sequencing using a 2x250 V2 kit. The sequence data has been submitted to the NCBI SRA database under accession number SRP051109.

2.4 Sequence Analysis

Sequencing outputs were processed using the QIIME toolkit (v1.8.0) (Caporaso *et al.*, 2010) including demultiplexing, quality filtering and operational taxonomic unit (OTU) assignment. Forward and reverse Illumina reads were joined using the join_paired_ends.py script in QIIME. Reads were truncated after a stretch of three low-quality bases and reads shorter than 211 bp were discarded. Quality data was not obtained from four samples, which were discarded from analysis ultimately rendering n = 6, 5, and 5 at Chimney and n = 9, 8, and 8 at Niwot for green, red, and grey phase trees respectively. Reads were assigned to OTUs using UCLUST (Edgar, 2010) and taxonomy assigned using the RDP classifier v2.2 and the Greengenes v13.8 database filtered at 97% identity (DeSantis *et al.*, 2006). UCLUST was chosen for OTU picking as it is more universally used due to accessibility and open distribution and hence deemed better for comparing to other studies as opposed to UPARSE though debates on reproducibility and reliability for these different approaches continue (Schmidt *et al.*, 2014).

The resultant OTU table was rarefied so each sample contained 4283 reads (the smallest number in a given sample). All subsequent analyses were done on the rarefied OTU table except for differential abundance statistical tests. Recently, it has been suggested that normalizing rather than rarefying data is the best approach for clade differential abundance analyses as statistical power increases (McMurdie and Holmes, 2014). Along these lines, the unrarefied OTU table was normalized using the MetagenomeSeq v1.6.0 package (Paulson *et al.*, 2013) that implements a cumulative sum normalization method able to control for biases in measurements across taxonomic features and a zero-inflated Gaussian distribution mixture model that accounts for biases in statistical testing resulting from undersampling of the bacterial

community (Paulson et al., 2013). This normalized OTU table was then used for differential abundance analyses as describe below.

Alpha diversity metrics were calculated and plotted using the phyloseq package (v1.9.13) (McMurdie and Holmes, 2013) in R v3.1.0 (Team, 2008) using two different metrics: the Shannon diversity index and OTU richness. Shannon diversity increases as the number of species increases and the distribution of individuals among species becomes even whereas OTU richness looks at the number of individual OTUs found in each sample but does not take into account abundances. As alpha diversity can be sensitive to uneven sampling depths, all diversity measures were calculated from the rarefied OTU table. Weighted and unweighted UniFrac matrices (Lozupone *et al.*, 2010) were created in QIIME. It is important to take into account both the weighted and the unweighted UniFrac matrices as the weighted UniFrac matrix can be more informative for revealing community differences due to changes in relative abundances, particularly in nutrient-limited (or nutrient-altered) scenarios (Lozupone *et al.*, 2007) analogous to the changing carbon and nitrogen dynamics often observed after a bark beetle infestation (Mikkelsen et al., 2013a).

2.5 Statistical Tests

Differential abundance analyses were conducted on the normalized OTU table using the ‘fitZig’ test in MetagenomeSeq on all OTUs with a variance threshold greater than 30 (Paulson et al., 2013). Results focus on classified clades (unless at the OTU level) with a relative abundance greater than 1% in at least one phase and all p-values were adjusted for multiple comparisons using the false discovery rate (Benjamini and Hochberg, 1995).

ADONIS was used to compare microbial community composition under green, red and grey phase trees using both weighted and unweighted UniFrac distance matrices. Mantel tests were used to test for significant correlations between edaphic parameters and the total bacterial community composition under all tree phases at both sites using both the weighted and unweighted UniFrac distance matrices. All above-mentioned statistical tests were run in QIIME. Spearman correlations run in R were used to

determine correlation between the relative abundance of bacterial clades found to be significantly different between phases and edaphic parameters. Principal coordinate analysis was used to determine similarities among bacterial community composition under different tree phases using the phyloseq package in R (McMurdie and Holmes, 2013). The Friedman test was run in R for multiple sample comparisons of soil physicochemical parameters and alpha diversity followed by the post-hoc non-parametric Wilcoxon test with the Bonferroni's adjustment of the p-value. All p-values were considered significant if at or below the 0.05 threshold.

3. Results

3.1 Changes in soil edaphic properties

During the 2013 sampling, multiple edaphic parameters were distinct at the heavily impacted Chimney site (85% mortality) when contrasting soils beneath trees of different stages (Fig.1, Table S1). Friedman tests revealed significant differences in soil OM, DOC and DON (Fig.1, Table S1). The post-hoc Wilcoxon test showed significant differences between the green and red phase ($p = 0.022$) and red and grey phase ($p = 0.045$) for OM, the green and red phase ($p = 0.024$) and the green and grey phase ($p = 0.039$) for DOC, and the red and grey phase ($p = 0.041$) for DON. A trend was observed where soil OM, DOC, and DON each decreased with tree death (red phase) to approximately half that of healthy (green phase) trees followed by a potential rebound during the grey phase. In contrast at Niwot, the Friedman test only revealed a significant effect of tree phase on WC (Table S1); post-hoc tests did not reveal any significant differences when doing multiple comparisons between the different phases.

Interestingly, seasonal differences in soil physicochemical parameters were only found at Niwot and not at Chimney (Table S2). Friedman test results showed that sampling date had a significant effect on WC, TN and pH at Niwot. The post-hoc Wilcoxon test verified that there was a significant difference in WC and pH between the June and August sampling dates and a significant difference in pH between the July and August sampling dates. pH decreased from 4.57 ± 0.18 in June to 4.56 ± 0.24 in July to 4.30 ± 0.06 in August while WC increased from $10.4 \pm 1.6\%$ in June to $14.0 \pm 5.7\%$ in August. However, post-

hoc tests did not reveal any significant differences in TN soil concentrations with respect to sampling date when doing multiple pairwise comparisons.

Despite similar soil types at both sites, local soil physicochemical properties were not universally equivalent. When comparing the averages of all edaphic properties between these two sites, it was found that OM and pH did not vary significantly whereas WC was significantly higher at Chimney than at Niwot. In contrast, soil DOC and DON were both higher at Niwot than at Chimney (Table S3).

3.2 Taxon distribution and microbial diversity analysis

After joining and quality filtering the Illumina reads and removing those smaller than 211 bases, a total of 826,655 paired-end sequences were obtained from 42 samples. The number of sequences varied from 4,823 sequences to 48,485 sequences per sample and the average number of sequences was 19,682. All subsequent results except for differential abundance analyses are based on a rarefied OTU table with an even sampling depth of 4,283 sequences. At both sites, 66 different bacteria phyla were found; however, only 10 bacterial phyla were present at a relative abundance of 1% or greater (Fig. S1).

We found that overall bacterial community composition was significantly altered after beetle-induced tree mortality at Chimney but not at Niwot. Bacterial alpha diversity (both OTU richness and Shannon) was significantly higher in soils under grey phase trees at Chimney (Fig. 2). Friedman tests revealed a significant effect of phase on Shannon diversity at Chimney Park ($X^2 = 6.4$, $p = 0.041$) and the post-hoc Wilcoxon test revealed a significant difference between the green and grey phase ($p = 0.039$). Friedman tests also revealed a significant effect of tree phase on OTU richness ($X^2 = 6.4$, $p = 0.041$) and the post-hoc Wilcoxon test showed a significant difference between the green and grey phase ($p = 0.025$). PCoA of the soil samples based on their 16S rRNA gene sequences using weighted and unweighted UniFrac further revealed significant clustering as a function of tree phase at Chimney but not at Niwot (Fig. 3). ADONIS indicated a significant association between differences in bacterial diversity and tree phase with unweighted ($p = 0.003$, $R^2 = 0.19$) but not weighted UniFrac distance matrices ($p = 0.096$, $R^2 = 0.18$) at Chimney. Interestingly, tree phase did not significantly change alpha diversity or PCoA

clustering at Niwot. In addition, ADONIS tests verified that seasonality (*i.e.* sampling date) did not significantly alter bacterial community structure at either site.

As total bacterial community structure differed in association with tree death at Chimney, further analysis was conducted to investigate the individual bacterial clades that were driving this shift. Overall, 69 OTUs significantly differed between the green and grey phases at Chimney, five of which were above 1% relative abundance and 33 OTUs significantly differed between the green and red phases, four of which were above 1% relative abundance (Table 1). The prominent clades (above 1% relative abundance) that were significantly different under trees of different phases all decreased in abundance after tree death. These clades belonged to the Acidobacteria, Actinobacteria, Proteobacteria (Beta and Gamma), and Verrucomicrobia phyla. Of the rare clades (less than 1% relative abundance) that differed in association with tree death (grey phase), the majority increased in relative abundance after tree death (39 out of 64 clades); 18 of those were not present under green phase trees and presumably developed in association with tree decay (grey phase). The rare clades that were only present under grey phase trees typically belonged to the Proteobacteria (Gamma), OP3 and Verrucomicrobia phyla. For comparison purposes, the same differential abundance tests were run on all phase combinations at Niwot and no significant differences were found.

3.3 Links between microbial community composition and edaphic properties

When analyzing correlations between bacterial community structure and edaphic parameters, Mantel tests revealed significant relationships between both soil DOC and DON and bacterial communities at Chimney (Table S4 – unweighted UniFrac) while none were observed at Niwot. Interestingly and in accordance with Mantel tests, none of clades that significantly differed between phases at Chimney (Table 1) were significantly correlated with either pH or WC (Figures S2 and S3); however, all of the clades were positively correlated to OM content and DOC concentrations (Fig. 4 and 5), while three of the OTUs were significantly correlated to DON concentrations (Fig. S4).

4. Discussion

During and after bark beetle infestation, soil physicochemical parameters under impacted trees can change due to altered hydrobiogeochemical processes, which in turn can influence the cycling of carbon, nitrogen and metals (eg. Kurz et al., 2008; Kaňa et al., 2012; Mikkelsen et al., 2013a; Rhoades *et al.*, 2013). The controlled investigation of trees of different stages (which represent a 4+ year succession) across two sites with moderate versus extensive tree mortality over an entire snow-free season provided the opportunity to explore the evolution of soil physicochemical parameters and near surface bacterial communities without the complications of annual variations in temperature or precipitation. As there is a distinct temporal progression of biogeochemical and hydrologic changes that continue changing for decades after beetle infestation (Edburg et al., 2012), sampling two years after Ferrenberg et al. (2014) provides insight into the longer-term temporal ramifications of beetle-kill. At the heavily infested site (Chimney), the soils under beetle-impacted trees experienced significant changes in OM, DOC and DON concentrations with the succession of tree stages (Fig.1 and Table S1) but not sampling date (Table S2). In the Rocky Mountains, during most years the majority of snow melts in May and June increasing soil moisture with July and August being the hottest months of the year. It is possible that seasonal differences often seen in soil physicochemical parameters below the tree canopy (Xu and Qi, 2001; Chen *et al.*, 2003) were trumped by the influence of beetle-induced tree death. OM and DOC were highest in the green phase, suggesting that terrestrial carbon inputs from healthy trees trumped competing inputs from pulsed needle drop during the red to grey phase transition.

Soil organic matter can broadly be defined as the combination of litter input, microbial biomass and plant and animal derived organic compounds (Kuzyakov *et al.*, 2000; Weintraub *et al.*, 2007), whereas soil DOC is often highest in the spring and early summer indicating a strong influence of release from active root systems (Högberg and Högberg, 2002; Weintraub et al., 2007). Hence it would appear that for these lodgepole pines in the Colorado/Wyoming Rocky Mountains, organic carbon contributions from the green tree root system represent a large input to the subsurface and contrast sharply with the red phase cessation of root exudates. During the red phase, annual needle release ceases and most needles

remain attached to the tree. The soils under grey phase trees, recorded within 1-2 years after the needles have fallen, did have more OM and DOC than soils under red phase trees, suggesting that this litter pulse can increase soil organic carbon, but not to pre-infestation concentrations. This may be due to the decomposition of roots or the addition of other woody material to the forest floor as skeletal trees begin to degrade and fall down. Though shown to broadly correlate to microbial shifts in soils (King et al., 2010), pH did not appear to change under beetle-impacted trees. While initially counterintuitive when considering the acidic nature of needles, this observation is consistent with other studies that have independently measured soil chemical properties beneath beetle-killed trees in Arizona and Central Europe (Morehouse et al., 2008; Štursová et al., 2014).

At the moderately infested site (Niwot), beetle infestation did not appear to alter edaphic properties; a result similar to what was observed at an adjacent site exploring soil biogeochemical properties beneath limber pines two years prior (Ferrenberg et al., 2014). With approximately 18% mortality, the majority of trees surrounding red and grey phase trees were not beetle-killed; hence a possible explanation is that bulk soils beneath the red and grey phase trees did not experience effects from a larger-scale loss of canopy and cumulative changes in root exudate and litter input. The theory that a ‘threshold canopy gap’ must be surpassed before soil biogeochemical processes are impacted has been studied in relation to forestry practices (Parsons *et al.*, 1994; Prescott *et al.*, 2003) but to the best of our knowledge has not yet been equated to microbial profiles nor bark beetle infestations. While we recognize that there are differences between the impacts of tree harvesting and bark beetle infestation, some analogies can be drawn due to similar changes in canopy cover and the cessation of key ecological processes such as transpiration (Adams et al., 2012). Prior work in a lodgepole pine forest where the median tree age was 95 years old (similar to the age at which trees are most susceptible to bark beetle attack) found that the threshold number of tree harvests in an 8m radial plot to incur a change in soil nitrogen biogeochemical transformations was 15-30 trees (Parsons et al., 1994). A subsequent study found that soil nitrate concentrations did not change beneath single-cut trees and only began to change when harvested tree gaps were established that were larger than 0.1 ha (Prescott et al., 2003). With this in

mind, we suggest that in order to witness physicochemical and microbiological changes beneath beetle-killed trees, a particular threshold of beetle-killed trees is needed. Alternatively or in concert, it is also possible that the higher DOC and DON present at Chimney could also play a role.

Microbial populations can be sensitive to changes in local edaphic properties, particularly carbon inputs, following various forms of forest disturbance (Hackl et al., 2004; Lauber et al., 2008). As rhizodeposits of carbon cease following tree mortality in a coniferous forest, the driver for rhizospheric heterotrophic microbial respiration switches from a more labile (Grayston et al., 1997) to a more refractory and recalcitrant organic carbon associated with decomposing needle litter (Gholz *et al.*, 2000) and other terrestrial inputs. However, the extension of the rhizosphere's influence on bulk soil microorganisms is not well understood. It has been hypothesized that filamentous fungi are able to carry the legacy of rhizospheric inputs and plant traits to the bulk soil (Urbanová et al., 2015) suggesting that we might witness changes to bulk soil microorganisms as rhizospheric processes shift in association with beetle-kill. In addition to carbon inputs, soil moisture (Schimel *et al.*, 1999) and temperature (Fierer *et al.*, 2003) which would be altered as the tree canopy decreases have also been shown to be drivers of microbial diversity supporting the overarching hypothesis that microbial populations would change after a large-scale beetle infestation. However, a recent study has shown that despite beetle infestation, the bacterial community beneath beetle-killed trees is not significantly different than the community beneath healthy trees (Ferrenberg et al., 2014) and our findings at our moderately-impacted adjacent site support those results. Bacteria have been known to be resilient and less responsive to forest disturbance than fungal populations (Hartmann et al., 2012; Štursová et al., 2014), which supports our observations at Niwot. This makes the contrast between Niwot and Chimney all the more interesting as to our knowledge the present study is the first to show a bacterial response in bulk soils associated with beetle-induced tree mortality.

Notably, our study reveals that bacterial community composition (Fig. 2 and 3) is different under beetle-infested trees when compared to healthy trees in a conserved watershed, and this ecological succession is coupled to edaphic parameter changes. Interestingly, alpha diversity (representative of the

number of species and/or distribution of individuals among species) also increased in soils under beetle-killed trees in this scenario (Fig. 2). A possible explanation is that community diversity increased as bacterial populations able to decompose more recalcitrant organics (*i.e.* from needles as opposed to root exudates) increased in relative abundance while populations associated with rhizospheric processes are still present but metabolically dormant (Stenström *et al.*, 2001; Lennon and Jones, 2011; Yarwood *et al.*, 2013). Alternatively, increased diversity could enhance the metabolic degradation potential of the microbial community and has been witnessed as a function of exposure to refractory versus more bioavailable DOC in saturated aquifer sediments in both the laboratory and field (Li *et al.*, 2012; Li *et al.*, 2013). As the total bacterial community composition changed (Fig. 3) and became more diverse (Fig. 2) beneath beetle-impacted trees, particular clades were found as the primary contributors to structural differences between tree phases (Table 1). Clades within the Actinobacteria, Acidobacteria, Proteobacteria, and Verrucomicrobia phyla were all found to significantly differ between tree phases and decrease in relative abundance with tree death (Table 1). Hence, as alpha diversity increased in association with tree death and these six abundant clades (at the OTU level) appear to decrease in numbers with tree death, it is probable that the increase in diversity is due to the introduction of rare clades (39 clades with less than 1% relative abundance increased after tree death) and the distribution of individuals evening out across clades.

While pH has been shown as a primary driver of bacterial community composition across various landscapes (Janssen, 2006; Will *et al.*, 2010; Heiko Nacke *et al.*, 2011), in absence of its change, other factors such as soil OM and DOC content appear to play a large role in driving bacterial community composition (Pfeiffer *et al.*, 2013). Despite the differences in soil DOC levels between Niwot and Chimney (Figure 1 and Table S3), the pre-beetle or green phase microbial communities were similar in composition (Figure S1) suggesting that the observed progression of DOC levels after beetle attack at Chimney (Figure 1) and not the difference in soil characteristics between sites is responsible for the observed microbial shift at Chimney. The species found to be significantly more abundant under alive, green phase trees at Chimney belonged to the *Acidobacteriaceae*, *Burkholderiaceae*, *Mycobacteriaceae*,

and *Sinobacteraceae* families, all of which belong to phyla whose functionalities are associated with carbon cycling. Acidobacteria can comprise up to 19% of the rhizosphere bacterial community under lodgepole pines (Chow *et al.*, 2002) and are often more abundant in rhizosphere soils with low organic availability (Fierer *et al.*, 2007) and less abundant in soil amended with low molecular weight organic carbon (Eilers *et al.*, 2010) that is representative of root exudates (Strickland *et al.*, 2010). Interestingly, our study revealed that *Acidobacteriaceae* positively correlate to OM and DOC content in soils from Chimney and were more abundant under healthy trees (Fig. 4 and 5). This could be due to the fact that while collected near the root zone, our samples were from the bulk soil and less influenced by changes in rhizospheric processes. However, the correlation to OM and soil DOC further supports the importance of soil carbon input to bacterial community response, and suggests this family of bacteria might grow more readily on carbon derived from root exudates as opposed to litter inputs. Along the same lines, clades within Proteobacteria (Janssen, 2006; Heiko Nacke *et al.*, 2011) and Actinobacteria are often correlated to changes in carbon availability (Fierer *et al.*, 2007; Goldfarb *et al.*, 2011; Pfeiffer *et al.*, 2013). Proteobacteria are the dominant bacteria in the rhizosphere of lodgepole pine trees (Chow *et al.*, 2002) and multiple studies have observed an increase in γ -Proteobacteria (Fierer *et al.*, 2007; Eilers *et al.*, 2010) and β -Proteobacteria (Goldfarb *et al.*, 2011) in response to the addition of more labile, low molecular weight organic carbon compounds (Padmanabhan *et al.*, 2003; Cleveland *et al.*, 2007), along with an increase in the abundance of the *Mycobacteriaceae* family of the Actinobacteria phylum. These findings are in accordance with the results of this study, as clades within both β and γ -Proteobacteria (specifically the *Sinobacteraceae* and *Burkholderiaceae* families) and Actinobacteria decreased in relative abundance in association with tree death (when rhizodeposits of C have ceased and more recalcitrant C inputs are common).

In addition to many microbial groups decreasing in abundance with tree death, there were 39 different clades at Chimney that significantly increased in abundance from the green to grey phase; however, their relative abundances were under 1%. Of these clades, many belonged to the *Rhodospirillaceae* family in the α -Proteobacteria class, the *Opitutus* genus of the Verrucomicrobia phyla,

and the Sphingobacteriales order of the Bacteroidetes phyla. Members of the *Rhodospirillaceae* family are often found in moist terrestrial environments with an autotrophic dependence on CO₂ as a carbon source (Johnson and Henderson, 1995). As WC did increase (although not significantly) under grey phase trees at Chimney it is possible that the wetter conditions contributed to an increase in the abundance of this family. *Rhodospirillaceae* are also known for their nitrogen fixation abilities (Madigan *et al.*, 1984) and their abundance could be influenced through alterations in nitrogen cycling which has been observed beneath beetle-killed trees (eg. Griffin *et al.*, 2011; Edburg *et al.*, 2012; Mikkelsen *et al.*, 2013a). *Opitutus* have also often been observed in anoxic terrestrial environments (Schlesner *et al.*, 2006), similar to what might be found in high moisture conditions under grey phase trees. In addition, strains of *Opitutus* have been successfully grown on plant structural polysaccharides (amorphous cellulose and pectin) (Schlesner *et al.*, 2006) indicating that they could play a role in the degradation/decay of beetle-kill woody debris. Bacteria related to Sphingobacteriales have been found capable of assimilating a wide variety of root exudates (el Zahar Haichar *et al.*, 2008), are more often found in agricultural soils as compared to pine forests (Kuramae *et al.*, 2012) and are associated with a decreasing C:N ratio (Kuramae *et al.*, 2012). In the grey phase at Chimney, the decrease in canopy cover and C:N ratio could have created conditions more conducive to species within this order.

5. Conclusions

Changes in microbial populations have been investigated as indicators of disturbance as they often respond faster and are potential drivers of soil biogeochemical parameters (Smith *et al.*, 2008). However, a recent study in the Colorado Rockies has shown that bacterial communities are able to resist perturbation and maintain their processes after beetle infestation of limber pines (Ferrenberg *et al.*, 2014), and our study at an adjacent field plot (Niwtot) parallels this observation for the lodgepole species while drawing a contrast with another more heavily impacted site in the region (Chimney). This suggests that there may be a threshold level of disturbance needed in order for bulk soil bacterial communities and thus their processes to shift as both edaphic parameters and the bacterial footprint changed at that site with

~85% beetle-induced tree mortality. As our study did not sample rhizospheric soils, future studies are needed to determine mechanistically whether or not rhizospheric inputs and processes extend beyond the rhizosphere to impact microbial populations in bulk soil under the tree canopy. It is also possible that there are larger changes occurring to the metabolically ‘active’ microbial community throughout a bark beetle infestation and it would be insightful to compare the progression of the metabolically ‘active’ microbial community to the progression of the ‘inactive’ or dormant microbial community during and after a bark beetle infestation.

On a larger scale, recent findings have shown that in a warming climate, additional carbon inputs (such as could be found from beetle-killed tree decay) will not lead to increased soil organic carbon storage but rather to increased soil respiration and CO₂ release into the atmosphere (Giardina et al., 2014) providing a positive feedback to global warming. Given the current extent of tree mortality, it is therefore important to determine if the additional carbon input from the decay of millions of beetle-killed trees will be rapidly decomposed, released as CO₂ into the atmosphere and/or transported through the subsurface hydrologic pathways as opposed to accumulating in the subsurface soil organic carbon stores. With respect to aqueous transport, the implications of altered carbon decomposition extend to our domestic water supply, where bark beetle infestations have been shown to detrimentally impact water quality as the organic carbon entering water treatment facilities in beetle-killed watersheds appears to have changed in composition and is increasing disinfection byproduct levels (Mikkelsen et al., 2013b). Further understanding of how terrestrial heterotrophic respiration and the capabilities, evolution, and rebound of microbial communities will respond to climate-induced landscape perturbations could enable us to prepare for these types of larger scale ramifications.

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Table and Figure Legends

Table 1: Differential abundance tests of significance for taxa with greater than 1% abundance between green and grey and green and red phase trees at Chimney Park.

* Only clades found significantly different between tree phases are listed; unknown clades were not tested except at the OTU level. Values under the tree phase are relative abundance and based off of the normalized OTU table. All listed p-values were adjusted using the Benjamini-Hochberg correction (false discovery rate) for multiple comparison testing.

Figure 1: The measured edaphic parameters under green, red and grey phase trees with the dots representing outliers. OM = soil organic matter (%), WC = water content (%), DOC = dissolved organic carbon (mg/l), DON = dissolved organic nitrogen (mg/l). Parameters that were statistically different between phases are indicated with a star. Data and statistical test results associated with this figure can be found in Supplementary Table S1.

Figure 2: Alpha diversity metrics of green, red and grey phase trees at Chimney Park and Niwot Ridge. Whisker lengths correspond to the 1.5*IQR (interquartile range) above and below the third and first quartile respectively and points represent outliers.

Figure 3: Principal coordinate analysis based on weighted (A,B) and unweighted (C,D) UniFrac distances showing the change in bacterial community composition under green, red and grey phase trees at the two field sites: Chimney Park (A and C) and Niwot Ridge (B and D).

Figure 4: The relationship between soil organic matter content and the relative abundance of OTUs that significantly differed between phases at Chimney Park. The p-values were generated using the Spearman

731 test of correlation along with the underlying rho statistical value and were considered significant if at or
732 below the 0.05 threshold.

733

734 **Figure 5:** The relationship between soil dissolved organic carbon concentration and the relative
735 abundance of OTUs that significantly differed between phases at Chimney Park. The p-values were
736 generated using the Spearman test of correlation along with the underlying rho statistical value and were
737 considered significant if at or below the 0.05 threshold.

738 **Table 1:** Differential abundance tests of significance for taxa with greater than 1% abundance between green and grey and green and red phase
739 trees at Chimney Park.

Level	Clade	p-value: Green - Grey	p-value: Green - Red	Relative Abundance		
				Green	Red	Grey
Phylum	Actinobacteria	0.006	NS	6.1	3.1	3.1
Class	Actinobacteria; Actinobacteria	0.014	0.047	3.8	1.5	1.4
Order	Actinobacteria; Actinobacteria; Actinomycetales	0.023	NS	3.8	1.5	1.4
Family	Acidobacteria; Acidobacteriia; Acidobacteriales; Acidobacteriaceae	0.031	NS	5.6	2.1	2.2
	Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae	0.042	NS	1.8	0.8	0.8
	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Sinobacteraceae	0.040	NS	6.4	2.4	2.8
Genus	Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia	0.033	NS	1.8	0.8	0.8
Species: OTU level	Acidobacteria; Acidobacteriia; Acidobacteriales; Acidobacteriaceae; unknown; denovo131590	0.028	0.047	2.0	0.7	0.7
	Acidobacteria; Acidobacteriia; Acidobacteriales; Acidobacteriaceae; unknown; denovo64498	0.011	0.043	1.2	0.4	0.4
	Actinobacteria; Actinobacteria; Actinomycetales; Mycobacteriaceae; Mycobacterium; denovo102623	0.029	0.020	1.1	0.3	0.5
	Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia; denovo68170	0.029	NS	1.5	0.5	0.5
	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Sinobacteraceae; unknown; denovo83393	0.037	NS	5.6	2.0	2.3
	Verrucomicrobia; Methylocidiphilae; Methylocidiphilales; unknown; unknown;denovo37640	NS	0.047	1.1	0.4	1.1

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742 * Only clades found significantly different between tree phases are listed; unknown clades were not tested except at the OTU level. Values under
743 the tree phase are relative abundance and based off of the normalized OTU table. All listed p-values were adjusted using the Benjamini-Hochberg
744 correction (false discovery rate) for multiple comparison testing.

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Figure 1

Chimney

Niwot

Figure 2

Figure 3

Figure 4

Figure 5