

Aquatic Microbial Community Structure and Function Across a Gradient of Logging, Fire, and
Industrial Watershed Disturbances

by

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Abstract

The role of microbial communities in the recovery of aquatic ecosystems from watershed disturbance has received little attention despite their important role in energy and nutrient cycling. This study investigates the structure and function of microbial communities on a standardized substrate (alder leaves) in small streams across a wide gradient of watershed disturbances. Microbial communities exhibited variation with disturbance regime with lower hydrolase enzyme activities at all disturbed streams compared to undisturbed streams, and the lowest rates of microbial decomposition, fungal biomass, and differences in microbial community composition at the most severely disturbed streams. Forest and wetland cover were identified as important watershed features that provide DOC to fuel microbial activity in aquatic ecosystems. Increasing road density within the watershed was identified as having a negative impact or association on microbial activity that appeared to be linked to inputs of inorganic solutes that were measured through increased levels of specific conductance in stream water samples. This study is one of the first of its kind and it provides some important evidence that leaf litter associated microbial communities can be influenced by factors linked to watershed disturbance and as such may be useful as indicators of watershed disturbance and potentially the state of recovery of aquatic ecosystems.

Keywords: Land-water linkages, fungi, bacteria, microbial decomposition, microbial ecology, watershed disturbance, aquatic ecosystem recovery

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List of Abbreviations

Abbreviation	Meaning
DOM	Dissolved organic matter
DOC	Dissolved organic carbon
α -G	α -glucosidase
β -X	β -xylosidase
LAP	Leucine aminopeptidase
PHOS	Phosphatase
POX	Phenol oxidase
PER	Peroxidase
CQI	Carbon quality index
HIX	Humification index
PMA	Propidium monozone
OTU	Operational taxonomic unit
LWD	Large woody debris
AICc	Second order Akaike information criterion
CCA	Canonical correspondence analysis
VIF	Variance inflation factor

Chapter 1

1 General Introduction

Microbial communities in aquatic ecosystems provide essential ecosystem services through the decomposition of organic matter, and the cycling of energy and nutrients (Azam *et al.*, 1983; Bärlocher, 2005; Gessner *et al.*, 2007). In aquatic environments streams are actively metabolizing systems that provide a corridor for the incorporation and transfer of allochthonous materials into both the stream and near shore littoral zones (Figure 1) (Fisher *et al.*, 2004), and allochthonous sources of leaf litter and dissolved organic matter (DOM) are important sources of energy to aquatic organisms in forested low-order streams (Wallace *et al.*, 1997; Mulholland, 1997). In order to better understand the aquatic ecosystem and the recovery of aquatic ecosystems from disturbance, more research on stream microbial communities that process this important allochthonous organic matter is needed.

Studies looking at the contribution of microbial communities, namely fungal and bacterial members, to leaf litter decomposition have found microbial communities to be responsible for around 10-30 % of a leaf's total mass loss in freshwater streams (Hieber & Gessner, 2002; Kreutzweiser *et al.*, 2008), and 35-50 % in nutrient enriched streams (Pascoal & Cassio, 2004). Fungi rapidly colonize submerged leaf litter and initiate the biotic breakdown of leaf litter in streams (Gessner *et al.*, 2007). Fungal decomposition of leaf litter releases fine particulate organic matter and DOM into the aquatic ecosystem, and plays an important role in the conditioning of leaf matter for further decomposition by stream invertebrates. This conditioning renders the leaf matter more palatable, and increases its nutritional value as fungi degrade the

more complex molecules, and enrich the leaf matter with nutrients (Bärlocher, 1985; Suberkropp, 1992; Graça, 2001). The breakdown of complex molecules by fungi also assists bacteria by providing more readily available products, which enhances bacterial growth and reproduction (Mille-Lindblom & Tranvik, 2003). However, fungi and bacteria have also been shown to have an antagonistic relationship, as bacterial communities can have a negative effect on fungi by out-competing the fungi for substrate (Mille-Lindblom & Tranvik, 2003; Romani *et al.*, 2006). Fungi are the main microbial contributors to the initial break down of leaf litter comprising of 88-99.9 % of the microbial biomass on decomposing leaves, and while bacterial communities do contribute to the breakdown of coarse particulate leaf litter, they play a greater role in the processing of fine particulate organic matter and DOM (Mille-Lindblom *et al.*, 2006; Gessner *et al.*, 2007).

Fungal and bacterial extracellular enzymes are required to breakdown high molecular weight compounds into compounds that are small enough to cross the cell membrane and enter the cell for energy generation and cell growth (Chróst, 1991). Extracellular enzymes play a key role in the processing of allochthonous particulate organic matter and DOM (Azam *et al.*, 1983; Kirchman *et al.*, 2004; Brookshire *et al.*, 2005). Fungi and bacteria together produce enzymes capable of breaking down plant and animal derived compounds such as lignin, cellulose, hemicellulose, pectin, proteins, lipids, starch, and other aromatic compounds (Gessner *et al.*, 2007; Sinsabaugh *et al.*, 2008). These extracellular enzymes contribute to the decomposition of leaf litter in streams, and the processing of DOM.

DOM is an important subsidy to aquatic food webs, providing energy and nutrients for microbial communities and ultimately higher organisms such as aquatic plants, invertebrates and fish (Pace *et al.*, 2004; Petrone, 2008). DOM is also important to habitat conditions having the ability to

bind metals (McGeer *et al.*, 2003), influence light availability and temperature (Snucins & Gunn, 2000), and decrease UV penetration (Scully & Lean, 1994). The quantity and quality (ie. bioavailability and chemical composition) of DOM that enters a stream can be affected by watershed characteristics such as riparian vegetation, soil type, hydrology, disturbance (Petrone, 2007a; Petrone, 2007b), as well as wetland cover, urbanization and agriculture (Dillon & Molot, 1997a; Dillon & Molot, 1997b; Williams *et al.*, 2010). Changes to the quantity and quality of DOM have in turn been found to influence microbial community structure (Eiler *et al.*, 2003; Roiha *et al.*, 2011) and the use of DOM by microbes (Kawahigashi *et al.*, 2004).

Along with alterations to the quantity and quality of DOM there are many other important aquatic habitat characteristics that can be influenced by changes in the watershed. For example, watershed disturbance such as forest cover removal (i.e. fire and logging activity), and acidification and metal deposition (i.e. industrial activity) can influence aquatic organisms through changes to water chemistry and hydrology (Carignan & Steedman, 2000; Kreutzweiser *et al.*, 2008), temperature (Johnson & Jones, 2000), and particulate organic matter quantity and transport (Wesolek *et al.*, 2010; Szkokan-Emilson *et al.*, 2011; Tanentzap *et al.*, 2013). Time since disturbance is also an important factor when considering water chemistry recovery and the recovery of aquatic organisms from disturbance. Carignan and Steedman (2000) found that the time it takes for chemistry to return to a pre-disturbance state can vary across studies and is dependent on many factors, and in acidified and metal contaminated water bodies it has been shown that even after water chemistry improves biological recovery is not immediate (Keller *et al.*, 2007; Wesolek *et al.*, 2010).

Studying the response of aquatic organisms to disturbance is ideal not only for observing changes in aquatic communities and the resulting implications for aquatic ecosystem health, but

also for developing a better understanding of the process of aquatic ecosystem recovery.

Reclamation and monitoring strategies have tended to focus on the response and integrity of macroorganisms such as stream invertebrates (E.g, Wesolek *et al.*, 2010; Szkokan-Emilson *et al.*, 2011) given their sensitivity, importance in the aquatic ecosystem, and relative ease at which they can be collected and characterized (Rosenburg & Resh, 1993). However, given the known importance of microorganisms such as fungi and bacteria to the cycling of energy and nutrients within the aquatic ecosystem (Figure 1-1), the response of microbial community function and structure to watershed disturbance may also be important, and remains an important area for investigation.

Recent advances in DNA sequencing techniques have allowed for the rapid characterization of microbial communities (Shokralla *et al.*, 2012), and these sequencing techniques can be used to better characterize microbial communities associated with leaf litter decomposition and the processing of DOM (Findlay, 2010). Along with the better characterization of stream microbial communities, more work is needed to understand how changes in the aquatic environment influence microbial community composition and the essential ecosystem services that these microbial communities provide (Findlay, 2010). Using next generation sequencing techniques and a range in watershed disturbance types, from undisturbed, to logging and fire disturbed, to industrially disturbed streams, the aim of my study is to better describe stream microbial communities associated with leaf litter in low-order boreal shield streams, and to look for signs of recovery in microbial communities that are linked to changes in the watershed. The characterization of the abundance and composition of both bacterial and fungal members of the leaf associated microbial communities in combination with ecosystem level functional measures

provides a more detailed characterization of leaf litter associated microbial communities, and their potential role in aquatic ecosystem recovery. The specific objectives of my study are to:

- Describe and compare the structure and function of microbial communities across a gradient of low to high watershed disturbance (Chapter 2a).
- Explore the resulting implications for energy and nutrient cycling in the aquatic ecosystem (Chapter 2b).
- Assess which watershed and habitat characteristics are most associated with differences in microbial community structure and function (Chapter 3).

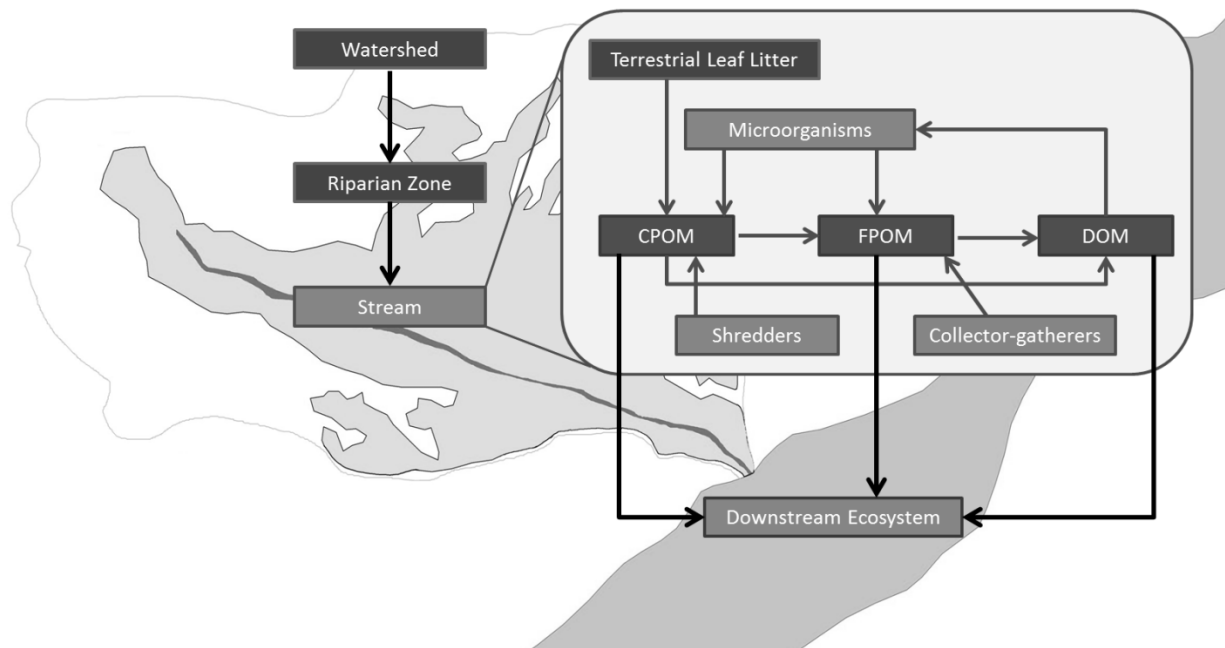


Figure 1-1 Summary of the role of microorganisms in the cycling of energy and nutrients in the aquatic ecosystem (based on Bärlocher, 1985 and Kirchman, 2012)

Chapter 2

2 Microbial Community Structure and Function Across a Gradient of Logging, Fire, and Industrial Watershed Disturbances

2.1 Abstract

Leaf litter associated microbial communities are known to perform key ecosystem functions, but rarely have the microbial communities themselves been assessed as indicators of ecosystem health. Streams in the Sudbury and White River areas that covered a wide range of logging, fire, and industrial watershed disturbance were examined to assess differences in leaf litter associated microbial community structure (i.e. microbial biomass, and fungal and bacterial community composition) and microbial community function (i.e. microbial leaf litter decomposition, and microbial extracellular enzyme activities). Microbial communities at industrially disturbed streams were found to be the most impacted, with the lowest rates of microbial leaf litter decomposition, a significantly decreased fungal biomass, a fungal community with a greater abundance of taxa from the class Eurotiomycetes, and a unique bacterial community composition with key taxa from the genera *Pseudomonas*, *Luteibacter*, *Amantichitinum*, *Burkholderia*, and *Enterobacter* missing or in very low abundance. Additionally, both industrially and logged and fire disturbed streams had overall lower hydrolase enzyme activities, and a higher ratio of aromatic compound to simple compound degradation when compared to undisturbed streams suggesting a slower cycling of energy and nutrients even years after logging, fire, and industrial watershed disturbance.

2.2 Introduction

Stream microbial communities play an important role in the cycling of energy and nutrients in aquatic ecosystems via the breakdown of particulate organic matter (Bärlocher, 2005; Gessner *et al.*, 2007) and the processing of DOM (Azam *et al.*, 1983). Due to their key functional roles studies have started to explore the response of fungal and bacterial communities to aquatic ecosystem disturbance. For example, studies have found that increased stream temperatures and nutrient enrichment can increase microbial decomposition (Martínez *et al.*, 2014; Fernandes *et al.*, 2013; Gulis & Suberkropp, 2003; Pascoal & Cássio, 2004; Gulis *et al.*, 2006), while increased metal concentrations can reduce microbial decomposition (Duarte, Pascoal & Cassio, 2004; Duarte *et al.*, 2008). Recent studies have also found microbial extracellular enzyme activities to increase with increasing temperatures, decrease outside of an optimal pH range, and to respond to changes in elevated nutrient concentrations (Cunha *et al.*, 2010; Hill *et al.*, 2012).

Along with the use of microbial functional measures, studies have attempted to characterize the fungal and bacterial members that colonize decomposing leaf litter using estimates of microbial biomass (e.g. Findlay & Arsuffi, 1989; Pascoal & Cássio, 2004; Gulis *et al.*, 2006; Lecerf & Richardson, 2010), and characterization of fungal and bacterial communities using coarse methods such as DGGE (denaturing gradient gel electrophoresis) and tRFLP (terminal restriction-fragment length polymorphism) (e.g. Das *et al.*, 2007; Duarte *et al.*, 2010; Kominoski *et al.*, 2011; Fernandes *et al.*, 2013). Although these techniques are valid, next generation sequencing provides higher resolution of the microbial community structure and has proven useful for the identification of microbial communities in environmental samples (Shokralla *et al.*, 2012). A more detailed characterization of microbial communities in environmental samples may help to explain how microbial communities can contribute to stream ecological processes and

responses by better linking microbial community structure to the essential ecosystem services that microbial communities provide (Findlay, 2010).

In this study, I therefore used microbial communities collected on standardized leaf litter in streams across a gradient of watershed disturbance and recovery to assess high resolution structural characteristics (i.e. fungal and bacterial biomass, and community composition using next generation sequencing) along with functional characteristics (i.e. microbial decomposition, and extracellular enzyme activities). Using these structural and functional microbial characteristics across different disturbance types ranging in disturbance severity the objectives were to: 1) assess major differences in microbial communities between disturbance types, and 2) explore the resulting implications for energy and nutrient cycling in the aquatic ecosystem.

2.3 Materials & Methods

Study streams

A total of 24 low-order streams were used for this study. The selected streams were of 3 types: 1) streams that have been largely undisturbed for at least the last 50 years, 2) streams impacted by fire or logging 7-15 years earlier, or 3) streams in an industrial area recovering from the effects of severe metal deposition and acidification over approximately 30-35 years, with a 90 % reduction in deposition in recent years (Gunn and Keller, 1990). Undisturbed (n=6), logged (n=5), and fire (n=7) streams were located near White River, Ontario and industrial streams (n=6) were located in Sudbury, Ontario. Three of the 6 industrial streams were designated industrial/urban because they were located closer to the city center, and as a result, experienced urban as well as industrial disturbance. All of the streams were located on the boreal shield, and surrounded by mixedwood forest (varying proportions of white and black spruce (*Picea glauca*,

Picea mariana), balsam fir (*Abies balsamea*), white, red, and jack pine (*Pinus* spp.), trembling aspen (*Populus tremuloides*), and white birch (*Betula papyrifera*)).

Grab samples of water from each stream were collected on 1 or 2 occasions during leaf pack incubation and were analyzed for selected chemical parameters (i.e. DOM concentration measured as dissolved organic carbon (DOC), dissolved inorganic carbon, nutrients, cations and metals) in the Canadian Forest Service water chemistry laboratory following standard methods (Beall *et al.*, 2001). Temperature and pH were measured in the field with a HI991003 portable meter and HI 1296 probe (Hanna Instruments, Rhode Island, USA), and conductivity was also measured in the field with a Primo 3 TDS Tester (Hanna Instruments, Rhode Island, USA) during leaf pack incubation. Two grab samples were taken from the Sudbury streams and the data averaged for use in statistical analysis, but only 1 grab sample was taken for each stream in White River. Stream habitat surveys were performed on a 50 m reach surrounding the leaf packs in the summer of 2010 for White River streams, and the summer of 2012 for Sudbury streams. Physical habitat characteristics were collected including depth, the abundance of large woody debris ($/\text{m}^2$), sedimentation rates ($\text{g}/\text{day}/\text{m}^2$), and percent organic matter in the sediment. Large woody debris was characterized as being > 10 cm in diameter and > 1 m in length. To calculate sedimentation rates deposited sediment was collected in each stream at approximately 10 cm from the stream bottom in 7 upright falcon tubes held in position by a brick. After collection the sediment was dried and weighed to calculate the rate sediment deposited per unit area (i.e. surface area of the tube opening). The collected sediment was combusted at $500\text{ }^{\circ}\text{C}$ for 2 h to estimate the amount of inorganic and organic material present.

Collection of microbial communities & functional characterization

Fine mesh (0.5 mm) leaf packs containing alder leaves (*Alnus incana*) were incubated in the study streams for 6 weeks during the summer of 2012 to collect microbial communities and measure microbial decomposition rate. Alder leaves were selected because Alder is a common tree species found by the streams in this study. All the alder leaves used in these leaf packs were collected at the same time and from the same trees. Each leaf pack contained 3 sections. The first section contained 1 leaf for estimation of the microbial decomposition rate based on percent mass loss. The leaves used for estimation of microbial decomposition were first leached for 24 h, cut into a 15 mm diameter disc, air-dried (30 °C for 48 h), and then weighed prior to in-stream incubation. The other 2 mesh bag compartments contained 8 and 10 leaves for characterizing microbial community structure and potential extracellular enzyme activity. Six replicate leaf packs were used at each site.

After collection leaves from the first compartment were gently washed, dried and re-weighed to calculate mass loss of that time period. The fine mesh prevented grazing by macroinvertebrates and therefore can be used to estimate only the effects of microbial decomposition. Leaves from the second compartment were stored immediately in a -20 °C freezer prior to DNA extraction, fungal biomass estimates, and extracellular enzyme activity assays, and leaves from the third compartment were immediately stored in 2 % formaldehyde in a -4 °C fridge prior to flow cytometry bacterial cell counts.

The activity of the microbial communities was measured using the extracellular enzyme activities of 6 commonly measured enzymes including: the hydrolases α -glucosidase (α -G), β -xylosdiase (β -X), leucine aminopeptidase (LAP), and phosphatase (PHOS), and the oxidases phenol oxidase (POX) and peroxidase (PER) (Table 2-1). These enzyme activities were

measured under controlled conditions (i.e. same pH, temperature, and buffer solution) using engineered substrates. Therefore, the extracellular enzyme activities were a measurement of potential activity and the concentration of extracellular enzyme found in each sample rather than in situ extracellular enzyme activity. Potential hydrolase and oxidase extracellular enzyme activities were measured based on existing protocol (Saiya-Cork *et al.*, 2002; Findlay, 2007). Briefly 6 replicates from 6 different leaf packs per stream were prepared by mixing 0.5 g of leaf material with 60 mL of acetate buffer for 30-60 s. The sample slurry was then placed in a petri dish and mixed each time before dispensing into 200 uL microplate wells. Dark hydrolase microplates were set up with a negative control (acetate buffer and substrate), standard (acetate buffer and MUB/ MUC standard), buffer (acetate buffer), quench (MUB/ MUC standard and sample), and blank (sample and acetate buffer) to account for fluorescence not produced by the transformation of substrate by extracellular enzymes. Assays (i.e. wells containing sample and substrate) contained 8 replicates per sample. For oxidases, the plate set up contained 2 negative control columns (acetate buffer and substrate), a buffer (acetate buffer), a blank for each sample (sample and acetate buffer), and 8 wells per sample for both POX and PER assays. Fluorescence for hydrolases and absorbance for oxidases were read on a BioTek Synergy H1 Hybrid Reader (BioTek, Vermont, USA). Fluorescence was read using an excitation 365 nm, and emission of 450 nm. Absorbance was read through a 460 nm filter. For hydrolases, 10 uL of 0.5 M NaOH was added to each well after the 4 h incubation period to raise the pH above 7.5 and allow for a fluorescence reading to be obtained. Fluorescence was measured exactly 1 min after NaOH addition because fluorescence has been found to increase with time after addition of NaOH (German *et al.*, 2011). The extinction coefficient was calculated for absorbance assays (Appendix-1), and time trials were performed to measure fluorescence and absorbance at 0, 30,

60, 90, 120, 150, 180, 210, 240 min to identify the optimal incubation time for each enzyme. Based on the enzyme activity over time, the optimal incubation time for all enzymes was 4 h (Appendix-2). Potential enzyme activity data was then used to calculate the total sum of potential hydrolase activity for each site, and C:N and N:P ratios (Sinsabaugh *et al.*, 2009). The Carbon Quality Index (CQI) was also calculated based on the ratio of labile carbon acquisition to recalcitrant carbon acquisition (Sinsabaugh & Follstad Shah, 2011; Hill *et al.*, 2012). In this study CQI was calculated using the ratio of hemi-cellulose degradation (i.e. β -X activity) to lignin and aromatic compound degradation (i.e. POX activity).

The quantity of DOM was measured as DOC concentration in the water samples taken at each stream. Additionally, fluorescence scans measured DOM quality across a range of excitation from 225 nm to 450 nm, reading emissions from 300 nm to 600 nm. The resulting excitation emission matrices were then used along with other samples in a unique parallel factor analysis (PARAFAC) to identify key structural characteristics of the DOM based on operationally defined DOM pools. The PARAFAC model was developed by Szokan-Emilson *et al.* (2014) and included 1216 samples from Boreal lakes, streams and rivers. In total 6 fractions were identified including 2 fulvic (C1, C2), 2 humic (C3, C4) and 2 protein-like components (Tyrosine-like C5, Tryptophan-like C6). Ratios of summed fulvic, humic and protein components for the 24 streams in this study are presented. The Humification Index (HIX) was also calculated from excitation emission matrices (Zsolnay *et al.*, 1999).

Bacterial and fungal biomass & community composition

Flow cytometry was used to count the bacterial cells present on each incubated leaf sample. Each sample was vortexed and subsampled into a 15 mL tube, taking 10 mL of liquid and ¼ of 1 leaf (approx. 0.1g). To detach bacteria from the leaf matter without damaging the cells samples were

first sonicated with an ultrasonic probe (Fisher Scientific Sonic Dismembrator 500) connected to a 13 mm tip at a low setting of 15 % for 4x20 seconds with 5 second breaks (Buesing & Gessner, 2002). The sample was then vortexed and a 1 mL subsample taken. Finally, the sample was centrifuged for 10-12 seconds at 1000 x g and the supernatant removed. Samples were then processed following the manufacturer's protocol for the Invitrogen Bacteria Counting kit for Flow Cytometry (Invitrogen, California, USA). Cell counts were performed on a BD FACSCanto II flow cytometer (BD Biosciences, USA), and the results gated in FlowJo 10 software (FlowJo, Oregon, USA). Green fluorescence (FL1) was collected at 530 ± 30 nm. The specific gain setting was 505 nm at a low speed to ensure counts did not exceed 1000 cells/ s. Cells that had a fluorescence signal of less than 10^2 FSC were excluded along with cells that were larger than the microbeads (approx. 6 nm), to remove background noise and larger organisms such as algae and eukaryotes from the counts. This may have also excluded some small and large bacterial cells from the counts. Gates to count cells were set and then adjusted for each sample manually. Results were then converted from cells/ mL to cells/ g according to the approximate amount of leaf matter analyzed. Cell counts were also converted into a rough estimate of biomass (mg/ g) using a conversion factor of 58 fg/ cell as calculated by Frossard *et al.* (2012) on stream bacterial communities.

To estimate fungal biomass, the incubated leaves were cut into 2 sets of 5 disks using an 11.2 mm diameter cork borer. The first set was dried at 60 °C for 48 h and then weighed for dry mass. The second set was submerged in 0.14 M KOH-methanol for 10-12 h. The ergosterol extractions were then performed following protocol from Gessner (2005). Briefly, samples were heated at 80°C for 30 min and cooled. Lipid content was then extracted onto an SPE cartridge and eluted into a vial to be analyzed for ergosterol concentration using reverse-phase high performance

liquid chromatography on a HP1100 liquid chromatograph (Agilent, Hewlett Packard Canada Ltd., Mississauga, Ont.). Ergosterol concentration was then used to calculate fungal biomass based on the final extraction volume, leaf dry mass and a conversion factor for hyphomycetes of 5.5 mg/ g (Gessner & Chauvet, 1993). A control leaf spiked with ergosterol standard was included with each set of samples to calculate extraction efficiency. The average extraction efficiency was 74 % (± 10 %). Bacterial biomass (mg/ g) was then used in combination with fungal biomass to calculate the ratio of fungal to bacterial biomass present on the leaf matter in each stream.

DNA was extracted from incubated leaf samples using the MO BIO PowerSoil DNA Isolation kit and protocol (MoBio, Carlsbad, USA). The following alterations were made to the protocol. Samples were first treated with Propidium Monozide (PMA, Biotium, California, USA), a photo reactive binding dye that alters exposed DNA from dead cells preventing it from being extracted and identified (Nocker *et al.*, 2006). A total of 50 μ M of PMA was added to each sample containing 0.2 g of leaf and 1.5 mL of sterile water. Samples were then left in the dark for 5 min, followed by 2 x 2 min exposure to a 500 W halogen light. The samples were vortex briefly before and in between light treatments. The samples were then centrifuged for 10 min at 5000 x g and the supernatant taken for DNA extraction. Extracted DNA samples were sent to MR.DNA's sequencing facility (Molecular Research and Testing LP) in Shallowater, Texas for 16S and 18S rRNA gene pyrotag sequencing. Pyrotag sequencing was done on the Roche 454 platform following Dowd *et al.* (2008) protocol.

Qiime (Caporaso *et al.*, 2010) was used to process raw sequence data. A summary of sequence statistics is included in Appendix-3. Sequences were first filtered for low quality and ambiguous reads using Qiime defaults (min quality score = 25, min/ max length= 200/ 1000). The

USEARCH package (Edgar, 2010) was then used to perform denovo and reference based chimera checking using UCHIME (Edgar *et al.*, 2011), cluster sequences at 97 % similarity, and remove singletons. After representative sequences were selected taxonomy was assigned to 16S Operational taxonomic units (OTUs) using GreenGenes database 13/5 with the RDP classifier (Wang *et al.*, 2007), and 18S sequence taxonomy was assigned using the Silva database 111 with a re-trained RDP classifier. All assignments were made with a minimum confidence of at least 0.8. OTU biome tables were generated using Qiime default settings.

Data analysis

All statistical analyses were performed in R 3.0.1 (R Core Team, 2013). The study involved a nested design with 5-7 streams nested in each disturbance type and 6-7 replicates nested in each stream. Rather than taking stream averages to make comparisons between disturbance types for functional and structural measures, a simple mixed model of a nested analysis of variance was used. A mixed model is necessary for a nested design because it allows for variation explained by known random variables (i.e. variation resulting from differences between streams within disturbances) to be accounted for, while examining the variation explained by a fixed variable (e.g. mass loss between disturbance types), and avoids inflated degrees of freedom and pseudoreplication. Mixed models were run in the package lme4 (Bates *et al.*, 2014), and Tukey's Honest Significant Difference (HSD) post hoc tests run in the package multcomp (Hothorn, Bretz & Westfall, 2008) when significant differences were found. For measures of DOM quality, and bacterial and fungal diversity a simple one-way ANOVA was performed as only one sample was analyzed per stream, followed by Tukey's Honest Significant Difference (HSD) when required. Variables were transformed using logarithmic and square root transformations to meet normality assumptions when necessary.

Community analyses for bacterial OTUs were performed on a rarefied dataset (Appendix-4) to remove differences in community composition based on uneven sampling depth. For the fungal dataset further filtering was required because the 18S rRNA gene region targeted by the sequence primers is not fungi specific, and so other eukaryotes were also sequenced. After rarefaction of the complete 18S rRNA dataset (Appendix-4) non-fungal OTUs were filtered out and relative values for fungal abundance were used for all further analyses. Overall patterns in community composition for both fungi and bacteria were examined by ordination using detrended correspondence analysis in the vegan package (Oksanen *et al.*, 2013) to see if communities clustered by disturbance. Diversity and richness were also calculated based on the Shannon index and the number of OTUs present in each sample. Bar plots and heat maps were used to look more closely at patterns of OTU occurrence and abundance across disturbance types. Bar plots for fungal OTUs were built in the gplots package (Warnes *et al.*, 2013), and a heat map for bacterial OTUs was built using the Hmisc (Harrell & Dupont, 2013) package. For the heat map the rarefied bacterial dataset was first filtered for low abundance and low frequency. Specifically, only OTUs that occurred at ≥ 3 streams for at least one disturbance type, with a percent abundance of $\geq 1\%$ for at least one of the occurrences were kept. Filtering by disturbance for OTU frequency and abundance allowed for the visualization of taxonomic differences within the dominant community across disturbance types.

Co-occurrence networks were used in the visualization of bacterial community composition. Co-occurrence networks were based on strong ($r \geq 0.7$), and significant ($p \leq 0.01$) correlations between OTUs across streams, and showed any shared community composition across disturbance types. The rarefied bacterial data set ($n = 5000$), was first filtered to remove very low abundance ($< 0.1\%$), and low frequency OTUs (occurring at < 3 streams). Checkerboard

analysis was then run using the bipartite package (Dormann, Gruber & Fruend, 2008) to test for non-random patterns of co-occurrence in the data set based on the C-score (Stone and Roberts 1990). Spearman's correlations were then run using the Hmisc package (Harrell & Dupont, 2013), and the matrix filtered for strong ($r \geq 0.7$), and significant ($p \leq 0.01$) correlations setting all others to zero. The matrix was then converted to a network object using the igraph package (Csardi & Nepusz, 2006), and viewed in the program Gephi (Bastian, Heymann & Jacomy, 2009).

Results from the co-occurrence network were also combined with results from indicator species analysis. Indicator species analysis is based on the frequency and abundance of each OTU across streams and was used to identify any strong and significant associations between each OTU and streams from each disturbance type. Indicator species analysis was run using the indicpecies package (De Caceres & Legendre, 2009) with 1000 permutations. Industrial/urban and industrial streams were grouped into one industrial category to explore associations based purely on industrial disturbance. OTUs in the co-occurrence network were then labelled by any strong and significant associations with a disturbance type. Strong and significantly disturbance associated fungal and bacterial OTUs were also noted on their own as potential indicators of disturbance. Potential bacterial indicators were combined into a phylogenetic tree built in Qiime (Caporaso *et al.*, 2010) using Muscle alignment (Edgar, 2004).

2.4 Results

Stream habitat

Water depth was similar across streams from different disturbance types and ranged from 0.1 (± 0.03) to 0.16 (± 0.11) m. However, there were differences in some other key stream habitat characteristics between undisturbed, logged, fire, and industrially disturbed (i.e. industrial and industrial/urban) streams. The abundance of large woody debris for example was slightly greater

on average in streams with logging and fire disturbance, slightly lower in streams with industrial disturbance, and 3 times lower in streams with industrial/urban disturbance (Table 2-2). A 2-3 times higher sedimentation rate was also found at logged, fire, and industrial streams, while industrial urban streams had a sedimentation rate 10 times higher when compared to undisturbed streams. Higher sedimentation rates were coupled with increasing inorganic matter content of the sediment. Water chemistry also varied across disturbance types. In particular, logged streams had slightly lower temperatures, and higher conductivity about 2 times the conductivity of undisturbed streams. Fire streams also had conductivity about 2 times higher than undisturbed streams, but a larger range in temperature from 10.4-19.4 °C. Industrial streams in the Sudbury area were marked by increased metals (i.e. Ni, Zn, Cu) and temperature, slightly decreased DOC concentrations, and a 1 unit decrease in pH from undisturbed streams. Industrial/urban streams also showed increased metals and temperature, and decreased DOC concentrations, but additionally had a 3-4 times increase in nutrients (i.e. TN and TP), and a 12 times increase in conductivity when compared to undisturbed streams (Table 2-2).

Microbial community function

The average percent mass loss (i.e. microbial decomposition) of the leaf disks across streams ranged from 0.1- 29.2 % ($0-0.0095 \text{ k day}^{-1}$). There were no statistically significant differences in percent mass loss between disturbance types, but overall there was a general decreasing trend from undisturbed to industrial streams. Logged streams had a slightly higher average rate of decomposition compared to undisturbed streams, fire a slightly lower average decomposition rate, and industrial/urban and industrial streams the lowest average decomposition rate (Figure 2-1).

POX and PER exhibited the greatest potential activities of all of the enzymes measured (Figure 2-2). Enzyme activities for combined hydrolase activities (i.e. β -X + α -G + LAP + PHOS) were lower in logged, fire, and industrially disturbed streams than in undisturbed streams. β -X enzyme activities were significantly lower under all disturbances, except industrial streams, when compared to undisturbed streams (Table 2-3). α -G, LAP, and PHOS activities were not significantly different between undisturbed, logged and industrial streams, but were significantly lower in fire and industrial/urban impacted streams (Table 2-3). For the oxidases (i.e. POX and PER) there were no significant differences in average activity across disturbance types. However, on average POX activities tended to be higher for logged, fire, and industrial streams when compared to undisturbed and industrial/urban streams, while PER activities tended to be higher for logged and fire streams and lower for industrial and industrial/urban streams when compared to undisturbed streams. The ratio of labile to recalcitrant carbon acquisition (i.e. CQI) revealed a lower CQI at all disturbance types when compared to undisturbed streams (Figure 2-2). There were no significant differences in carbon and nutrient limitations across disturbance types based on the C:N and N:P potential extracellular enzyme ratios (Figure 2-2).

The quantity and quality of DOM varied across streams. Industrial/urban and industrial streams had a lower concentration of DOC of $5.7 (\pm 1.89)$ and $6.9 (\pm 2.81)$ mg/ L respectively compared to undisturbed, logged and fire streams which had average DOC concentrations of $10.3 (\pm 3.09)$, $9.1 (\pm 2.84)$, and $9.54 (\pm 4.24)$ mg/ L respectively (Table 2-2). Although not statistically significant, there appeared to be differences in the quality of the DOM pool across disturbances. HIX values showed a decreasing trend across disturbance types with a higher average HIX value for logged streams, and a lower HIX value for fire, industrial/urban and industrial streams (Figure 2-3b). DOC concentration was positively correlated with HIX across the streams in this

study ($r = 0.55$, $p\text{-value} = < 0.01$). Ratios of humic, fulvic, and protein PARAFAC components suggested further differences in DOM quality across disturbances. Logged streams were very similar in composition to undisturbed streams, fire streams tended to have higher humic to fulvic signal, and industrial/urban and industrial streams had relatively higher proportions of protein-like compounds reflected in higher protein to humic and protein to fulvic ratios (Figure 2-3).

Bacterial and fungal biomass & community composition

Bacterial biomass was similar across disturbance types with the exception of industrial/urban streams (Figure 2-4) which had significantly higher biomass compared to all other disturbances (Table 2-3). Bacterial biomass was found to be positively correlated with conductivity ($r = 0.75$, $p = < 0.001$), N ($r = 0.5$, $p = < 0.05$), and P ($r = 0.41$, $p = < 0.05$), but not significantly with temperature ($r = 0.4$, $p = > 0.05$) across streams. Fungal biomass at industrial/urban and industrial streams was significantly lower compared to all other streams (Figure 2-4, Table 2-3).

Regardless of differences in fungal and bacterial biomass, fungal biomass made up the vast majority (99.95-99.99%) of total microbial biomass (i.e. fungal + bacterial biomass) on leaves across all streams.

A total of 14348 unique OTUs of bacterial taxa were identified. The total number of unclassified OTUs at the phylum level was 1209 (8%). All of the classified OTUs were identified at $\geq 80\%$ confidence, of which 10037 (76%) were identified at $\geq 90\%$ confidence using the GreenGenes database. Alphaproteobacteria was the most dominant class and Proteobacteria was the most dominant phylum across all disturbances (Figure 2-5).

Although total bacterial diversity was not significantly different across disturbance types (Figure 2-6, Table 2-3), overall bacterial community composition at industrial and industrial/urban

streams was found to be unique with significantly different OTU presence and abundance, when compared to undisturbed, logged, and 5 of the 6 fire stream bacterial communities (Figure 2-7). Bacterial communities from undisturbed and logged streams were most similar in overall bacterial community composition, and bacterial communities from 4 of the 6 fire streams were also more similar to undisturbed bacterial communities (Figure 2-7). The differences in bacterial community composition at industrial/urban and industrial streams were largely due to the absence or low abundance of key bacterial taxa (Figure 2-8). These taxa include 20 OTUs from the genera *Pseudomonas*, *Luteibacter*, *Amantichitinum*, *Burkholderia*, and *Enterobacter* (Table 2-4). OTUs from the genera *Pseudomonas*, *Luteibacter*, and *Amantichitinum* on average comprised of > 1 % of undisturbed, logged and fire bacterial communities, and < 0.1 % of industrial/urban and industrial bacterial communities (Table 2-4). *Burkholderia*, and *Enterobacter* comprised > 1 % of the bacterial community at undisturbed streams, with abundances comprising < 1 % across all other disturbance types (i.e. logged, fire, industrial/urban, and industrial) (Table 2-4).

From co-occurrence network analyses two distinct clusters of co-occurring bacterial OTUs were identified. Initial checkerboard analysis confirmed non-random co-occurrence patterns in the data set (C-score=19.33, P-value=<0.01), and overall the co-occurrence network consisted of 897 nodes (ie. OTUs), and 2466 edges (i.e. strong and significant correlations). The two clusters of OTUs consisted of co-occurring taxa from the families Pseudomonadaceae, Enterobacteriaceae, Xanthomonadaceae, and Neisseriaceae (Figure 2-9a). Combining network analysis with results from indicator species analysis showed that the predominant co-occurring taxa at all other streams were largely missing from industrial and industrial/urban streams (Figure 2-9a, b).

There were potential bacterial indicators of disturbance for each disturbance type. The most significantly associated OTUs defined at the Family level for each disturbance type, were as follows: Neisseriaceae and Rhodospirillaceae for undisturbed streams, Hyphomicrobiaceae and Solirubrobacteraceae for logged streams, an unclassified OTU for fire streams, and Micromonosporaceae and Oxalobacteraceae for industrial and industrial/urban streams (Figure 2-10).

Of the 2349 unique OTUs identified 1083 (46 %) of the OTUs were classified as fungal, 799 (34 %) other eukaryotes, and 467 (20 %) unclassified. All classified fungal OTUs were identified at a ≥ 80 % confidence level, of which 688 (64 %) were identified at a ≥ 90 % confidence level. Of the total rarefied dataset (n=1030) fungal sequences made up an average of 50- 72 % of sequences across disturbance types.

Fungal community composition showed no significant differences between disturbance types (Figure 2-11). The average number of fungal OTUs increased at all disturbances when compared to undisturbed streams. Logged, fire, industrial/urban, and industrial fungal OTUs increased by 4.2 %, 15.3 %, 34.7 %, and 11.8 % respectively (Table 2-5). When evenness and richness were considered together using the Shannon diversity metric, slight increases in average diversity occurred for only logged and industrial/urban fungal communities (Figure 2-12), but these increases were not statistically significant. Across all disturbance types fungal communities were dominated by the phylum Ascomycota (Figure 2-13). Although differences in overall community composition were not detected in the detrended correspondence analysis, some differences in fungal taxa across disturbance types were apparent at the class level for fire, industrial/urban, and industrial fungal communities in contrast to undisturbed and logged streams. In particular, fungal communities at fire streams contained the fungal group LKM11 in greater abundance than all

other streams, and the class Eurotiomycetes was found in greater abundance at both industrial/urban and industrial streams (Figure 2-13). Seven potential fungal indicators of industrial disturbance (i.e. industrial/urban and industrial streams) were the only indicators of disturbance type identified based on strong (statistic ≥ 0.8) and significant (p-value < 0.01) OTU associations. Six of the OTUs associated with industrial streams were from the class Eurotiomycetes and either the genera *Aspergillus* or *Penicillium*, and the last industrial associated OTU was an unidentified environmental fungus (Table 2-6).

2.5 Discussion

Microbial community function across disturbance types

Across the disturbance types ranging from logging to industrial/urban disturbed streams there was a decrease in at least one key aspect of microbial community function when compared to undisturbed streams, suggesting that even years after disturbance the microbial communities at the base of the aquatic food web are still recovering. Microbial decomposition at streams with logging and fire disturbed watersheds seemed to be less impacted compared to microbial decomposition at streams with industrially disturbed watersheds, agreeing with the greater signs of disturbance reflected in industrial stream habitat characteristics. Average decomposition rates for logged and fire streams were very similar to average decomposition rates for undisturbed streams with the exception of one logged and one fire stream that were outliers. The logged stream outlier had a much higher decomposition rate when compared to all other streams. An elevated decomposition rate at this logged stream could be due to elevated levels of N at this stream (0.683 mg/ L), as previous studies have found that increased nutrients can stimulate decomposition (Gulis & Suberkropp, 2003; Gulis *et al.*, 2006). An industrial/urban stream was the only other stream with a higher N value, but this stream did not have a higher decomposition

rate probably due to high metal concentrations that have been found to decrease microbial activity, and overall decomposition in other stream ecosystems (Niyogi *et al.*, 2001; Carlisle & Clements, 2005). The fire stream outlier had a low decomposition rate, low nutrient concentrations (N 0.0051 mg/ L, P 0.151 mg/ L) and a low temperature of 10.4 °C. Martínez *et al.* (2014) found that a decrease in water temperatures can also slow decomposition rates. Therefore, combined low nutrient concentrations and low temperatures could help to explain the low decomposition rate at the fire outlier stream. The consistently slower rate of microbial leaf litter decomposition in industrial/urban and industrial streams could slow the cycling of energy and nutrients within these aquatic ecosystems, and the growth and development of organisms up the food web (Bärlocher & Kendrick, 1975; Suberkropp *et al.*, 1976).

Overall microbial decomposition rates for alder leaves at undisturbed streams were similar to previous studies done in the same region (Kreutzweiser *et al.*, 2008), but on the lower end of what other studies have found (Hieber & Gessner, 2002; Feio *et al.*, 2010; Duarte *et al.*, 2010). Lower decomposition rates could have been due to location on the boreal shield with typically lower temperatures and nutrient concentrations. However, lower values could also be explained by slight differences in the methodology to calculate microbial decomposition of leaf litter. Firstly, in this study and Kreutzweiser *et al.* (2008) initial leaf weight was taken after a 24 h leaching period excluding mass loss that occurred as a result of the initial leaching process from microbial decomposition calculations. Pre-leaching of fallen, air-dried leaves is a standard practice because physical leaching that occurs after emersion in water can contribute up to 30% of a leaf's total mass loss (Bärlocher, 2005). Previous studies that leached for a shorter time period potentially track microbial decomposition plus varying degrees of the mass loss attributed to initial leaching (Feio *et al.*, 2010; Duarte *et al.*, 2010). Secondly, some studies such as Hieber

and Gessner (2002) calculated microbial contribution to decomposition based on microbial biomass instead of actual leaf mass loss. Hieber and Gessner (2002) mention that this could lead to an over estimate of microbial decomposition as bacteria could be taking up DOM instead of contributing to leaf litter decomposition.

Similar to decomposition rates the total potential hydrolase enzyme activities (i.e. β -X + α -G + LAP + PHOS) were lower at industrially disturbed streams. However, despite the apparent severity of industrial disturbance, logged and fire disturbed streams also had overall lower potential hydrolase enzyme activities compared to undisturbed streams. An overall decrease in hydrolase activities at logged, fire, and industrially disturbed streams could influence the cycling of C, N, and P within the aquatic ecosystems and the aquatic food web (Arnosti, 2003).

A higher activity of aromatic compound degradation (i.e. POX and PER potential activities), compared to the hydrolysis of less complex compounds (i.e. hydrolase potential activities) found across all the streams in this study and in other stream ecosystems (Frossard *et al.*, 2012; Hill *et al.*, 2012) suggests that leaf litter stream microbial communities are well adapted for the breakdown of aromatic DOM. Aromatic compounds are predominantly of terrestrial origin because land plants require more complex molecules, such as lignin, for structural support (McKnight *et al.*, 2001). Allochthonous organic matter is known to be an important subsidy to forested stream ecosystems (Kaushik & Hynes, 1968; Fisher & Likens, 1973), and microbial communities attached to substrate in streams have been found to have a greater response in activity to terrestrial rather than in-stream DOM sources (Kreutzweiser & Capell, 2003). A higher activity of aromatic compound degradation across streams suggests that despite watershed disturbance leaf litter associated microbial communities are still largely dependent on terrestrial inputs.

Although microbial communities at all streams showed higher activity in aromatic compound degradation compared to simple compound degradation, a lower ratio of potential simple to complex carbon compound acquisition (i.e. CQI) for microbial communities at logged and fire streams when compared to undisturbed streams may suggest that leaf litter microbial communities in logged and fire disturbed streams depend more heavily on terrestrial inputs than those in undisturbed streams. This greater focus on aromatic compound degradation may be a reflection of the DOM pool delivered to streams from those watersheds (Sinsabaugh & Follstad Shah, 2011). On average, DOM at logged streams had a higher HIX value compared to undisturbed streams indicative of a more humified, or aromatic (terrestrial origin) DOM pool. At fire streams there was a slight decrease in HIX compared to undisturbed streams, but there was also an increase in the proportion of humic to fulvic compounds. HIX measured the overall composition of both humics and fulvics, while PARAFAC analysis distinguished between humics and fulvics for a more detailed description of the DOM pool. Humic compounds contain a greater proportion of aromatic groups, while fulvics are a product of plant and animal remains and contain more fatty acids (Aitkenhead-Peterson *et al.*, 2003). At fire streams a higher humic to fulvic ratio suggests that the DOM pool may in fact be more aromatic in nature. Therefore, compared to undisturbed streams it appears that in logging and fire disturbed streams there was a greater availability of terrestrial DOM to microbial communities, coupled with a greater microbial focus on the utilization of terrestrial DOM.

However, at industrial/urban and industrial streams the DOM pool was more dominated by protein-like compounds despite a potentially greater focus on complex carbon degradation when compared to undisturbed streams. The relationship between the DOM pool and microbial community enzyme activity may not always be straight-forward. Studies have found that the

quality and quantity of DOM can influence the structure and function of microbial communities (Eiler *et al.*, 2003; Roiha *et al.*, 2011), while microbial community processing also influences the quantity and quality of the DOM pool (Wipfli *et al.*, 2007). Describing this relationship in a lotic system is very complex, with spatial and temporal variation as DOM enters and is processed while it moves downstream (Wipfli *et al.*, 2007). It could be speculated however, that the apparent disconnect between aromatic DOM focused enzyme acquisition, and a more protein dominated DOM pool at industrial/urban and industrial streams could be explained by industrially disturbed watersheds that have little soil organic matter and low DOM exports to streams. DOC concentration and HIX (i.e. relative amount of humic matter in DOM pool) were positively associated across streams, so that when DOC concentrations decreased so too did HIX. At industrial/urban and industrial streams there was a lower concentration of DOC and HIX. An increased protein signal in the DOM pool could be due to the measurement of actual microbial biomass and extracellular enzymes, which are rich in protein, and in higher concentration than the comparatively low available DOC.

Bacterial and fungal structure across disturbance types

The characterization of microbial community structure across disturbance types can help explain differences in microbial community function, and lend to the exploration of microorganisms as indicators of ecosystem health. Across the gradient of disturbance and recovery bacterial biomass on leaf matter was similar except for at industrial/urban streams. At industrial/urban streams there was a significant increase in bacterial biomass that could be coupled with increased nutrients. Nitrogen, and P concentrations at industrial/urban streams were elevated and ranged from 0.358-3.89 mg/ L, and 0.016-0.038mg/ L, respectively. Previous studies have found increased bacterial biomass on leaves to be associated with increased nutrients (Gulis &

Suberkropp, 2003; Pascoal & Cassio, 2004), and in this study bacterial biomass was positively correlated with N, and P across streams. Additionally, conductivity ranging from 22.7-1020.3 ppm/ C was also positively correlated with bacterial biomass. Therefore, it appears that increased bacterial biomass at industrial/urban streams could be the result of increased urban influences as indicated by increased conductivities, and elevated nutrient concentrations. Increased abundance of bacteria at industrial/urban streams was not coupled with an obvious increase in the extracellular enzyme activities measured in this study. However, average percent mass loss was slightly higher at industrial/urban streams when compared to industrial streams suggesting that urban influences (i.e. increased bacterial biomass, conductivities, and nutrients) may have contributed to greater microbial decomposition at industrially disturbed streams.

Across disturbance types bacterial communities were all dominated by the phylum Proteobacteria, and the class Alphaproteobacteria. The class Alphaproteobacteria is ubiquitous in freshwater ecosystems, and comprises a great diversity of taxa that can live under many different environmental conditions (Newton *et al.*, 2011). Looking at a finer taxonomic scale key differences were found in the composition of the dominant (> 1 %) bacterial community across disturbance types. The abundance of seven OTUs from the genus *Amantichitinum* comprised in total 12 % of the undisturbed bacterial community, < 5 % of logged and fire bacterial communities, and ≤ 0.01 % of industrial/urban and industrial bacterial communities.

Burkholderia, and *Enterobacter* were also a part of the dominant community at undisturbed streams, but had a lower abundance (< 1 %) at logged, fire, industrial/urban and industrial streams. These three genera are known to contribute to the cycling of energy and nutrients within the aquatic ecosystem via the breakdown of chitin by *Amantichitinum* sp. (Gooday, 1990), the breakdown of aromatic compounds such as lignin by aquatic *Burkholderia* sp. (Woods & Sokol,

2006), and the breakdown of sugars from cellulose and hemi-cellulose hydrolysis by *Enterobacter* sp. (Grimont & Grimont, 2006). A lower abundance of species from these genera across logged, fire, and especially among industrial/urban, and industrial streams could indicate a degree of energy and nutrient cycling impairment within these disturbed watersheds.

Despite differences in the dominant bacterial community across all disturbance types compared to the undisturbed, the majority of shared, co-occurring OTUs across streams were not significantly associated with bacterial communities at industrial/urban and industrial streams, marking a more significant shift in the bacterial community at industrially disturbed streams. In particular 7 OTUs represented by *Pseudomonas* spp., and 2 OTUs from *Luteibacter* spp. were absent or in very low abundance at industrial/urban and industrial streams. The absence or very low abundance of these generally ubiquitous heterotrophs could impair the cycling of energy and nutrients in industrial streams. In particular, *Pseudomonas* spp. are thought to be key contributors to the mineralization of DOM in aquatic ecosystems (Moore *et al.*, 2006).

Along with differences in the dominant bacterial community across disturbance types, there were also significant differences in the rare bacterial biosphere (i.e. bacteria comprising < 1 % of the total community). Unique taxa within the rare biosphere were found to be associated with each disturbance type as potential indicators of past disturbance. The rare biosphere has been viewed as a seed bank, and a mechanism for the maintenance of microbial community diversity (Pedró – Alió, 2006; Sogin *et al.*, 2006). However, recent studies have also noted considerable activity of rare biosphere taxa (Jones & Lennon, 2010; Campbell *et al.*, 2011), and an opportunistic response of these taxa to exploit disturbance situations and contribute to ecosystem functioning (Sjöstedt *et al.*, 2012; Wilhelm *et al.*, 2014). OTUs from the rare biosphere were identified as potential indicators of disturbance for each disturbance type. These unique disturbance

associated low abundance taxa may be an indication of the past influences of logging, fire and industrial activity on species sorting and bacterial community composition, and may have the potential to contribute to ecosystem functioning. They may also be useful indicators of watershed recovery or reclamation success, with communities significantly associated with *Magnetospirillum*, and *Amantichitinum* potentially indicating a return to the undisturbed condition.

As previous studies have shown (e.g. Gessner *et al.*, 2007) fungi made up the vast majority of microbial biomass on leaf packs and this was true in all of my streams as well. However significant decreases in overall fungal biomass were found at industrial/urban and industrial streams. These results agree with the findings of Sridhar *et al.* (2001) and Solé *et al.* (2008) who also found significant decreases in fungal biomass on leaf litter at industrially disturbed (i.e. metal contaminated) streams. Decreases in fungal biomass could have a negative impact on macro-invertebrate communities, and the decomposition and cycling of organic matter in the aquatic ecosystem. Fungi are usually the first to colonize submerged leaf litter, and initiate decomposition by conditioning leaf litter and making it more palatable for macro-invertebrate consumption (Bärlocher, 1985; Suberkropp, 1992; Graça, 2001). Conditioning of the leaf litter also benefits bacterial colonization and uptake as the breakdown of the more complex plant molecules creates more readily available compounds (Mille-Lindblom & Tranvik, 2003). Fungal biomass in my study exhibited a strong positive correlation with microbial decomposition consistent with previous study findings (Gessner & Chauvet, 1994; Dangles & Chauvet, 2003; Pascoal & Cássio, 2004; Duarte *et al.*, 2010). Along with being important contributors to the decomposition process, fungal biomass itself is also a labile, readily available food source for macroinvertebrates. Previous studies have found preferential feeding by macroinvertebrates on

leaf litter containing fungal biomass (Bärlocher, 1985), and even preferential feeding on leaf matter with specific fungal species (Gonçalves *et al.*, 2014). Decreases in fungal biomass are therefore, not only important to microbial decomposition, but could also reduce macro-invertebrate contribution to leaf litter decomposition, and macroinvertebrate growth at industrial/urban and industrial streams (Bärlocher & Kendrick, 1975; Suberkropp *et al.*, 1976).

Compared to large differences in fungal biomass, overall differences in fungal community composition across disturbance types were minimal. Across streams and disturbance types Ascomycota was the most dominant fungal phylum. Ascomycota has been identified as the dominant and perhaps most important fungal phylum on decomposing leaves in streams (Bärlocher, 1992). Although there were no significant differences in overall fungal diversity, increases in fungal richness were found for all disturbance types with an increase in the number of fungal OTUs of 4.2 %, 15.3 %, 34.7 %, and 11.8 % at logged, fire, industrial/urban, and industrial streams respectively. Higher fungal richness at disturbed streams compared to undisturbed streams may be explained by the intermediate disturbance theory, which states that intermediate disturbance allows for the co-occurrence of both native competitors and opportunists, and therefore leading to greater community richness (Connell, 1978; Townsend & Scarsbrook, 1997). Streams in this study experienced varying degrees of disturbance from logging and fire to industrial influences, and varying recovery times from disturbance (≥ 7 years). Therefore, it is quite possible that increases in fungal richness are due to intermediate disturbance, where some lingering influences of disturbance remain and allow for the co-occurrence of both natural competitors and opportunists.

Distinct differences in fungal community composition were found at fire, industrial/urban, and industrial streams. At fire streams the group LKM11 made up a greater proportion of the fungal

community compared to all other disturbance types. LKM11 is a highly diverse environmental group that was first sequenced in an experimental freshwater system (van Hanne, 1999). Since its discovery further phylogenetic analysis has identified LKM11 as being a very close relation to the parasitic fungi *Rozella* (Lara, Moreira & Garcia, 2010). Jones et al. (2011) also found LKM11 cell walls to be devoid of chitin, and cellulose, and named the LKM11 group cryptomycota because of its lack of fungal characteristics and largely unknown behaviour. Its close relation to *Rozella* and lack of chitin and cellulose composed cell walls suggest that like *Rozella* species, species of the group LKM11 may be parasitic to other fungi, algae, and protists (Jones & Richards, 2011). If taxa from the group LKM11 are parasitic their high abundance at fire streams could indicate their specific response to conditions created by fire (natural) disturbance.

At industrial/urban and industrial streams, the class Eurotiomycetes was in much greater abundance when compared to all other disturbance types. The class Eurotiomycetes includes a large range of fungi including saprotrophs, parasites, pathogens, and lichens (Geiser *et al.*, 2006). OTUs classified under the class Eurotiomycetes were identified as potential indicator species at industrial/urban and industrial streams. These potential indicators included species from the genera *Aspergillus*, and *Penicillium*. *Aspergillus* and *Penicillium* are characterized as generally ubiquitous molds found in both terrestrial and aquatic habitats (Webster & Weber, 2007). A study looking at fungi in acid mine drainage found members from the class Eurotiomycetes to be in greater abundance (Baker *et al.*, 2004), highlighting the greater ability of some taxa from the class Eurotiomycetes to withstand metal contamination and low pH. Although the conditions at industrially disturbed streams in this study are not nearly as severe as in acid mine drainage, slightly lower pH and a higher concentration of Ni, Zn, and Cu could be providing conditions

that can support this particular fungal community composition at industrial/urban and industrial streams.

2.6 Summary & Conclusions

Microbial communities on leaf litter in streams appeared to exhibit variation following changes in their environment related to disturbance type. Industrial/urban and industrial streams with most physical and chemical stream habitat disturbance, exhibited the most impacted microbial community. Industrial/urban and industrial streams had the lowest microbial decomposition, a significantly decreased fungal biomass, a fungal community with a greater abundance of taxa from the class Eurotiomycetes, and a unique bacterial community composition with key taxa missing or in very low abundance. These differences point to a potentially slower cycling of energy and nutrients within industrially disturbed streams.

There were however also signs of disturbed microbial communities across all streams from all disturbance types, with lower overall potential hydrolase activities, and CQI values for logged, fire, industrial/urban, and industrial streams when compared to undisturbed streams. At logging, fire, and industrially disturbed streams lower potential hydrolase extracellular enzyme activities could result in the slower cycling of C, N, and P within these disturbed aquatic ecosystems, while a lower CQI may suggest either an overall decreased availability of varied carbon sources, or an increased dependence on terrestrial DOM rich in aromatic compounds. Furthermore, an increase in fungal richness at logged, fire, industrial/urban and industrial streams may be the result of intermediate disturbance where influences of past disturbance allow for the co-existence of competitive native species and opportunists.

Overall, it appears that at all logging, fire, and industrially disturbed streams there were signs of decreased microbial function many years after disturbance indicating that there may be an intimate connection between land disturbances and aquatic microbial communities on leaf litter. Land-water linkages have been previously described as the response of macroorganisms such as fish and macroinvertebrates to changes in their watershed. Chapter 3 will look more closely at land-water linkages by exploring potential connections between watershed characteristics, stream habitat characteristics and leaf litter stream microbial communities.

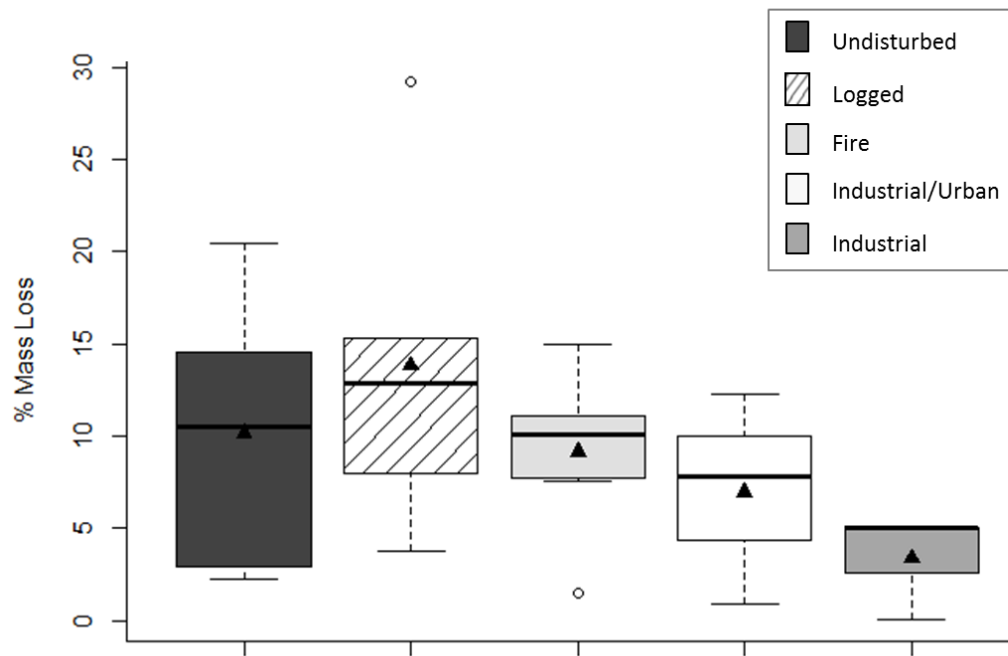


Figure 2-1 Percent mass loss of leaves across disturbance types with 95% confidence intervals. The black line represents the median and the triangle represents the mean.

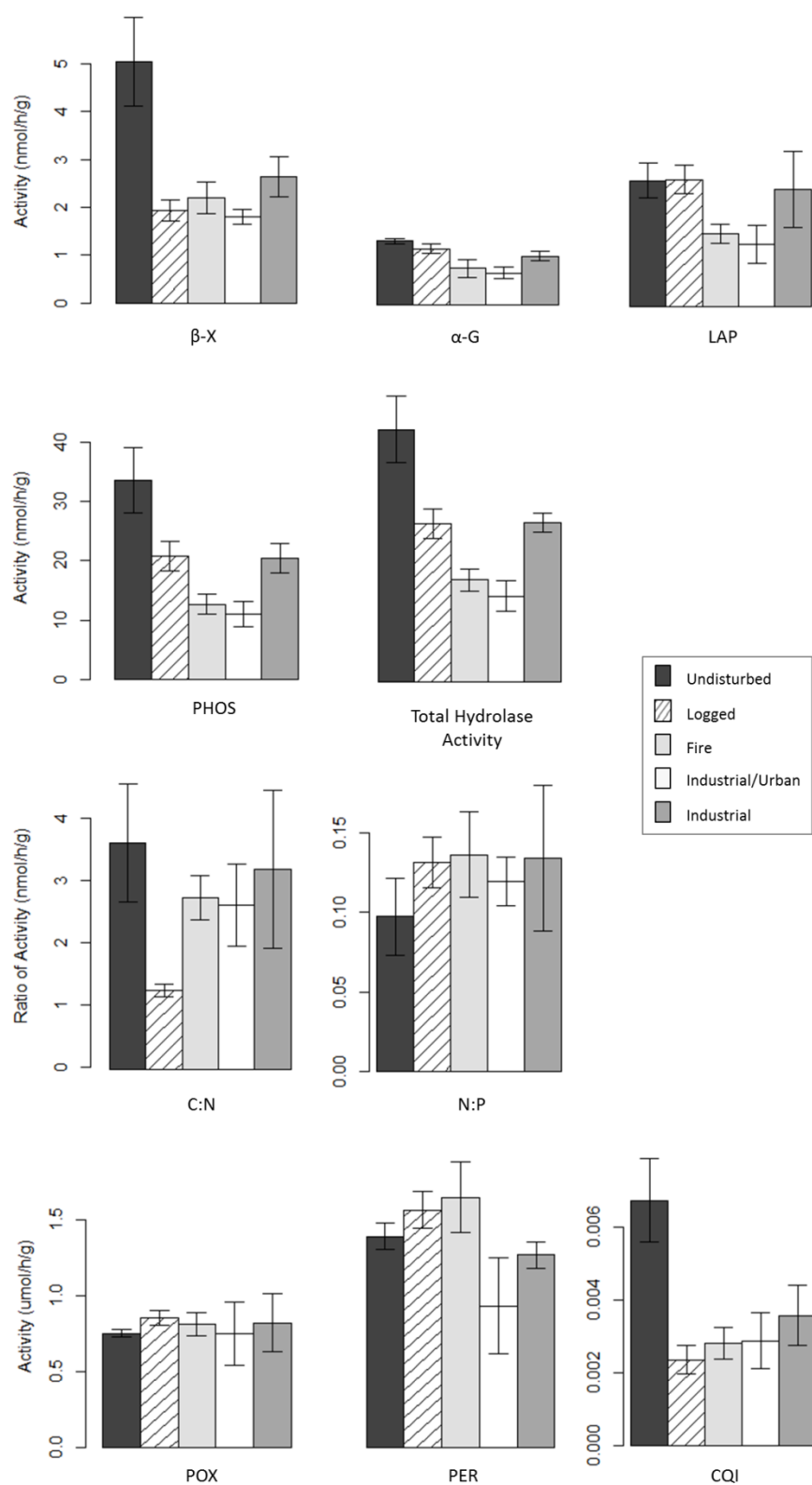


Figure 2-2 Average extracellular enzyme activities of microbial communities from each disturbance type. Error bars represent standard error. POX and PER are in $\mu\text{mol/h/g}$, while all others are in nmol/h/g .

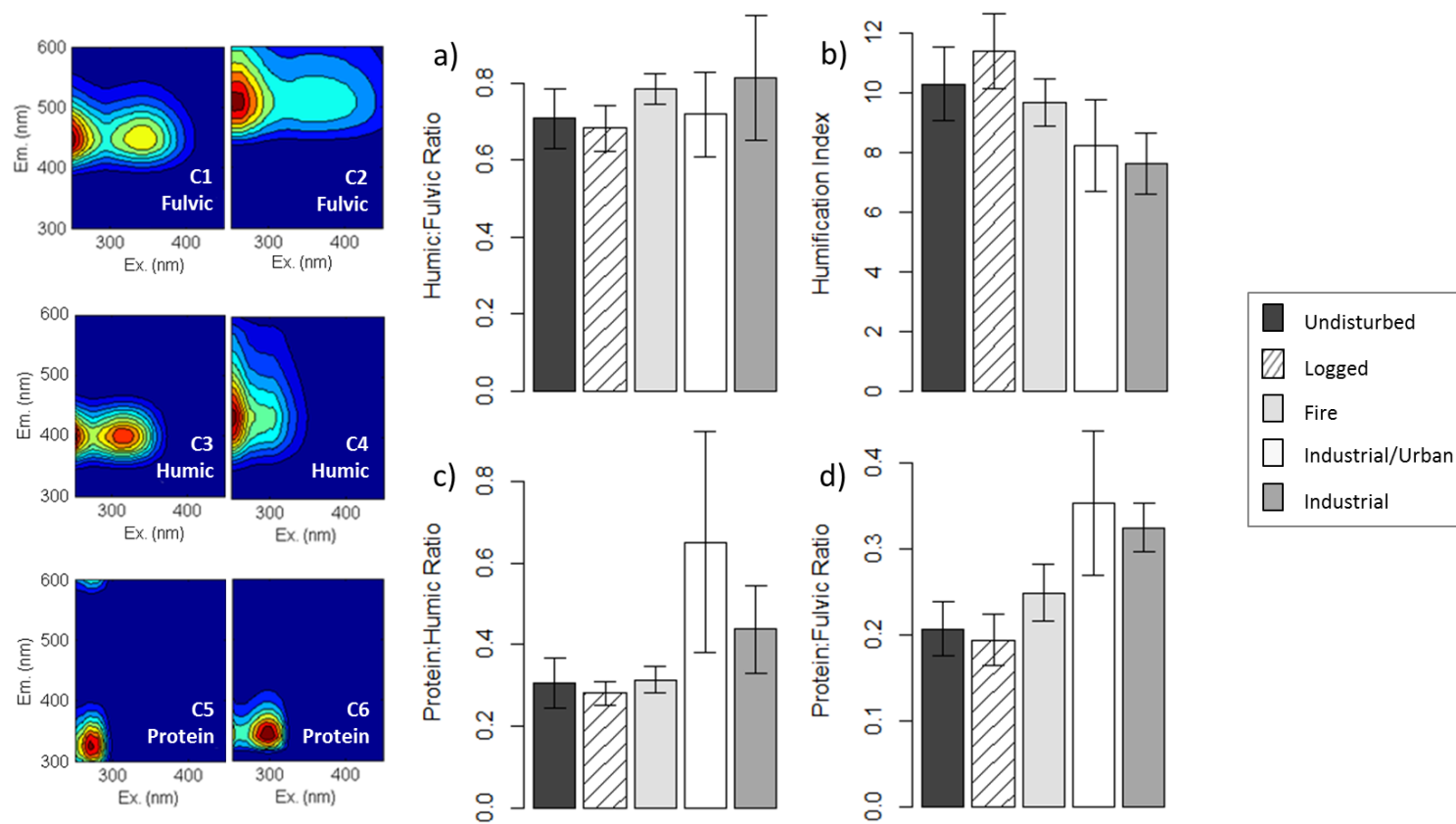


Figure 2-3 Quality of DOM described using the HIX (b), and the average ratios of humic:fulvic (a), protein:humic (c), and protein:fulvic (d) across disturbance types derived from PARAFAC analysis. PARAFAC analysis revealed two fulvic components (C1&C2), two humic components (C3&C4), and two protein components (C5&C6). Error bars represent standard error.

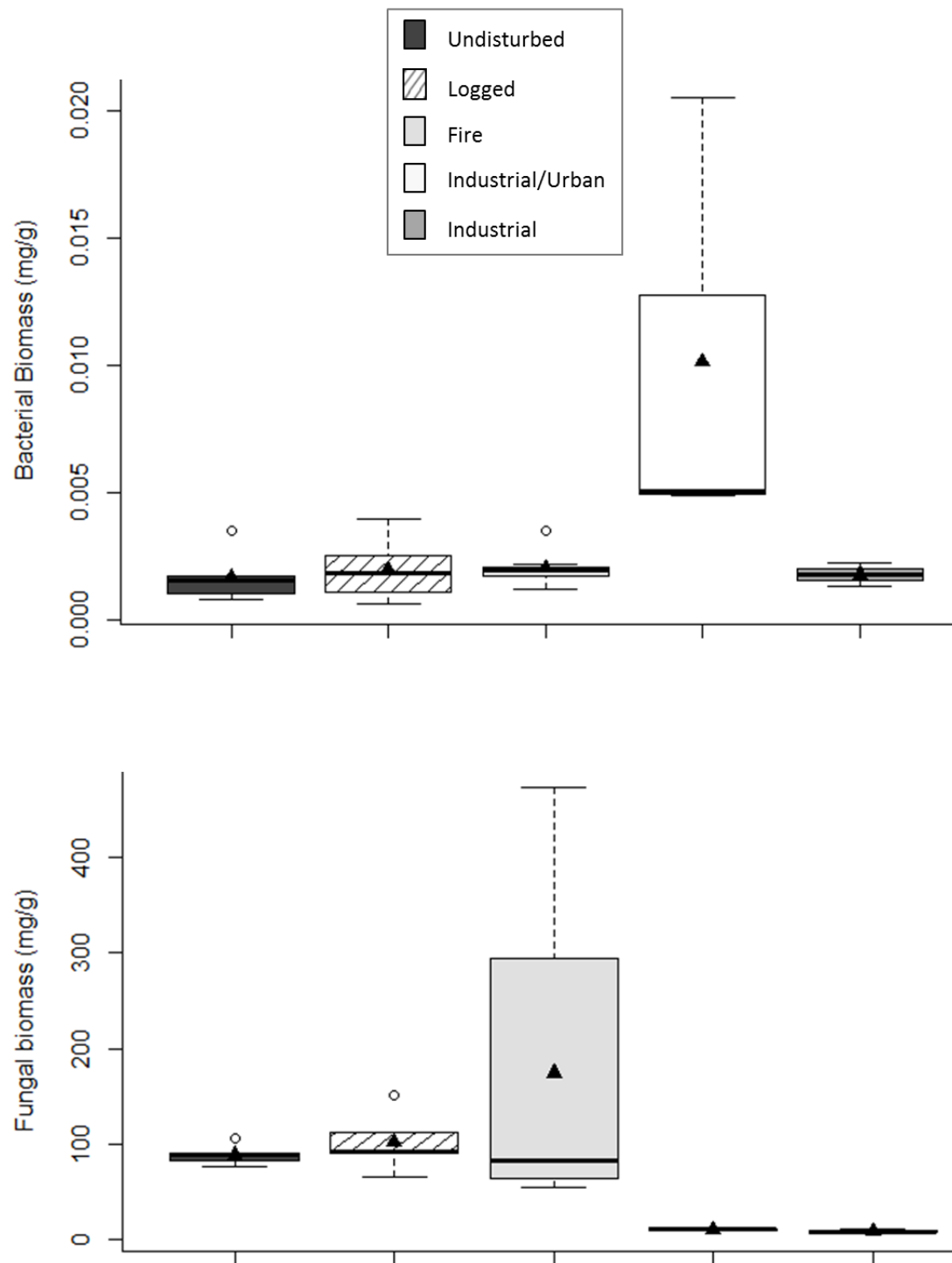


Figure 2-4 Average bacterial (top), and fungal (bottom) biomass (mg/g) on leaves in streams from each disturbance type with 95% confidence intervals. Solid black lines represent the median and triangles represent the mean.

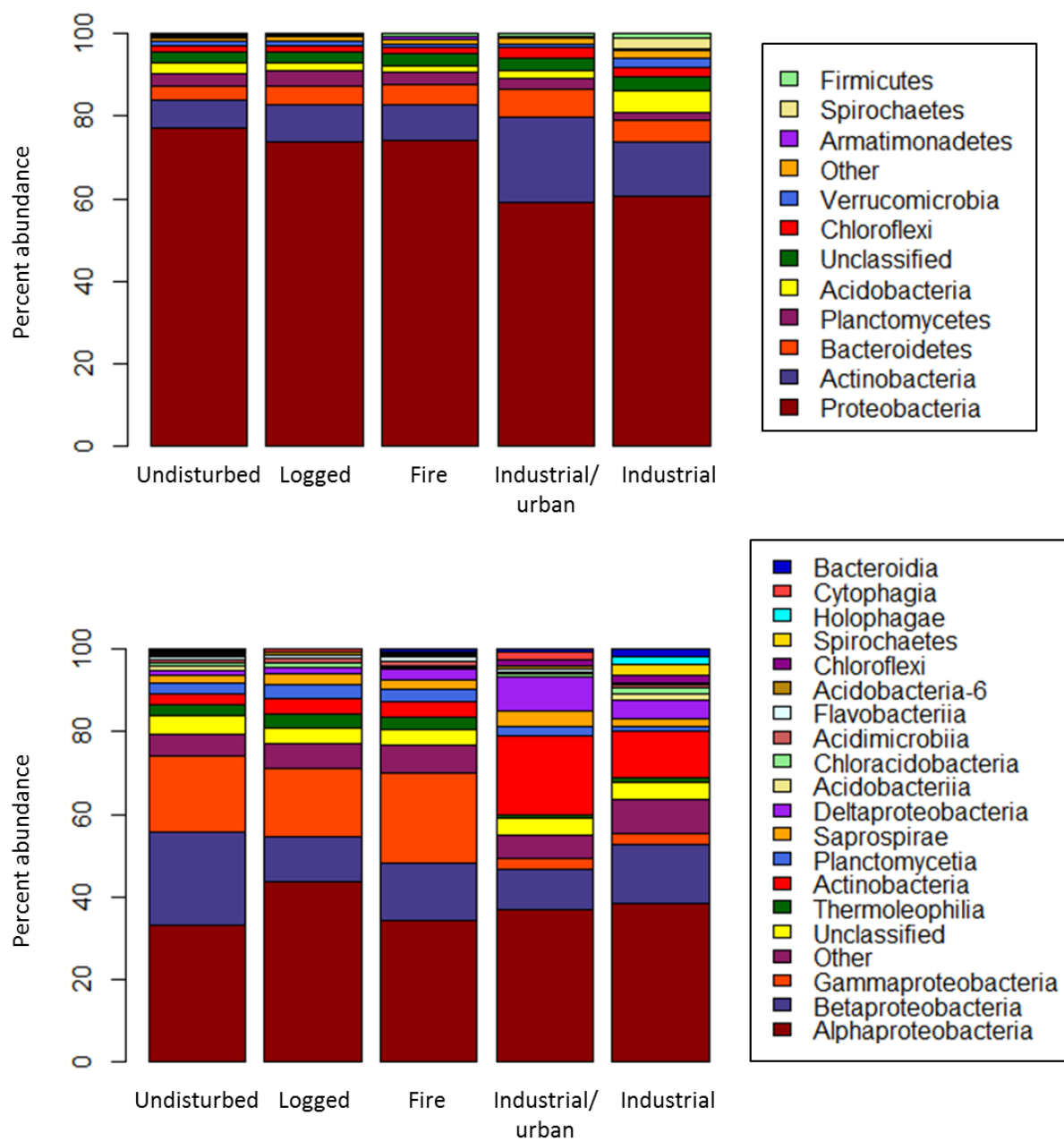


Figure 2-5 Average percent abundance of bacterial phyla (top), and classes (bottom) for each disturbance type.

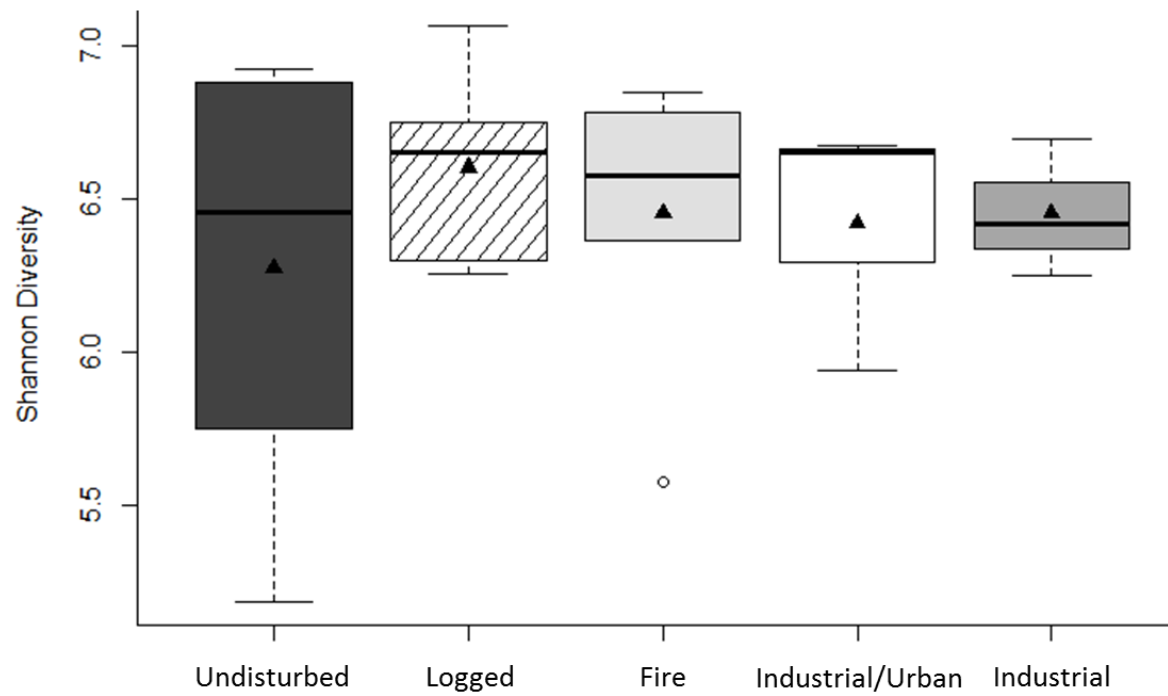


Figure 2-6 Shannon diversity of bacterial communities across disturbance types with 95% confidence intervals. The solid black lines represent the median and the triangles represent the mean.

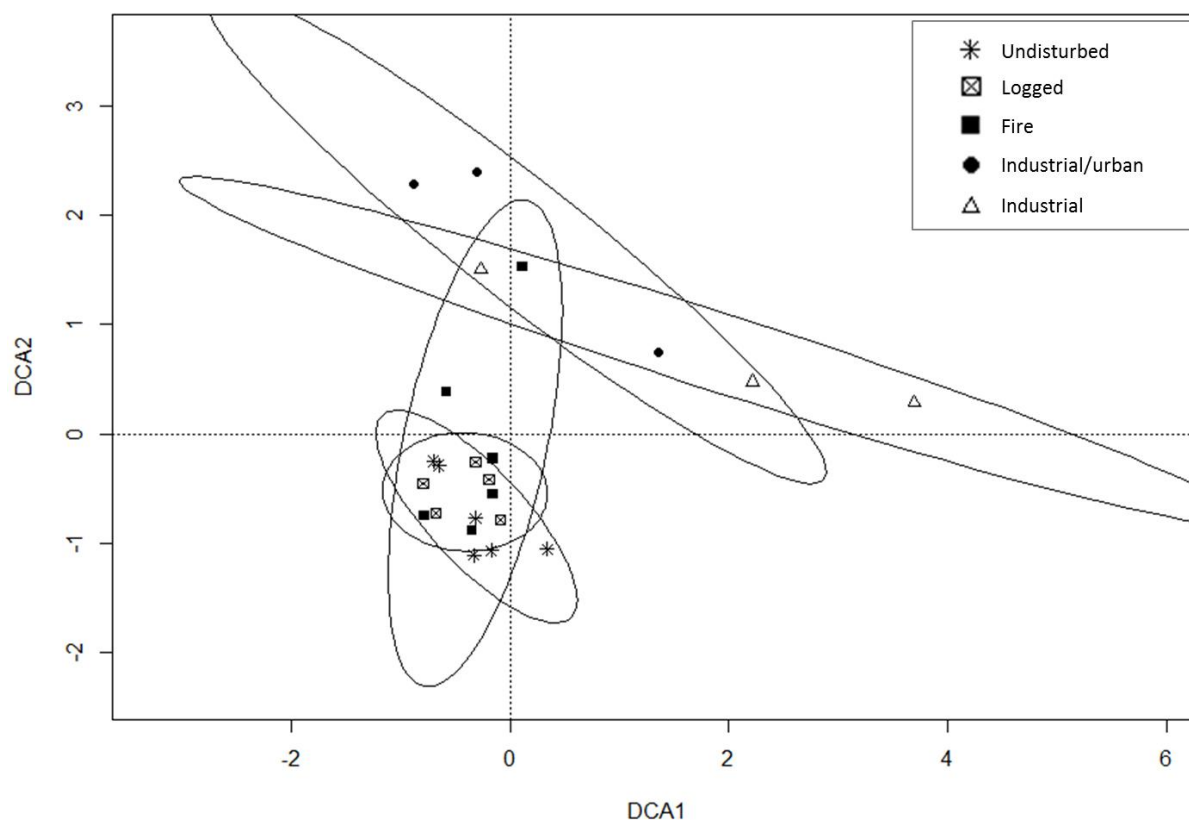


Figure 2-7 Detrended correspondence analysis based on the abundance of bacterial OTUs from each stream. Each stream is labelled by its disturbance type and ellipses represent 95% confidence intervals.

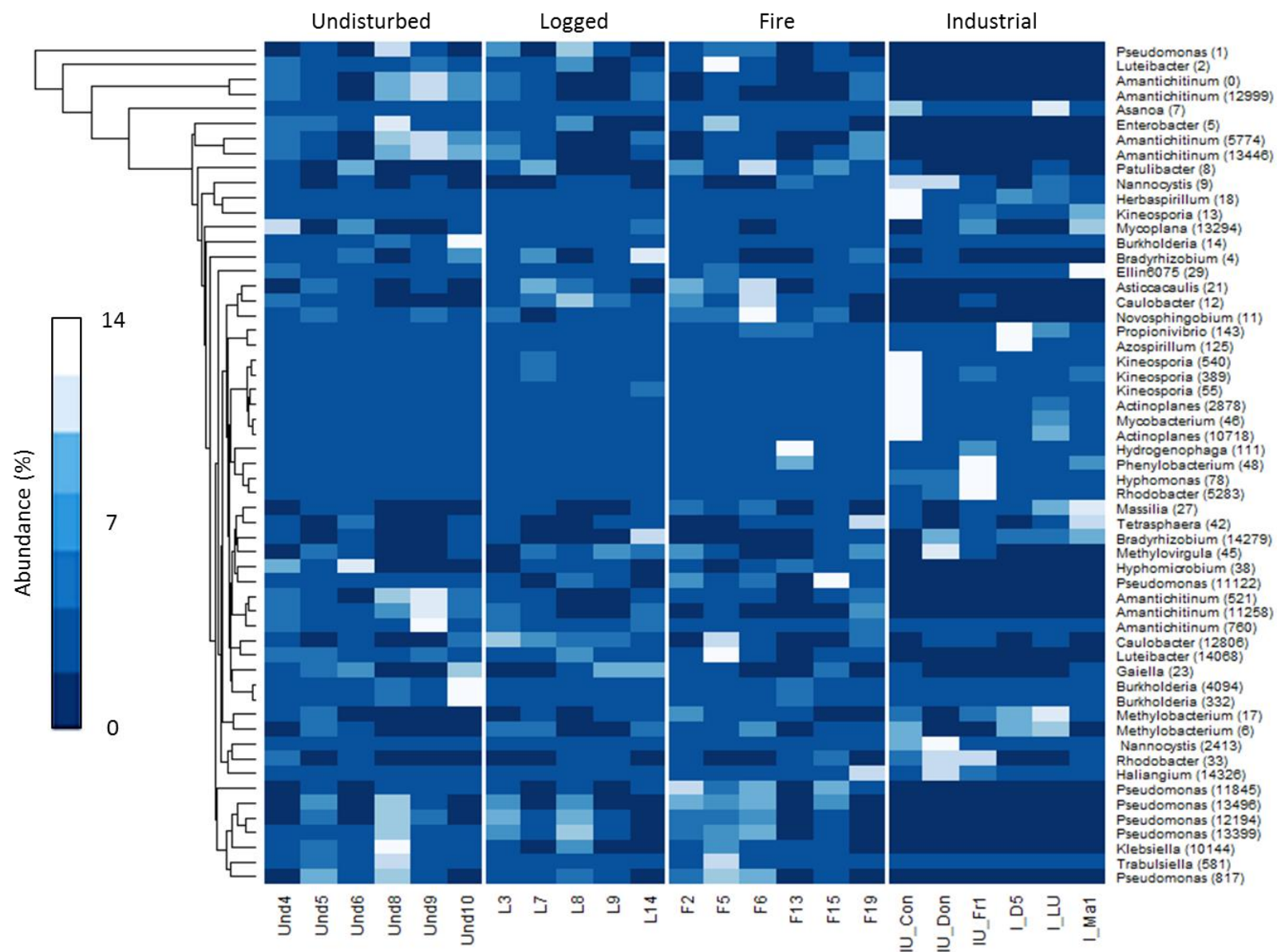


Figure 2-8 Heat map of dominant and frequently occurring OTUs (rows) presented across sites from each disturbance type (columns) and sorted by hierarchical clustering. Abundance increases from dark (0%) to light (14%), and OTUs are labelled by their GreenGenes assigned family with OTU number in brackets. Four families were identified based on closest blast hit.

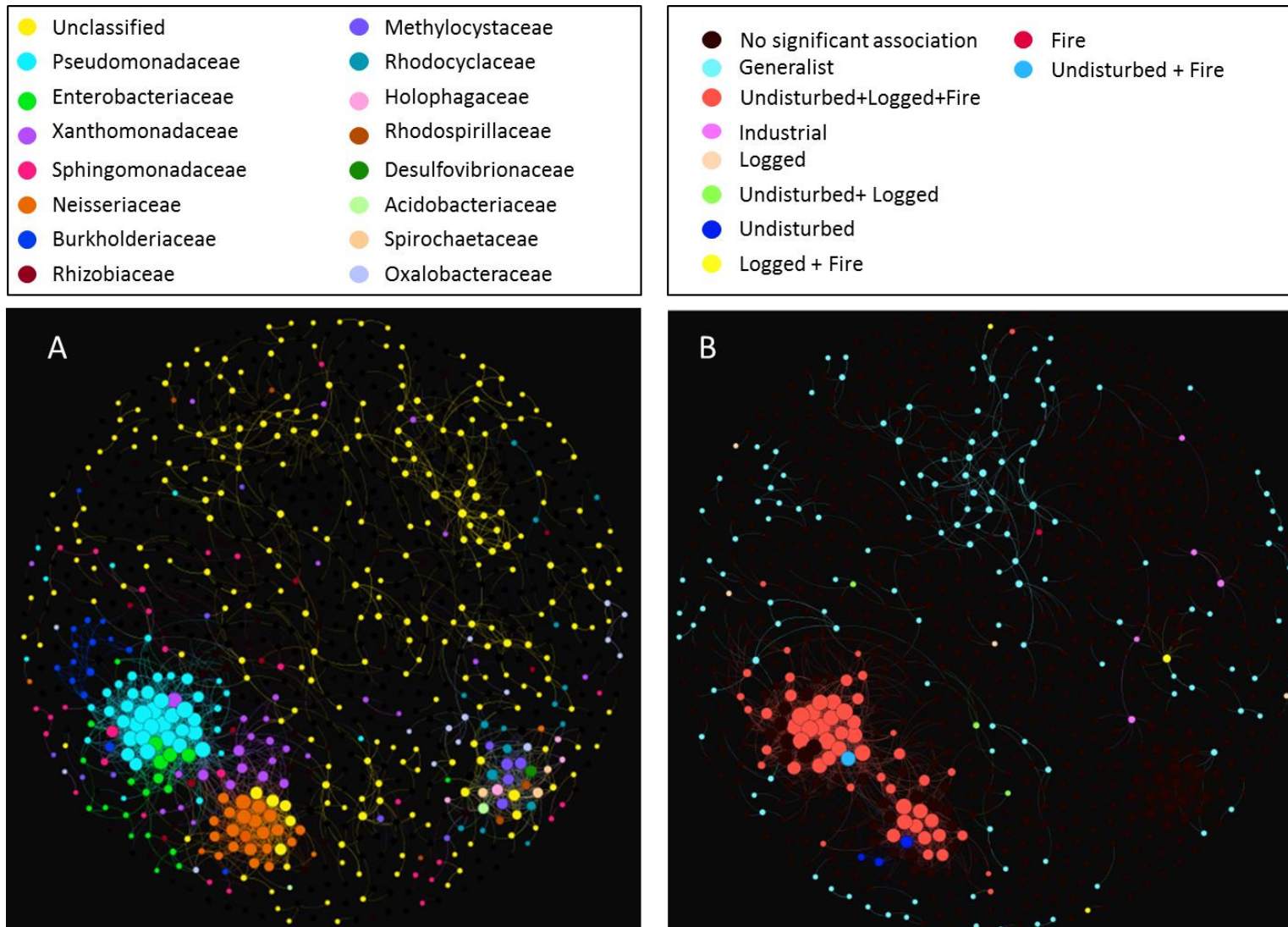


Figure 2-9 Co-occurrence network of bacterial OTUs based on strong ($r>0.7$) and significant ($p<0.01$) correlations. Co-occurring OTUs coloured by their respective bacterial families (A, left), and Co-occurring OTUs coloured by significant ($p<0.01$) associations with a disturbance type(s) based on indicator species analysis (B, right). Node (i.e. OTU) size is representative of connectivity, where the larger the node the more nodes it co-occurs with.

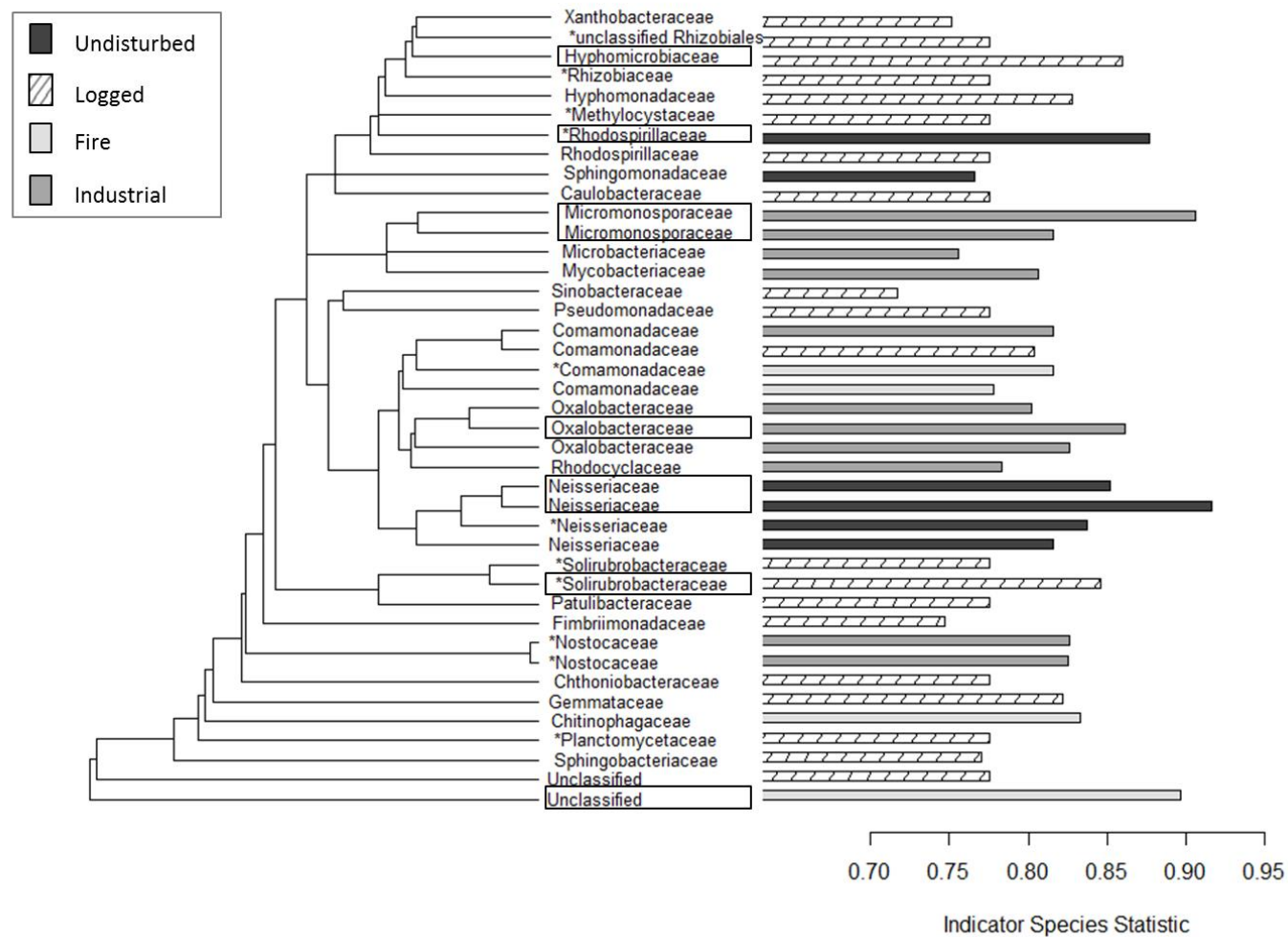


Figure 2-10 Phylogenetic tree of bacterial taxa significantly ($p \leq 0.01$) associated with each disturbance type based on abundance and frequency across streams (indicator species statistic). The larger the indicator species statistic the stronger the association, and taxa with an indicator species statistics of >0.85 are highlighted. Industrial includes both industrial/urban and industrial streams.

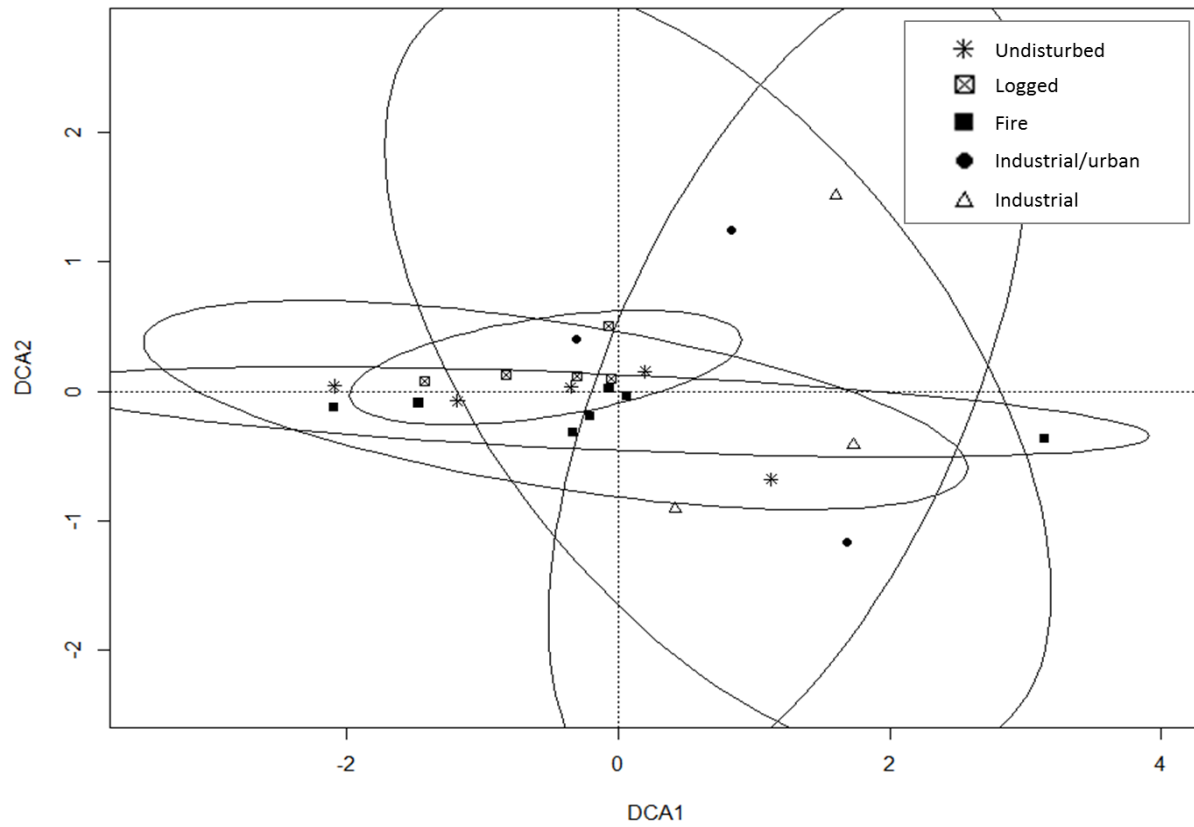


Figure 2-11 Detrended correspondence analysis based on the relative abundance of fungal OTUs from each stream. Streams are labelled by disturbance type, and ellipses represent 95% confidence intervals.

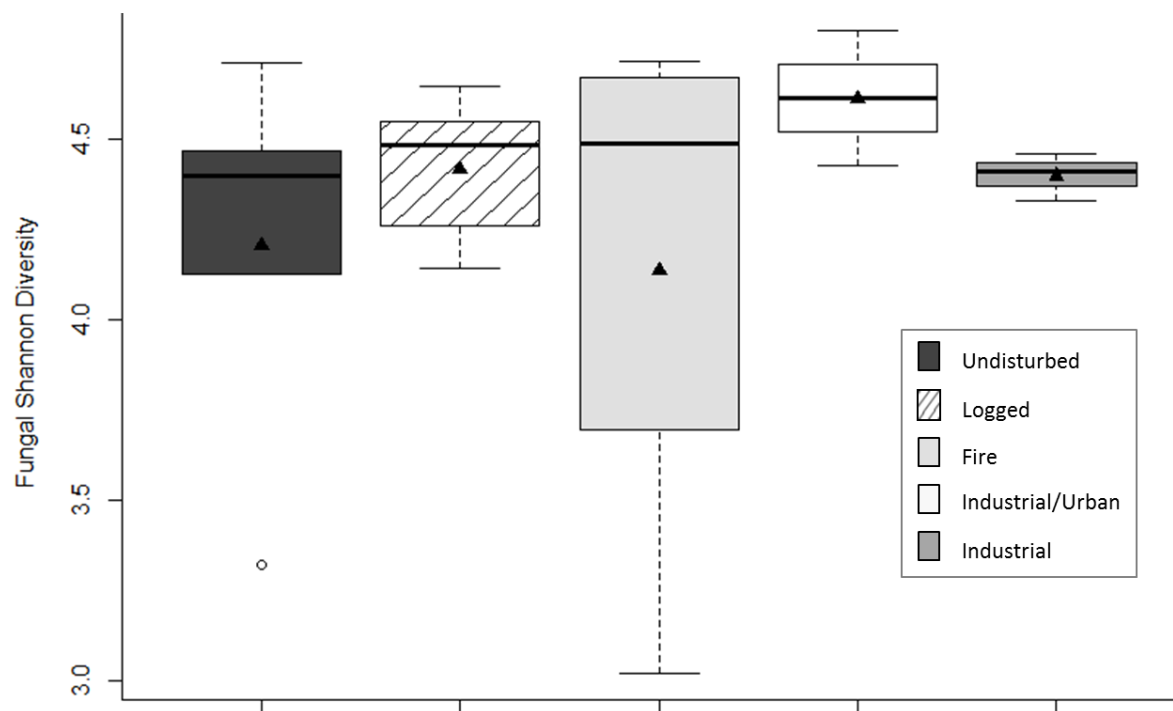


Figure 2-12 Shannon diversity of fungal communities across disturbance types with 95% confidence intervals. The solid black lines represent the median and the triangles represent the mean.

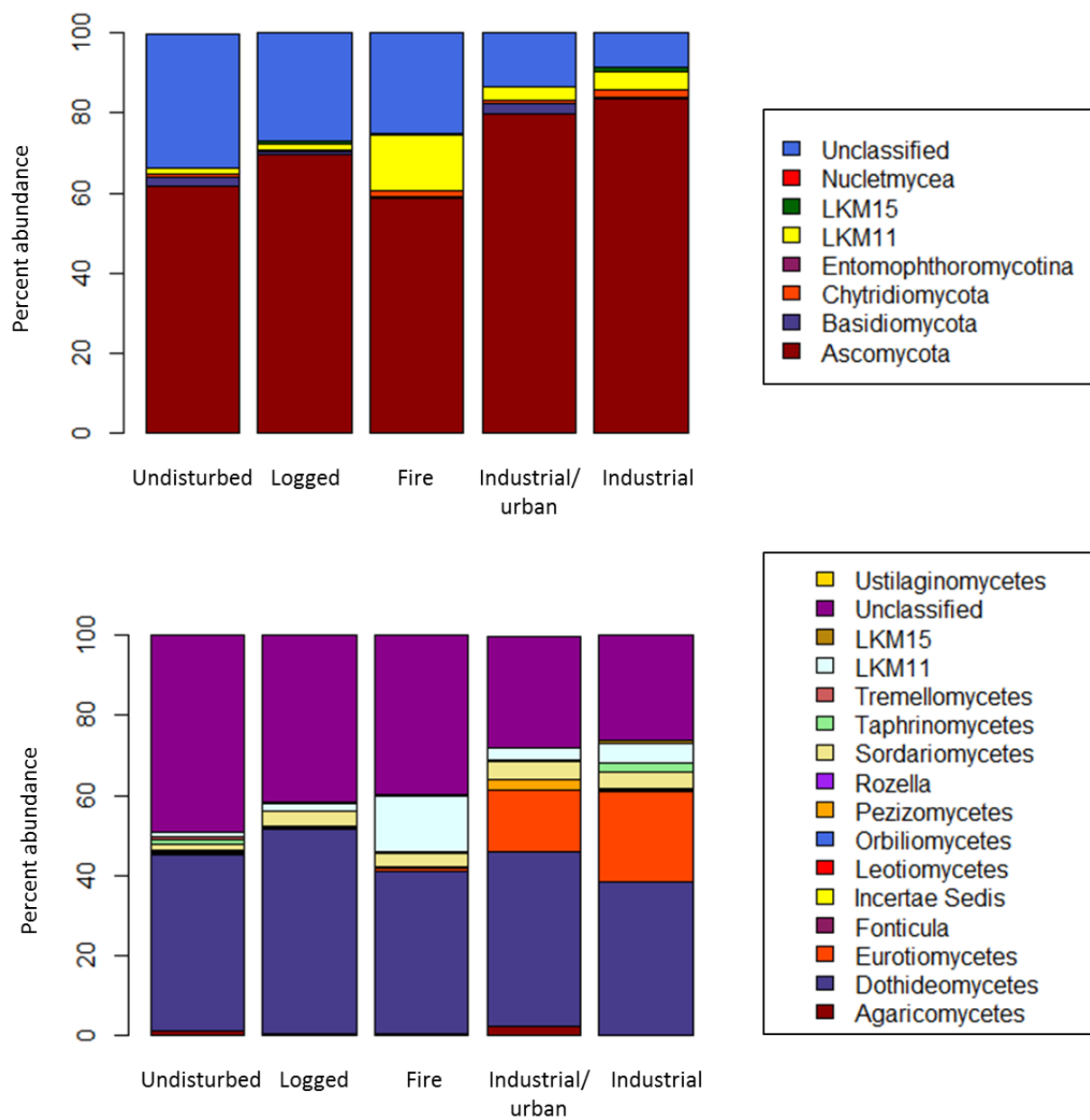


Figure 2-13 Average percent abundance of fungal phyla (top), and classes (bottom) for each disturbance type.

Table 2-1 Measured enzyme activities, substrates used, and function of enzymes (Sinsabaugh et al., 2008; Kirchman, 2012).

Enzyme	Substrate Used	Function
α -Glucosidase (α -G)	4-MUB- α -D-glucopyranoside	Starch and disaccharide degradation
β -Xylosidase (β -X)	4-MUB- β -D-xylopyranoside	Hemi-cellulose degradation
Phosphatase (PHOS)	MUB-Phosphate	Phosphomonoester degradation
Leucine Aminopeptidase (LAP)	L-leucine-7-amido-4-methylcoumarin	Peptide and protein degradation
Phenol Oxidase (POX)	LDOPA	Polyphenol oxidation (E.g. lignin)
Phenol peroxidase (PER)	LDOPA+ peroxide	Polyphenol oxidation (E.g. lignin)

Table 2-2 Average stream habitat characteristics across disturbance types with standard deviation

	Undisturbed		Logged		Fire		Industrial/urban		Industrial	
	<i>Avg.</i>	<i>SD</i>	<i>Avg.</i>	<i>SD</i>	<i>Avg.</i>	<i>SD</i>	<i>Avg.</i>	<i>SD</i>	<i>Avg.</i>	<i>SD</i>
Water Depth (m)	0.100	±0.03	0.110	±0.04	0.130	±0.03	0.150	±0.040	0.160	±0.11
LWD abundance (#/m²)	0.0600	±0.03	0.080	±0.03	0.100	±0.06	0.020	±0.010	0.050	±0.050
Sedimentation g/day/m²	0.260	±0.11	0.830	±0.65	0.500	±0.44	2.60	±1.42	0.450	±0.200
% Sediment organic matter content	58.0	±18.95	40.0	±19.7	46.0	±8.52	7.00	±1.18	17.0	±3.63
TN (mg/L)	0.520	±0.135	0.376	±0.167	0.328	±0.123	1.63	±1.61	0.479	±0.142
TP (mg/L)	0.0070	±0.002	0.007	±0.001	0.009	±0.006	0.027	±0.009	0.026	±0.01
Temperature (°C)	14.6	±1.46	13.7	±1.36	15.3	±2.80	19.5	±1.26	18.3	±1.98
Conductivity (ppm/C)	63.4	±37.94	117	±78.2	146	±58.7	754	±249	70.4	±31.5
Ni (mg/L)	0.001	±0.0006	0.001	±0.00	0.001	±0.00	0.146	±0.088	0.093	±0.079
pH	6.53	±0.246	6.14	±0.488	6.51	±0.467	6.16	±0.135	5.44	±0.652
DOC (mg/L)	10.4	±3.09	9.10	±2.84	9.54	±4.24	5.70	±1.89	6.87	±2.81

Table 2-3 Summary of results from ANOVA on key microbial community functional and structural characteristics. P-values are the statistical significance for the overall ANOVA, and NS stands for no significant difference. Shared letters represent no significant difference between disturbance types.

Variable	Undisturbed	Logged	Fire	Industrial Urban	Industrial	F- statistic	P- value
Mass loss	-	-	-	-	-	-	NS
Xylosidase EA	a	b	b	b	a,b	4.87	**0.0071
Glucosidase EA	a	a	b	b	a,b	4.35	*0.0115
LAP EA	a	a	b	b	a,b	4.32	*0.0122
Phosphatase EA	a	a	b	b	a	8.1	***0.0005
Total Hydrolase EA	a	b	c	c	b	9.4	***0.0002
C:N	-	-	-	-	-	-	NS
N:P	-	-	-	-	-	-	NS
Phenol Oxidase EA	-	-	-	-	-	-	NS
Peroxidase EA	-	-	-	-	-	-	NS
CQI	a	b	b	b	a,b	4.25	*0.0127
Humic: Fulvic	-	-	-	-	-	-	NS
Protein:Humic	-	-	-	-	-	-	NS
Protein:Fulvic	-	-	-	-	-	-	NS
HIX	-	-	-	-	-	-	NS
Bacterial Biomass	a	a	a	b	a	5.41	**0.0044
Fungal Biomass	a	a	a	b	b	26.26	***0.0000
Bacterial Diversity	-	-	-	-	-	-	NS
Fungal Diversity	-	-	-	-	-	-	NS

Table 2-4 Percent abundance of key OTUs from the heat map summed by bacterial genus and averaged across streams by disturbance type. Confidence intervals in brackets represent standard error.

	<i>Amantichitinum</i>	<i>Burkholderia</i>	<i>Enterobacter</i>	<i>Luteibacter</i>	<i>Pseudomonas</i>
Undisturbed	12.24 (±3.74)	1.41 (±0.97)	1.22 (±0.51)	1.87 (±0.39)	5.31 (±2.54)
Logged	4.42 (±1.37)	0.21 (±0.08)	0.54 (±0.23)	1.87 (±0.74)	5.88 (±2.44)
Fire	3.24 (±1.39)	0.30 (±0.12)	0.85 (±0.37)	3.51 (±2.1)	5.82 (±1.46)
Industrial Urban	0.00 (±0)	0.00 (±0)	0.02 (±0.01)	0.09 (±0.04)	0.04 (±0.03)
Industrial	0.01 (±0.01)	0.00 (±0)	0.05 (±0.03)	0.04 (±0.01)	0.11 (±0.01)

Table 2-5 Average richness of fungal OTUs across disturbances, and percent difference from undisturbed streams.

Disturbance	Average # OTUs	% Difference from Undisturbed
Undisturbed	144	-
Logged	150	(+) 4.2
Fire	166	(+)15.3
Industrial/ Urban	194	(+)34.7
Industrial	161	(+)11.8

Table 2-6 OTUs strongly (statistic ≥ 0.8) and significantly (p-value < 0.01) associated with a disturbance type according to indicator species analysis. Identification was based on closest blast hit.

OTU#	Associated Disturbance	Class	Genus	Species	Blast% identity, evalue	Indicator Species Statistic	Indicator Species P-value
316	Industrial	Eurotiomycetes	<i>Aspergillus</i>	<i>alliaceus</i>	97, 0	0.972	0.001
2157	Industrial	Eurotiomycetes	<i>Penicillium</i>	<i>uncultured</i>	94, 0	0.816	0.002
2221	Industrial	Eurotiomycetes	<i>Aspergillus</i>	<i>fumigatus</i>	98,5e-16	0.816	0.002
1934	Industrial	Environmental	-	-	94, 0	0.816	0.003
102	Industrial	Eurotiomycetes	<i>Aspergillus</i>	<i>alliaceus</i>	96, 0	0.816	0.004
13	Industrial	Eurotiomycetes	<i>Penicillium</i>	<i>charlesii</i>	97, 0	0.887	0.006
1775	Industrial	Eurotiomycetes	<i>Penicillium</i>	<i>charlesii</i>	96, 0	0.809	0.009

Chapter 3

3 Land-water Linkages Across a Gradient of Disturbance and Recovery: Key Watershed Characteristics Influencing Stream Microbial Communities

3.1 Abstract

The influences of land-water linkages in disturbed watersheds on stream microbial communities have received little attention despite the important role of microbial communities in energy and nutrient cycling in aquatic ecosystems and the rapid response of microorganisms to changes in their environment. Microbial communities that colonized standardized leaf litter were characterized across a gradient of logging, fire and industrial watershed disturbance to assess which watershed and habitat features were most associated with microbial community structure and function. Percent wetland cover along with % forest cover were associated with an increased concentration of dissolved organic carbon (DOC) in streams, and increased DOC concentration was associated with increased microbial decomposition of the standardized leaf litter, and increased α -glucosidase (α -G), phenol oxidase (POX), peroxidase (PER), and leucine-aminopeptidase (LAP) potential enzyme activities. Percent forest cover was also associated with an increased abundance of large woody debris in streams, and the abundance of large woody debris was positively associated with fungal biomass on leaf litter. Finally, increased road density was associated with increased stream water conductivity, and increased conductivity was associated with a decrease in β -xylosidase (β -X) and phosphatase (PHOS) potential enzyme activities. Overall, this study suggests that while roads can have a negative impact on microbial energy and nutrient cycling via elevated conductivity, wetlands and forests are important

providers of organic material that stimulates the microbial cycling of energy and nutrients within the aquatic ecosystem.

3.2 Introduction

Despite the important role of microbial communities in energy and nutrient cycling in the aquatic ecosystem (Azam *et al.*, 1983; Bärlocher, 2005; Gessner *et al.*, 2007) and the rapid response of microorganisms to changes in their environment (Paerl & Pickney, 1996) understanding the linkages between disturbed watersheds and stream microbial communities has received little attention. Instead the influence of watershed disturbance on aquatic ecosystems has largely been focused on the response of macroorganisms such as stream invertebrates and fish (e.g., Rosenberg & Resh, 1993; Wesolek *et al.*, 2010; Szkokan-Emilson *et al.*, 2011, Paller *et al.*, 2014). However, recent studies have started to explore the relationships between watershed features, and various measures of stream microbial communities. For example, Hill *et al.* (2012) found that percent forest cover was positively correlated with microbial extracellular enzyme activities for 1st-10th order streams across the USA, and Wang *et al.* (2011) found impervious surface cover to be a good indicator of urbanization and the sorting of denitrifying bacteria in streams. The community composition of aquatic fungi has also been proposed as a good indicator of anthropogenic disturbance (Solé *et al.*, 2008).

Because of the strong land-water linkages that couple aquatic ecosystems to their surrounding landscapes, the measurement of structural and functional microbial responses to watershed disturbances may help to provide improved insights into land-water linkages and help to identify key watershed characteristics that are associated with microbial responses. For example, extracellular enzyme activities and microbial leaf litter decomposition have been shown to be useful measures of the functional role of aquatic microbial communities because they are

indications of the cycling of energy and nutrients (Graça *et al.*, 2007; Cunha *et al.*, 2010).

Therefore, it is hypothesized that changes in the structure and function of fungal and bacterial communities may help describe the severity of watershed disturbance and provide an assessment of the degree to which structural and functional characteristics respond to disturbance. I addressed these hypotheses in a comparative study of microbial communities across a wide range of habitat conditions related to disturbance history. The main objective of my study was to assess which watershed and habitat characteristics were most associated with differences in microbial community structure and function across this gradient of disturbance.

3.3 Materials and Methods

Study design

A total of 24 low-order streams were used for this study. The gradient in disturbance severity ranged from no recent disturbance to high disturbance and was of 3 types: 1) streams that have been largely undisturbed for at least the last 50 years (no recent disturbance), 2) streams in watersheds impacted by fire or logging 7-15 years earlier (moderate to low disturbance), or 3) streams in an industrial area recovering from the effects of severe metal deposition and acidification over approximately 30-35 years, with a 90 % reduction in deposition in recent years (Gunn and Keller, 1990) (high disturbance). Undisturbed (n=6), logged (n=5), and fire (n=7) streams were located near White River, Ontario and industrial streams (n=6) were located in Sudbury, Ontario. Three of the 6 industrial streams were located closer to the city center, and as a result, experienced urban as well as industrial disturbance. These streams are referred to as industrial/urban. All of the streams were located on the boreal shield (similar underlying geology), and surrounded by boreal mixedwood forests (varying proportions of white and black

spruce (*Picea glauca*, *Picea mariana*), balsam fir (*Abies balsamea*), white, red, and jack pine (*Pinus* spp.), trembling aspen (*Populus tremuloides*), and white birch (*Betula papyrifera*)).

Microbial community characteristics

Microbial communities were collected using fine mesh leaf packs (0.5 mm) containing alder leaves (*Alnus incana*) incubated in the streams during the summer of 2012. The mesh size was selected to exclude macroinvertebrates and thus limit the measured decomposition and breakdown rates to only microbial communities. All the alder leaves used in the leaf packs were collected at the same time and from the same trees. Alder leaves were selected because Alder is a common tree species found by the streams in this study. Each leaf pack contained 3 sections. The first section contained 1 leaf for estimation of microbial decomposition rate (leaf mass loss). This leaf was pre-leached for 24 h, cut into a 15 mm diameter disc, air-dried (30 °C for 48 h), and then weighed prior to in-stream incubation. The other 2 leaf pack compartments contained 8 and 10 leaves for characterizing microbial community structure and potential extracellular enzyme activity. Six replicate mesh bag leaf packs were used at each stream and were anchored on the bottom at each site for an incubation period of 6 weeks.

After collection the leaf from the first compartment was gently washed (to remove sediments and biofilms without damaging the leaf material), dried and re-weighed to calculate mass loss.

Leaves from the second compartment were collected and then stored in a -20 °C freezer for later DNA extraction, extracellular enzyme activity assays, and fungal biomass estimation, while leaves from the third compartment were stored in 2 % formaldehyde in a -4 °C fridge prior to bacterial cell counts.

Fungal biomass was estimated by ergosterol extractions. For ergosterol extractions leaf samples were removed from the freezer and cut into 2 sets of 5 disks using an 11.2 mm diameter cork borer. One of the sets was dried at 60 °C for 48 h and weighed for an estimate of dry mass, while the second set was put in 0.14 M KOH-methanol and returned to the freezer for 10-12 hr. The ergosterol extractions were then performed following Gessner (2005) protocol. The concentration of ergosterol in the final extract was measured using reverse-phase high performance liquid chromatography on a HP1100 liquid chromatograph (Agilent, Hewlett Packard Canada Ltd., Mississauga, Ont.) , and fungal biomass calculated using a conversion factor of 5.5mg/g for hyphomycetes (Gessner & Chauvet, 1993). A control spiked with ergosterol standard was also run to calculate extraction efficiency. Across samples the average extraction efficiency was 74 % (± 10).

Flow cytometry cell counts were used to estimate bacterial biomass. To detach bacteria from the leaf matter without damaging the cells samples were first sonicated with an ultrasonic probe (Fisher Scientific Sonic Dismembrator 500, Massachusetts, USA) connected to a 13 mm tip at a low setting of 15 % for 4x20 seconds with 5 second breaks. The sample was then vortexed, subsampled taking 7 % of the volume, and centrifuged at 1000 x g for 12 seconds to remove particulate matter. The protocol for the Invitrogen Bacteria Counting Kit for Flow Cytometry (Invitrogen, California, USA) was then followed, and the samples were run on a BD FACSCanto II flow cytometer (BD Biosciences, USA). Green fluorescence (FL1) was set to 530 ± 30 nm with a gain of 505 nm at a low speed to ensure a limit of 1000 cells per second was not exceeded. Results were then gated using Flo Jo 10 software (FlowJo, Oregon, USA) to quantify microbeads and cells < 6 nm in size. Cells with a fluorescence of $< 10^2$ FSC were also excluded to remove potential background noise. Cell counts were then converted into cells/ g based on the

approximate amount of leaf matter analyzed and a conversion factor of 58fg/ cell used to convert cell counts into an estimate of bacterial biomass (Frossard *et al.*, 2012).

Bacterial and fungal community composition was assessed using 454 pyrosequencing of the 16S and 18S rRNA genes. Samples were first treated with Propidium Monozide (PMA, Biotium, California, USA) to prevent exposed DNA from being extracted and identified (Nocker *et al.*, 2006). A total of 50 uM of PMA was added to each sample of 0.2 g leaf matter suspended in 1.5 mL of sterile water. Samples were then left in the dark for 5 min followed by 2x2 min exposures of 500 W halogen light. Samples were briefly vortexed in between light exposures. After PMA treatment samples were centrifuged for 10 min and the supernatant taken for DNA extraction. DNA was extracted using the MO BIO PowerSoil DNA isolation kit and protocol (MoBio, Carlsbad, USA). Extracted DNA samples were sent to MR.DNA's sequencing facility (Molecular Research and Testing LP) in Shallowater, Texas for 16S and 18S rRNA gene pyrotag sequencing. Pyrotag sequencing was done on the Roche 454 platform following Dowd *et al.* (2008) protocol.

Raw pyrosequence data was processed in Qiime (Caporaso *et al.*, 2010). Sequences were first filtered for low quality and ambiguous reads using Qiime defaults (min quality score=25, min/max length=200/1000). The USEARCH package (Edgar, 2010) was then used to perform denovo and reference based chimera checking using UCHIME (Edgar *et al.*, 2011), cluster sequences at 97 % similarity, and remove singletons. After representative sequences were selected taxonomy of Operational Taxonomic Units (OTUs) was assigned with the 16S rRNA gene GreenGenes database 13/5 with the RDP classifier (Wang *et al.*, 2007), and 18S sequence taxonomy was assigned using the Silva database 111 with a re-trained RDP classifier. All assignments were made with a minimum confidence of at least 0.8. OTU tables were made using

Qiime default settings. Bacterial and fungal OTU tables were then rarefied to remove bias from uneven sample depth (Appendix-3). For the fungal dataset further filtering was required because the 18S rRNA gene region is not fungi specific, and so other eukaryotes were also sequenced. After rarefaction of the complete 18S dataset non-fungal OTUs were filtered out and relative values for fungal abundance used for further analyses.

Extracellular enzyme activities of 6 commonly measured enzymes were measured for 6 replicates per stream. The 6 extracellular enzymes included the 4 hydrolases: α -glucosidase (α -G), β -xylosidase (β -X), Leucine amino-peptidase (LAP), and phosphatase (PHOS), and the 2 oxidases: phenol oxidase (POX), and peroxidase (PER) (Table 3-1). These enzyme activities were measured under controlled conditions (i.e. same pH, temperature, and buffer solution) using engineered substrates. Therefore, the extracellular enzyme activities were a measurement of potential activity and the concentration of extracellular enzyme found in each sample rather than in situ extracellular enzyme activity. Potential hydrolase and oxidase extracellular enzyme activities were measured based on existing protocol (Saiya-Cork *et al.*, 2002; Findlay, 2007). Samples were prepared by mixing 0.5 g of leaf material with 60 mL of acetate buffer (pH 5.5) for 30-60 seconds. The sample slurry was then placed in a petri dish and mixed each time before dispensing into 200 uL microplate wells. Dark hydrolase microplates were set up with a negative control, standard, buffer, quench, and blank to account for fluorescence not produced by the transformation of substrate by extracellular enzymes. Assays (i.e. wells containing sample and substrate) contained 8 replicates per sample. For oxidases the plate set up contained 2 negative control columns, a buffer, a blank for each sample, and 8 wells per sample for both POX and PER assays. Fluorescence for hydrolases and absorbance for oxidases were read on a BioTek Synergy H1 Hybrid Reader (BioTek, Vermont, USA). Fluorescence was read using an excitation

of 365 nm, and emission of 450 nm. Absorbance was read through a 460 nm filter. For hydrolases, 10 uL of 0.5 M NaOH was added to each well after the 4 h incubation period to raise the pH above 7.5 and allow for a fluorescence reading to be obtained. Fluorescence was measured exactly 1 min after NaOH addition because fluorescence has been found to increase with time after addition of NaOH (German *et al.*, 2011). The extinction coefficient was calculated for absorbance assays (Appendix-1), and time trials were performed to measure fluorescence and absorbance every 30 min for 4 h to identify the optimal incubation time (4 h) for each enzyme (Appendix-2).

Watershed and Stream Habitat Attributes as Explanatory Variables

Stream habitat measurements were performed on a 50 m reach surrounding the section of stream where the leaf packs were installed. Stream habitat measurements were collected in the summer of 2010 for White River streams, and the summer of 2012 for Sudbury streams. Physical habitat characteristics measured included depth, width, the abundance of large woody debris/ m² (LWD), sedimentation rates (g/ day/ m²), and percent organic matter in the sediment. Large woody debris was characterized as being > 10 cm in diameter and > 1 m in length. To calculate sedimentation rates deposited sediment was collected in each stream at approximately 10cm from the stream bottom in 7 upright falcon tubes held in place by bricks. After collection the sediment was dried and weighed to calculate the rate sediment was deposited per unit area (i.e. surface area of the tube opening). The collected sediment was combusted at 500°C for 2 h to estimate the amount of inorganic and organic material present. Water chemistry analyses (i.e. Dissolved organic matter (DOM) concentration measured as dissolved organic carbon (DOC), dissolved inorganic carbon, nutrients, cations and metals concentrations) were also performed on 1 or 2 grab samples from each stream collected during leaf pack incubation in the summer of 2012, and were analyzed in

the Canadian Forest Service water chemistry laboratory following standard methods (Beall *et al.*, 2001). Two grab samples were taken from the Sudbury streams with an average used for analyses, but only one grab sample was collected for streams in White River. Temperature and pH were measured in the field with a HI991003 portable pH meter (Hanna Instruments, Rhode Island, USA) and HI 1296 probe, and conductivity measured in the field with a Primo 3 TDS Tester (Hanna Instruments, Rhode Island, USA).

Watershed characteristics included percent cover type (i.e. forest, open wetland, and open water), the roughness coefficient (variation in ground height across watershed), road density (road length (m)/ watershed area (m^2)), watershed area (m^2), system length (water line through watershed including lakes and streams (m)), and drainage density (total stream length (m)/ watershed area (m^2)), and were calculated using ArcGIS software based on the White River Sustainable Forest management Plan 2008 (sfmmO8), the Sudbury Forest Management Unit 2010 (889), and the Ontario Base Topographic Map. Watersheds were delineated using Digital Elevation Models or Enhanced Flow Accumulation Grids from Land Information Ontario (LIO). Riparian vegetation surveys were conducted for each stream. Briefly, 2, 26 m transects parallel to the stream were marked 5 m from the streams edge on both sides for a total of 4 transects per stream. Along each transect 50 cm diameter circular plots were examined to record the density (i.e. number of stems originating from within the plot) for each species of woody stemmed vegetation in the plot.

Statistical Analyses

Selection of the best multiple regression model using second order Akaike Information criterion (AICc) complimented with hierarchical partitioning, and canonical correspondence analysis (CCA) were used to explore if key stream and watershed characteristics (i.e. explanatory variables) explained variation in microbial community structure and function (i.e. response

variable) across a gradient of disturbance and recovery (Mac Nally, 2000). Prior to analyses variables that were not normally distributed were transformed using logarithmic or square root transformations, or arc sin square root transformations for percentages. All statistical analyses were performed in R 3.0.1 (R Core Team, 2013).

Before AICc selection, hierarchical partitioning, and CCA, explanatory variables were first selected from the suite of collected variables to avoid problems of multicollinearity. Variables were removed based on ecological theory and a variance inflation factor (VIF) cut off of > 5 (O'Brien, 2007). VIF was implemented in the package car (Fox & Weisberg, 2011). Stream and watershed variables were grouped into ecologically significant categories where applicable (e.g. water quality, nutrients, metals, ions). When there were problems of multicollinearity expressed by high VIF values redundant, less ecologically relevant variables were removed (Table 3-2). In total, 9 stream habitat variables with VIF values < 5 were kept. The stream variables included pH, P (measured as TP), N (measured as TN), DOC concentration, Ni, conductivity, sedimentation rate, abundance of large woody debris (LWD), and temperature. The seven selected watershed variables included: Shannon diversity of riparian woody stem vegetation, % forest cover, % open wetland cover, % open water, the roughness coefficient, road density, and drainage density (Table 3-2). These watershed variables all had VIF values of < 3 .

AICc was used for multiple regression model selection. Akaike information criterion is based on likelihood instead of p-values and penalizes for model complexity to select the simplest model that explains the most variation, and AICc is a corrected version of AIC that accounts for a small sample size to prevent over fitting (Burnham & Anderson, 2002). Multiple regression with AICc model selection was used to identify potential key habitat and watershed variables associated with microbial community biomass, decomposition, and extracellular enzyme activities.

Watershed conditions can influence stream habitat characteristics, which can in turn influence aquatic organisms (Likens & Bormann, 1974; Paller *et al.*, 2014), therefore AICc multiple regressions were performed in two separate models. AICc multiple regressions were first performed to identify key habitat variables (i.e. explanatory variables) associated with each microbial characteristic (i.e. response variable), and then a second round of AICc multiple regressions was run to identify key watershed variables (i.e. explanatory variables) associated with each key habitat variable (i.e. response variable), thus identifying relationships from microbial community to stream habitat, and stream habitat to watershed. AICc selection was performed using the function ‘stepAICc’ in R (Scherber, 2011). Akaike weights (w_i) were calculated to compare the strength of the ‘best’ model with all other models generated in the AICc step process (Burnham & Anderson, 2002).

Hierarchical partitioning was used to complement multiple regression. Hierarchical partitioning calculates the individual influence of each variable in a global model by calculating the individual and joint influence of each variable in all possible model combinations, and then taking the average (Chevan & Sutherland, 1991; Mac Nally, 2000). This approach moves away from single model inferences and in combination with multiple regression provides insight into the relative influence and relationship of key variables. Hierarchical partitioning was performed in the package hier.part (Walsh & Mac Nally, 2013).

CCA was used to examine the influence of environmental factors on fungal and bacterial community composition. CCA is a direct gradient analysis that constrains species composition data by environmental variables, and is essentially the combination of ordination and multiple regression techniques. Fungal and bacterial OTU abundance and selected stream habitat variables were used in a CCA to examine if environmental variables explained any variation in

fungus and bacterial community composition. Stepwise selection with AIC was performed using the function `ordistep` in the `vegan` package (Oksanen *et al.*, 2013) to select key variables from the group of 9 stream habitat variables that best explained variation in fungus and bacterial community composition.

3.4 Results

Gradient of disturbance and recovery

The gradient of disturbance and recovery was reflected in watershed, stream habitat, and microbial community characteristics across streams in this study. Watershed characteristics ranged from 40-97 % for forest cover, 0-13 % for wetland cover, 0-27 % for open water cover, 0-0.005 for road density, 0.0002-0.0036 for drainage density, 0.023-0.097 for the roughness coefficient, and 0.7-2.45 for Shannon riparian diversity (Figure 3-1). The most severely disturbed industrial/urban and industrial streams had watersheds with the lowest % forest cover and lowest riparian diversity compared to all other disturbances. Industrial/urban streams also had watersheds with the greatest road density representative of urban influences (Figure 3-1).

Water chemistry variables ranged from 3.20-18.6 (mg/ L) for DOC, 4.59-14.7 for HIX, 0.004-0.04 (mg/ L) for P, 0.151-3.90 (mg/ L) for N, 0.001-0.245 for Ni, 0.06-4.30 (g/ h/ m²) for sedimentation rate, 0.003-0.190 (#/ m²) for the abundance of LWD, 12.0-20.9 (°C) for water temperature, 4.9-6.9 for pH, and 22.7- 1020.3 (ppm/ C) for conductivity (Figure 3-2). Out of the main water chemistry elements measured conductivity was found to be most strongly associated with Mg ($r = 0.866$, $p\text{-value} = <0.001$) and Ca ($r = 0.853$, $p\text{-value} = <0.001$) (Table 3-3). At the most severely impacted industrial/urban and industrial streams temperature, and P and Ni concentrations were the highest, and LWD, DOC, and HIX were the lowest compared to all other disturbance types (Figure 3-2). Although only DOC concentration was used in analyses in this

study, DOC concentration was found to be positively correlated with HIX across streams ($r = 0.55$, $p\text{-value} = <0.01$).

A range in microbial community characteristics were also found across streams in this study with leaf litter mass loss (i.e. microbial decomposition) ranging from 0.08-29.21 %, fungal biomass from 6.67- 473 (mg/ g), bacterial cell counts from 1.10-8.68 ($\times 10^7$), α -G activity from 0.38-1.85 (nmol/ h/ g), β -X activity from 1.14- 7.82 (nmol/ h/ g), LAP activity from 0.57- 3.94 (nmol/h/g), PHOS activity from 6.40-47.9 (nmol/h/g), POX activity from 0.45-1.23 (umol/h/g), and PER activity from 0.40-2.79 (umol/h/g) (Figure 3-3). Industrial/urban and industrial streams had the lowest decomposition and fungal biomass, while industrial/urban streams had the highest bacterial biomass (Figure 3-3).

Stream habitat associations with microbial community function

DOC, conductivity, LWD, sedimentation, pH, temperature, P, N, and Ni were the principal variables that proved useful in explaining variation in microbial community function. Across all measures of microbial function (i.e. microbial decomposition, and extracellular enzyme activities) significant relationships with either conductivity or DOC concentration were found (Table 3-4). For microbial decomposition a positive relationship with conductivity and DOC concentration, and a negative relationship with P and pH occurred (adjusted $R^2 = 0.449$, $p\text{-value} = < 0.01$). For the extracellular enzyme activities the following relationships were found: β -X activity had a positive relationship with N and pH, and a negative relationship with conductivity (adjusted $R^2 = 0.274$, $p\text{-value} = < 0.05$), α -G activity had a positive relationship with DOC concentration and N, and a negative relationship with conductivity (adjusted $R^2 = 0.585$, $p\text{-value} = < 0.001$), LAP activity had a positive relationship with DOC concentration, and a negative relationship with pH, P, and LWD (adjusted $R^2 = 0.332$, $p\text{-value} = < 0.05$), PHOS activity had a

positive relationship with N and pH, and a negative relationship with conductivity (adjusted $R^2 = 0.474$, p-value = < 0.01), POX activity had a positive relationship with DOC concentration, temperature and sedimentation rate, and a negative relationship with N (adjusted $R^2 = 0.539$, p-value = < 0.001), and finally PER activity had a positive relationship with DOC concentration, temperature and LWD, and a negative relationship with N (adjusted $R^2 = 0.736$, p-value = < 0.001) (Table 3-4).

DOC concentration and conductivity were the variables that had the greatest influence on microbial functions based on hierarchical partitioning. DOC concentration was the most influential stream habitat variable for microbial decomposition, α -G activity, LAP activity, POX activity, and PER activity with an independent contribution of 23.7 %, 43.2 %, 21.2 %, 29.6 %, and 30.9 % respectively, while conductivity was the most influential variable for β -X activity and PHOS activity with an independent contribution of 27.3 %, and 48.9 % respectively (Figure 3-4). These key variables identified by hierarchical partitioning agreed with the results of AICc multiple regression for all response variables except for microbial decomposition and POX activity. For microbial decomposition conductivity explained the most variation, and for POX N explained the most variation instead of DOC concentration in the AICc selected 'best' multiple regression model. DOC concentration did however explain the second most amount of variation for both microbial decomposition and POX activity in these models.

Stream habitat associations with microbial community structure

A number of stream habitat characteristics were also found to explain variation in microbial community structure. Fungal biomass was found to be positively related to LWD and pH, and negatively related to Ni based on the 'best' AICc selected multiple regression model (adjusted $R^2 = 0.668$, p-value = < 0.001) (Table 3-4). For the fungal community composition, P was the best

stream habitat explanatory variable across streams ($p\text{-value} = < 0.01$) explaining 9 % of the unconstrained variation in the relative abundance of fungal OTUs (Figure 3-6), and the abundance of taxa from the class Eurotiomycetes was found to be associated with elevated P concentrations across streams ($\rho = 0.652$, $p\text{-value} = < 0.001$). Bacterial biomass had a positive relationship with conductivity, sedimentation rate, and LWD (adjusted $R^2 = 0.667$, $p\text{-value} = < 0.001$) (Table 3-4), and for bacterial community composition conductivity also explained variation along with N, P and Ni. Conductivity, N, P, and Ni explained a total of 25 % of the unconstrained variation in the relative abundance of bacterial OTUs across streams ($p\text{-value} = < 0.01$) (Figure 3-7), and the abundance of Beta-proteobacteria was found to be negatively associated with conductivity ($r = -0.536$, $p\text{-value} = < 0.01$). Further correlation between Beta-proteobacteria and enzyme activities revealed positive relationships between the abundance of Beta-proteobacteria and PHOS ($r = 0.633$, $p\text{-value} = < 0.01$) and $\beta\text{-X}$ ($r = 0.679$, $p\text{-value} = < 0.001$) activities (Appendix-5).

Hierarchical partitioning returned the same key habitat variables described by multiple regression for fungal and bacterial biomass. LWD had the largest individual contribution of 27.3 % for fungal biomass, and conductivity the largest individual contribution of 33.5 % for bacterial biomass (Figure 3-4).

Watershed characteristics associated with stream habitat

A clear link back from stream characteristics to watershed characteristics was also evident in the data set. In particular % forest cover was found to explain variation in all stream habitat variables except for N. Increasing % forest cover was related to increasing LWD, DOC concentrations, pH, and decreasing sedimentation, Ni, P, temperature, and conductivity. In relation to water chemistry DOC concentration had a positive relationship with % forest cover, % wetland cover

and road density, and a negative relationship with % open water cover (adjusted $R^2 = 0.521$, p-value = < 0.01), conductivity had a positive relationship with road density and riparian vegetation diversity, and a negative relationship with % forest cover (adjusted $R^2 = 0.576$, p-value = < 0.001), pH had a positive relationship with % forest cover and road density, and a negative relationship with % open water cover and drainage density (adjusted $R^2 = 0.446$, p-value = < 0.01), P had a negative relationship with % forest cover (adjusted $R^2 = 0.563$, p-value = < 0.001), N had a positive relationship with road density (adjusted $R^2 = 0.299$, p-value = < 0.01), and Ni had a positive relationship with drainage density, and a negative relationship with % forest cover, % open wetland cover, riparian woody stemmed diversity and the roughness coefficient (adjusted $R^2 = 0.911$, p-value = < 0.001) (Table 3-5). Physical stream habitat characteristics such as temperature, LWD, and sedimentation rate also showed predictable relationships with watershed characteristics. Temperature was found to be positively related to road density and negatively related to % forest cover (adjusted $R^2 = 0.467$, p-value = < 0.001), LWD was found to be positively related to % forest cover and drainage density (adjusted $R^2 = 0.275$, p-value = < 0.05), and sedimentation rate was found to be negatively related to % forest cover, % open water cover and the roughness coefficient (adjusted $R^2 = 0.392$, p-value = < 0.01) (Table 3-5).

When I applied hierarchical partitioning percent forest cover and road density were the variables that proved to be the most influential of stream habitat conditions. Percent forest cover explained the most variation in LWD (I= 47.1%), sedimentation rate (I= 43.1 %), P (I= 66.8 %), and Ni (I= 54.9 %), and road density explained the most variation for conductivity (I= 56.7 %), temperature (I= 38.4 %), and N (I= 42.3 %). Riparian woody stemmed diversity was the most influential variable for pH (I= 29.2 %), although closely followed by % forest (I= 23.4 %) (Figure 3-5).

Results from hierarchical partitioning concurred with results from multiple regression with a few exceptions. For DOC concentration % wetland cover was identified as the most important variable by hierarchical partitioning, while percent forest cover was identified as the most important variable by the ‘best’ AICc multiple regression model. However, in both analyses the difference between percent wetland cover and percent forest cover was very small with slopes of 0.612 and 0.674 respectively in the DOC concentration ‘best’ AICc multiple regression model, and an independent contribution of 27 % and 26.7 % respectively based on hierarchical partitioning. Therefore, both percent wetland cover and percent forest cover were identified as the most important variables for DOC concentration across streams (Figure 3-5). For pH riparian diversity which was identified as the most important variable by hierarchical partitioning did not enter the ‘best’ AICc multiple regression model. However, % forest cover which was the second most influential variable according to hierarchical partitioning, explained the most variation based on multiple regression. This suggests that there was an important association between % forest cover and pH.

3.5 Discussion

Key stream habitat characteristics associated with microbial communities

DOC was the most important factor explaining the microbial processes in these study streams and was associated with increased microbial decomposition, and α -G, LAP, POX, and PER potential enzyme activities. Along with DOC concentration the quality of DOM has also been found to influence microbial activity (Findlay, 2003; Hosen *et al.*, 2014). Humified organic matter is typically of terrestrial origin (McKnight *et al.*, 2001), and forest stream ecosystems, like the streams in this study, are known to be heavily dependent on terrestrial inputs (Kaushik & Hynes, 1968; Fisher & Likens, 1973). A positive relationship between DOC concentration and

HIX suggests that streams with a higher concentration of DOC in this study therefore have more terrestrial input and represent a DOM pool that is more humified. However, increases in humic material would likely contribute little to increases in the “quality” of the DOM (Findlay, 2003), therefore, it is likely that DOC concentration was the main stimulator of microbial decomposition, α -G, LAP, POX, and PER potential activities in my study. In particular, low DOC concentrations appeared to represent or were associated with a stressor in industrially disturbed streams because industrial/urban and industrial streams had lowest DOC concentrations, decomposition rates, and PER potential activities.

Unlike DOC, elevated specific conductance or conductivity appeared to hinder the production of some extracellular enzymes having a negative association with potential β -X and PHOS activities. This negative relationship may be explained by structural changes in the bacterial community driven by conductivity. Across the streams in this study, conductivity was found to be the most important variable explaining variation in bacterial biomass, and was also found to explain variation in bacterial community composition, specifically in the abundance of Betaproteobacteria. Betaproteobacteria was also found to be positively associated with PHOS and β -X activities. Therefore, a lower abundance of Betaproteobacteria associated with increasing conductivity across streams may help explain the negative impact of conductivity on β -X and PHOS potential activity. Bacterial community composition has been found to influence β -X and PHOS potential activities in previous studies. For example, a study by Kirchman *et al.* (2004) found a positive relationship between an increasing abundance of Betaproteobacteria and PHOS activity, along with a relationship between the abundance of Alphaproteobacteria and β -X activity in the Hudson River. At industrial/urban streams conductivity appeared to have the most impact where extremely high conductivities were coupled with the lowest Betaproteobacterial

abundance, and β -X and PHOS potential activities when compared to all other disturbances. Conductivity in streams was most strongly associated with the main constituents of road salts (i.e. Ca and Mg) suggesting that increased export of road salts from urban watersheds may have been responsible for extremely high conductivities in industrial/urban streams.

Previous studies have also found that stream bacterial community composition was strongly affected by nutrient enrichment (Olapade & Leff, 2006; Fierer *et al.*, 2007; Rubin & Leff, 2007), and metal contamination (Lawrence *et al.*, 2004; Vishnivetskaya *et al.*, 2011). However, although N, P, and Ni did explain some variation in bacterial community composition, N, P, and Ni did not appear to be as important as conductivity in structuring bacterial communities given the strong associations found between conductivity, and bacterial biomass and Betaproteobacterial abundance.

Unlike bacterial communities, fungal community composition was found to be most influenced by P concentrations. Nutrient enrichment has been reported to influence fungal community composition in different ways. Studies have found nutrient enrichment to increase fungal diversity (Suberkropp, 1995; Gulis & Suberkropp, 2003), have no effect on fungal composition (Ferreira, Gulis & Graça, 2006), and to decrease fungal diversity in highly eutrophied systems (Lecerf & Chauvet, 2008; Duarte *et al.*, 2014). Regardless, 9 % explained variation attributed solely to P highlights inorganic nutrients as a potentially important factor for fungal community composition across the streams in my study. In particular, high P concentrations found at industrial/urban and industrial streams were associated with a high abundance of fungi from the class Eurotiomycetes. The fungal class Eurotiomycetes could therefore be a potential indicator of elevated nutrients.

Fungi are primary decomposers in the aquatic ecosystem rapidly colonizing, reproducing and making up the majority of microbial biomass on decomposing leaf litter (Gessner *et al.*, 2007). Fungal biomass has been found in several studies to be positively associated with leaf litter decomposition rates (Chapter 2, Gessner & Chauvet, 1994; Dangles & Chauvet, 2003; Pascoal & Cássio, 2004; Duarte *et al.*, 2010), and is known to enhance leaf litter as a food source to macroinvertebrates (Bärlocher, 1985; Suberkropp, 1992; Graça, 2001). In this study fungal biomass was found to be most influenced by the abundance of LWD in streams. LWD is an important retention mechanism in lotic systems because it traps leaves and other organic matter allowing for microbial colonization (Bilby & Likens, 1980). Streams with a greater abundance of LWD can therefore potentially house a greater standing stock of fungi, which could have contributed to our measure of fungal biomass on leaf material. The relatively low abundance of LWD in industrial/urban and industrial streams is in accordance with the lowest fungal biomass and decomposition rates among my study streams.

Although Ni and pH did explain some variation in fungal biomass, they were not the most important variables for either fungal biomass or fungal community structure. This is not surprising as aquatic fungi have been observed to have a large optimal pH range from 5-7 (Bärlocher, 1987), and some aquatic fungi have been found to be resilient to metal contamination (Sridhar *et al.*, 2008, Krauss *et al.*, 2011). Furthermore, the levels of Ni concentration and acidity at industrial streams in this study though high in some cases, were not that of extreme environments like acid mine drainage, making it plausible that the reduced substrate availability (LWD) and elevated nutrient concentrations were the more influential factors for fungal community structure in the industrial/urban and industrial streams.

Key watershed characteristics associated with stream habitat

Forests were identified as a provider of essential DOC and retention structures to the aquatic ecosystem agreeing with previous studies that identified large woody debris as an important retention mechanism allowing for microbial colonization (Bilby & Likens, 1980), and fallen leaf litter as fuel for the aquatic food web (England & Rosemond, 2004; Szkokan-Emilson *et al.*, 2011; Tanentzap *et al.*, 2014). Furthermore, decreases in forest cover have been found to increase weathering rates and sedimentation, change hydrology, increase water temperatures, and alter the export of nutrients and contaminants from the soil into the aquatic ecosystem (Binkley & Brown, 1993; Hazlett *et al.*, 2007). These relationships between forest cover and stream habitat quality were also identified in this study where forest cover was found to mitigate sedimentation rates, and reduce conductivity, water temperature, and nutrient and metal concentrations (i.e. P, Ni). Industrial/urban streams were the most impacted by decreased forest cover in the watershed where the lowest % forest cover coupled with the lowest DOC concentrations and abundance of LWD, were associated with the highest sedimentation rates, conductivities, temperatures, and metal and nutrient concentrations across streams.

Along with forests, wetlands were also identified as providers of essential DOC stimulating activity in the aquatic ecosystem. Wetlands have been identified as an important source of DOM to the aquatic ecosystem because they are rich in vegetation, water and microbial decomposition (Mulholland & Kuenzler, 1979; Dillon & Molot, 1997). Wetlands also appeared to help decrease sedimentation rates and Ni concentrations in streams agreeing with previous studies on the retention of contaminants and sediment in wetlands (Johnston, 1991).

Unlike forests and wetlands, road density was suspected to have a negative influence on stream habitat quality and microbial activity. Across the streams in this study road density was found to

influence water chemistry through increases in temperature, N concentrations and conductivity consistent with previous studies on road influences (Trombulak & Frissell, 2000). These influences were most evident at industrial/urban streams where road density was the highest.

3.6 Summary and conclusions

This study demonstrates that watershed disturbance may influence the essential ecosystem services that microbial communities provide. Firstly, microbial decomposition and enzyme activities appeared to be fuelled by DOC supplied by forests and wetlands. Secondly, fungal biomass seemed to be stimulated by the presence of large woody debris supplied by forests acting as an important retention mechanism of OM in streams, and finally, some microbial enzyme activities appeared to be hindered by a decrease in Betaproteobacterial abundance caused by elevated conductivity associated with road density (Figure 3-8). Therefore, the restoration of forests and wetlands in industrially-disturbed watersheds appears to be important in providing the supply and transport of DOC to fuel the microbial cycling of energy and nutrients within the aquatic ecosystem. Conversely, roads should be taken into consideration as having a potentially negative impact on the aquatic ecosystem given the potential of elevated conductivities from road salts to alter microbial community structure and hinder the cycling of energy and nutrients.

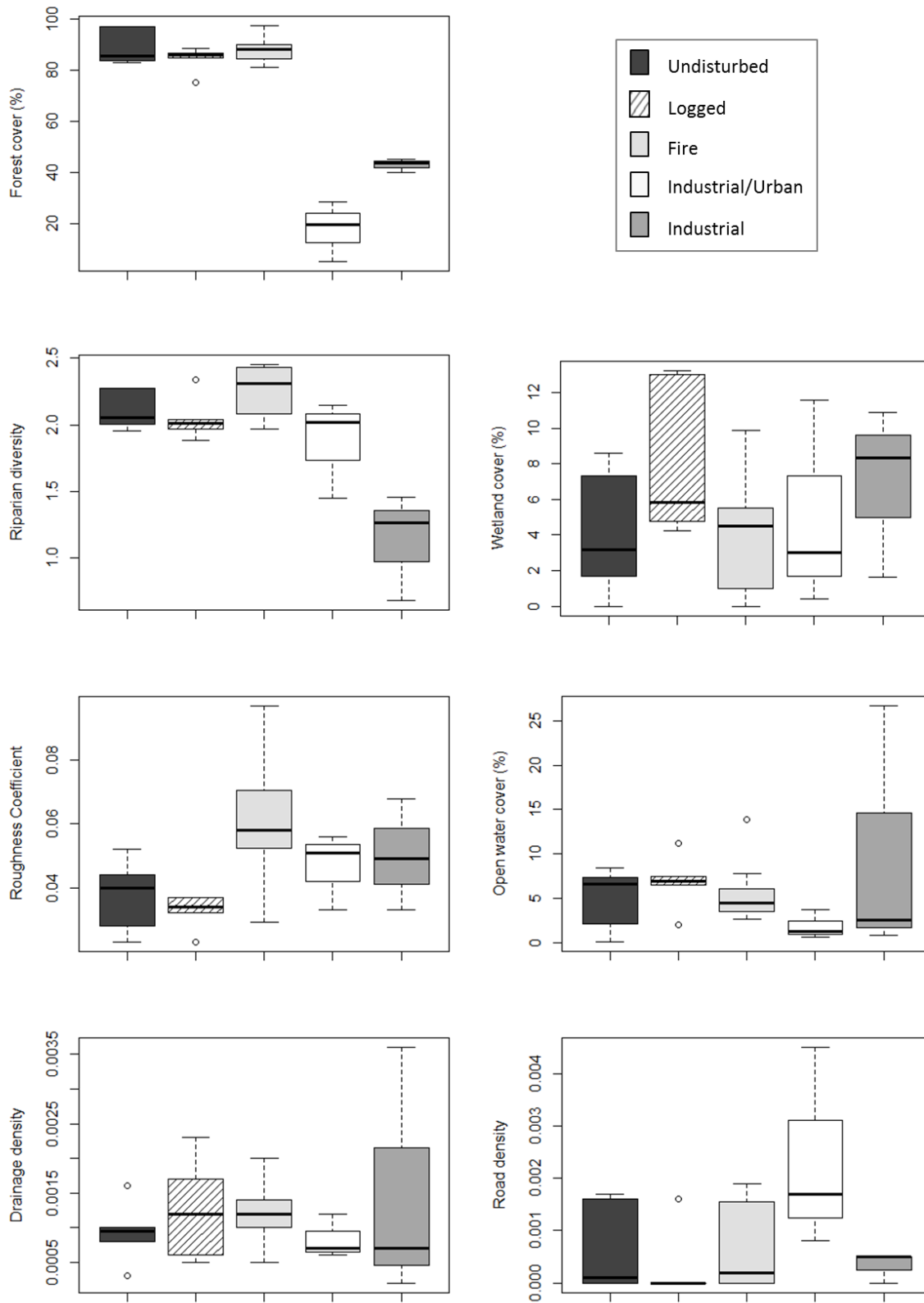


Figure 3-1 Watershed characteristic boxplots by disturbance type with median values and 95% confidence intervals displayed.

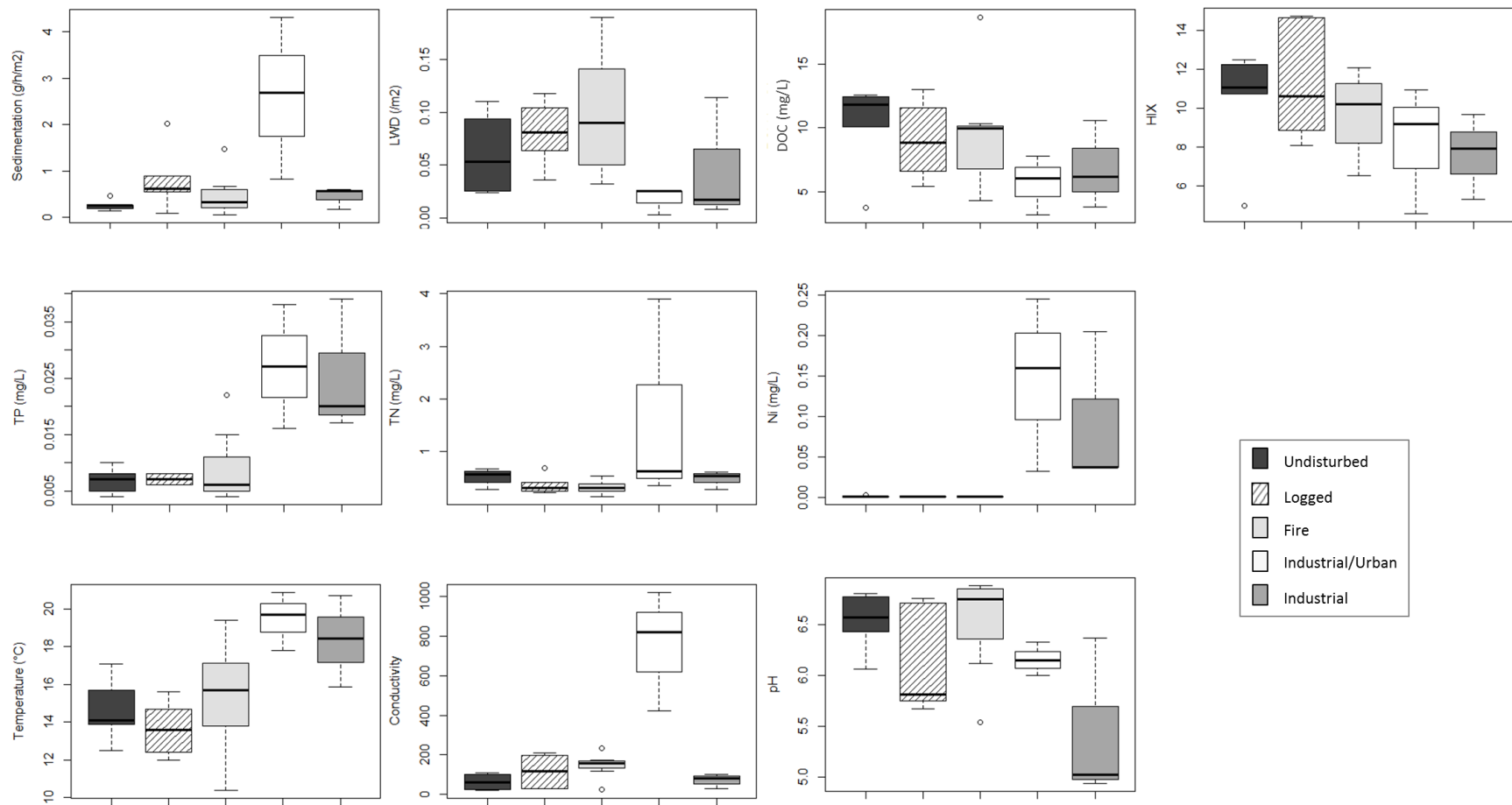


Figure 3-2 Stream habitat characteristic boxplots by disturbance type with the median and 95% confidence intervals displayed.

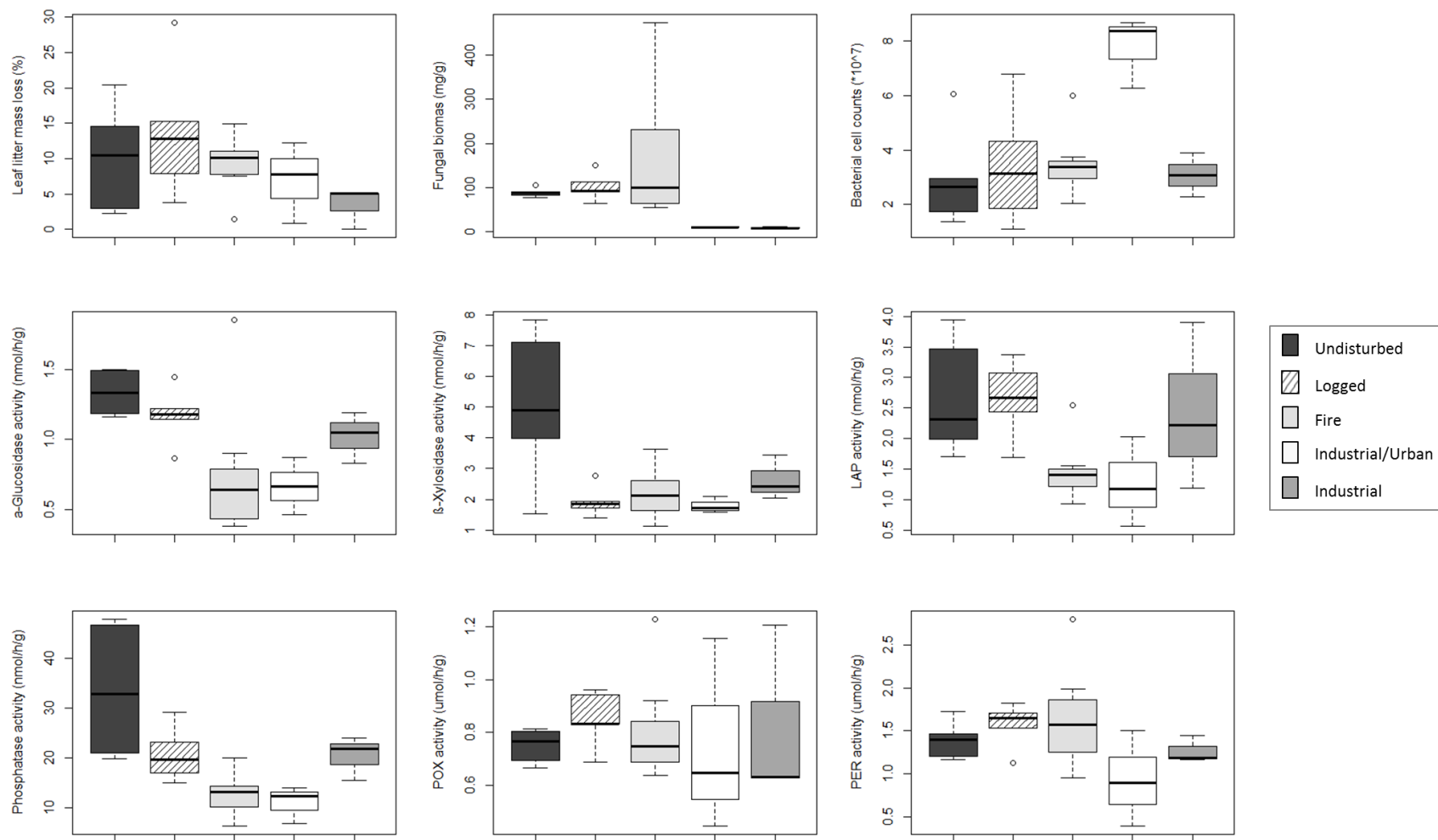


Figure 3-3 Microbial community characteristic boxplots by disturbance type with median and 95% confidence intervals displayed.

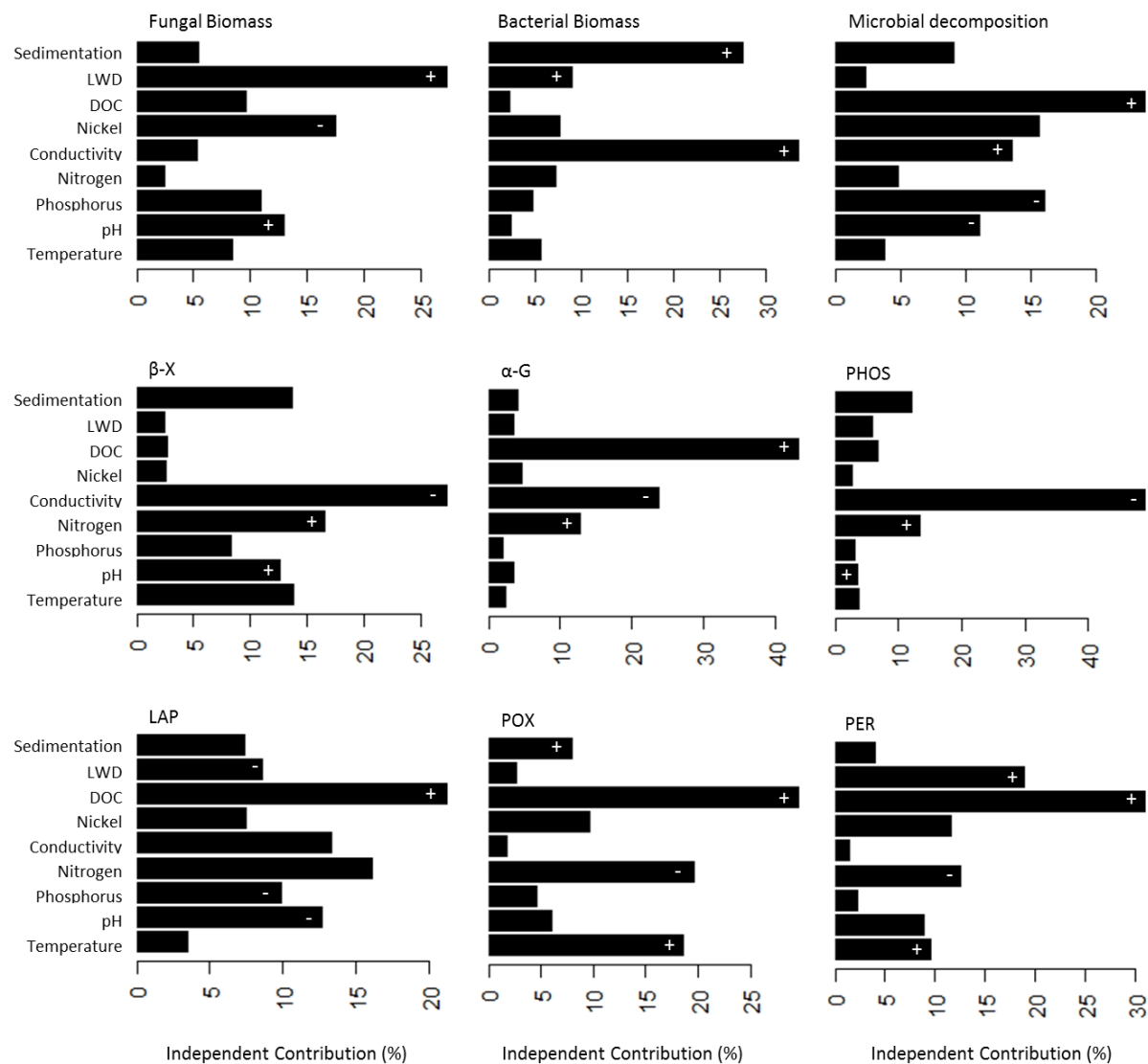


Figure 3-4 Independent contribution (%) of each stream habitat explanatory variable to each microbial community response variable based on hierarchical partitioning. +/- signs are on variables that explained variation in multiple regression and represent the direction of that relationship.

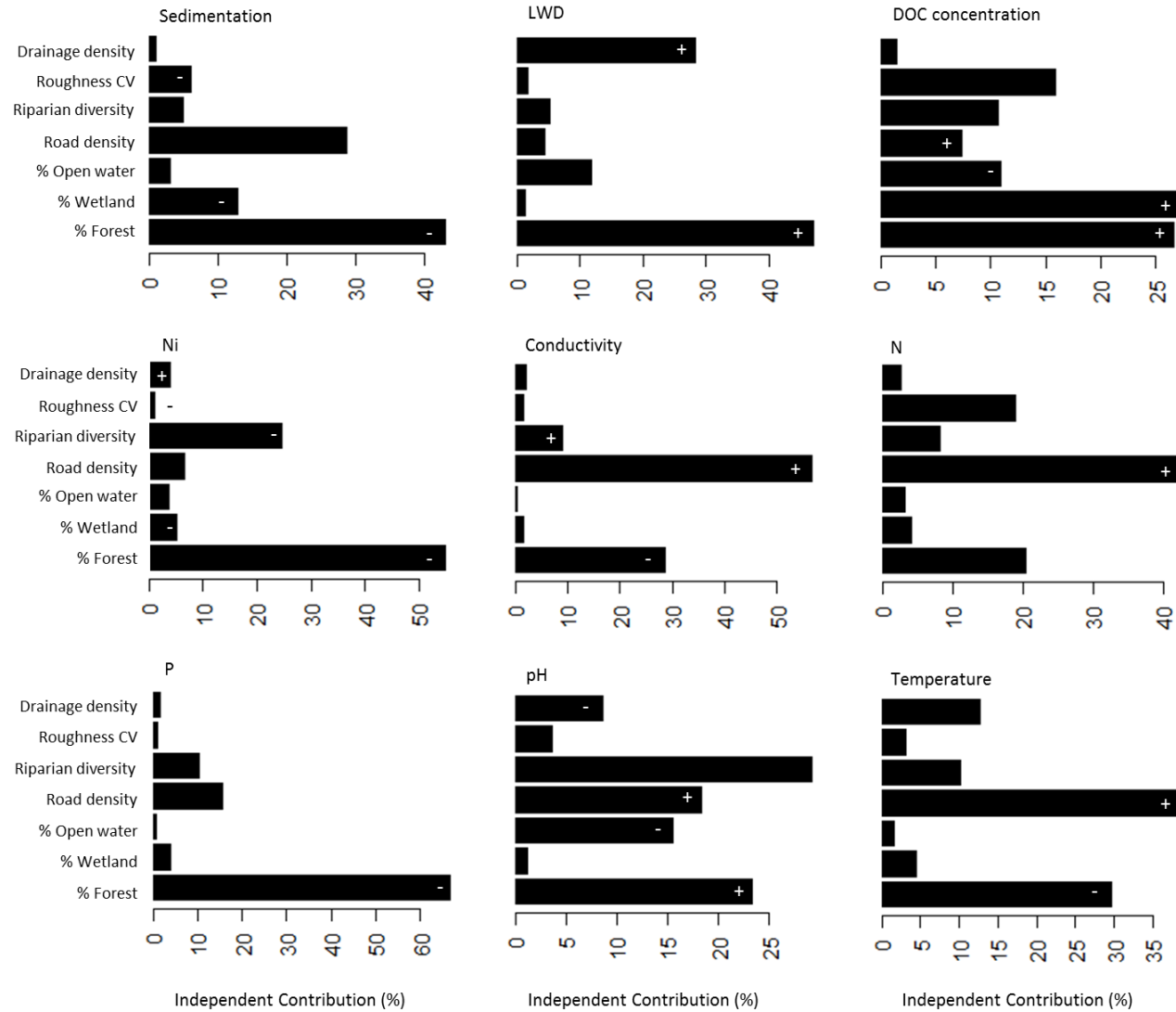


Figure 3-5 Independent contribution (%) of each watershed explanatory variable to each stream habitat response variable based on hierarchical partitioning. +/- signs are on variables that explained variation in multiple regression and represent the direction of that relationship.

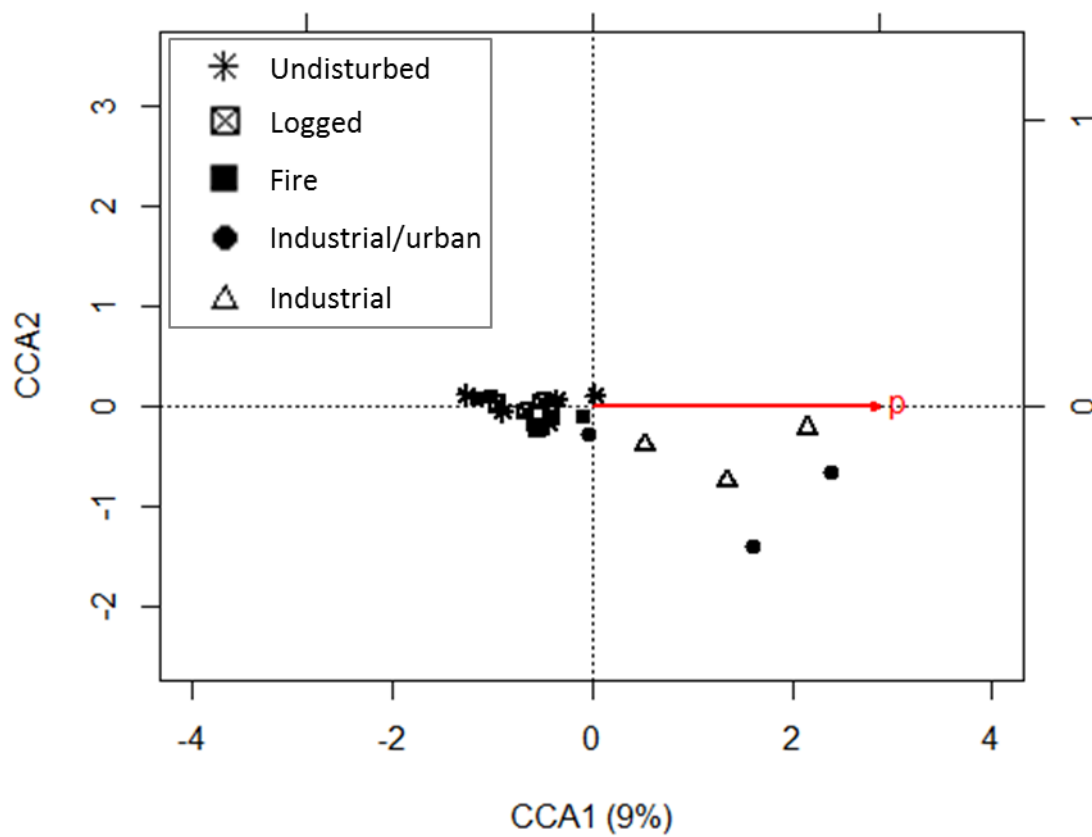


Figure 3-6 CCA (p-value $ < 0.01$) of the relative abundance of fungal OTUs constrained by water chemistry variables. The total explained variation by all axes was 9%.**

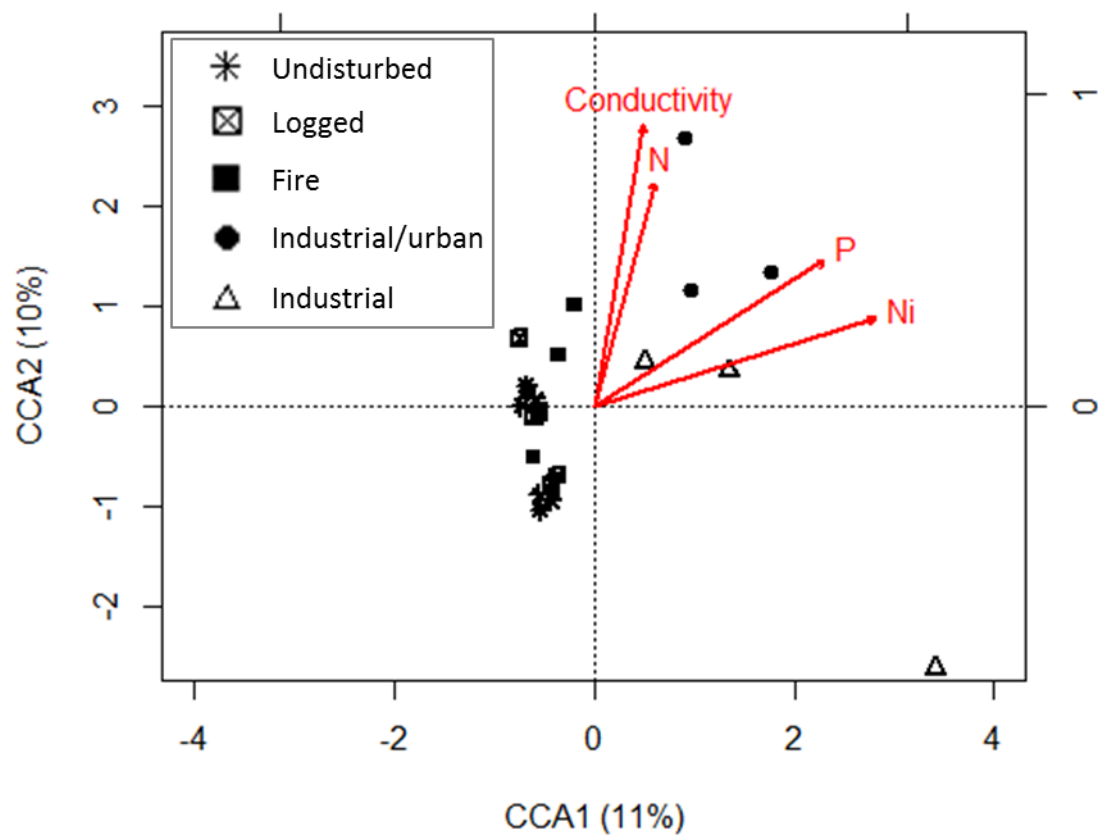


Figure 3-7 CCA (p-value= **<0.01) of the relative abundance of bacterial OTUs constrained by water chemistry variables. The total variation explained by all axes and selected variables was 25%.

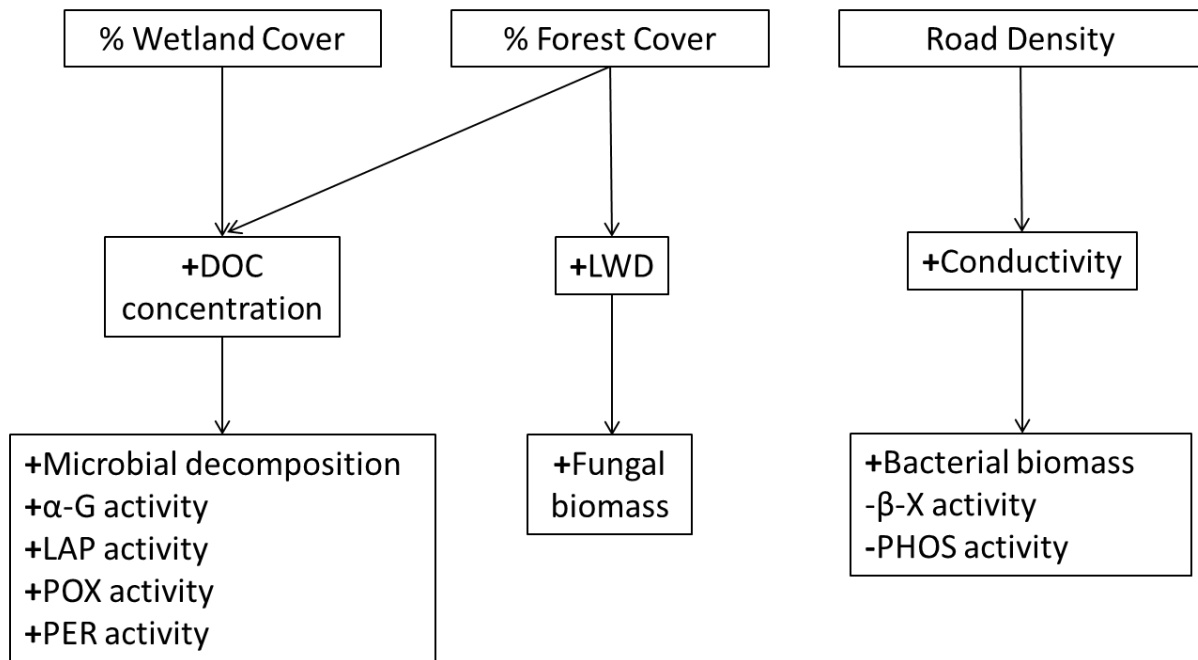


Figure 3-8 Land-water linkages flow chart highlighting the main linkages between the watershed and stream habitat, and stream habitat and microbial communities found in this study. The direction of each relationship is marked as + positive / - negative.

Table 3-1 Measured enzyme activities, substrates used, and function of enzymes (Sinsabaugh et al., 2008; Kirchman, 2012).

Enzyme	Substrate Used	Function
α -Glucosidase (α -G)	4-MUB- α -D-glucopyranoside	Starch and disaccharide degradation
β -Xylosidase (β -X)	4-MUB- β -D-xylopyranoside	Hemi-cellulose degradation
Phosphatase (PHOS)	MUB-Phosphate	Phosphomonoester degradation
Leucine Aminopeptidase (LAP)	L-leucine-7-amido-4-methylcoumarin	Peptide and protein degradation
Phenol Oxidase (POX)	LDOPA	Polyphenol oxidation (E.g. lignin)
Phenol peroxidase (PER)	LDOPA+ peroxide	Polyphenol oxidation (E.g. lignin)

Table 3-2 All variables collected, and variables selected for analyses based on the variance inflation factor (VIF) and grouping by ecological relevance.

	Variables Collected	Variables Selected
Stream Habitat Characteristics	pH, Alkalinity	pH
	NUTRIENTS: TP, TN, SO4, DOC, DIC	TP, TN, DOC
	INDUSTRIAL METALS: Ni, Zn, Cu	Ni
	IONS: Ca, Mg, Cl, Na, K, Fe, Mn, Al, conductivity	conductivity
	OTHER: Sedimentation, % organic in sediment, abundance of LWD/m2, HIX, depth, width, temperature	sedimentation, LWD, temperature
Watershed Characteristics	RIPARIAN VEGETATION: diversity, density	riparian diversity
	COVER TYPE: % forest, % open wetland, % open water	%forest, %open wetland , %open water
	OTHER: Roughness Coefficient (CV), road density, watershed area, system length, drainage density	roughness coefficient road density drainage density

Table 3-3 Pearson correlations between conductivity and stream water ions and nutrients

Variable	Conductivity	
	r	p-value
Mg	0.866	<0.001
Ca	0.853	< 0.001
K	0.736	<0.001
Cl	0.625	<0.01
Na	0.605	<0.01
DIC	0.578	<0.01
TN	0.527	<0.01
Al	-0.487	<0.05
TP	0.386	ns
Ni	0.371	ns
Zn	0.241	ns
Cu	0.173	Ns
Mn	-0.105	Ns
Fe	-0.312	Ns

Table 3-4 AICc multiple regression results with stream habitat characteristics as the explanatory variables and microbial community characteristics as the response variables. The variable that explains the most variation for each response variable is in bold, and +/- signs represent a positive/ negative relationship respectively.

	Fungal Biomass		Bacterial Biomass		Microbial Decomposition	
	slope	p-value	slope	p-value	slope	p-value
DOC conc.	-	-	-	-	+0.67	**<0.01
LWD	+0.496	***<0.001	+0.263	*<0.05	-	-
Sedimentation	-	-	+0.393	*<0.05	-	-
pH	+0.301	*<0.05	-	-	-0.496	ns
Temperature	-	-	-	-	-	-
Phosphorus	-	-	-	-	-0.669	**<0.01
Nitrogen	-	-	-	-	-	-
Nickel	-0.381	*<0.05	-	-	-	-
Conductivity	-	-	+0.543	**<0.01	+0.73	**<0.01
AICc		50.61		50.7		65.13
w_i		0.763		0.696		0.713

*AICc=AIC value of selected model, w_i =Akaike weight

Table 3-4 continued. AICc multiple regression results with stream habitat characteristics as explanatory variables and microbial community characteristics as the response variables. The variable that explains the most variation for each response variable is in bold, and +/– signs represent a positive/ negative relationship respectively.

	β -X EA		α -G EA		LAP EA		PHOS EA		POX EA		PER EA	
	Slope	p-value	slope	p-value	slope	p-value	slope	p-value	slope	p-value	slope	p-value
DOC conc.	-	-	+0.632	***<0.001	+0.76	**<0.01	-	-	+0.631	***<0.001	+0.571	***<0.001
LWD	-	-	-	-	-0.48	*<0.05	-	-	-	-	+0.376	*<0.05
Sedimentation	-	-	-	-	-	-	-	-	+0.37	Ns	-	-
pH	+0.339	ns	-	-	-0.614	*<0.05	+0.285	ns	-	-	-	-
Temperature	-	-	-	-	-	-	-	-	+0.437	*<0.05	+0.403	*<0.05
Phosphorus	-	-	-	-	-0.537	*<0.05	-	-	-	-	-	-
Nitrogen	+0.407	ns	+0.471	**<0.01	-	-	+0.473	*<0.05	-0.699	**<0.01	-0.439	**<0.01
Nickel	-	-	-	-	-	-	-	-	-	-	-	-
Conductivity	-0.762	**<0.01	-0.593	**<0.01	-	-	-0.938	***<0.001	-	-	-	-
AICc		69.38		55.95		69.76		61.63		60.86		47.51
w_i		0.688		0.746		0.742		0.31		0.454		0.467

*AICc=AIC value of selected model, w_i =Akaike weight

Table 3-5 AICc multiple regression results with watershed characteristics as explanatory variables and stream habitat characteristics as response variables. The variable that explains the most variation for each response variable is in bold, and +/ – signs represent a positive/negative relationship respectively.

	DOC concentration		Conductivity		LWD		Sedimentation		pH	
	slope	p-value	slope	p-value	slope	p-value	slope	p-value	slope	p-value
% Forest Cover	+0.674	***<0.001	-0.459	*<0.05	+0.439	*<0.05	-0.61	**<0.01	+0.637	**<0.01
% Wetland	+0.612	***<0.001	-	-	-	-	-0.418	*<0.05	-	-
% Open water	-0.367	*<0.05	-	-	-	-	-	-	-0.433	*<0.05
Roughness Coeff. (CV)	-	-	-	-	-	-	-0.312	ns	-	-
Road Density	+0.443	*<0.05	+0.537	**<0.01	-	-	-	-	+0.472	*<0.05
Drainage Density	-	-	-	-	+0.34	ns	-	-	-0.346	ns
Riparian Diversity	-	-	+0.34	ns	-	-	-	-	-	-
AICc		61.79		56.5		67.3		65.12		65.26
w _i		0.836		0.805		0.745		0.732		0.576

*AICc=AIC value of selected model, w_i=Akaike weight

Table 3-5 continued. AICc multiple regression results with watershed characteristics as explanatory variables and stream habitat characteristics as response variables. The variable that explains the most variation for each response variable is in bold, and +/- signs represent a positive/negative relationship respectively.

	Temperature		Phosphorus		Nitrogen		Nickel	
	Slope	p-value	slope	p-value	slope	p-value	slope	p-value
% Forest Cover	-0.442	*<0.05	-0.763	***<0.001	-	-	-0.8	***<0.001
% Wetland	-	-	-	-	-	-	-0.275	***<0.001
% Open water	-	-	-	-	-	-	-	-
Roughness Coeff. (CV)	-	-	-	-	-	-	-0.141	ns
Road Density	+0.409	*<0.05	-	-	+0.574	**<0.01	-	-
Drainage Density	-	-	-	-	-	-	+0.244	**<0.01
Riparian Diversity	-	-	-	-	-	-	-0.25	**<0.01
AICc		59.89		53.35		64.7		24.21
w _i		0.598		0.651		0.48		0.695

*AICc=AIC value of selected model, w_i=Akaike weight

Chapter 4

4 General Conclusions

This study is one of the first of its kind and provides promising evidence for the use of leaf litter associated microbial communities as indicators of watershed disturbance. Microbial communities appeared to exhibit variation associated with changes in their environment with lower hydrolase enzyme activities at all disturbed streams compared to undisturbed streams. Streams with the most severe disturbance from industrial activity also had the lowest rates of microbial decomposition, a significantly decreased fungal biomass, a fungal community with a greater abundance of taxa from the class Eurotiomycetes, and a unique bacterial community composition with key taxa from the genera *Pseudomonas*, *Luteibacter*, *Amantichitinum*, *Burkholderia*, and *Enterobacter* missing or in very low abundance.

The relationships between the structure and function of microbial communities found in this study present the potential of fungal biomass and abundance of Betaproteobacteria to act as indicators of energy and nutrient cycling in the aquatic ecosystem. Fungal biomass and microbial decomposition were significantly and positively associated, and the abundance of Betaproteobacteria and the activity of β -xylosidase and phosphatase were also significantly and positively associated. Relationships between microbial community structure and function are not always found due to functional redundancy. Therefore, it is interesting that these relationships were found but further studies are certainly needed to explore these relationships in more detail.

Along with evidence that microbial communities responded to changes in their environment, forest cover, wetland cover, and road density were identified as key watershed characteristics

associated with microbial community structure and function. Watershed disturbance in this study were caused by a wide range of logging, fire, industrial and urban activities and these disturbances no doubt resulted in multiple stressors on the aquatic ecosystem, beyond even the long list of variables that I found to be significantly different in my analysis (i.e. decreased abundance of large woody debris, decreased dissolved organic matter concentrations, increased sedimentation, increased temperature, increased conductivity, nutrient enrichment, and metal contamination). Therefore, it is quite instructive that three variables, forest cover, wetland cover, and road density emerged as being associated with microbial community structure and function across the gradient of disturbance. Specifically, forest cover was found to be important to the cycling of energy and nutrients, and leaf litter decomposition in streams through the supposed export of DOC to the aquatic ecosystem and the supply of large woody debris as a retention mechanism for organic matter. Percent wetland cover was also associated with increased DOC concentrations in streams. Therefore, the restoration and retention of forest and wetland cover should be taken into consideration to maintain essential DOC exports to the aquatic ecosystem.

Along with the positive influence of forest and wetland cover, road density was found to have a potentially negative impact on the aquatic ecosystem by increasing conductivity. Increases in conductivity appeared to be mainly due to increased export of road salts (i.e. Ca and Mg), and conductivity appeared to influence bacterial community composition and decrease β -xylosidase, and phosphatase potential activities. The negative impact of roads should also be taken into consideration for aquatic ecosystem management and restoration.

In conclusion, this study was the first to explore leaf litter associated fungal and bacterial communities in structural and functional detail, and in the context of land-water linkages. The description and identification of differences in fungal and bacterial community structure and

function across a gradient of disturbance severity illustrated the potential use of fungal and bacterial communities as indicators of watershed disturbance. The land-water linkages identified between forest cover, wetland cover, road density, and fungal and bacterial community structure and function also have potential to be used to direct watershed management strategies and restoration efforts. Future studies could build on these findings to examine if other patterns in microbial community structure and function exist across broader spatial scales. There is always a need to conduct controlled lab studies to resolve the correlation/causation debate and to better understand the mechanisms by which disturbances alter microbial communities. Results of such expanded surveys and lab experiments would then increase the potential use of both healthy and disturbed microbial communities as strong indicators of disturbance at a more regional level, and further contribute to our knowledge of leaf litter associated microbial communities and the essential aquatic ecosystem services that they provide.

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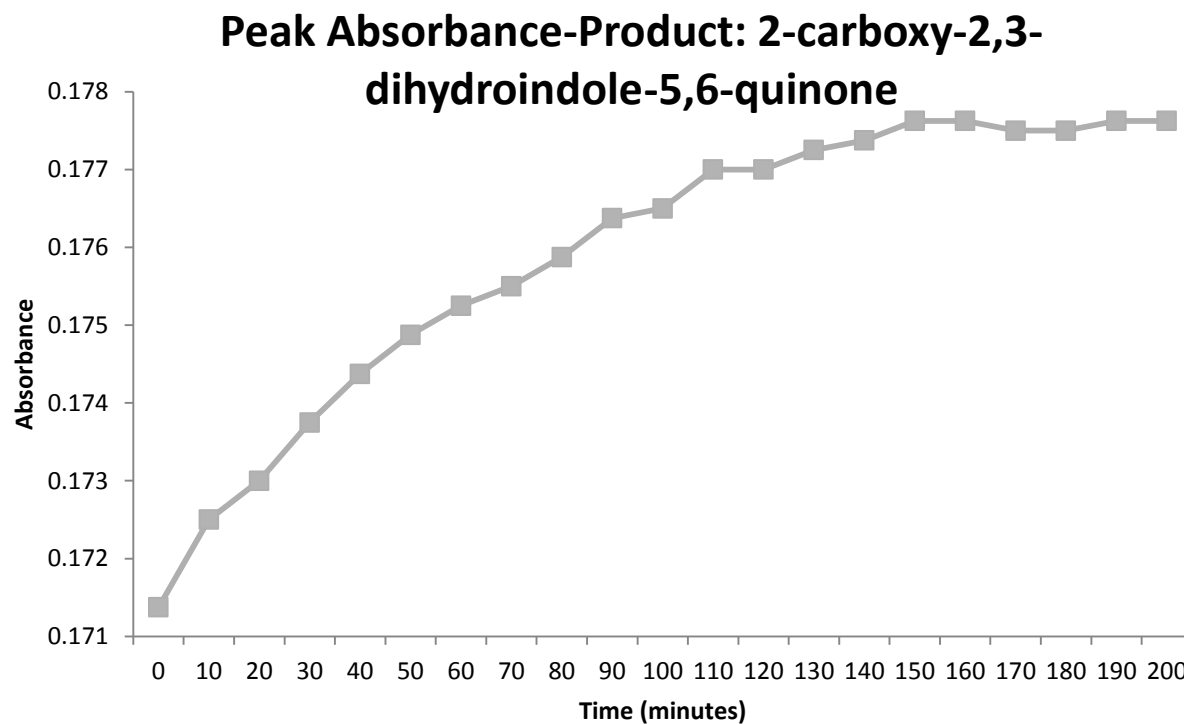
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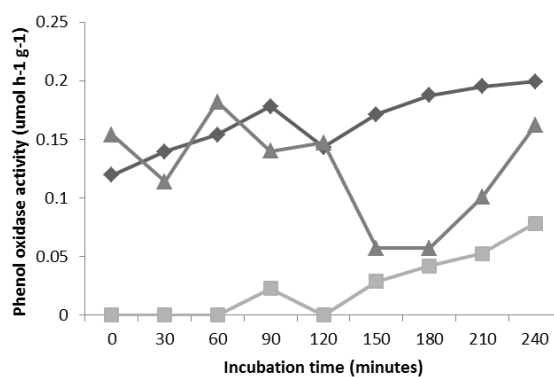
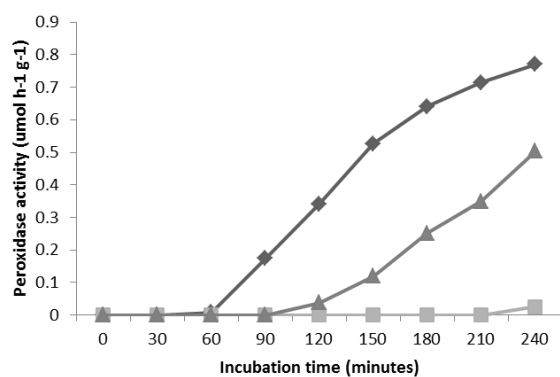
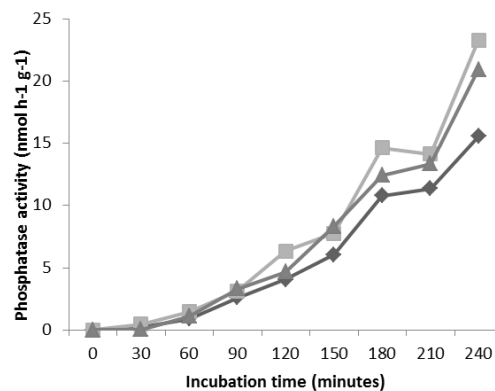
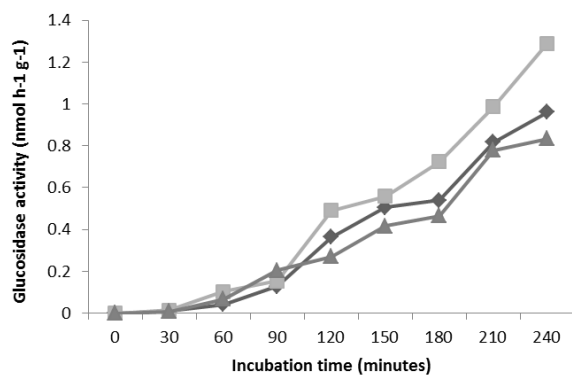
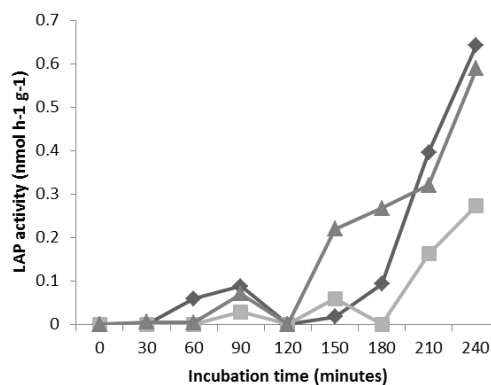
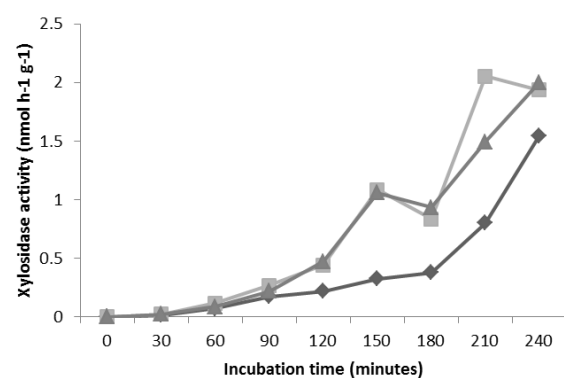
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Appendices



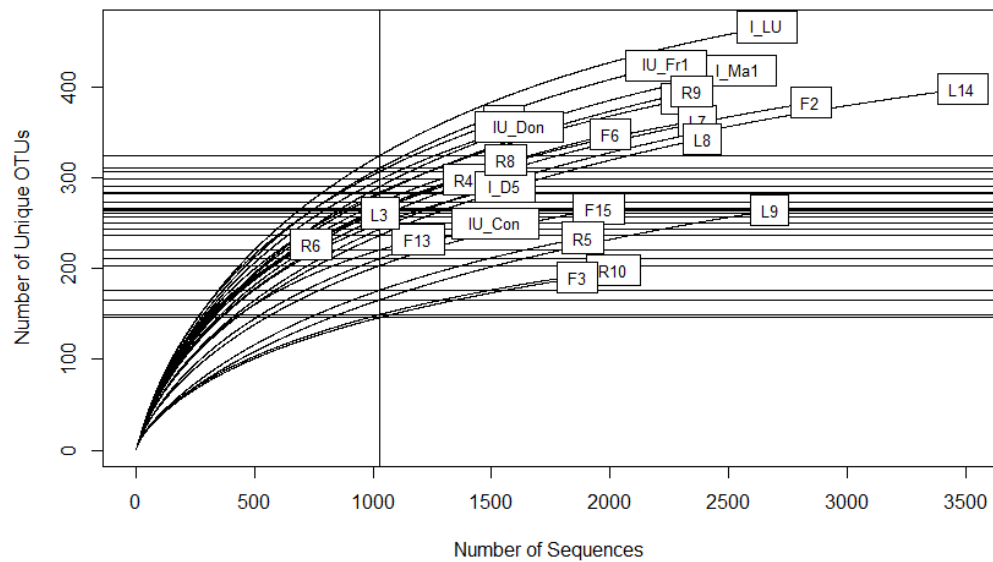
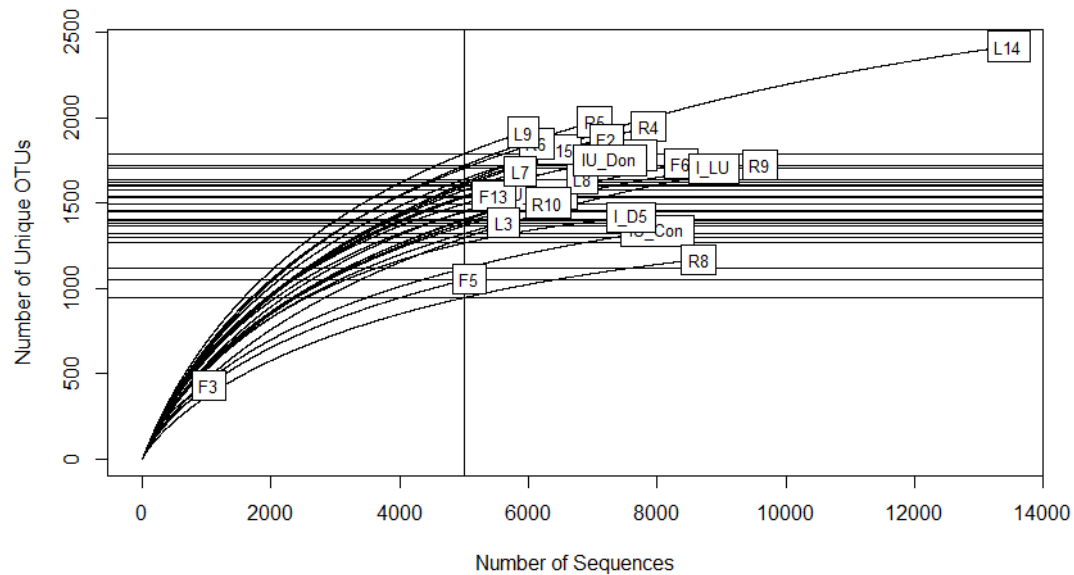
Appendix-1. Peak absorbance curve (product 2-carboxy-2,3-dihydroindole-5,6-quinone) measured to calculate extinction coefficient for oxidase assays. The extinction Coefficient= $OD/c \cdot l$, where OD= peak absorbance, $c = 1$ (1% solution extinction coefficient), $l =$ length of light path. 1% solution extinction coefficient= $0.177625 / (1 \cdot 0.676)$.



Appendix-2. Extracellular enzyme activity time trial assays for an Industrial urban (circle), fire (square), and logged (triangle) stream leaf sample.

Appendix-3. Sequence statistics data for fungal and bacterial sequence processing. Raw sequences represent the number of sequences before filtering, clustered OTUs represent the number of OTUs after quality filtering, chimera filter, and filtering for singletons. Clustered fungal OTUs represent the number of OTUs identified as strictly fungal after filtering OTU clustering and rarefaction (n=1030).

#SampleID	Raw 16S rRNA bacterial sequences	Clustered bacterial OTUs	Raw 18S rRNA Sequences	Clustered 18S rRNA OTUs	Clustered fungal OTUs
Con	9863	7997	1713	1515	614
D5	10032	7595	1812	1556	607
Don	9497	7260	1981	1618	1036
Fire13	7047	5460	1353	1189	704
Fire15	8518	6475	2084	1953	1204
Fire19	7846	6416	2745	2325	1865
Fire 2	9422	7218	3291	2848	1881
Fire3	1366	1032	1996	1862	1636
Fire5	5946	5077	1834	1551	1068
Fire6	10800	8368	2334	2002	1665
Fr1	8311	6066	2797	2238	1911
L14	16108	13462	3882	3487	3128
L3	6882	5617	1210	1030	333
L7	7561	5875	2712	2370	1617
L8	8686	6839	2642	2389	974
L9	7884	5920	2867	2674	484
LU	11372	8878	3147	2662	640
Ma1	9718	7508	3042	2541	2208
Und10	7889	6306	2129	2016	1827
Und4	9881	7866	1628	1382	1204
Und5	9581	7029	2032	1887	347
Und6	7922	6123	924	738	449
Und8	10084	8643	1765	1561	394
Und9	11935	9595	2725	2343	1910



Appendix-4. Rarefaction curve of bacterial communities (top) and eukaryotic communities (bottom) from each stream based on 16S and 18S rRNA 454 pyrotag sequencing results after all filtering and processing. Vertical line represents where the dataset was rarified (i.e. 5000 sequences for bacterial, 1030 sequences for fungal per sample) for equal sampling depth before analyses.

Variable	Alphaproteobacterial abundance		Betaproteobacterial abundance		Gammaproteobacterial abundance	
	r	P-value	r	P-value	r	P-value
Conductivity	0.235	ns	-0.536	<0.01	-0.288	ns
N	0.071	ns	-0.183	ns	-0.256	ns
P	0.006	ns	-0.04	ns	-0.555	<0.01
Ni	0.099	ns	-0.13	ns	0.401	ns
α -G	0.019	ns	0.377	ns	-0.209	ns
β -X	-0.444	<0.05	0.679	<0.001	0.034	ns
PHOS	-0.256	ns	0.634	<0.01	-0.058	ns
LAP	0.341	ns	-0.09	ns	-0.028	ns
POX	0.155	ns	-0.181	ns	-0.293	ns
PER	-0.025	ns	0.143	ns	-0.098	ns

Appendix-5. Results of Pearson correlations between the abundance of the top three Proteobacterial classes (Alpha, Beta, and Gamma), and the top explanatory variables for bacterial communities composition, and enzyme activities.