DIVERSE ALLOCHTHONOUS RESOURCE QUALITY EFFECTS ON HEADWATER STREAM COMMUNITIES THROUGH INSECT-MICROBE INTERACTIONS

By

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ABSTRACT

DIVERSE ALLOCHTHONOUS RESOURCE QUALITY EFFECTS ON HEADWATER STREAM COMMUNITIES THROUGH INSECT-MICROBE INTERACTIONS

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Freshwater resources are vital to environmental sustainability and human health; yet, they are inundated by multiple stressors, leaving aquatic communities to face unknown consequences. Headwater streams are highly reliant on allochthonous sources of energy. Riparian trees shade the stream, limiting primary production, causing macroinvertebrates to consume an alternative food source. Traditionally, leaf litter fallen from riparian trees is the primary allochthonous resource, but other sources, such as salmon carrion associated with annual salmon runs, may also be important. An alteration in the quantity or quality of these sources may have far reaching effects not only on the organisms that directly consume the allochthonous resource (shredders), but also on other functional feeding groups. Allochthonous resources directly and indirectly change stream microbial communities, which are used by consumers with potential changes to their life histories and behavior traits. The objective of my research was to determine the influence allochthonous resources have on stream communities of macroinvertebrates and microbes using two systems: salmon carrion decomposition and emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) invasion. It was hypothesized that with an alteration in allochthonous resource quantity and/or quality, the aquatic community would be altered.

When salmon carcasses, a heterotrophic allochthonous resource, are introduced to a stream, the macroinvertebrate and microbial (bacteria and microeukaryotes) communities changed compared to a control stream reach without salmon carcasses over time. Specifically, *Heptagenia* (Heptageniidae: grazer) density was five times higher in the salmon reach compared to the control.

In the salmon reach during year one, Stramenopiles (i.e., eukaryotic microbes) decreased in biofilm communities after two weeks of decomposition. Although unique microbial taxa, introduced to the naïve stream via salmon carrion, persisted in biofilms on benthic substrate and internal to insects during both years, those taxa represented <2% of the relative abundance in microbial communities. These results highlight the importance of allochthonous carrion resources in the microbial ecology of lotic biofilms and macroinvertebrates.

Mortality of ash trees along stream corridors as a result of EAB invasion can result in canopy light gaps, which potentially alter subsequent organic matter subsidies into streams. We characterized the coarse woody debris, leaf litter and their associated bacterial communities (terrestrial and aquatic), and macroinvertebrate communities upstream, downstream, and at the center of one EAB-related canopy gap in six headwater streams of Michigan. Downstream locations had significantly lower dissolved oxygen and macroinvertebrate diversity, but we did not detect watershed and gap location effects on aquatic leaf litter. These findings reveal EAB invasion impacts stream ecosystems through indirect routes downstream of canopy gaps, yet leaf litter subsidies are resilient. Decomposition rates and macroinvertebrate colonization in leaf packs of four species (ash, oak, buckthorn, and cotton control) upstream, downstream, and at the center of one EAB-related canopy gap were assessed. There was no gap effect on decomposition rates. Shredder genera were more abundant on ash compared to buckthorn leaves, and macroinvertebrate diversity was significantly higher in the gap, compared to upstream and downstream. Our findings suggest a shift in macroinvertebrate communities in response to EAB by indirect routes of leaf litter subsidies and light availability. From these results, it can be concluded that allochthonous resources were a significant contributor to stream biodiversity patterns, and my dissertation research represents a significant contribution of knowledge on community assembly in streams.

Dedicated to my father, Loren Larson, and my friend, Shengpan Lin, who both showed me the joy found in lakes, streams, and rivers.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Eric Benbow, on his inspiration and guidance throughout my degree. I also thank my graduate committee; Michael Kaufman, Deborah McCullough, Jennifer Pechal, and Jan Stevenson; for their support through my studies. Additionally, gratitude is expressed to Rich Merritt for reviewing materials and sharing advice learned over years of studying Michigan's streams. I would like to thank Patrick Engelken for his work on riparian forests affected by EAB, without which this research would not be possible. For funding this work I thank the National Science Foundation Graduate Research Fellowship Program; Great Lakes Fishery Trust; Hutson Memorial Endowment Fund; Gordon E. Guyer Endowed Fellowship in Aquatic Entomology; Merritt Endowed Fellowship in Entomology; MSU EEBB Summer Fellowship; KBS Graduate Student Research Fellowship; T. Wayne and Kathryn Porter Scholarship; Society for Freshwater Science General Endowment Fund; MSU CANR; AgBioResearch; and the College of Osteopathic Medicine. Countless MSU student employees and volunteers contributed to this work including Caleb Armstrong, Nicholas Babcock, Benjamin Bejek, McKinley Brewer, Nikki Cavalieri, Hepsiba Chepngeno, Saavas Constantinou, Rebecca Drenth, Emmy Fedor, Juanjuan Guo, Rachel Harvey, Daniel Hulbert, Katie Kierczynski, Heather Kittredge, Kelsie Kroll, Shengpan Lin, Sydney Manning, Jason Matlock, Mack McGinn, Nell McGuan, Robert Mobley Jr., Michael Orbain, Rachel Osborn, Emilie Parkansky, Dustin Phelps, Joseph Receveur, Connie Rojas, Katelyn Smiles, Brianna Timmons, Ariana Troia, Ryan Walquist, Courtney Weatherbee, Nicole Wonderlin, Allie Yackley, and Alison Zahorec, for assistance with fieldwork, lab work, and copy editing. Finally, I would like to thank my friends and family for their support.

PREFACE

In this preface, I must address the current events that are challenging our world and hindering the goals of this dissertation. As of submission, There are 20.9 million cases of COVID-19 caused by SARS-CoV-2, resulting in 760,000 deaths, and society has drawn to a halt to stop the spread (WHO, 2020). The MSU community was under a “Stay at home, stay safe” executive order from March 24 – June 1, 2020, effectively stopping all on campus research activities. Writing this dissertation during the COVID crisis was a challenge, and the closures directly impacted my ability to complete research goals for my dissertation. Specifically, DNA samples required to complete Chapters 3 and 4 were not able to be sequenced by the Michigan State University (MSU) genomics core. Chapter 3 uses a reduced bacterial dataset (nine samples missing), and Chapter 4 does not have any bacterial data associated with it at this time. The genomics core officially reopened on June 9, 2020, and eventual peer-reviewed publications of these chapters will include the complete bacterial datasets.

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KEY TO ABBREVIATIONS

AFDM - ash free dry mass

ALL - aquatic leaf litter

ASV - amplicon sequence variant

BA - basal area

Baet - *Baetis brunneicolor*

bp - base pair

Carc - carcass

CPOM - coarse particulate organic matter

CWD - coarse woody debris

DBH - diameter at breast height

df - degrees of freedom

EAB- emerald ash borer

FFG - functional feeding group

FPC - Flood Pulse Concept

FPOM - fine particulate organic matter

ha - hectare

Int - introduced

ISA - indicator species analysis

KEGG - Kyoto Encyclopedia of Genes and Genomes

LJ - log jam

LL - leaf litter

MS - mean sum of squares

MSE - mean standard error

NMDS - nonmetric multidimensional scaling

NPH - Network Position Hypothesis

OTU - operational taxonomic unit

P/R - production to respiration ratio

PD - phylogenetic diversity

POM - particulate organic matter

RCC - River Continuum Concept

RIV - Relative Importance Value

RPM - Riverine Productivity Model

RWC - River Wave Concept

SS - sum of squares

Steg - *Stegopterna mutata*

TLL - terrestrial leaf litter

Treat - treatment

Y1 - year 1

Y2 - year 2

CHAPTER 1: THE RIVER CONTINUUM CONCPET: LESSONS FROM THE PAST AND PERSPECTIVES FOR THE FUTURE

Doretto, A, E Piano, and CE Larson, Can J Fish Aquat Sci. (in press as of July 24th, 2020)

Abstract

The River Continuum Concept (RCC) is a milestone in stream ecology because of its comprehensive evaluation of the structure and function of lotic ecosystems. Linking stream physical and geo-morphological attributes with patterns in biodiversity, functional traits, and metabolism dynamics, this theory describes downstream gradients in community composition and ecosystem processes. The aim of this review is to evaluate how the RCC, 40 years from its publication in the Canadian Journal of Fisheries and Aquatic Sciences, has influenced basic and applied research in stream ecology, focusing on the most important contributions and recent developments. This work puts into perspective the historical significance of the RCC in the scientific process and integrates past and recent theories including metacommunity and metaecosystem theories and the river network perspective to predict taxonomic and functional diversity of benthic communities. Thus, this review provides a unifying overview of the historical context of the field for exploring basic and applied ecological questions to the next generation of stream ecologists.

Introduction

The scientific process tends to be slow and steady, punctuated by times of rapid growth and change in the field, as Thomas Kuhn once described as a “scientific revolution” (1962). This process comes to mind when reflecting on one of the most important (and most commonly cited: 5768 citations – Web of Science, July 10th, 2020, Figure 1.1) stream ecology papers. The River Continuum Concept (RCC) (Vannote et al. 1980) was published in the Canadian Journal of

Fisheries and Aquatic Sciences 40 years ago. This concept integrated important theoretical foundations of that time, such as the habitat template theory (Southwood, 1977) and the entropy theory (Leopold and Langbein, 1962). At the same time, it revolutionized the research approach in stream ecology, because with the mechanistic inter-relations between the environmental parameters, energetic inputs, and biota composition postulated by this theory; the field of stream ecology shifted from a descriptive to a predictive based approach (Minshall et al., 1985). Another reason for its role in revolutionizing stream ecology is because of its marked interdisciplinarity that encompassed several aspects of the riverine systems, ranging from geomorphology, biology, and biogeochemistry. As a consequence, in the last four decades the RCC has become a key conceptual framework among river ecologists for testing basic and applied hypotheses related to species distributions and community structure, as well as energetic flow in riverine habitats, and many new ideas have been formed. Yet, many of the RCC's predictions were proven circumstantial, with limited applicability (Table 1.1).

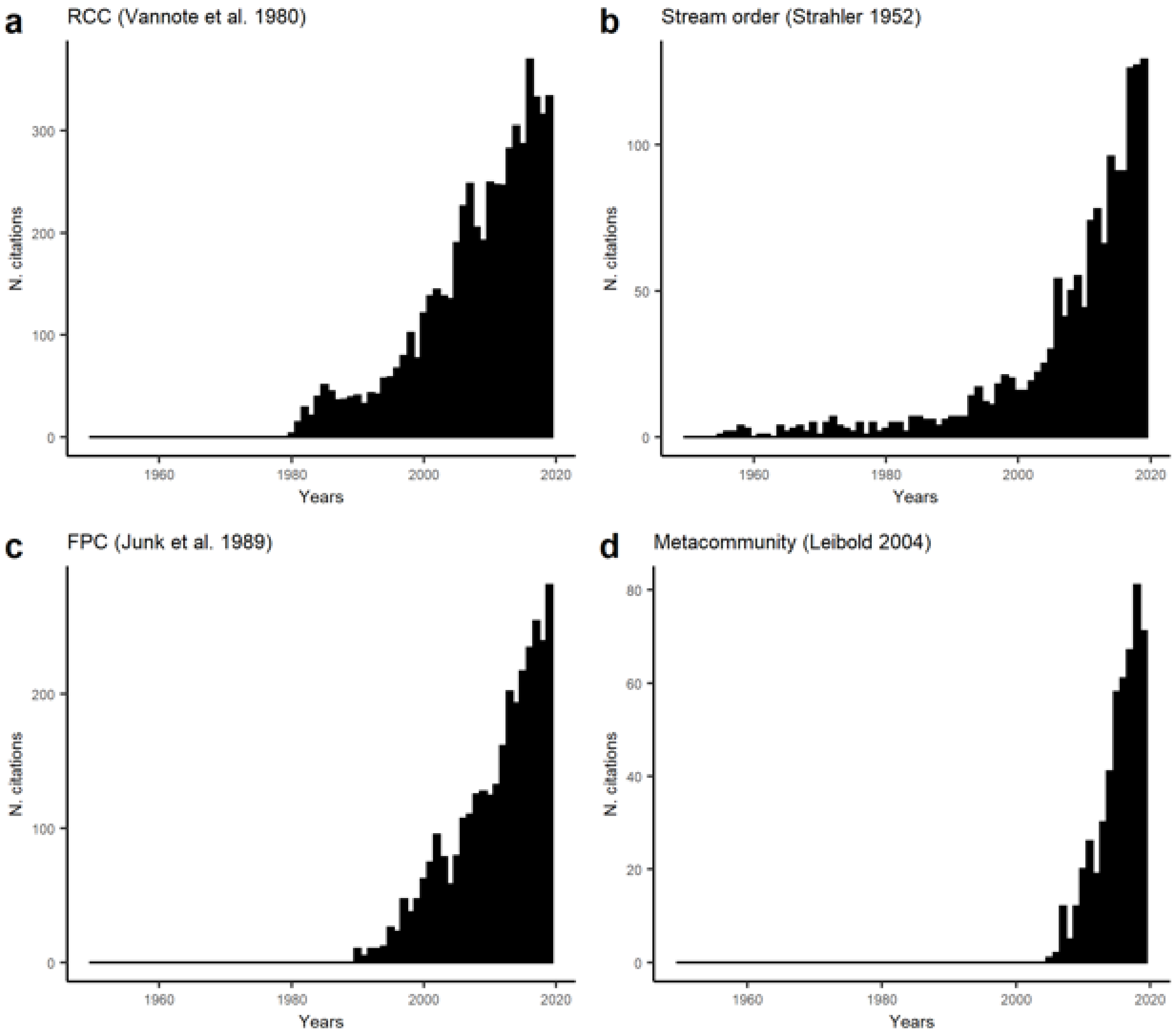

Figure 1.1. Number of citations over time for a) The River Continuum Concept (Vannote et al. 1980), b) Hypsometric (area-altitude) analysis of erosional topography (stream order; Strahler 1952), c) The Flood Pulse Concept in River-Floodplain Systems (Junk et al. 1989), and d) The metacommunity concept: a framework for multi-scale community ecology (Leibold et al. 2004) in publications also having “streams” or “rivers” as key words. These data sets were extracted from Web of Science on July 10th, 2020.

Table 1.1. Selected list of citations testing predictions generated by the RCC.

Continent	Citations
Africa	Chakona et al. 2008, Bredenhand and Samways 2009, Masese et al. 2014, Dalu et al. 2017, Masese et al. 2018
Asia	Qu et al. 2005, Xiaocheng et al. 2008, Lau et al. 2009, Jiang et al. 2010, Jiang et al. 2011, Gao et al. 2014, Fu et al. 2016
Australasia	Winterbourn et al. 1981, Barmuta and Lake 1982, Winterbourn 1982, Lake et al. 1985, Ryder and Scott 1988, Harding et al. 1999, Thompson and Townsend 2000
Europe	Heino et al. 2005, Maiolini and Bruno 2006, Manfrin et al. 2013, Dossi et al. 2018
North America	Clup and Davies 1982, Perry and Schaeffer 1987, Grabaugh et al. 1996, Delong and Brusven 1998, Rice et al. 2001, Rosi-Marshall and Wallace 2002, Grubs and Taylor 2004, Greathouse and Pringle 2006, Lessard et al. 2009, Holt et al. 2015, Rosi-Marshall et al. 2016, Yates et al. 2017, Curtis et al. 2018
South America	Afonso et al. 2000, Callisto et al. 2004, Tomanova et al. 2006, Tomanova et al. 2007, Miserendino 2009, Brasil et al. 2014, Ramírez and Gutierrez-Fonseca 2014
Global	Statzner and Higler 1984, Minshall et al. 1985, Covich 1988, Minshall 1988, Sedell et al. 1989, Bretschko 1995, Montgomery 1999, Ward and Tockner 2001, Wantzen and Wagner 2006, Boulton et al. 2008, Winemiller et al. 2010, Xenopoulos et al. 2017, Jager and Borchardt 2018

Now is a good time to reflect on the key role the RCC has played in shaping stream ecosystem research, as it has reached the 40-year milestone, because it is an important case study on the ecological scientific process. This manuscript is not aimed to assess the validity of the predictions of the RCC: a large body of evidence currently exists in scientific literature and several studies have been carried out worldwide with this regard (Winterbourn et al., 1981; Barmuta and Lake, 1982; Minshall et al., 1983). It is therefore impossible to provide, here, an exhaustive and synthetic overview on the validity of the RCC across river typologies and geographical areas. Instead, the aim of this review manuscript is to highlight and discuss the key features that make the RCC a still valid conceptual framework (if not globally, empirically accurate) that led to an abundance of studies on changes in the biodiversity and ecosystem processes in rivers. Although the RCC has been tested using a variety of distinct biological communities (Ruiz-González et al., 2015; Bolpagni et al., 2016; Feio et al., 2017; Curtis et al., 2018), particular attention is paid to the taxonomical and functional composition of macroinvertebrate communities, which were the taxonomic group for which this theory was originally developed. At the same time, we elucidate the main criticisms to the RCC, demonstrating how limitations in the predictions of the RCC have contributed to the development of new conceptual theories and approaches to study patterns in biodiversity and river functionality. Therefore, the relationships between the RCC and recent ecological frameworks, including metacommunity theory and the river network perspective, are examined to provide to the next generation of stream ecologists a unifying overview for exploring basic and applied ecological questions.

Stream ecology pre-1980 and the development of the RCC

Classifications and conceptual models are widely used by ecologists, and streams appear to be especially prone to such an approach due to their spatial and hierarchical structure. Zonations of lotic ecosystems were pioneered by early river ecologists (Huet, 1949, 1954; Margalef, 1960; Illies and Botosaneanu, 1963), but the RCC provided a multidisciplinary view of the riverine system that links its physical attributes with the availability of food resources. The combination of these factors ultimately determines the composition of aquatic communities, both in structure and function. Compared to previous classifications, which subdivided streams into distinct zones that were often empirically indistinguishable, the most useful novelty of the RCC was the shift to a continuum-oriented view of river systems along a longitudinal gradient of abiotic factors and biotic response, which allowed greater precision in making ecological predictions. The inadequacy of strict zonations was already noticed by Hynes (1975) in his influential paper “The stream and its valley”. Because each stream possesses a unique combination of land-use, topographic and climatic conditions, the author pointed out that “the valley rules the stream in every respect” (Hynes 1975) and many of the RCC foundations were built on the idea that streams are strongly influenced by the physical attributes of the surrounding terrestrial areas. According to the RCC, the position of a stream reach in its river network, measured as stream order (Strahler, 1952, 1957), is considered a proxy of multiple environmental conditions including substrate composition and size, river slope, and canopy cover, which in turn influences water temperature, chemistry, and light penetration.

All these factors influence food availability, in the form of primary production and particulate organic matter (POM), and act as environmental filters on aquatic taxa, depending on their feeding strategy. In particular, the RCC related the variation of environmental parameters and energetic inputs along the longitudinal gradient with the trophic structure of benthic macroinvertebrates. Prior to the development of the RCC, the formulation of feeding categories for macroinvertebrates were established. A functional analysis of invertebrate feeding based on morpho-behavioral mechanisms of food acquisition was led K. W. Cummins to define functional feeding groups (FFGs); highlighting a direct correspondence between the categories of nutritional resources present in the environment and adaptations to efficiently harvest a given food resource (Cummins, 1973, 1974; Anderson and Cummins, 1979; Cummins and Klug, 1979). This classification, which clusters macroinvertebrates taxa into five main trophic groups (Table 1.2), was incorporated into the RCC to predict the energy flow through the longitudinal gradient (Vannote et al. 1980). The use of FFGs, rather than taxonomic metrics, served to better elucidate the RCC predictions of ecosystem flow pathways, establishing trophic relationships at the core of this ecological theory (Grubaugh et al., 1996).

Table 1.2. Definitions of the Functional Feeding Groups (FFGs) and their preferred food items.

FFG	Preferred food item
Shredders	Coarse particulate organic matter (CPOM): fragments of organic matter larger than 1 mm; such as leaves, plant tissue, and wood debris
Scrapers	Periphyton: biofilm mainly composed of benthic primary producers; such as diatoms, green algae, and cyanobacteria; but may also contain polysaccharidic substances, fungi, and bacteria
Collector-gatherers	Fine particulate organic matter (FPOM): the finer fractions of organic matter (50 µm - 1 mm) created by the biological or physical degradation of CPOM, as well as fecal pellets
Collector-filterers	Very fine particulate organic matter (< 50 µm) and micro-organisms delivered by the water current
Predators	Small invertebrates, meio-benthos and other animals

The main predictions of the RCC are as follows. Headwater (low-order) streams are characterized by a narrow width and coarse substrates and are strongly shaded by the surrounding riparian vegetation, which provides a large input of coarse particulate organic matter (CPOM) in the form of falling leaf litter. CPOM represents the main food resource in headwater systems, making them heterotrophic with a ratio between gross in-stream primary production and respiration (P/R) less than 1. The macroinvertebrate communities of headwater streams mainly depend on the detritus food chain and are dominated by those invertebrates that directly feed on the CPOM (shredders), followed by the collector-gatherers and filterers that consume shredded CPOM (the fine particulate organic matter, FPOM, Figure 1.2).

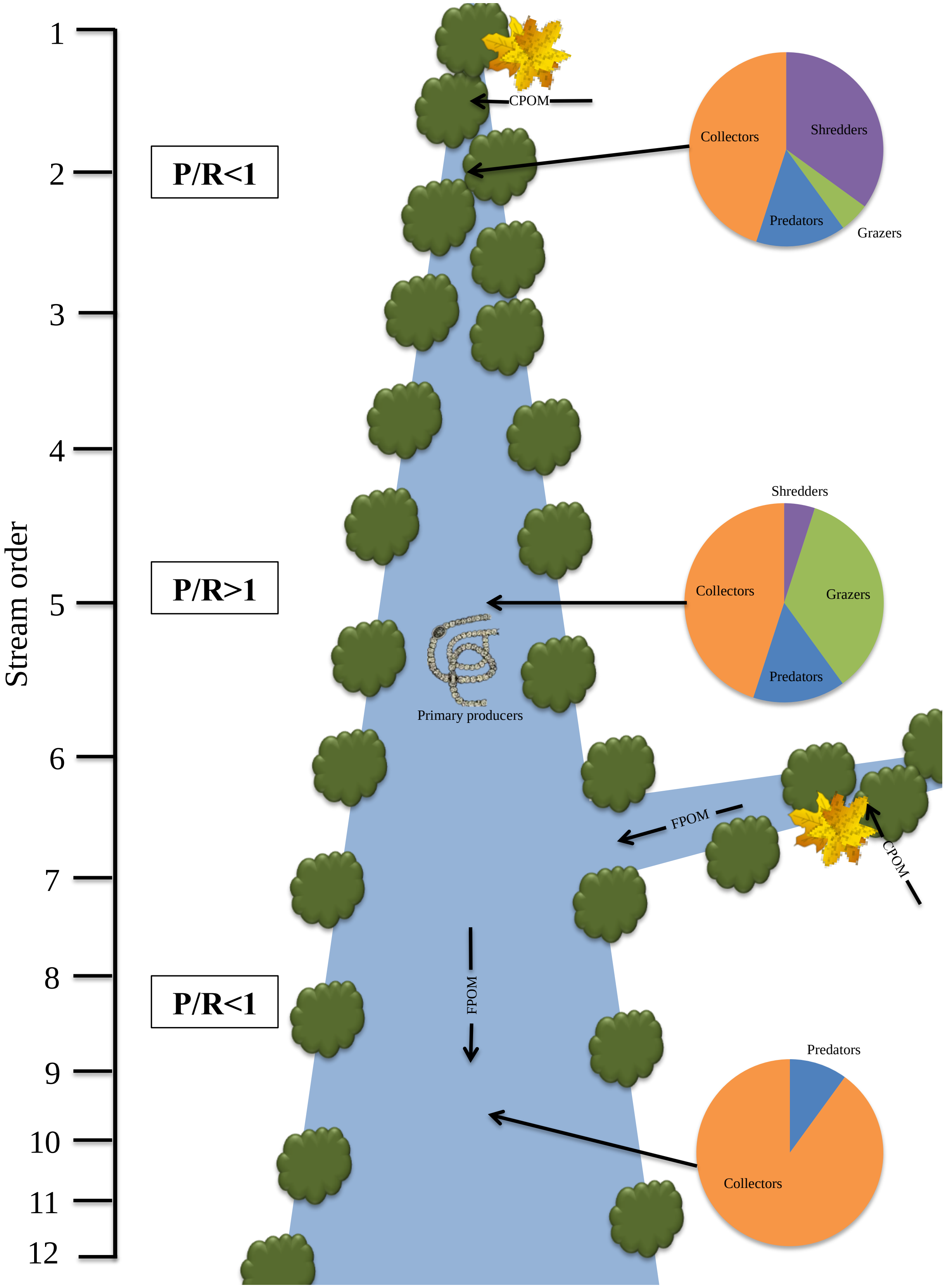

Figure 1.2. Illustration of the River Continuum Concept predictions (from Vannote et al. 1980).

With stream size and width increases in mid-order streams, the influence of shading and CPOM input by riparian vegetation decreases and allows greater light penetration into the stream. As a consequence, the contribution of primary producers becomes more important, and the mid-order streams are considered autotrophic systems (P/R > 1). The community composition of benthic macroinvertebrates is, in turn, altered by the proportion of scrapers, consumers feeding on the abundant periphyton usually peaking at these locations. Collector-gatherers also remain abundant due to their generalist strategy and ability to feed on FPOM from autotrophic sources (Figure 1.2).

Furthest downstream (in large rivers), the steam size further increases so the influence of the riparian vegetation in relation to the wetted width is nearly negligible. Moreover, in these sections light and primary production by benthos is strongly limited by water depth and turbidity, which inhibits light penetration. From an energetic point of view, such sections are again heterotrophic (P/R < 1) and depend on the fine particulate organic matter (FPOM) delivered into the water column from upstream shredding. Here benthic macroinvertebrate communities are almost entirely composed of collector-gatherers and filterers, while the proportion of shedders and scrapers is markedly reduced (Figure 1.2).

Since its publication, the power of the RCC in predicting stream conditions was well recognized, yet river ecologists have strongly argued over time on the full range of its applicability (Winterbourn, 1982; Statzner and Higler, 1985; Ryder and Scott, 1988; Sedell et al., 1989). In fact, the RCC was originally developed for temperate, near-pristine, and forested headwater streams and several authors have demonstrated its validity for such type of watercourses (Culp and Davies, 1982; Grubaugh et al., 1996; Rosi-Marshall and Wallace, 2002; Yates et al., 2017). Its transferability to other biomes was merely speculated (Table 1). As a result, great effort has been made to set the domain of applicability of the RCC for comparing an array of streams worldwide based on their size (Minshall et al., 1992; Cushing et al., 2006), hydrology (Greathouse and Pringle, 2006; Xenopoulos et al., 2017), and climate conditions (Bott et al., 1985; Minshall, 1988; Jiang et al., 2011).

At the same time, some criticisms were raised to the theoretical background of the RCC. First, RCC focuses almost entirely on the dynamics occurring in the mainstem of streams, without framing them within the stream network and underestimating the types and sources of discontinuities that can occur along the longitudinal gradient of a stream. It has to be said that the same authors anticipated a few exceptions to their predictions; for instance, recognizing the transition from heterotrophy to autotrophy may depend on the stream type (e.g., xeric and deeply-incised). Similarly, the authors mentioned the localized effects of tributaries based on their amount and type of energetic inputs. However, these examples are too few to draw general rules and are mostly discussed from the mainstem standpoint without a comprehensive view of their role in the stream network. Second, the composition of biotic communities is only determined by environmental heterogeneity, while other aspects, such as dispersal capability of organisms, are disregarded. The following sections illustrate how these criticisms of the original RCC and exceptions to its predictions have contributed to the development of new theories and conceptual approaches to evaluate patterns in biodiversity and composition of macroinvertebrate communities.

The role of natural discontinuities

Although constrained and low-order streams are expected to follow the predictions of the RCC because the longitudinal connection between succeeding river sections is the dominant dimension (*sensu* Ward 1989) influencing these streams, one of the main criticisms to the RCC is that this theory underestimates the importance of natural discontinuities along a stream's longitudinal gradient. We define natural discontinuities as spatial elements and/or temporal processes that alter the linear downstream variation in abiotic and biotic parameters and modify the longitudinal connectivity (Perry and Schaeffer, 1987; Jones, 2010).

Discontinuities and their related deviations from predictable longitudinal changes can emerge at any scale of investigation, depending on the abiotic and/or biological parameters considered. For instance, lakes and tributaries interrupt the longitudinal continuum at the basin scale by changing key abiotic parameters at the local scale, such as nutrient concentration, substrate composition, solid transport, and organic matter availability. The downstream section of a lake (Ward and Stanford, 1995; Stanford and Ward, 2001; Jones, 2010) as well as the junctions between the main stem and tributaries (Rice et al., 2001; Torgersen et al., 2008; Milner et al., 2019) are highly diverse zones, with distinct biological communities different than riverine main stem sites. Bruns et al. (1984), for example, investigated the longitudinal variation in FFGs in the Salmon River (Idaho, USA) comparing sampling reaches with and without tributaries and found that tributaries perturbed the downstream patterns in FFGs and organic matter with a magnitude that was proportional to their stream size. However, these spatial discontinuities were largely ignored by the RCC: there were no references to lakes in the original publication, while tributaries were mostly regarded as collectors and deliverers of CPOM from the drainage basin to the main channel.

Specifically, Vannote et al. (1980) provided only one example about stream confluences: a third-order tributary entering a mid-order or large stream, anticipating localized effects on the mainstem community based on the nature and amount of energetic inputs. However, the influence of tributary junctions in other parts of the stream network position was ignored. Ward and Stanford (1995) proposed the "Serial Discontinuity Concept" to assess the effects of lentic water bodies on the downstream reaches of a watercourse. This concept highlights how the effects of these lentic habitats on the benthic communities and ecosystem processes vary according to their longitudinal position in the river network, which has been extensively applied to evaluate the effects of anthropogenic water bodies, such as dams and reservoirs (Ellis and Jones, 2013; Guareschi et al., 2014; Mellado-Díaz et al., 2019).

At local scale, discontinuities can be also created by the direct action of some riverine species. For instance, beavers are widely recognized as ecological engineers that create woody dams thus affecting flow and nutrient dynamics (Robinson et al. 2020). Although beaver populations have been reduced in some geographical areas, where numerically abundant they can strongly modify the channel morphology by creating beaver complexes that disrupt the longitudinal continuum and have important repercussions on different components of lotic ecosystems, including organic matter subsidies (Catalán et al. 2017), algae (Rodríguez et al. 2020) and macroinvertebrates (Anderson and Rosemond 2007, Bush et al. 2018).

Spatial discontinuities can also be context dependent, with stream communities responding to variation in reach-scale geomorphology and natural elevation gradients, especially in mountain areas. Depending on the type of rocks, the valley topography and geometry can generate a complex arrangement of confined and floodplain river segments, which punctuate the longitudinal gradient with relatively pronounced deviations from ideal patterns (Bellmore and Baxter 2014). Moreover, for mountain lotic ecosystems, the longitudinal continuum is intimately related to elevation gradients, with inseparable consequences on environmental variables, such as temperature, oxygen, and riparian vegetation. The latter is one of the core aspects of the RCC, because riparian vegetation is a subsidy of CPOM for rivers (Cummins et al. 1989). However, herbaceous vegetation, rather than forest, naturally characterizes alpine and glacial streams at high altitude (i.e., above the tree line), due to their very low temperature and high slope. As a consequence, there is reduced input of terrestrial organic matter into alpine streams, which does not adequately support shredders (Milner et al. 2001; Füreder et al. 2003). Similarly, the temperature is too low and water force too erosive to allow for the growth of primary producers, limiting grazers as well. For instance, Fenoglio et al. (2014) observed that streams located above the tree line have a reduced input of allochthonous organic matter in autumn, because the organic matter input becomes trapped under ice and snow cover during the entire cold season, reaching streams during late spring, when meltwater collects and transports particles throughout the catchment. In this case, the food web depends on in-stream primary production, and benthic communities are characterized by a greater proportion of scrapers and species with higher trophic plasticity than those of downstream sections (Körner 1998; Fenoglio et al. 2014).

Some authors filled the knowledge gap on the functioning of lotic ecosystems above the tree line in glacial streams, since the RCC did not deal with this aspect. For instance, Craig (2002) introduced a modification to Vannote et al. (1980) stating that glacial headwater streams in alpine areas were predominately host collector-gatherers, which feed on inorganic detritus produced by glacial and snow melt, and are characterized by accentuated temporal variations in trophic inputs. In a recent review, Brighenti et al. (2019) showed that water source and glacial influence are the main factors affecting habitat typology and ecology of highland lotic ecosystems.

Lowland rivers, instead, are typically characterized by spatial and temporal connectivity between the main channel and the floodplain. Variation in discharge generates a complex assortment of habitats, such as the main channel, side channels, backwaters, and pools. Although these habitats strongly contribute to the biodiversity and functionality of these rivers by providing refuges for aquatic species and acting as metabolically active areas (Burgazzi et al., 2017), they were largely ignored in the original version of the RCC. Moreover, some studies have demonstrated that the floodplain areas have the potential to provide substantial inputs of CPOM (Thorp and Delong, 1994; Chauvet, 1997), and that large rivers may not depend exclusively on the FPOM generated in the upper sections and delivered downstream, as postulated by Vannote et al. (1980).

An additional source of spatial and temporal discontinuity is represented by the flow regime, which is defined by Poff et al. (1997) as “the master variable” for rivers, because it affects all the other environmental variables through five components: magnitude, frequency, duration, timing and flashiness. Yet, similar to the above-mentioned spatial discontinuities, temporal variation in flow regime and connectivity between stream reaches was not considered in the original version of the RCC because it was developed for perennial rivers. In fact, many of the RCC predictions are based on the continuous transport, processing and release of organic matter from up- to downstream, which is facilitated by permanent flow (i.e. “downstream communities are structured to capitalize energetic leakages from upper sections” – Vannote et al. 1980). This assumption was also conceptualized in a way known as “nutrient spiraling” (Webster and Pattens 1979; Webster 2007). Spirals are used to describe the coupled process of transport and uptake of nutrients, with the length and width of the spirals depending on the rates of exchange and retention between the abiotic and biological components of lotic ecosystems.

However, at global scale the proportion of intermittent and temporary rivers is larger than previously thought (Larned et al. 2010). Naturally intermittent rivers occur in the Mediterranean regions of Europe and North America (Bonada et al., 2006, 2007; Vannucchi et al., 2013) as well as in the arid and semi-arid areas around the globe. Under ongoing climate change, the redistribution of precipitation and enhanced frequency of extreme events (i.e., droughts and floods) are altering the flow regime of many watercourses, increasing the number of rivers that experience drying conditions (Lake, 2003; Arthington, 2012; Ledger and Milner, 2015; Woodward et al., 2016). Flow cessation results in the loss of the longitudinal connectivity and isolation of stream reaches with consequences on population survival, the diversity of aquatic communities and transport and storage of organic matter subsidies (Datry et al., 2016b; Doretto et al., 2020). Such adverse conditions may be exacerbated by the effect of anthropogenic-induced hydro-morphological alterations at the local scale, such as damming and water abstraction (Grubbs and Taylor, 2004; Holt et al., 2015; Doretto et al., 2019; Piano et al., 2019). Regardless of the nature of these alterations (i.e. natural or human-induced), RCC poorly adapts to lotic ecosystems experiencing changes in flow regime, with deviations from its predictions probably depending on the components of the flow regime that are mostly modified (i.e. magnitude, frequency, duration, timing and flashiness).

Moreover, flow-related modifications are not the only way by which anthropogenic activities generate deviations from the RCC predictions. In fact, similar effects are expected anywhere human modifications act on the key parameters conceptualized by the RCC, especially the riparian vegetation and channel morphology. Discrepancies between the observed and theoretical longitudinal patterns have been reported in relation to deforestation (Batalla Salvarrey et al. 2014), agricultural practices (Delong and Brusven 2001) and urbanization (Fu et al. 2015).

From the longitudinal gradient to patchy-structured systems

The awareness of such spatial and temporal complexity of riverine systems, including channel morphology, the branching structure of river networks, distance between tributary-main stem junctions and the shifting flow regime, has led a growing number of authors to reject the linear and predictable longitudinal changes of the RCC and support instead an idea of rivers as discontinuum- or patchy-structured systems (Townsend, 1989; Bretschko, 1995; Kiffney et al., 2006; Thorp et al., 2006; Winemiller et al., 2010). This shift in frameworks has been strongly influenced by developments in the field of landscape ecology, as well as the growing attention toward the hierarchical structure of lotic ecosystems (Ward and Tockner, 2001; Duarte et al., 2019). According to this approach, which considers the spatial and temporal dynamics of lotic ecosystems, the river network can be seen as a mosaic of different patches, typically defined as geomorphological units (Datry et al., 2016a), and the distribution of aquatic taxa can be predicted as a function of the spatial and temporal availability of these patches (Montgomery, 1999).

Based on this assumption, several theories have been proposed to integrate the RCC with the new concepts and provide a holistic view not limited to the main stem (Benda et al., 2004; Thorp et al., 2006). For instance, given the inability of the RCC to accurately model large and braided rivers, the Flood Pulse Concept (FPC) was developed to provide a framework for this type of lotic ecosystem (Junk et al., 1989; Junk, 1999). This theory emphasizes the pivotal role of dynamic interplay between the river and its floodplain in terms of nutrients, water, sediment, and organisms. Unlike headwater streams, the lateral dimension is the main factor that drives the ecological processes in large rivers, where fluctuations in discharge create a complex of spatially and/or temporally connected habitats. Poole (2002) suggested both the RCC and FPC are valid concepts, but should be interpreted in the context of fluvial landscape ecology because in each concept the biotic response can be predicted as a function of ecological connectivity (i.e., longitudinal and lateral, respectively), which is affected by the physical stream structure. Therefore, their relevance is dependent on the dominant connectivity dimension at the scale of investigation.

In addition, Thorp and Delong (1994) proposed the Riverine Productivity Model (RPM) to better elucidate the energetic pathways in shallow and constrained large rivers, with firm substrate and the presence of a photic zone. According to these authors, both the RCC and FPC fail to predict the functional response of benthic communities in this specific type of river, because they are biased toward the centrality of the main channel, ignoring the nearshore areas and underestimate the contribution of in-stream primary production and direct inputs of leaf litter from riparian areas. The authors argue that the downward leakage of FPOM as the main source of organic carbon in large rivers (as postulated by the RCC) is overemphasized compared to the in-stream primary production. Moreover, direct inputs of leaf litter from riparian vegetation constitute near-continuous carbon subsidies (i.e. not limited to flood pulses as predicted by FPC) with higher nutritional value than FPOM delivered from upstream. Therefore, in this type of river, the secondary production and food webs are simultaneously supported by both in-stream production and riparian-derived inputs of leaf litter, especially in the nearshore areas were macroinvertebrate density usually peaks (Thorp and Delong 1994).

Humphries et al. (2014) combined the RCC, FPC, and RPM into a unifying theory: The River Wave Concept (RWC). According to the RWC the spatial and temporal variation in river flow can be described as a wave in terms of shape, magnitude, wavelength, and frequency. Basin-specific characteristics, such as climate and geomorphology, determine the attributes of the waves, which in turn affect the production, storage, transformation, and transport of material and energy. When looking at waves, three different positions can be identified: troughs, crests, and ascending/descending limbs, which correspond to different ecosystem stages. For example, troughs consist of phases in which local autochthonous and allochthonous inputs predominate, while the ascending and descending limbs of the wave represent stages of high flow, governed by the longitudinal transport of allochthonous organic matter from the upstream reaches (like the RCC predicts). On the contrary, the crests indicate stages where the energy and material from the adjacent floodplain is the dominant feature (like the FPC predicts). Integrating these ideas serves an important role in stream ecology moving forward, as the relative role of these conceptual models, such as the RCC, play in stream systems may elucidate many of the context dependencies that make the models, on their own, applicable in certain systems, but not others.

Stream metacommunities as a conceptual framework for large-scale studies

The RCC identifies environmental heterogeneity as the main driving force structuring the composition of biotic communities, while dispersal capability of organisms is not addressed in the model. Yet, dispersal is known to be a key driver of ecological communities, as pointed out as early as the 1960's from MacArthur's ecological theories (Chase and Myers, 2011). Therefore, river ecologists need a theoretical framework beyond the RCC to evaluate patterns in biodiversity and make predictions on the role of environmental heterogeneity, as well as dispersal, in explaining the observed composition of benthic communities. Although not developed in aquatic environments, the metacommunity concept has been applied to stream ecosystems to add on the role of dispersal to what the RCC already found in relationship to environmental heterogeneity. The general aim of the metacommunity theory is thus to quantify the role of different mechanisms (e.g. dispersal/connectivity, environmental filters) on the composition of biological communities. This represents the main novelty of this concept, which makes it a useful predictive tool for basic and applied stream ecology (Brown et al., 2011).

Metacommunities may be represented by one of the four paradigms defined by Leibold et al. (2004) over a gradient of dispersal and environmental heterogeneity. The species sorting and mass effect paradigms postulate that local sites are environmentally heterogeneous, so that different species might be favored at different sites. While dispersal capacity of a species is assumed to be high enough to permit species to track their preferred environmental conditions under the species sorting perspective (Leibold, 1995). The mass effect paradigm states that individuals from suitable patches can colonize low-quality patches and guarantee species survival in these habitats (Pulliam, 1988). The patch dynamics and neutral theory paradigms assume patches to be homogeneous in terms of environmental parameters. The presence of a species in a patch is mainly driven by a trade-off between its dispersal capacity and its competitiveness (patch dynamics) or community composition is determined by stochastic demographic factors (neutral theory) (Hanski, 1994; Hubbell, 2001). However, the four paradigms may not be mutually exclusive, since deterministic processes associated with environmental filtering can occur simultaneously with ecological drift and dispersal-related processes within a metacommunity (Leibold and Chase, 2017).

Of the four paradigms, the most commonly tested in stream ecosystems are mass effects and species sorting (Logue et al., 2011). An abundance of work in stream metacommunity ecology is currently being done, demonstrated by the recent Freshwater Biology special issue “Metacommunities in river networks: The importance of network structure and connectivity on patters and processes” (2018). In general, the RCC supports a species-sorting paradigm of stream macroinvertebrate community structuring, due to the dominance of environmental heterogeneity, in the form of POM and light availability, and in structuring local communities (de Mendoza et al., 2018). Yet, how this varies over taxonomic groups and diverse stream conditions, influencing dispersal capability, remains an active area of study (Göthe et al., 2017).

Naturally and recently induced intermittent rivers represent a good study system for the implementation of metacommunity-related concepts (Datry et al., 2017; Burgazzi et al., 2018). In this case, dispersal capabilities of stream invertebrates influence the metacommunity structuring (Cañedo-Argüelles et al., 2015). Many invertebrates in these systems use overland travel including flight for dispersal with aquatic refugia interspersed, demonstrating the need for these refugia for overall fragmented system sustainability and conservation.

Empirical tests on the application of metacommunity theory on stream macroinvertebrates can be highly dependent on the breadth of taxonomic groups studied (e.g. a specific order of insects vs. all benthic macroinvertebrates) and basin location (Heino et al., 2010; Tonkin et al., 2016). A meta-analysis of studies over a global latitudinal gradient has shown there is only a weak relationship between environmental factors and beta-diversity of stream insect groups (Heino et al., 2015). This weak relationship may be because the phylogenetic scope of the meta-analysis was limited, due to incompatibility of datasets. The groups studied are interacting with other diverse groups and have terrestrial dispersal pathways, neither of which is accounted for, which limits the applicability of metacommunity concepts (Seymour et al., 2016; Tonkin et al., 2018). The RCC did a better job of accounting for this taxonomic diversity by using the entire benthic community of macroinvertebrates in its predictions.

Although metacommunity theory has become a “hot topic” in stream ecology, many efforts are being made to move beyond it, because of its limited view of benthic community structuring. For instance, a three-component metacommunity framework, whose structuring forces are represented by species equivalence, in addition to habitat heterogeneity and dispersal, has been proposed to work better for streams, rather than four paradigms (de Mendoza et al., 2018). Yet, according to these authors, habitat heterogeneity is expected to be most important factor in structuring stream communities, similar to the RCC predictions. Another way stream ecologists are enhancing metacommunity studies is by integrating the classical taxonomic approach with trait-based approaches, which, again, reiterates the importance of FFGs discussed in the RCC. More recent trait-based stream studies have shown that local environmental factors, such as riparian and in stream habitat, have greater influence on FFG variation than geographic factors related to dispersal capabilities (Heino et al., 2007), confirming past RCC insight.

Metacommunity dynamics have been previously shown to affect ecosystem processes, highlighting the need to incorporate that feedback into a conceptual model. Streams studied as metaecosystems, defined by Loreau et al. (2003) as "a set of ecosystems connected by spatial flows of energy, materials and organisms across ecosystem boundaries" (Figure 1.3), rather than metacommunities are more complete, due to the vital importance of terrestrial subsidies in structuring stream communities as discussed in the RCC. In fact, Massol et al. (2011) cites the RCC as an example of a spatially structured food web/ecosystem where the coupling medium is material (leaf fall) and spatial grain is rare and far (transport down the longitudinal gradient). Adding the dispersal of invertebrates, both via consistent downstream drift and annual, multidirectional terrestrial dispersal, represents a unique, well-suited system to integrate differing patterns of dispersal into metaecosystem models, as suggested by Gounand et al. (2018), to take the field of metaecosystem ecology into a more realistic, field-based approach. Metaecosystem ecology represents an innovative pathway towards a better bridging of stream network dynamics within the larger field of ecology.

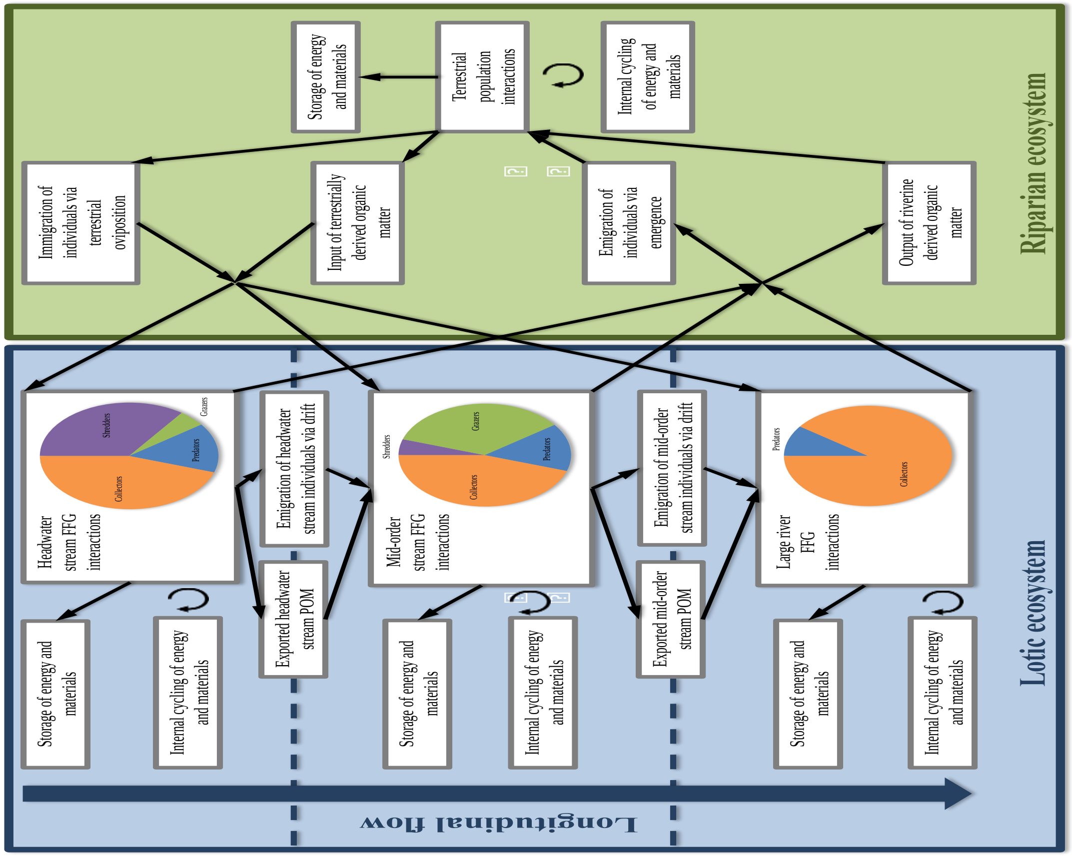

Figure 1.3. Conceptual diagram of the River Continuum Concept revised to represent a metaecosystem perspective (modified from Leibold and Chase 2017). Contained within each box are local ecosystems: headwater streams, mid-order streams, and large rivers representing a larger lotic metaecosystem; and the adjacent, terrestrial riparian ecosystem. The dashed lines delineate boundaries between the three local, lotic ecosystems, although these boundaries represent a gradient along the longitudinal flow. Within each local ecosystem, there are local communities with FFGs predicted by the RCC, as well as the internal cycling and storage of energy and materials, which is modified by interactions with the local community. Individuals in each local aquatic community emigrate downstream, via drift, or emerge as adults to disperse terrestrially. Likewise, terrestrial dispersing invertebrates may disperse via oviposition to local aquatic ecosystems. Terrestrially derived organic matter that is transported to lotic ecosystems via leaf fall is then exported downstream in the form of particulate organic matter (POM). This diagram represents the basic predictions of the RCC, and deviations in riverine metaecosystems are common and often context dependent.

Assessing the position in the river network and its effects on aquatic biodiversity

According to Altermatt (2013), it should be acknowledged that both the RCC and the recent advances in metacommunity theories have elucidated the role of spatial dimension on macroinvertebrate communities and processes in lotic ecosystems. In contrast to the limited view of lotic systems described in the RCC, river networks should be viewed as dendritic systems, where river reaches are branches, and confluences and other discontinuities represent the nodes. Because aquatic biodiversity and ecosystem processes are affected by such spatial structure, river ecologists have recognized that the position of a sampled reach/site in the river network should be accounted for in lotic biodiversity studies (Altermatt, 2013; Brown et al., 2018; Henriques-Silva et al., 2019). However, this implies some fundamental questions not addressed by the RCC: Are we able to thoroughly describe the dendritic structure of rivers? Are our spatial variables suitable for interpreting biological responses?

While the longitudinal gradient of the RCC seems inadequate because of its focus on mainstem sites, even the stream Strahler order may not be the best indicator for describing the positions of sampling sites in the river network (Ryder and Scott, 1988; Altermatt, 2013). This inadequacy is because the stream Strahler order alone does not give information on some important attributes, such as the connectivity, hydrological, and habitat heterogeneity, that in turn affect the dispersal of aquatic taxa and biodiversity.

New spatial metrics and descriptors are therefore necessary, as well as new experimental and statistical approaches (Landeiro et al., 2011; Erős and Lowe, 2019). Interestingly, recent developments arise from the scientific literature: for instance, Larsen et al. (2019) investigated the longitudinal variation in macroinvertebrate FFGs of the entire Adige River basin. To consider the position of the sampling sites in the river network and their spatial connectivity, beyond the mainstem view that the RCC employs, the authors used spatially explicit models using three different measures of distance: overland, flow connected, and flow-unconnected. They found that downstream changes in FFGs were generally consistent with the predictions of the RCC, but this longitudinal variation was mostly patchy-discontinued rather than a gradient (Larsen et al. 2019). Moreover, the spatial autocorrelation of spatially explicit modelling generally improved the performance of the statistical analysis.

Similarly, the direct and indirect effects of local and regional factors on the functional composition of macroinvertebrates were evaluated by Harvey and Altermatt (2019) in the Rhine River basin. Thirty-eight environmental variables acting at local and regional scales were considered as predictors of the composition in FFGs of macroinvertebrate communities from 364 sampling sites. By means of an integrative path analysis, authors identified the most significant spatial descriptors across scales and assessed how they directly and indirectly affected each FFG, with repercussion at river network scale (Harvey and Altermatt 2019). Although different statistical approaches were used, these two publications clearly illustrate that one of the main aspects of the RCC, the prediction of spatial patterns in FFGs, continues to be a main objective of river ecologists. However, these two papers also provide a more detailed characterization of the spatial dimension than the RCC's longitudinal focus.

A conceptual synthesis of the RCC, metacommunity theory, and the river network structure has been recently proposed by Schmera et al. (2018) into the Network Position Hypothesis (NPH). According to this theory, headwater stream communities are more influenced by species sorting due to their relatively isolated position in the river network. In contrast, downstream and main stem communities are mainly influenced by dispersal related dynamics (i.e. mass effect) because of higher centrality and/or connectivity within the river network (Schmera et al. 2018).

The NPH integrates the main paradigm of the metacommunity theory and recognizes that the driving processes acting on stream communities differ in relationship to the position in the river network. To date, empirical tests of this theory are scarce, and hence, this topic represents a promising field of investigation in the future. For instance, Henriques-Silva et al. (2019) evaluated the validity of the NPH predictions on fish assemblages from 3353 sites in France, and their results weakly supported the NPH predictions. However, one of the more interesting findings was that when new network metrics that describe the river network position (e.g. node metrics to quantify centrality and connectivity between sites) were used in the statistical analysis, the evidence for NPH support was higher compared to the distance from source or upstream drainage area spatial variables that represent the upstream-downstream longitudinal gradient used in RCC studies.

Conclusions

Our work reveals that the River Continuum Concept (Vannote et al. 1980) since its publication has significantly advanced the basic and applied knowledge in stream ecology. Although the RCC does not apply to all river types and biomes, its worldwide implementation has been and continues to be important. In fact, a number of relevant theories and concepts blossomed from its application, which aimed at accounting for context-dependent conditions and the importance of the spatial complexity, filling the gap on the variability of lotic ecosystems at large spatial scales (i.e. river network). In addition, a breadth of studies have adopted a functional approach by measuring the deviation of abundances and/or relative proportions of FFGs from the RCC predictions (Bredenhand and Samways, 2009; Miserendino, 2009; Manfrin et al., 2013; Fierro et al., 2015). The predictive power of the RCC has provided a multidisciplinary view of lotic ecosystems for testing hypotheses on the taxonomic and functional organization of benthic communities. In particular, the most innovative ideas of the RCC were that biological communities of lotic ecosystems vary in their functional composition along the river continuum (spatial gradient), and this shift is due to the predictable effects of the environmental conditions, such as type of organic matter, channel morphology and light penetration (habitat sorting). Such ideas have been incorporated and integrated by metacommunity, metaecosystem, and river network approaches, which are currently candidate frameworks to investigate patterns in biodiversity and functional processes in spatially nested and dendritic systems like rivers.

Finally, much remains to be done to better understand the complexity of lotic systems. With these regards, we identify the following topics as research areas of primary importance for future studies:

1) New geo-statistical and conceptual approaches are needed to relate observed taxonomic and functional biodiversity with the position in the river network, especially in relationship to key mechanisms like dispersal, genetic flow, habitat sorting, and connectivity (Tornwall et al., 2015; Harvey and Altermatt, 2019). Advances in this direction, allow a better characterization of the spatial structure of lotic ecosystems, limiting the context-dependency of the results, and promoting shared approaches.

2) Empirical tests on the validity of new theories, such as the Network Position Hypothesis, are needed to enhance our knowledge. In particular, a multi-taxa approach may be valuable in this context, because it allows for a better understanding of the spatial constraints of aquatic biota based on the taxon-specific size and dispersal ability.

We hope that reflecting on the RCC will spur novel approaches to examine stream ecosystems that have the potential to result in a paradigm shift in stream ecology towards river network modelling along environmental gradients.

CHAPTER 2: MICROBIAL COMMUNITY RESPONSE TO A NOVEL SALMON RESOURCE SUBSIDY

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Preface

In this already published chapter, microbial communities refer to bacterial and microeukaryotic communities determined by sequencing of the 16S rRNA gene amplicon sequencing. This excluded fungi from our analyses, which are a significant aspect of microbial communities. Preliminary analyses of sequences from the ITS rRNA gene region for fungal community characterization revealed no difference in biofilm communities in salmon vs. control reaches and only 13% of sequence reads could be identified to the phyla level, due to limited reference datasets. Although we were not able to include fungi in this manuscript, future studies of microbial community response to salmon carrion introduction should include fungi, due to their dominant role in decomposition.

Additionally, pseudoreplication was an issue in this study, and results should be interpreted cautiously. The study was conducted in only one creek in Michigan and serves as an excellent case study for how carrion allochthonous resources influence stream communities. Yet, additional studies are needed to gain the statistical power necessary to make generalizations to the entire range of Great Lakes salmon runs. Voucher specimens were deposited in the Albert J. Cook Arthropod Research Collection, Michigan State University (Appendix S1).

Abstract

Salmon decomposition is traditionally viewed through the lens of energy and nutrient subsidies, but not as a potential “microbial subsidy.” Microbial communities residing on and within spawning salmon are directly introduced into streams after host death. This incorporation

takes the form of microbes sloughing off and integrating into substrate biofilms, or indirectly, by macroinvertebrates facilitating dispersal via consumption. The objective of this study was to determine the effects of salmon carcass-derived microbial communities on stream biofilms and macroinvertebrates during an experimental salmon carcass addition in a naïve stream (i.e., no evolutionary history of salmon). Microbial communities [epilithic biofilms and within macroinvertebrates (internal)] were sampled at treatment and control sites before (September), during (October), and after (November to following August) a salmon carcass subsidy introduction in two successive years (September 2014-August 2016). We found a significant interaction between carcass addition and time on microbial and macroinvertebrate communities. *Heptagenia* (Heptageniidae: grazer) density was five times higher in the salmon reach compared to the control. In the salmon reach during year one, Stramenopiles (i.e., eukaryotic microbes) decreased in biofilm communities after two weeks of decomposition. The internal microbiome of *Stegopterna mutata* (Simuliidae: collector-filterer) varied between years but was significantly different between reaches over time during year two of the study, with four times greater abundance of melanogenesis functional pathways (function determined *in silico*) in the control reach. Although unique microbial taxa, introduced to this naïve stream via salmon carrion, persisted in biofilms on benthic substrate and internal to insects during both years, those taxa represented <2% of the relative abundance in microbial communities. These results highlight the importance of allochthonous carrion resources in the microbial ecology of lotic biofilms and macroinvertebrates. Furthermore, this study contributes to previous research into the complex interkingdom interactions in stream communities in response to a novel allochthonous resource.

Introduction

Headwater streams are highly reliant on allochthonous organic matter as an energy base for consumers. Shading from riparian trees restricts the amount of sunlight to most headwater streams thereby limiting autochthonous primary production (Vannote et al., 1980). Therefore, stream trophic networks rely on organic matter decomposition from outside sources. Organic matter decomposition has traditionally been viewed through the lens of carbon and nutrient subsidies (e.g., leaf litter) into the system in ways that alter macrobenthic communities (Polis and Strong, 1996; Hagen et al., 2012; Benbow et al., 2018). Allochthonous organic matter may also act as a “microbial subsidy” source to streams, by transferring novel microbes from one ecosystem to another (Steffan et al., 2017; Figure 2.1). Due to the high diversity of microbes on Earth, each allochthonous resource has an individual microbial community residing on and within it (Lindström and Langenheder, 2012; Locey and Lennon, 2016; Thompson et al., 2017). These novel microbes are hypothesized to be introduced into streams through the addition and transport of the allochthonous resources from adjacent or upstream habitats (e.g., riparian zones or tributaries) (Ruiz-González et al., 2015), but energy, nutrients, and microbes can also arrive in the form of decomposing heterotrophic biomass, such as carrion (Pechal and Benbow, 2016; Benbow et al., 2018).

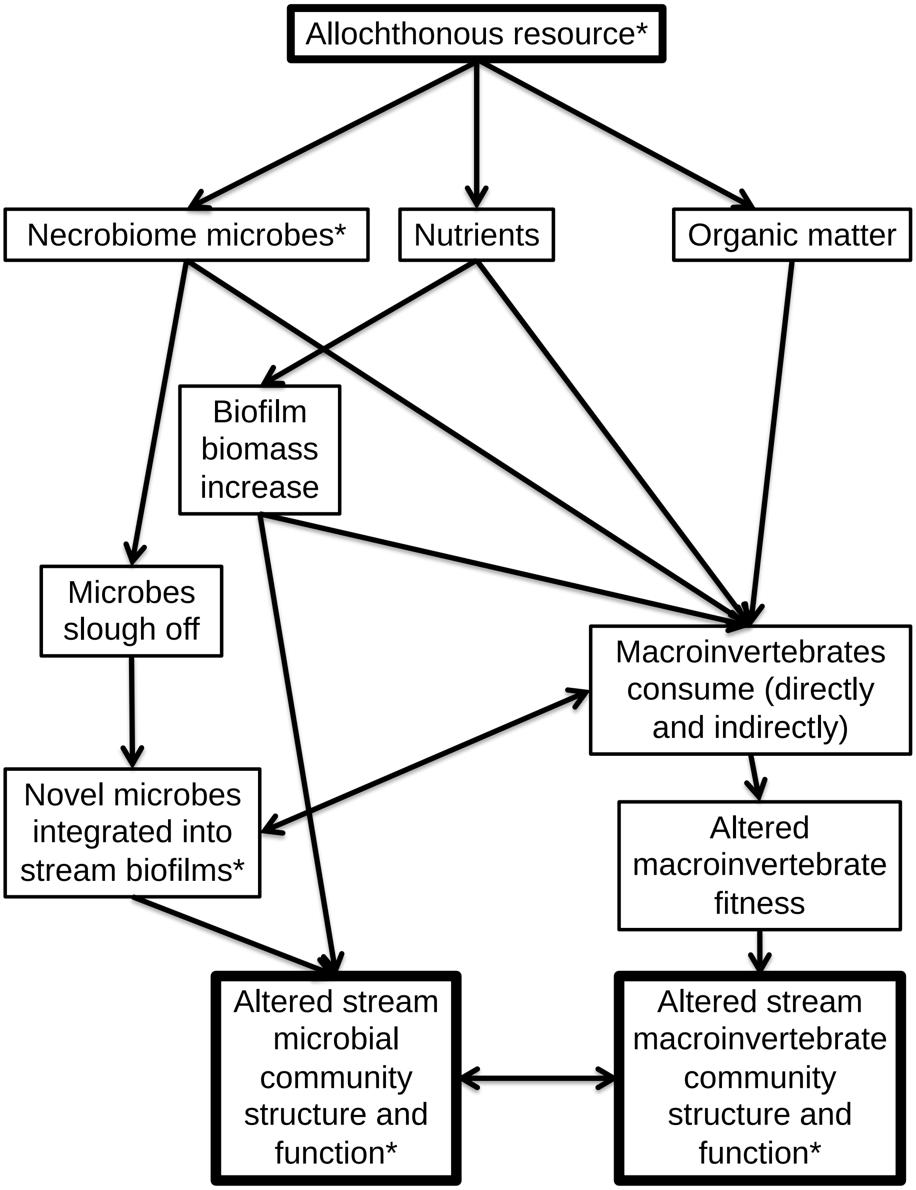


Figure 2.1. Conceptual framework of allochthonous resources altering microbial and macroinvertebrate communities via nutrients, organic matter, and microbes. Arrows represent directional links of effects. An asterisk represents factors directly measured in this study, while those without asterisks are hypothesized. Hypotheses on the overall importance of each linked agent are not offered, as these may vary over time and space, and the overall figure represents how these components fit into the larger ecosystem context.

Macroinvertebrate consumers may directly ingest and subsequently disperse allochthonous resource microbes throughout a stream (McEwen and Leff, 2001). Insects are vectors of microbes from diverse substrates involved in decomposition, transferring the microbes they come into contact from one environment to another [e.g., insects on food: Blazar et al., 2011; blow flies (Diptera: Calliphoridae) on agar: Junqueira et al., 2017]. For example, in a microcosm experiment, dispersal of marked microbes by mayfly (*Baetis* sp.), stonefly (*Pteronarcys* sp.), dragonfly (Aeshnidae) nymphs, and glass shrimp (*Palaeomonetes* sp.), exhibited considerable variation in the abundance of macroinvertebrate-associated bacteria transferred to other surfaces (Leff et al., 1994). In some trials, there was a large pulse of marked microbes dispersed, and in others, no marked microbes were detected. It remains unknown how these occasional releases of macroinvertebrate-associated bacteria ultimately influence microbial structure on downstream surfaces. Stream macroinvertebrates also act as important consumers of biofilms and can alter microbial community structure by selectively consuming taxa (Mulholland et al., 1991; Feminella and Hawkins, 1995; Rosemond et al., 2000; Lang et al., 2015). Alternatively, microbes may slough off the host resource and become integrated into the water column and benthic biofilm microbial communities (Leff et al., 1998; Crump et al., 2012). These changes to microbial communities alter the functional base of the aquatic food web, which may have far reaching effects throughout the stream network (Hall and Meyer, 1998; Meyer et al., 2007). Therefore, it is important to understand how microbial subsidies associated with allochthonous resources influence stream communities, from riparian leaf litter to carrion generated through mass mortalities, such as annual salmon runs.

Pacific salmon (*Oncorhynchus* spp.) are an important annual input of allochthonous nutrients and organic matter into streams. Particularly in the Pacific Northwest and Alaska, which have a several thousand year long history of native salmon, and other watersheds in North America where they have been anthropogenically introduced as recreational and economic fisheries (Cederholm et al., 1999; Gende et al., 2002; Moore et al., 2004). Benthic biofilms increase in biomass and have lower nutrient limitation when salmon carrion is present (Wipfli et al., 1998;

Johnston et al., 2004; Mitchell and Lamberti, 2005; Rüegg et al., 2011). Similarly, macroinvertebrate community structure, function, and growth rates are influenced by decomposing salmon organic matter (Chaloner and Wipfli, 2002; Chaloner et al., 2002; Lessard and Merritt, 2006), often with contrasting effects within different regions and streams (Bilby et al., 1996; Claeson et al., 2006; Janetski et al., 2009, 2013). Temporal dynamics are important in mediating the influence of carcass additions. For example, peak salmon derived nutrient enrichment (determined by stable isotopes) in grazers occurred one to two months after carcass introduction, while it occurred two to three months after introduction in predatory macroinvertebrates (Morley et al., 2016). Microbes associated with salmon carcasses were detected in aquatic macroinvertebrates located within Alaskan salmon-bearing streams (Pechal and Benbow 2016), which demonstrates the potential for salmon carcasses to act as conduits for new microbe introduction into streams. Yet, this potential has not been tested in streams without a historical salmon run, such as those found in the Laurentian Great Lakes watershed, where Pacific salmon have been introduced.

Since first introduced to the Great Lakes region in 1966-1970 to control invasive alewife (Parsons, 1973), chinook (*O. tshawytscha*) and coho (*O. kisutch*) salmon have been naturalized to many tributary streams of the watersheds where they now spawn and die. Therefore, salmon carrion has been a non-native resource subsidy to some Michigan streams for only the last 50 years. After spawning and death, the resulting carcasses may structurally and functionally impact the aquatic communities residing in naïve headwater streams (Cederholm et al., 1989). These annual pulses of salmon increase the nutrients and organic matter inputs to recipient streams, which can have far reaching effects on both aquatic and terrestrial ecosystems throughout the food web (Bilby et al., 1996; Schuldt and Hershey, 1995). The salmon resource may be directly used by both stream macroinvertebrates and microbes, as well as indirectly through nutrient and dissolved organic matter subsidy pathways (Collins et al., 2011; Levi and Tank, 2013; Levi et al., 2013). However, salmon do not migrate to all Michigan streams, such as those with dams, providing an opportunity to investigate salmon carrion effects on microbial and macroinvertebrate communities in historically naïve systems through carrion subsidy introduction and monitoring.

The objective of this study was to evaluate allochthonous salmon carcass resource subsidy effects on aquatic macroinvertebrate and microbial communities in a naïve Michigan stream. We postulated these communities would demonstrate short- and long-term responses to introduced salmon carcasses. Specifically, we predicted in carcass-introduced habitats that: 1) macroinvertebrate communities would be initially dominated by shredders and transition to an increase in grazers and collectors; 2) biofilm communities would be dominated by heterotrophic bacteria compared to the control habitats; 3) the internal macroinvertebrate microbiomes would be supplemented with salmon carcass-associated microbes after carcass introduction; and 4) salmon carcasses would introduce microbes to the stream, some of which would persist and become more abundant, while others diminish.

Materials and Methods

Experimental design

This study was conducted in Hunt Creek on the property of the Hunt Creek Fisheries Research Station near Lewiston, Michigan, USA (44.86, -84.16). Hunt Creek is a groundwater fed second-order stream in the Thunder Bay River watershed and has never received an annual salmon run (Grossman et al., 2012). Several barriers to upstream movement of fish preclude colonization of Hunt Creek by salmon, which were reasonably abundant in Lake Huron before a major decline in the early to mid-2000s (Cwalinski et al., 2006). A Before-After-Control-Intervention field study design was implemented for this study (Stewart-Oaten et al., 1986). Chinook and Coho (n = 120; 50/50 species split) salmon carcasses were introduced into the same salmon “treatment” reach in October 2014 and October 2015 (the typical timing of Michigan salmon runs; Gerig et al., 2018) using loading rates (~1 kg m^{-2} of stream) approximate to that of a typical salmon run in a Lake Michigan tributary (Janetski et al., 2012). Michigan Department of Natural Resources hatcheries were the source of the salmon carcasses, and salmon died of natural causes. For around one year, carcasses were frozen to prevent inadvertent disease introduction to waters that do not have migratory fish runs. Carcasses were then brought to ambient temperature before being staked with rebar in reach habitats, including pools, undercut banks, and debris jams, as has been performed in similar salmon carrion studies (Tiegs et al., 2011). A control reach lacking salmon carcass introduction was located 600 m upstream of the salmon reach (Figure S2.1). Both control and salmon reaches were 90 m long. The average width and depth of the control reach were 0.18 m and 3.21 m, and the average width and depth of the treatment reach were 0.19 m and 3.61 m, respectively.

Field sample collections

Prior to salmon carcass introduction, epinecrotic microbial communities of each carcass were aseptically sampled with sterile and DNA-free cotton swabs using the methods of Pechal and Benbow (2016). Swab samples were individually stored in 200 µL of molecular grade ethanol (>96%) at -20°C. Internal salmon carcass microbial samples were not sampled, so as to not influence the decomposition process by physically altering the salmon carcasses. Microbial and macroinvertebrate communities were sampled at three sub-reaches within the treatment and control reaches: once before (September), once during (October), and four times after (March through August) carcass introduction each year. Sterilized hexagonal unglazed ceramic tiles (29.25

cm^2) were deployed in the stream to characterize epilithic microbial communities (Lang et al., 2016). Six tiles were secured to a brick using a silicone adhesive; five bricks were placed along a transect perpendicular to stream flow in the center of each sub-reach. Bricks were introduced into the stream two weeks prior to the first sample collection to establish baseline communities in both reaches. During each collection, the bricks were removed from the stream, a tile was collected and placed in a sterile 188 mL WhirlPak bag (Nasco, Fort Atkinson, WI, USA), kept on ice during transport, and stored at -20°C until DNA extraction. Bricks with the remaining tiles were returned to the same location within the stream. After all tiles were collected, the biofilms were scraped from tiles in the laboratory using autoclaved sterile and decontaminated razor blades into a 2 mL microcentrifuge tube for immediate DNA processing. We did not quantify the amount of microbial growth on any of the collected samples, due to the small amount of growth. During each sampling event, water chemistry parameters of dissolved oxygen (mg/L), pH, conductivity (mS/cm), and temperature (°C) were determined using a YSI 6-Series multiparameter water quality 6600 V2-4 sonde (Table S2.1).

Macroinvertebrates were sampled using a modified Hess sampler (Merritt et al., 2008). At each sub-reach, three riffle habitats were sampled for 30 seconds each and combined into a single composite for that location (total area=0.3 m^2). Individual specimens that represented dominant taxa over a variety of feeding groups were hand-picked from the composite Hess sampler collection to ensure adequate sample sizes from representative groups to obtain internal microbial communities; samples were immediately stored in molecular grade ethanol for subsequent internal microbial community analysis. The remainder of the composite Hess sample was stored in 70% ethanol and hand-sorted in the laboratory. Macroinvertebrates were identified to the lowest taxonomic level (genus), except for those used for internal microbiome analyses, which were identified to species (Merritt et al., 2008; Bright, 2016). Functional feeding group was also determined using Merritt et al. (2008) (Table S2.2).Three species were used for internal microbiome analysis due to their abundance and to represent different functional feeding groups: *Heptagenia flavescens* (Walsh) (Ephemeroptera: Heptageniidae; grazer), *Baetis brunneicolor* McDunnough (Ephemeroptera: Baetidae; collector-gatherer), and *Stegopterna mutata* (Malloch) (Diptera: Simuliidae; collector-filterer).

DNA processing and targeted 16S rRNA gene amplicon sequencing

For insects, three identified individuals were pooled into one sample and surface sterilized using a 10% bleach rinse followed by three sterile deionized water rinses (Ridley et al., 2012). The insects were air-dried and ground in a 1.7 mL tube using a sterile pestle. DNA extraction was performed with the Qiagen PowerSoil DNA extraction kit® (Qiagen, Inc, Valencia, CA, USA) using a modified manufacturer's protocol: 20 mg mL^{-1} of lysozyme was added during the lysis step and the final DNA was eluted in 50µL of C6. DNA quantification was performed using the Quanti-iT dsDNA HS Assay kit and a Qubit 2.0 (Grand Island, NY, USA); a concentration of 0.1 ng µL^{-1} was used as a minimum threshold for subsequent sequencing procedures. All DNA preparations were stored at -20°C.

Illumina MiSeq 16S library construction (2 x 250 bp paired-end reads) and sequencing was performed at the MSU Genomics Core using a modified version of the Illumina MiSeq protocol (Caporaso et al., 2011a). The variable region 4 of the 16S rRNA gene was amplified with region-specific primers, 515F/806R (5'-GTGCCAGCMGCCGCGG-3', 5'-TACNVGGGTATCTAATCC-3') (Claesson et al., 2010; Caporaso et al., 2011b, 2012). The resulting 16S rRNA amplicon sequencing data were assembled, quality-filtered, and demultiplexed using QIIME2 version 19.1 (Kuczynski et al., 2012). Default settings were used, unless specified in the following methods. DADA2 was used to discard chimeric reads and other sequencing artifacts (Callahan et al., 2016). Taxonomy was assigned using a Naïve Bayes classifier trained using the 16S rRNA region, primer set, read length, and Greengenes 99% reference set version 13.8 (DeSantis et al., 2006; McDonald et al., 2012; Werner et al., 2012), including taxonomy for chloroplasts from eukaryotic microbes. Singletons were removed and samples rarefied to 2,500 sequences, which was the highest sequencing depth that included all biofilm samples (Figure S2.2). Relative abundance was determined by the number of reads in the rarefied dataset. Five samples (four carcass and one internal *H. flavescens*) were excluded due to insufficient sequence reads as a result of extraction or sequencing errors. Sequence files and metadata for all samples used in this study have been deposited in the NCBI SRA under number PRJNA526072.

Carrion-introduced operational taxonomic units (OTUs) in year one were determined by identifying those OTUs detected on salmon carrion prior to deposition in the stream, but not found in samples from either the control reach anytime during year one or the salmon reach before salmon were introduced (September). For year two, carrion-introduced OTUs were those OTUs not detected in year two in the control reach (background OTUs for year two) or during all of year one (both control and salmon reaches and carcasses – background OTUs resulting from any OTUs introduced in year one). The reasoning for excluding year one carcass-associated OTUs from year two carcass introduced, unique OTUs was to evaluate the integration of OTUs that the carrion introduces into biofilms and insects, rather than carrion associated OTUs themselves. Therefore, our strategy was to investigate the microbes completely naïve to the stream biofilms and internal insects during each year's salmon carcass introduction. These targeted sets of unique carrion introduced OTUs (year one and year two) were evaluated for presence in the downstream biofilms and internal insect microbiomes after carcass introduction. In addition, year one unique carrion introduced OTUs were evaluated for presence in year two carrion, to determine what OTUs not found in biofilms or internal insects were introduced both years.

Functional composition of the microbiome was predicted *in silico* using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) on the 16S rRNA amplicon sequences (Langille et al., 2013), using default settings in the online Galaxy version (http://galaxy.morganlangille.com/). PICRUSt analysis requires closed-reference OTU picking using the Greengenes database, thus clustering was conducted on 97% similarity OTUs using VSEARCH in QIIME2 (Rognes et al., 2016). OTUs were normalized by copy number, and predicted functional categories were assigned using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to predict KEGG orthologs, which were then collapsed at level 3 into hierarchical KEGG pathways by function (Kanehisa and Goto, 2000).

Statistical analyses

Mean ± standard error (MSE) was calculated for each individual taxon. Estimates of α-diversity in microbial communities [observed OTUs, Chao 1 richness, Shannon H’, and Faith’s phylogenetic diversity (PD) indices] were calculated in QIIME2 based on OTU sequence read matrices (Caporaso et al., 2010). Statistical analyses were performed using R version 1.1.442 (R Core Team, 2018). Estimates of α-diversity in macroinvertebrate communities (genus richness, Simpson’s diversity index) were calculated using the vegan 2.5-2 library diversity function in R (Oksanen et al., 2019). Differences in α-diversity metrics for each year’s carcass epinecrotic microbial community prior to deposition were tested using unpaired, two-tailed t-tests, while differences in α-diversity metrics in each reach over time were tested separately using repeated measures ANOVA. Residuals versus fit and normal probability plots were examined to determine if the assumptions of each statistical test were met. When assumptions were not met a Poisson distribution (count data) or $\log_{10}$ transformation (non-count data) was used. Confidence intervals (95%) were determined for coefficient estimates for $\log_{10}$-transformed data. Population densities of the three genera used for internal microbiome testing were examined using the same methods as α-diversity assessments.

Variation in microbial and macroinvertebrate community composition was visualized using nonmetric multidimensional scaling (NMDS) and statistically evaluated for β-diversity metrics with PERMANOVA, a phylogeny based matrix (weighted UniFrac) for microbial communities and Bray-Curtis dissimilarity matrix for macroinvertebrate communities (standard for macroinvertebrate community analysis) using 99,999 permutations in the vegan 2.5-2 library "adonis" function in R (Anderson, 2001). For those samples where stream reach was statistically significant, we identified taxa (genus level for macroinvertebrates and family level for microbes) found in the salmon reach over time via indicator species analysis (ISA) with Indicator Value (IndVal) Index and its significance using 99,999 permutations in the "signassoc" function in the R package indicspecies, with p-value adjustments for multiple comparisons using the Sidak method (Dufrêne and Legendre, 1997; Cáceres and Legendre, 2009; Cáceres et al., 2010). All other statistical tests were considered significant at $\alpha=0.05$. Due to statistically significant differences in salmon carrion conditions during each year of the study (see results, Tables S3-S5), separate analyses were conducted for each year for all response variables.

Results

Macroinvertebrate community composition

A total of 13,730 aquatic macroinvertebrates were identified comprising 49 taxa, with Chironomidae the most relative abundant and a mean of 17% (± 2%) (Table S2.6). No covariate (salmon treatment, time, or their interaction) was found to significantly influence macroinvertebrate density or richness in either year of the study ($p>0.05$). Although no covariate influenced diversity during year one, in year two macroinvertebrate diversity increased by 0.0015 (± 0.0005) each day ($p<0.01$), and there was a significant time x treatment interaction ($p=0.04$). Only time had significant effects on macroinvertebrate community structure during both years (PERMANOVA: $p<0.01$, Table 2.1). In year two, *Brachycentrus* (collector-filterer) density (individuals per 0.3 m^2) was lower in the salmon reach relative to the control reach [$\bar{x}_S = 0$ (±0), $\bar{x}_C = 6$ (±3), ISA: $p=0.03$, Figure S2.3]. *Heptagenia* population density significantly increased in year one and was also significantly higher in the salmon reach during both years [$\bar{x}_S = 14$ (±4), $\bar{x}_C = 3$ (±1), ANOVA: $p<0.02$, Figure S2.3]. *Baetis* density significantly increased each year, with a significant time x treatment interaction due to a higher abundance in the control reach nine months after salmon introduction during both years [$\bar{x}_S = 41$ (±14), $\bar{x}_C = 191$ (±41), $p<0.01$, Figure S2.3]. We found a significant time x treatment interaction influencing *Stegopterna* density in year one, due to decreased abundance in the salmon reach nine to eleven months after salmon introduction [$\bar{x}_S = 19$ (±6), $\bar{x}_C = 109$ (±44), $p<0.01$]. *Stegopterna* density also significantly increased over time during year two ($p<0.01$).

Table 2.1. PERMANOVA results testing benthic macroinvertebrate community structure based on Bray-Curtis distances for each year of the study with significant results (p<0.05) indicated by an asterisk. df=degrees of freedom; SS=sum of squares; MS=mean sum of squares.

Year One						
Factor	df	SS	MS	F	R$_2$	P
Treatment	1	0.35	0.35	1.63	0.03	0.11
Time	1	1.93	1.93	9.02	0.18	<0.01*
Treatment x Time	1	0.23	0.23	1.07	0.02	0.34
Residuals	38	8.15	0.21		0.76	
Total	41	10.66			1.00	
Year Two						
Factor	df	SS	MS	F	R$_2$	P
Treatment	1	0.39	0.39	1.63	0.05	0.11
Time	1	0.86	0.86	3.56	0.10	<0.01*
Treatment x Time	1	0.22	0.22	0.90	0.02	0.50
Residuals	32	6.99	0.24		0.83	
Total	35	8.46			1.00	

Salmon carcass epinecrotic community composition

A total of 11,219 microbial OTUs representing 51 phyla were identified in the carcass microbial communities. *Moraxellaceae* (γ-Proteobacteria) had the highest relative abundance [16% (±4%)]. While the diversity metrics Faith's PD and Chao 1 were not significantly different for each year of introduction (t-test: *p*>0.1), the epinecrotic microbial communities were different between years, both taxonomically (OTU level) and functionally (PERMANOVA: *p*<0.01, Table S2.3). Twenty microbial families were indicators of the year in the epinecrotic communities (ISA: *p*<0.05, Table S2.4). *Ruminocaccaceae, Geobaceracae, Succinivibrionaceae, Spirochaetaceae,* an unknown family in *Bacteroidales,* and an unknown family in *YS2* were the most significant indicator families (*p*<0.01) and were all only found in year one carcasses. The most abundant indicator family, *Sphingomonadaceae,* had 2.7 times higher relative abundance in year one carcasses than year two carcasses [$\bar{x}_{Y1}$ = 17% (±3%), $\bar{x}_{Y2}$ = 6% (±1%)]. Functionally, 135 KEGG orthologs were indicators of salmon carcass introduction year (ISA: *p*<0.05, Table S2.5). The most significant KEGG orthologs were caffeine metabolism, ether lipid metabolism, ethyl benzene degradation, isoflavonoid biosynthesis, mineral absorption and proteasome, all of which were greatest in year two carcass microbial communities ($p<0.01$). The most abundant salmon indicator KEGG ortholog was DNA repair and recombination protein, which was 1.4 times higher in year two carcasses [$\bar{x}_{Y1}$ = 41587 (±2865), $\bar{x}_{Y2}$ = 57353 (±3244), ISA: p=0.02]. Melanogenesis, a salmon indicator KEGG ortholog, was 3 times higher in year two [$\bar{x}_{Y1}$ = 8 (±2), $\bar{x}_{Y2}$ = 25 (±4), ISA: $p<0.01$].

Biofilm community composition

A total of 11,051 and 9,434 OTUs represented epilithic biofilm communities in year one and year two, respectively, from 72 total samples (36 per year). The most abundant family was an unnamed family in the order Stramenopiles, representing 17% (±2%) of the community. Faith's PD significantly decreased over time during both years (year one: 2%-3%, year two: 1%-2%, ANOVA: $p<0.01$), while Chao 1 richness increased over year one (0.6%-0.8%, $p<0.01$) and decreased in year two (0.4%-0.6%, $p<0.01$), but salmon treatment did not have an effect on Faith's PD or Chao 1 richness ($p>0.05$). Treatment, time, and a treatment x time interaction influenced community composition during year one (PERMANOVA, $p<0.05$, Table 2.2A, Figure S2.4), but only time was significant in year two.

Table 2.2. PERMANOVA results testing microbial community structure based on the weighted phylogenetic distance (UniFrac) matrix for β-diversity and Jaccard distance matrix for KEGG orthologs among the microbial communities from the two years' biofilms **(A)**, internal *B. brunneicolor* **(B)**, and internal *S. mutata* **(C)**, with significant results ($p<0.05$) indicated by an asterisk. Df=degrees of freedom; SS=sum of squares; MS=mean sum of squares.

A. Biofilm											
Year One	**OTUs**						**KEGG orthologs**				
Factor	**df**	**SS**	**MS**	**F**	**R$_2$**	**P**	**SS**	**MS**	**F**	**R$_2$**	**P**
Treatment	1	0.12	0.12	2.58	0.05	0.05*	0.04	0.04	2.87	0.07	0.05*
Time	1	0.69	0.69	14.75	0.27	<0.01*	0.08	0.08	5.16	0.12	<0.01*
Treat. X Time	1	0.22	0.22	4.66	0.09	0.01*	0.03	0.03	2.29	0.05	0.09
Residuals	32	1.50	0.05		0.59		0.48	0.02		0.76	
Total	35	2.53			1.00		0.64			1.00	
Year Two	**OTUs**						**KEGG orthologs**				
Factor	**df**	**SS**	**MS**	**F**	**R$_2$**	**P**	**SS**	**MS**	**F**	**R$_2$**	**P**
Treatment	1	0.12	0.12	1.59	0.04	0.15	0.03	0.03	1.07	0.03	0.32
Time	1	0.53	0.53	7.37	0.17	<0.01*	0.02	0.02	0.89	0.02	0.40
Treat. X Time	1	0.12	0.12	1.69	0.04	0.14	0.02	0.02	0.91	0.03	0.39
Residuals	32	2.32	0.07		0.75		0.82	0.03		0.92	
Total	35	3.09			1.00		0.89			1.00	
B. *B. brunneicolor*											
Year One	**OTUs**						**KEGG orthologs**				
Factor	df	SS	MS	F	R$_2$	P	SS	MS	F	R$_2$	P
Treatment	1	0.06	0.06	0.76	0.05	0.71	0.01	0.01	0.09	0.01	1.00
Time	1	0.17	0.17	2.16	0.13	0.07	0.03	0.03	0.30	0.02	0.80
Treat. X Time	1	0.05	0.05	0.59	0.03	0.81	0.02	0.02	0.16	0.01	0.95
Residuals	13	1.04	0.08		0.79		1.24	0.10		0.96	
Total	16	1.32			1.00		1.29			1.00	

Table 2.2. (cont’d)

Year Two	**OTUs**						**KEGG orthologs**				
Factor	**df**	**SS**	**MS**	**F**	**R_2**	**P**	**SS**	**MS**	**F**	**R_2**	**P**
Treatment	1	0.15	0.15	1.23	0.04	0.32	0.10	0.10	1.02	0.04	0.33
Time	1	0.44	0.44	3.54	0.11	0.02*	0.08	0.08	0.88	0.03	0.39
Treat. X Time	1	0.12	0.12	0.98	0.03	0.35	0.10	0.10	1.01	0.03	0.34
Residuals	26	3.21	0.12		0.82		2.45	0.09		0.90	
Total	29	3.92			1.00		2.72			1.00	
C. *S. mutata*											
Year One	**OTUs**						**KEGG orthologs**				
Factor	**df**	**SS**	**MS**	**F**	**R_2**	**P**	**SS**	**MS**	**F**	**R_2**	**P**
Treatment	1	1.92	1.92	5.69	0.58	0.09	0.03	0.03	0.84	0.08	0.49
Time	1	0.09	0.09	0.26	0.03	0.70	0.13	0.13	3.30	0.32	0.13
Treat. x Time	1	0.27	0.27	0.81	0.08	0.42	0.13	0.13	3.17	0.31	0.10
Residuals	3	1.01	0.34		0.31		0.12	0.04		0.29	
Total	6	3.29			1.00		0.41			1.00	
Year Two	**OTUs**						**KEGG orthologs**				
Factor	**df**	**SS**	**MS**	**F**	**R_2**	**P**	**SS**	**MS**	**F**	**R_2**	**P**
Treatment	1	0.10	0.10	1.36	0.06	0.15	0.02	0.02	0.19	0.01	0.89
Time	1	0.15	0.15	2.16	0.10	0.10	0.06	0.06	0.51	0.03	0.57
Treat. x Time	1	0.27	0.27	3.89	0.18	<0.01*	0.69	0.69	6.39	0.30	0.01*
Residuals	14	0.97	0.07		0.66		1.50	0.11		0.66	
Total	17	1.49			1.00		2.27			1.00	

Fifteen families were significant representatives of salmon reach biofilm communities in year one ($p<0.05$, Table S2.7). *Alteromonadaceae* was 3.5 times more abundant in the salmon reach [ISA: $p<0.01$, Figure 2.2]. Only two indicator families represented >10% of the community composition: *Saprospiraceae* and the abovementioned unnamed family in the order Stramenopiles. *Saprospiraceae* was 17 times lower in abundance in the salmon reach [0.7% (±0.2%)] compared to the control reach [12% (±3%)] two weeks after salmon introduction in year one, while an unnamed family in the order Stramenopiles exhibited the same pattern during both years, having 30 and 2.5 times lower abundance in the salmon reach in year one [$\bar{x}_S$ = 2% (±2%), $\bar{x}_C$ = 61% (±9%)] and year two [$\bar{x}_S$ = 25% (±13%), $\bar{x}_C$ = 60% (±4%)], respectively. Three indicator families identified from biofilms after carcass introduction were also indicator families of the salmon carcass microbial communities: *Sphingomonadaceae* [$\bar{x}_S$ = 1.8% (±0.4%), $\bar{x}_C$ = 0.9% (±0.1%)], *Geobacteraceae* [$\bar{x}_S$ = 0.12% (±0.04%), $\bar{x}_C$ = 0.04% (±0.02%)], and *Xanthomonadaceae* [$\bar{x}_S$ = 1.0% (±0.2%), $\bar{x}_C$ = 0.6% (±0.1%)], all of which had higher mean relative abundance in the salmon reach.

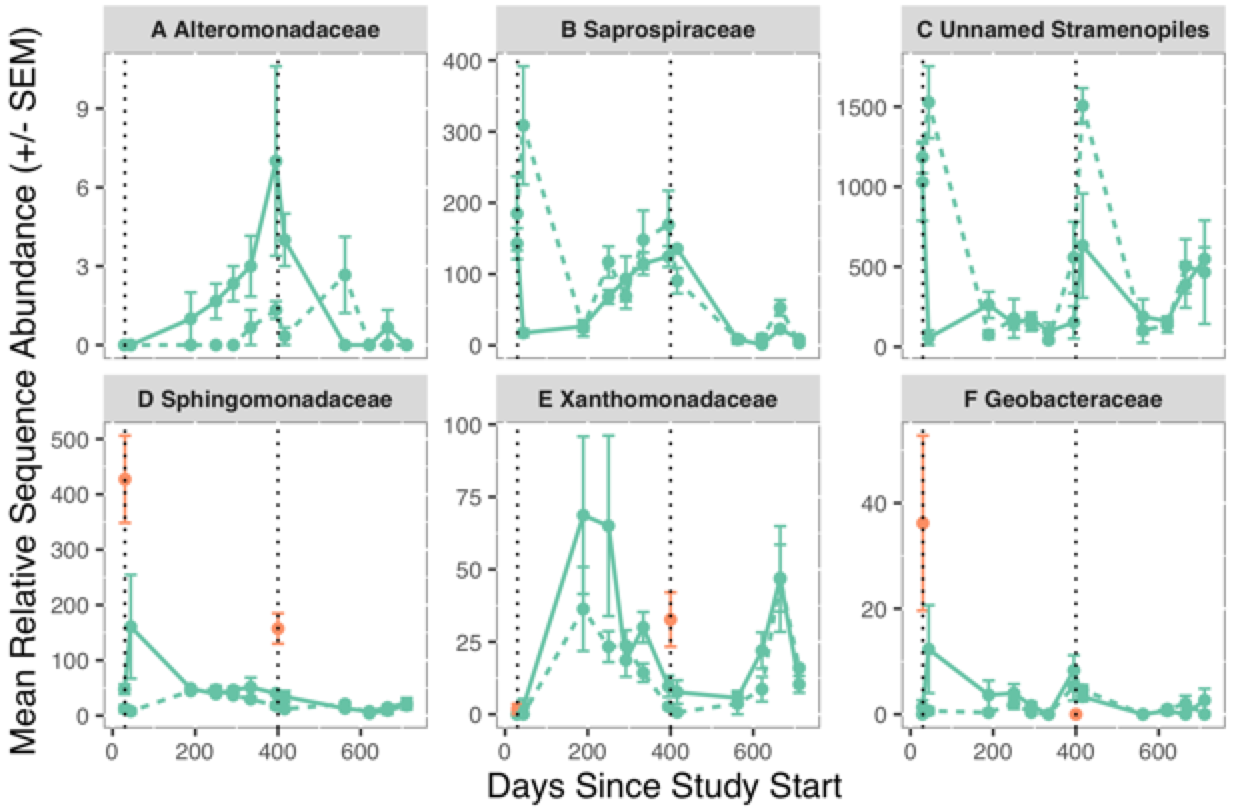


Figure 2.2. Mean relative sequence abundance (±SEM) of *Alteromonadaceae* **(A)**, *Saprospiraceae* **(B)**, Unnamed Stramenopiles family **(C)**, *Sphingomonadaceae* **(D)**, *Xanthomonadaceae* **(E)**, and *Geobacteraceae* **(F)** in biofilms (green) over time. *Sphingomonadaceae*, *Xanthomonadaceae*, and *Geobacteraceae* that were part of the carrion associated communities are shown in orange, as these families were also indicator taxa for year of salmon carrion addition within the salmon carrion microbial communities. The dashed line is the control reach, and the solid line is the salmon reach. Black, dotted vertical lines represent the day of salmon introduction. Relative sequence abundance is the number of reads in the rarefied (2,500 reads) dataset.

In year one, treatment and time (but not interaction) significantly influenced the composition of KEGG orthologs (PERMANOVA, *p*<0.05, Table 2.2A, Figure S2.4), yet there were no significant effects in year two. In year one biofilms, 113 indicator KEGG orthologs (*p*<0.05, Table S2.8) were identified, with the most significant carcass KEGG ortholog indicator being fluorobenzoate degradation [$\bar{x}_{S}$ = 1760 (±36), $\bar{x}_{C}$ = 1580 (±42), ISA: *p*<0.01], and the most abundant was the two-component system [$\bar{x}_{S}$ = 61151 (±148), $\bar{x}_{C}$ = 55800 (±154), ISA: *p*=0.04], both of which were higher in the control reach. A total of 41 KEGG orthologs indicated salmon treatment biofilm communities, as well as year of carcass introduction. Of those shared indicator

KEGG orthologs with higher abundance in the salmon reach, phosphotransferase system was the most abundant [$\bar{x}_S$ = 1805 (±106), $\bar{x}_C$ = 1411 (±163), ISA: $p<0.01$]. Another one of those shared KEGG orthologs was melanogenesis, which was 1.7 times higher in abundance in salmon reach biofilms [$\bar{x}_S$ = 17 (±3), $\bar{x}_C$ = 10 (±2), ISA: $p<0.01$].

Aquatic insect internal microbial community composition

In the mayfly *B. brunneicolor*, 1898 and 2269 OTUs were detected in year one and year two, respectively (47 total samples with 3 individuals each). In year one, an unnamed family in *Mollicutes* was the most relatively abundant family [13% (±4%)], while in year two the most abundant was *Pseudomonadaceae* [23% (±4%)]. Neither time nor treatment significantly influenced Chao 1 richness (ANOVA: $p>0.05$), yet Faith's PD decreased over time in year one (2.2%-9.3%, $p<0.01$) and year two (0.4%-1.7%, $p<0.01$). In year two, we also observed 99% lower Faith's PD in the salmon reach (31%-100%), and a significant time x treatment interaction ($p<0.05$).

Six families were indicators of internal microbial communities of *B. brunneicolor* in the salmon reach ($p<0.05$, Table S2.9). Of these six, the most significant and abundant was the aforementioned unnamed family in *Mollicutes*, which was five times greater in the control reach [$\bar{x}_S$ = 5% (±2%), $\bar{x}_C$ = 25% (±7%), ISA: $p<0.01$]. An unknown family in *Rhizobiales* (α-Proteobacteria) was an indicator of microbial communities in both internal *B. brunneicolor* from the salmon reach and from salmon carcass communities of year one, with 3.5 times higher abundance in the salmon reach [$\bar{x}_S$ = 7% (±2%), $\bar{x}_C$ = 2% (±1%), ISA: $p<0.01$]. Only time significantly influenced the microbial community composition of the *B. brunneicolor* internal microbiome during year two (PERMANOVA: $p=0.02$, Table 2.2B), while neither time nor treatment had significant effects in year one or influenced the KEGG orthologs detected in biofilms in either year ($p>0.05$).

In the black fly *S. mutata*, a total of 449 and 1224 OTUs were detected in year one and year two, respectively (23 total samples with 3 individuals each), with Firmicutes being the predominant phylum [32% (± 5%)]. Although no significant factors influenced Faith's PD in year one, in year two, mean diversity was 72 (± 33) times higher in internal *S. mutata* in the salmon reach compared to the control reach (ANOVA: $p=0.05$). Chao 1 richness was not influenced by time or treatment during either year ($p>0.05$).

Treatment, time, nor their interaction significantly affected the microbial composition or functional KEGG ortholog community composition of internal *S. mutata* in year one (PERMANOVA: $p>0.05$), In year two, the treatment x time interaction significantly influenced both the internal microbial community structure and function ($p<0.02$, Table 2.2C). An unnamed family in Streptophyta was an indicator family of treatment in year two, with four times greater relative abundance in the control reach internal *S. mutata* [$\bar{x}_S$ = 1.1% (±0.4%), $\bar{x}_C$ = 4% (±1%), ISA: $p<0.03$]. Melanogenesis was the only indicator KEGG ortholog for *S. mutata* internal communities from the salmon reach in year two, with four times greater relative abundance in the control reach [$\bar{x}_S$ = 21 (±4), $\bar{x}_C$ = 61 (±16), ISA: $p=0.05$], which was also an indicator KEGG ortholog in salmon carcasses and biofilms (Figure S2.5).

The internal microbiome of the mayfly *H. flavescens* could not be compared between treatment and control reaches due to low-yield microbial DNA and a low sample size (n=8).

Introduced salmon carcass microbes

Of the total 686 salmon carcass-derived OTUs introduced in year one, 645, representing an average relative abundance of 63% ± 3%, were not found in biofilm or internal insect samples in non-salmon reaches (unique) (Figure S2.6). During year two, 1786 [51% (± 6%)] of a total of 2196 were OTUs associated with introduced carcasses and not found in biofilm or internal insect samples anytime during year one, or in non-salmon reaches. Of unique OTUs introduced via carrion in the first year, 31 were detected in treatment biofilms (Table S2.10), of which 21 were only found in year one biofilms, six were only found in year two biofilms, and four were found in both years (Figure S2.6). Of the unique OTUs introduced via carrion in year two, 25 were detected in year two treatment biofilms. However, all unique OTUs introduced via salmon carrion and found in biofilms represented <2% of biofilm communities, except for a pulse two weeks after carcass introduction in year one when they increased to 5% (± 1%) (Figure 2.3B). Three year one, salmon carrion unique OTUs found in biofilms were found in the upstream control reach in year two.

Of the OTUs introduced via carrion in year one, nine were found in the internal *B. brunneicolor* communities collected in the salmon reach: three, five, and one OTU(s) were detected in year one, year two, and both years, respectively (Figure S2.7, Table S2.10). Four of these OTUs were also detected in treatment biofilms. Of those unique OTUs introduced via carrion in year two, eight persisted in year two treatment *B. brunneicolor* internal communities, none of which were found in treatment biofilms or *S. mutata*. However, these carrion-introduced unique OTUs represented <1% of the relative abundance of internal *B. brunneicolor* communities (Figure 2.3D); three year one introduced OTUs found in internal *B. brunneicolor* were also found in the upstream control reach in year two.

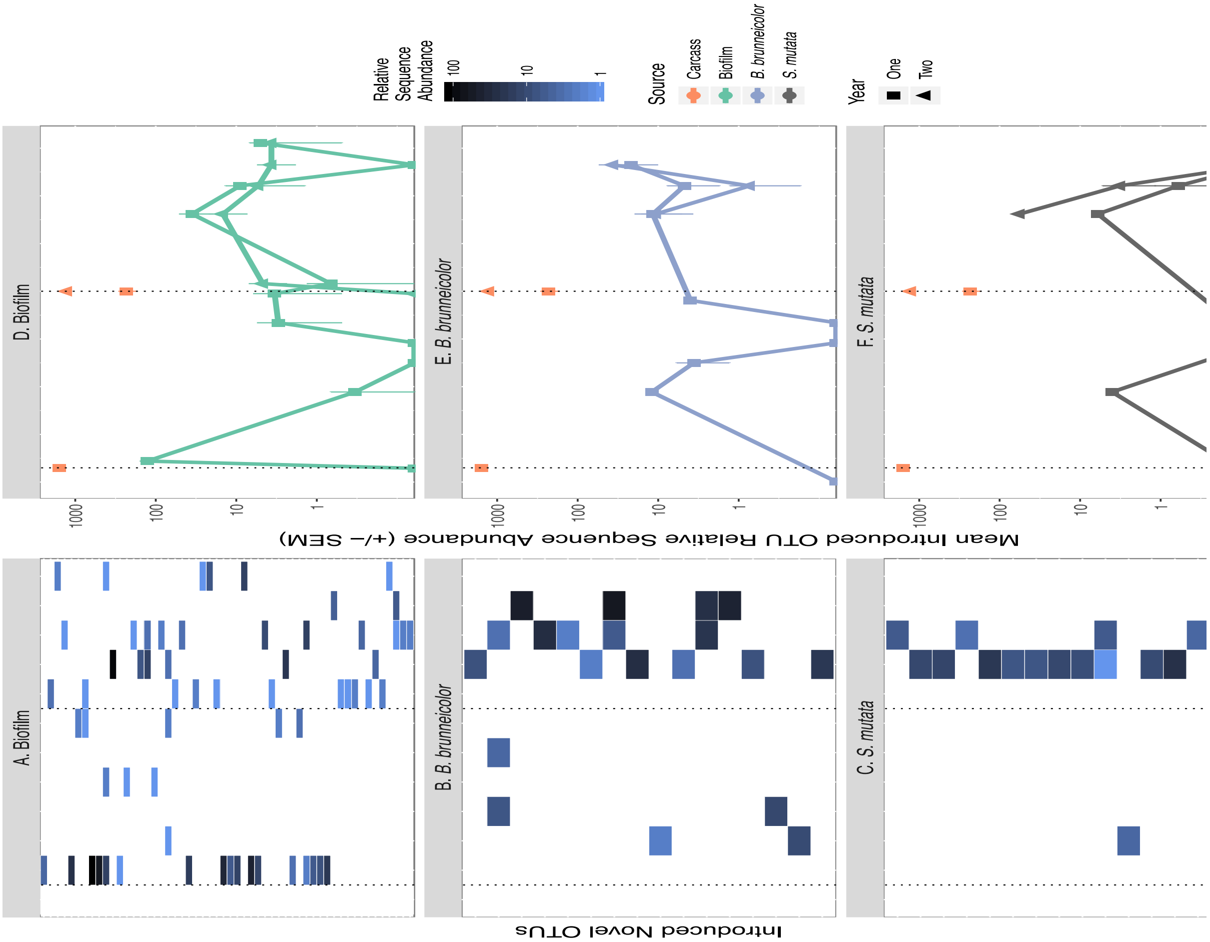


Figure 2.3. Heatmap of unique salmon carrion introduced OTUs found in treatment biofilm **(A)**, internal *B. brunneicolor* **(B)**, and internal *S. mutata* **(C)** after carcass introduction. Each y-axis row on the heatmap represents one unique salmon carrion introduced OTU. White color in heatmaps represents zero observations. Mean introduced OTU abundance (±SEM) in salmon carcasses and biofilms **(D)**, internal *B. brunneicolor* **(E)**, and internal *S. mutata* **(F)**, over time. The orange carcass points represent the unique OTUs introduced to the stream for that year. The carcasses added in year two contained OTU's previously introduced in year one (orange square), as well as new OTU's not previously found in biofilm or insect samples (orange triangle). The sample (biofilm, internal *B. brunneicolor*, or internal *S. mutata*) points (in green, purple or gray, respectively) represent the amount of that year's salmon introduced OTUs found in biofilm samples. Black, dotted vertical lines represent the day of salmon introduction. Relative sequence abundance is the number of reads in the rarefied (2,500 reads) dataset.

Three OTUs introduced by carrion in year one and not found in biofilm or internal insect samples in non-salmon reaches were detected in internal *S. mutata* collected in the salmon reach*:* one in year one and two in year two (Figure S2.7, Table S2.10). None of these OTUs were found in biofilms or *B. brunneicolor*. Of the unique salmon carrion OTUs introduced in year two, 14 were detected in year two treatment internal communities of *S. mutata*. Three of those fourteen were also detected in treatment biofilms in year two. These introduced OTUs from both years represented a small proportion (<1%) of internal *S. mutata* communities (Figure 2.3F). In year two, four year one introduced OTUs found in internal *S. mutata* were also found in the upstream control reach.

Discussion

Macroinvertebrate community composition

Specific metrics of α-diversity and population density of macroinvertebrate communities were altered by salmon carrion additions, which were influenced by the population dynamics of four taxa: *Brachycentrus*, *Baetis*, *Stegopterna*, and *Heptagenia*. Higher Brachycentridae abundance has been detected two to four weeks after salmon carcass introduction in Idaho (Kohler et al., 2008), and *Brachycentrus* has been observed feeding on salmon carcasses in Alaska (Kline et al., 1997). Yet, in our study, *Brachycentrus* density was lower in the salmon reach, never representing more than 1% of the community, which did not support the hypothesis that collectors would increase in the salmon treatment reach. In Michigan, *Brachycentrus* populations can be drastically reduced by a microsporidium parasite (Kohler and Hoiland, 2001), which has an unknown life cycle. It is possible that salmon carrion introduces microsporidium spores, and a local outbreak could have lowered *Brachycentrus* population in the salmon reach, although there is no direct historical evidence of this parasite in Hunt Creek (Wills et al., 2006), and the presence of this parasite or its spores were not directly measured in this study. Additional salmon carcass introduction studies in other Great Lakes streams with *Brachycentrus* are needed to determine whether these observed changes were due to natural environmental shifts that occurred upstream of the salmon reach or a treatment effect. Changes in the phenology of this insect may be more important in structuring the population than the availability of resources. Great Lakes region salmon research shows that salmon carrion has a much smaller impact on stream biota than it does in its native range in the Pacific Northwest and Alaska (Janetski et al., 2013), leaving phenological population changes to have a greater impact.

Although the *Brachycentrus* population was consistently higher in the control reach, *Baetis* and *Stegopterna* populations were higher only during a short time peak in the control reach compared to the salmon reach. Both taxa belong to the collector functional feeding group, and were found to increase in density or have no significant response to salmon carrion subsidies in Alaska (Wipfli et al., 1998, 1999; Minakawa and Gara, 1999; Chaloner et al., 2002, 2004; Lessard et al., 2009). In the few studies that show lower collector densities in salmon-bearing streams, this was attributed to benthic disturbance by live salmon spawning behavior (Honea and Gara, 2009; Collins et al., 2011), which was not a factor in this study, as we introduced salmon carcasses directly to a naïve stream. Earlier insect emergence in streams that experience annual salmon runs could be attributed to an insect evolutionary response to salmon spawning disturbance (Moore and Schindler, 2010). Alternatively, the salmon nutrient subsidy may also lead to earlier emergence because of increased production and faster growth rate in insects. The short 50 years of evolutionary history of salmon in Great Lakes streams may preclude such responses in taxa such as *Baetis* and *Stegopterna*.

Although *Brachycentridae, Baetis*, and *Stegopterna* populations had higher mean relative abundance in the control reach, *Heptagenia* were higher in the salmon reach. Mayfly grazers, such as heptageniids, have been found to consume periphyton containing salmon-derived nitrogen (Schuldt and Hershey, 1995). Therefore, a salmon nutrient subsidy may have had positive effects on the *Heptagenia* population, supporting the hypothesis that grazer macroinvertebrates would increase in abundance in the salmon treatment reach.

Microbial community structure

The microbial communities residing in benthic biofilms were altered by carcass introduction over time for both years of the study, but this impact differed in each year. The introduced carcasses supported different microbial communities between the two years, which may contribute to this variation. Specifically, melanogenesis, a pathway responsible for pigment production, was an indicator KEGG ortholog in salmon carrion microbial communities, as well as in biofilms and internal *S. mutata*, but with contrasting effects. Melanin pigment in microbes is associated with virulence in pathogens and protection against environmental stressors (Nosanchuk and Casadevall, 2003). Each year's salmon carcasses were raised in different environments, causing more melanogenesis in year two salmon epinecrotic microbial communities. Then, the melanogenesis performing microbes became integrated into salmon treatment biofilms in year two, so there was increased melanogenesis in salmon treatment biofilms compared to control sites. This functional pathway existed in the stream prior to salmon carrion introduction, but salmon could have enriched the OTUs already present in biofilms, leading to higher abundance in that treatment reach. In contrast, the internal microbial communities within *S. mutata* had elevated melanogenesis in the control reach. This elevation may be due to an environmental change in the treatment reach due to salmon introduction, such as increased dissolved organic carbon (Schuldt and Hershey, 1995; Collins et al., 2011), which may decrease the abundance of microbes that perform melanogenesis. It should also be noted that KEGG orthologs are predicted via *in silico* analysis of the microbial community datasets, and further studies directly measuring microbial functions are needed.

Another shift in biofilm composition involved an unnamed family in Stramenopiles in year one, which was lower in the salmon reach compared to the control reach two weeks after carcass introduction. Functionally, Stramenopiles are a dominant group of primary producers (Burliga and Kociolek, 2016). We would expect Stramenopiles to be more abundant after leaf fall, due to increases in light with less canopy cover (Sumner and Fisher, 1979). Leaf fall occurred at the same time that salmon carrion was introduced into the stream. Primary production only marginally increases due to nutrients released by salmon carrion in Great Lakes streams (Schuldt and Hershey, 1995; Hershey and Wold, 1999); however, this production can be altered by stream conditions, such as light availability, habitat structure, and organic material (Cederholm et al., 1999). Nutrient addition in a Tennessee stream increased primary production in the autumn with increased light availability, but this effect was significantly lower in the presence of grazers (Rosemond et al., 2000). We detected an increase in grazer *Heptagenia* in the salmon reach, and thus these grazer communities could have influenced the biofilm response and limited autotrophic microbes despite nutrient inputs from salmon carrion.

In contrast to Stramenopiles, the *Sphingomonadaceae* (α-Proteobacteria) were over twice as abundant in the salmon reach, suggesting that heterotrophic bacteria respond positively to salmon carrion subsidies. Some of this increase in heterotrophic microbes can be attributed to rare OTUs introduced via salmon carrion, but most are likely due to an increase in organic matter. Benthic biofilms in streams with higher dissolved organic carbon often have higher Proteobacteria relative abundance (Gao et al., 2005). Proteobacteria also was found to be in high relative abundance in the internal microbiome of a predator mayfly in salmon-bearing streams (Pechal and Benbow, 2016); we found a similar trend with an unknown family in *Rhizobiales* (α-

Proteobacteria), which had 3.5 times higher abundance in the internal microbiome of *B. brunneicolor* in the salmon reach of Hunt Creek.

Temporal dynamics

Early research into salmon carcass decomposition in streams have indicated that periphyton first use salmon nutrients followed by primary consumers (Juday et al., 1932; Mathisen et al., 1988). Salmon-derived nutrients were found to peak in insects directly feeding on carcasses at two weeks after introduction, while biofilms and insects that were indirectly affected by salmon carrion had a peak in salmon derived nutrients at two months after introduction (Claeson et al., 2006). We found that biofilms responded two weeks after introduction, integrating a small amount (<2%) of unique salmon introduced OTUs into these epilithic communities, suggesting a more direct uptake path. Additionally, we were only able to sample a small amount of the total introduced microbial diversity via salmon in our carcass surface swabs, because we were not able to monitor the introduction of microbes from the gastrointestinal (GI) tract. We speculate the GI microbes may also contribute to novel taxa found in biofilms and internal insects that were not detected in this small relative abundance. The macroinvertebrate communities shifted several months after introduction, suggesting indirect and lagged carcass resource use. In our study, the small number of microbes unique to carrion and integrated into biofilms are subsequently integrated into consumers, as nutrients would be integrated up the food chain. An alternative explanation is that naïve Hunt Creek does not contain the necrophilous invertebrates of a typical Pacific salmon stream, and future research should focus on the direct and indirect pathways of introduced microbes.

It should be noted that the number of reads in a sequencing dataset do not necessarily directly translate to abundance in the environment, but rather serves as a proxy. Additionally, the detection of OTUs in biofilms does not indicate living microbes, but that the DNA of those microbes was present. Residual DNA from the salmon may slough off and be retained in biofilms, without the bacteria reproducing and functioning in the environment. Further studies, using active, transcribed forms of DNA are necessary to mechanistically determine whether these unique OTUs play an environmentally significant role. Past studies have shown that rare microbial taxa may play vital roles in maintaining biodiversity and having functional roles (Shade et al., 2014; Lynch and Neufeld, 2015; Jousset et al., 2017). For example, rare taxa can provide a "seed bank" that may increase in abundance when there is a local extinction of more abundant taxa or immigrate to another habitat where it can outcompete other resident microorganisms. Therefore, despite the low abundances of carrion-introduced OTUs in our system, they may play a more disproportionate role in biodiversity and ecosystem functioning that future studies should investigate.

Conclusions

In this study, we contribute to knowledge on ecology of salmon carrion decomposition by investigating the microbial fauna of a naïve stream following a salmon carcass addition. These data provide evidence that salmon introduce microbial taxa to recipient streams, and a small amount become incorporated into the ecosystem. Further, these taxa may elicit a cascading effect that influences stream producer and consumer communities through direct and indirect pathways. Salmon migration may ignite complex interkingdom interactions in stream communities, necessitating additional field and laboratory studies on allochthonous sources of microbes and their potential importance and mechanisms to ecosystem function. Therefore, the functional roles of these salmon-associated microbial taxa represent a frontier for ecological research.

CHAPTER 3: EMERALD ASH BORER INVASION OF RIPARIAN FORESTS ALTERS ORGANIC MATTER AND BACTERIAL SUBSIDIES TO SOUTH MICHIGAN HEADWATER STREAMS

Abstract

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has killed millions of ash trees (*Fraxinus* spp.) since its introduction, propelling this invader to the forefront of natural resources concerns. Despite the prominence of this destructive forest invader, information about impacts of widespread ash mortality on terrestrial-aquatic linkages is scarce. Mortality of ash trees along stream corridors can result in canopy gaps, which increases light available to riparian plant communities and potentially affecting organic matter subsidies into streams. We postulated that composition of riparian and aquatic leaf litter and coarse woody debris (CWD) would shift in response to the changing riparian forest. Specifically, we anticipated an increase of CWD within EAB-related canopy gaps and a decrease in leaf litter taxon richness both at and downstream of these gaps. We hypothesized the bacterial (16S) communities colonizing leaf litter would also be influenced by the altered allochthonous riparian subsidies, with bacterial taxa in aquatic leaf litter being sourced by live leaves and terrestrial leaf litter microbes. In 2016, we identified EAB-related canopy gaps in six headwater streams of Michigan and characterized the CWD, leaf litter and their associated bacterial communities (terrestrial and aquatic), along with macroinvertebrate communities upstream, downstream, and at the center of one EAB-related canopy gap per stream before, during, and after autumnal leaf senescence. Clinton watershed, which had the earliest EAB-impact, had significantly greater ash relative abundance in CWD pieces than the Grand River watershed which was intermediate in EAB-invasion time We found stream sites downstream of EAB-related canopy gaps had significantly lower dissolved oxygen and macroinvertebrate diversity. We did not detect watershed and gap location effects on aquatic leaf litter richness or proportions of leaf taxa. Unique amplicon sequence variants to gap or forest were detected in live leaves, terrestrial leaf litter, and aquatic leaf litter; but in <1% relative abundance. Overall, these findings reveal EAB invasion negatively impacts stream ecosystems through indirect routes downstream of canopy gaps, yet leaf litter subsidies are resilient to the disturbance.

Introduction

Invasive species are a critical natural resources concern, due to their environmental and economic repercussions (Vitousek et al., 1997; McGeoch and Jetz, 2019). Forest invasive species cause an array of cascading ecological impacts, and those that occupy riparian forests have the potential to influence aquatic structure and function via cross-ecosystem interactions (Lovett et al., 2006, 2016; Ehrenfeld, 2010; Gandhi and Herms, 2010; Flower and Gonzalez-Meler, 2015; Morin and Liebhold, 2015; Liebhold et al., 2017). Similar to invasive plants, terrestrial invasive insects may indirectly affect aquatic ecosystems, but the research is lacking (Greene, 2014). In one example, at stream sites with severe eastern hemlock decline (>75% canopy loss) due to *Adelges tsugae* (hemlock woolly adelgid Annand) invasion, there were fewer overall logjams, but the logjams had higher coarse woody debris (CWD) volume compared to sites with moderate or no hemlock decline (<75% canopy loss) (Martin, 2012; Costigan et al., 2015). The alteration in CWD characteristics resulted in structural and functional shifts in stream macroinvertebrate communities (Diesburg et al., 2018).

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has killed hundreds of millions of ash trees (*Fraxinus spp.*) since it became established in the US during the early 1990s, thus propelling EAB to the forefront of natural resource concerns (Cappaert et al., 2005; Herms and McCullough, 2014; Siegert et al., 2014; McCullough, 2019). Emerald ash borer continues to spread and has become the most economically and ecologically destructive insect to invade North American forests (Aukema et al., 2011; Herms and McCullough, 2014; Lovett et al., 2016; McCullough, 2019). Emerald ash borer's deleterious impacts in forests, such as lower productivity and canopy gap formation, have been well documented (Flower et al., 2013; Burr and McCullough, 2014; Herms and McCullough, 2014; Klooster et al., 2014; Smith et al., 2015). Despite the prominence of this problem, information about the impact of widespread ash mortality on aquatic ecosystems is scarce (Nisbet et al., 2015). Green ash (*F. pennsylvanica*) and black (*F. nigra*) ash are common in riparian forests (Kennedy, 1990; Wright and Rauscher, 1990; Gucker, 2005b, 2005a). Mortality of overstory trees, such as ash, can result in canopy light gaps, which alter riparian plant community structure and subsequent organic matter subsidies into riparian forests (Engelken and McCullough, 2020; Engelken et al., 2020), and potentially into streams.

Headwater streams are highly reliant on allochthonous (external) sources of energy. Because riparian trees shade the stream and limit primary production, primary consumers must use leaf litter from the surrounding riparian trees as their food resource (Vannote et al. 1980). An alteration in the quantity or quality of these sources may have bottom-up effects on organisms that directly consume the allochthonous resource [microbial (bacteria, fungi, and microeukaryotes) communities and macroinvertebrate shredders] and on other functional feeding groups that directly and indirectly consume them, such as predators and collector-filterers (Wallace et al. 1997). Within EAB gaps in riparian zones, new plants, such as sedges, can be recruited (Engelken et al. 2020), potentially altering in-stream leaf decomposition, because these new plants have different leaf litter quality and quantity (Ehrenfeld et al., 2001; Knight et al., 2007; McNeish et al., 2012; Mineau et al., 2012). Additionally, dead ash can eventually result in an influx of CWD in forest ecosystems (Higham et al., 2017), and potentially into stream ecosystems, where CWD plays an important ecological role. Fallen logs provide habitat and affect hydrology of streams, from reach to basin scales (Orndorff and Lang, 1981; Maser and Sedell, 1994; Wohl et al., 2010), with consequential impact on microbial communities and consumers (Anderson et al., 1978; Tank and Webster, 1998; Dolloff and Warren, 2003; Scherer, 2004). Therefore, it is important to assess alterations in stream CWD in response to different riparian disturbance types.

Canopy gaps also influence leaf litter decomposition through increased light availability that may alter microbial decomposer communities residing on the litter. Models predict that a riparian disturbance event, such as an insect outbreak that results in defoliation and/or tree death, will increase light available to stream communities responsible for primary production and the processing of autochthonous (internal) carbon after the event (Warren et al., 2016). Duration of this increase is partially dependent on the stream bank width, with smaller streams having faster restoration to normal conditions, due to vegetation growth in the gap (Warren et al., 2016). In a field experiment, leaf packs conditioned in an unshaded stream reach had more chlorophyll *a* and less fungal biomass, and *Klapopteryx kuscheli* Illies (Plecoptera: Austroperlidae; shredder) larvae grew two times slower compared to litter conditioned in a shaded reach (Albariño et al., 2008). Therefore, light may mediate the effect of organic matter subsidies on aquatic communities at areas of EAB invasion.

Allochthonous organic matter has traditionally been viewed as carbon and nutrient subsidies, yet it may also act as a “microbial subsidy” by introducing microbes into streams from adjacent habitats. This change to stream microbial communities can alter the functional base of the food web, which may have far reaching effects throughout the stream network (Larson et al., 2020). Therefore, it is important to understand how microbial subsidies associated with riparian leaf litter influence stream communities. Past research has shown that soil microbial communities in ash vs. non-ash forest plots are significantly different from each other, with greater abundance of Acidobacteria in non-ash plots (Ricketts et al., 2018). Because aquatic microbial communities have a directional spatial structure, where dominant aquatic taxa are sourced from terrestrial soils (Ruiz-González et al. 2015), this change in riparian soil communities in EAB-related ash gaps may have cascading aquatic effects by introducing novel microbes that become more abundant in stream environments. Additionally, canopy gaps caused by EAB-related ash mortality are likely altering terrestrial living leaf and leaf litter microbiomes (Purahong et al., 2014; Laforest-Lapointe et al., 2017), which may consequently alter aquatic leaf litter microbiomes (Abelho and Descals, 2019). Aside from terrestrial microbial subsidies, changes in landscape features (e.g., riparian community composition) can alter water chemistry, thereby affecting leaf litter microbial communities (Emilson et al., 2017). Macroinvertebrate shredders, which serve as decomposers for the stream ecosystem, selectively feed on certain microbe taxa residing on leaf litter (Suberkropp, 1992; Graça, 2001). For example, Arsuffi and Suberkropp (1989) found that *Gammarus* sp. (Amphipoda: Gammardiae) and *Hesperophylax magnus* Banks (Tricoptera: Limnephilidae) preferentially fed on *Alatspora acuminata* Ingold (Helotiales: Leotiaceae) and *Flagellospora curvula* Ingold (Hypocreales: Nectriaceae) respectively. Therefore, a change to the leaf litter microbial community can functionally alter macroinvertebrate consumer communities and subsequent ecosystem functionality.

The objective of this study was to evaluate habitat, resource, and consumer relationships in southern Michigan streams following EAB-related ash mortality in the adjacent riparian forests. To do this, we quantified CWD in both riparian forest and adjacent aquatic habitats; characterized bacterial communities associated with live riparian leaves, terrestrial leaf litter, and submerged leaf litter; and measured benthic macroinvertebrate community responses along a chronosequence of EAB invasion across southern Michigan. We postulated the organic matter subsidies and bacterial and macroinvertebrate communities within EAB-related canopy gaps would undergo structural and functional shifts. Specifically, we predicted that in stream reaches adjacent to riparian ash canopy gap habitats:

1) there would be increased CWD;
2) aquatic leaf litter taxon richness and complexity would be lower at gap and downstream sites compared to upstream sites, due to the dominance of sedges and grass at the gap location;
3) aquatic leaf litter bacterial communities would have shared amplicon sequence variants (ASVs) with terrestrial live leaf and leaf litter samples that are unique to forested sites (i.e., never found in gap locations), representing pre-gap leaf-associated bacterial communities compared to gap leaf communities;
4) leaf-associated bacterial communities would shift in response to gap location, with higher abundance of primary producer microbes at gap locations compared to forested habitats; and
5) benthic macroinvertebrates would shift structurally and functionally in gap locations, with greater abundance of grazers responding to resources associated with increased light compared to upstream and downstream reaches.

Materials and Methods

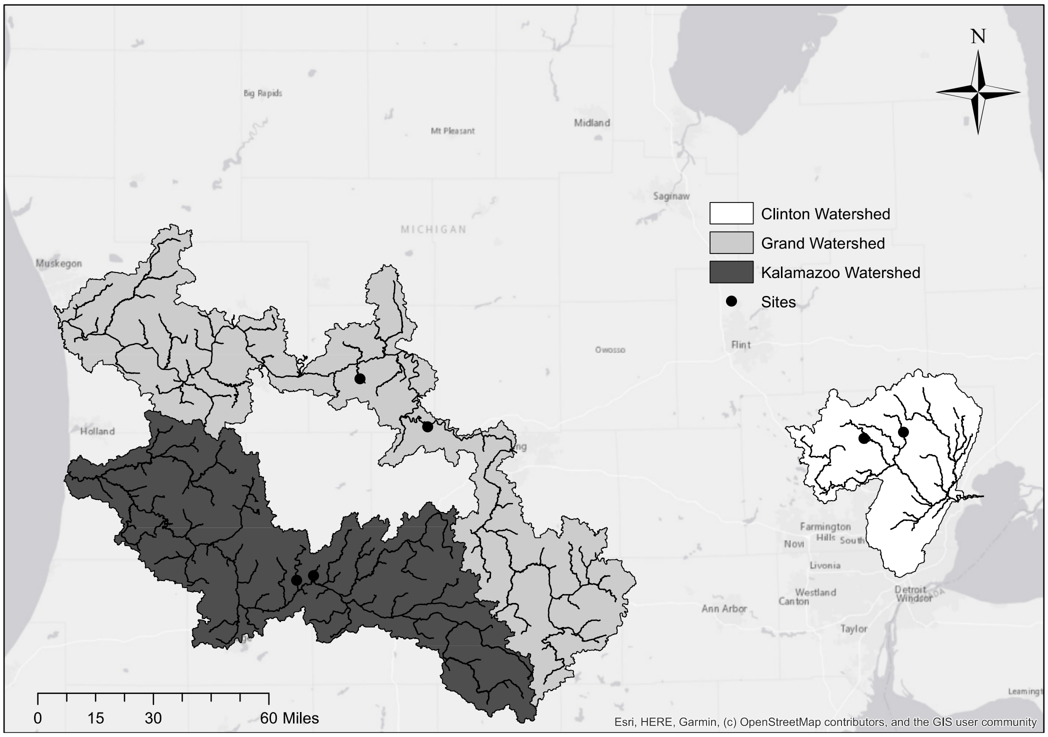
Riparian survey

This study was conducted at six streams selected to represent chronosequence of EAB invasion (Pickett, 1989; Burr and McCullough, 2014) in three watersheds of southern Michigan (Table 3.1, Figure 3.1). Two sites were chosen for each watershed, and sites were generally uniform in their stream physical characteristics and size of gap (Table 3.1). Riparian forest conditions on one side of the stream were assessed using a combination of linear transects and fixed radius plots as described in Engelken et al. (2020). The current study was concurrently undertaken on a subset of sites described in Engelken et al. (2020). Year of gap formation was estimated by Engelken et al. (2020) using leaf-on (summer) aerial images from the U.S. Geological Survey (earth-explorer.usgs.gov) and Google Earth (Google Earth Pro V 7.3.2) acquired between 2000 and 2017.

To determine differences in live leaf microbiomes in canopy gaps versus adjacent intact forests, leaf samples were collected from the five most dominant overstory tree species as determined by relative importance values (RIV) reported in Engelken et al. (2020). For each selected overstory species, two overstory trees (DBH >10.2 cm) were selected for sampling, one in the canopy gap and one in the intact forest surrounding the canopy gap, resulting in a total of ten samples. We pruned one branch tip consisting of 5-10 leaves from each cardinal direction of the mid-canopy from each tree, using a pole pruner from 18 August to 21 August 2016. Leaves were collected before they came in contact with the ground and stored in 3.79 L sized resealable polyethylene plastic bags. Disposable nitrile gloves were worn during sampling and were replaced between sampled trees, and pole pruner blades were sterilized with 70% ethanol between sampled trees.

Table 3.1. Location and characteristics of field sites sampled in southern Michigan. Three watersheds represent an east to west gradient, with two streams sampled in each watershed. Strahler stream order was determined by examining Google Earth images. Gap diameter, gap area, and year of gap formation were determined by Engelken et al. (2020). The year of gap formation is the year the gap was first apparent on aerial images.

Region	**Watershed**	**Stream**	**GPS coordinates**	**Stream Order**	**Gap diameter (m)**	**Gap area (ha)**	**Year of gap formation**
Southeast ↓ Southwest	Clinton	Stoney Creek	42.769, -83.075	3	277	0.40	2006
		Spring Creek	42.753, -83.225	3	315	0.69	2008
	Grand River	Sessions Creek	42.917, -85.117	2	224	0.32	2011
		Frayer Creek	42.788, -84.859	3	213	0.22	2011
	Kalamazoo	Seven Mile Creek	42.376, -85.290	3	254	0.32	2014
		Augusta Creek	42.362, -85.355	3	709	1.7	2014



Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community

Figure 3.1. Map of the 6 field site locations in 3 watersheds, across a chronosequence of EAB impacts. EAB-related canopy gaps were apparent in Clinton watershed in 2006 and 2008, Grand River watershed in 2011, and Kalamazoo watershed in 2014.

Leaf litter samples were collected after leaf senescence, but before the first snowfall in the winter (October to December 2016). For each site, we collected leaf litter samples from eight microplots, each 1 m2, on the forest floor. Four were in the canopy gap, and four in the surrounding forest up and downstream of the gap (Figure 3.2). Disposable nitrile gloves were worn for collection and replaced between samples. All four microplot samples were pooled for each gap and forest location. Overstory leaves and leaf litter samples were transported on ice and stored at -20 °C until processing.

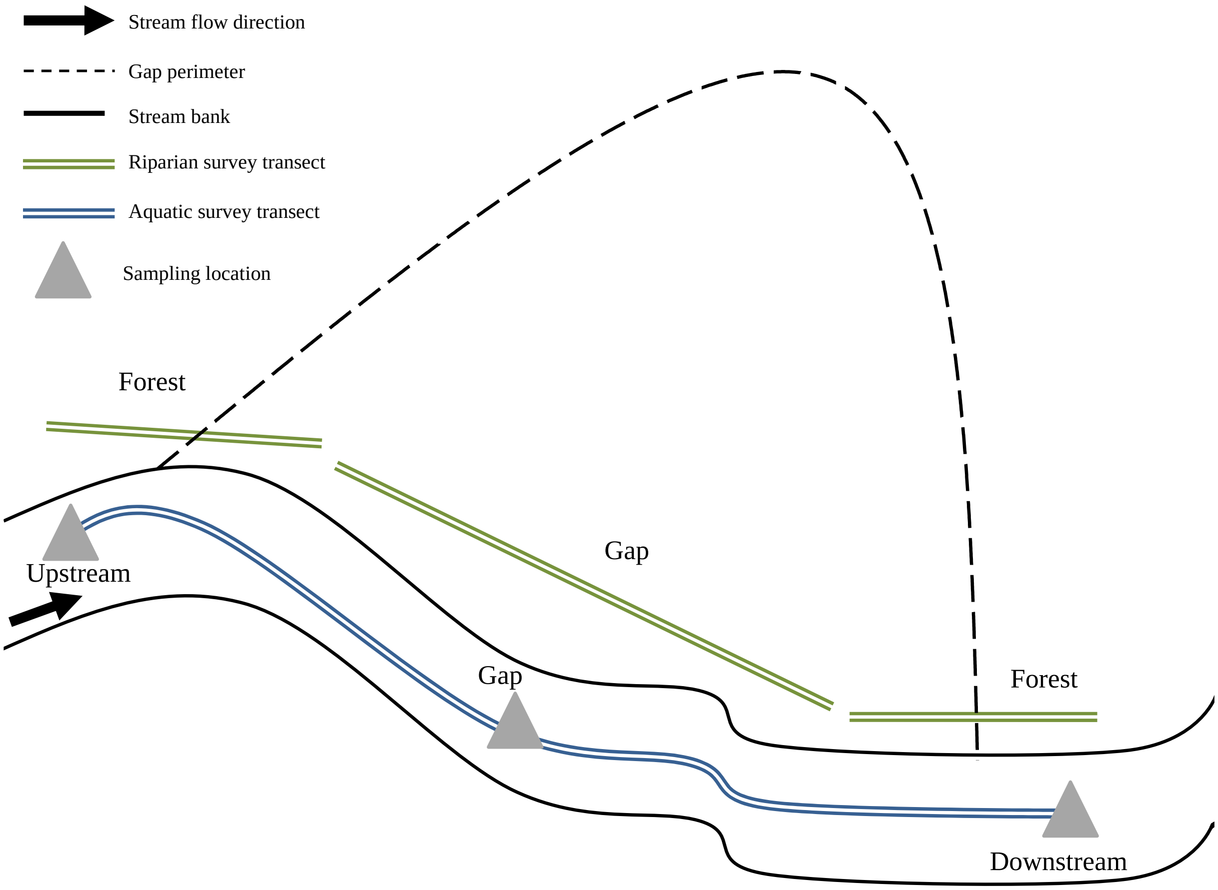


Figure 3.2. Survey scheme sampling for aquatic and riparian surveys of CWD, aquatic leaf litter, and bacterial and macroinvertebrate communities. Solid lines represent the stream bank. The black arrow represents stream flow direction. The EAB-related canopy gap perimeter is represented by a dashed line. Double lines represent transects in the stream (blue) and in the forest (green). Riparian (terrestrial) CWD transects were 50x2 m or 100x2 m, while the aquatic transects spanned from bank to bank, 20 m upstream of the gap perimeter to 20 m downstream of the gap perimeter. Aquatic leaf litter and macroinvertebrate sampling locations are represented by gray triangles.

Aquatic survey and sample collection

Further, in summer 2016, from 28 June to 23 August CWD was surveyed using similar methods as the terrestrial transect survey along the entire stream stretch adjacent to the selected riparian gaps, bank-to-bank, from 20m upstream of the gap perimeter, through the entire gap, and ending 20 m downstream of the gap perimeter (Figure 3.2). For each log encountered that was >7.6 cm in diameter, we recorded species (when possible), diameter, in-stream length, and full length. In addition, each piece of wood was classified as transported from upstream (entire piece within bankfull channel) or *in situ* (at least one end of the piece remained on the adjacent bank and above the bankfull channel), to determine which wood was being directly recruited from the adjacent riparian area compared to material transported from upstream (Wohl et al., 2010).

Aquatic chemistry, leaf litter, and macroinvertebrate sampling occurred at three times from 24 August 2016 and 3 March 2017, to represent a gradient of autumnal leaf fall. All sampling could not take place during 2016, due to sporadic flooding events that prevented field site access. At each stream, aquatic sampling was performed at the center of the gap, 20 m upstream, and 20 m downstream of the gap perimeter, hereafter called “gap”, “upstream reach”, and “downstream reach”, respectively (Figure 3.2). Aquatic macroinvertebrates were collected using a Hess sampler, with three cobbled sites per sampling location pooled into one composite sample that was then stored in 95% ethanol. For each collection, the benthos was disturbed for 30 seconds to dislodge invertebrates within the substrates for collection in the downstream net of the sampler. Aquatic invertebrates were identified to the family level using Merritt et al. (2008). Voucher specimens were deposited in the Albert J. Cook Arthropod Research Collection, Michigan State University (Appendix S1).

A ~40 mL subsample of submerged leaf litter was collected from a naturally occurring leaf pack in each reach, placed in a sterile 384 mL Whirl-Pak© bag and stored at -20 °C until processing (Benfield et al., 2017). Samples were dried at 55 °C in a drying oven, and leaves were identified to the lowest taxonomic level possible (genus or species) using Watts (1963). Proportions of each taxon represented in aquatic leaf litter samples were determined by taking taxon dry weight divided by the total sample dry weight. Samples were then homogenized using sterile scissors, and 150 mg were placed in a 1.7 mL sterile microcentrifuge tube (87003-294, WVR®, Radnor, PA, USA) for DNA processing and stored at -20 °C (Cline and Zak, 2015). All terrestrial and aquatic leaf samples were processed in the same manner: homogenized using sterile scissors with 150 mg placed in a 1.7 mL sterile microcentrifuge tube for DNA processing and stored at -20 °C.

Leaf litter bacterial DNA processing and targeted 16S rRNA gene amplicon sequencing

Prior to DNA extraction, leaf samples were ground in liquid nitrogen using a sterile pestle (Matulich et al., 2015). DNA isolation was performed with the Qiagen PowerSoil DNA extraction kit® (Qiagen, Inc, Valencia, CA, USA) using a modified manufacturer's protocol: 20 mg mL^{-1} of lysozyme was added during the lysis step and the final DNA was eluted in 50μL of C6. DNA quantification was performed using the Quanti-iT dsDNA HS Assay kit and a Qubit 2.0 (Grand Island, NY, USA); a concentration range of 1-2 ng μL^{-1} was used as a threshold for subsequent sequencing procedures (Table S3.1). All DNA preparations were stored at -20°C.

Illumina MiSeq 16S library construction (2 x 250 bp paired-end reads) and sequencing was performed at the Michigan State University Genomics Core using a modified version of the Illumina MiSeq protocol (Caporaso et al., 2011b). The variable region 4 of the 16S rRNA gene was amplified with region-specific primers, 515F/806R (5'-GTGCCAGCMGCCGCGG-3', 5'-TACNVGGGTATCTAATCC-3') (Claesson et al., 2010; Caporaso et al., 2011a, 2012). The resulting 16S rRNA amplicon sequencing data were assembled, quality-filtered, and demultiplexed using QIIME2 version 2019.10 (Kuczynski et al., 2012). Default settings were used except for the following modifications. To discard chimeric reads and other sequencing artifacts, we used DADA2 (Callahan et al., 2016). Taxonomy was assigned using a Naïve Bayes classifier trained using the 16S rRNA region, primer set, read length, and Greengenes 99% reference set version 13.8 (DeSantis et al., 2006; McDonald et al., 2012; Werner et al., 2012). Singletons and sequences belonging to chloroplasts or mitochondria were removed and samples rarefied to 1,100 sequences, which was the highest sequencing depth that included all samples, excluding 9 samples (3 terrestrial live leaves, 1 terrestrial leaf litter, and 5 aquatic leaf litter) that had insufficient sequence reads as a result of extraction or sequencing errors (Figure S3.1). Relative abundance was determined by the number of reads in the rarefied dataset. Sequence files and metadata for all samples used in this study have been deposited in the NCBI SRA under number PRJNA649297.

Fungi are the largest component of microbial decomposition of leaf litter (Gulis and Bärlocher, 2017), and terrestrial living leaf fungal community can play a large role in structuring stream fungal communities (Koivusaari et al., 2019). Also, eukaryotic algae are an important aspect of biofilm communities, which respond to light because they are photosynthesizers (Biggs, 1996). Yet we have not quantified the fungal or algal components of microbial communities in this study, as a result of using the bacterial community in our methods which excludes fungi and microeukaryotes. Only sequencing of the 16S rRNA gene occurred for this study due to limited funds and less comprehensive reference datasets for ITS rRNA genes. We recognize this is a significant limitation of our study, and future investigations on the microbial communities associated with riparian and aquatic leaves and leaf litter should include a fungal and microeukaryotic component.

Statistical analyses

Statistical analyses were performed using R version 1.1.442 (R Core Team, 2018). All QIIME 2 and R code can be found at github.com/BenbowLab/EAB.StReams. All statistical tests were considered significant at α=0.05 unless a specified adjustment for multiple comparisons was used as indicated below. Mean ± standard error (MSE) was calculated for each aquatic variable. The assumption of normality of residuals was determined by Shapiro-Wilk test and qqplots, and the assumption of homoscedasticity was determined by residuals versus fits plots.

To determine if CWD varied among the three watersheds, a one-way ANOVA test was run for each aquatic CWD variable (Table 3.2). For those tests that were significant, multiple comparisons of means were performed using Tukey's post-hoc analyses with a Tukey adjustment. To assess linear relationships between riparian (CWD and standing dead trees) and aquatic CWD volumes and number of pieces, Pearson correlation analysis was conducted. Shapiro-Wilk tests were conducted to confirm the assumption of normality was met. For those variables that were not normally distributed (number of terrestrial total and ash CWD pieces per ha), Spearman correlation analysis (a non-parametric alternative) was performed.

To determine whether water chemistry parameters of water temperature, conductivity, dissolved oxygen, pH, and turbidity were altered by EAB-related riparian changes, linear mixed models were built with gap location (upstream, gap, or downstream), watershed, and total live basal area of overstory trees as fixed effects and stream as a random effect. Variables that did not meet the assumption of normality were log10 transformed (turbidity).

Aquatic leaf litter richness and proportion of leaf taxa were assessed using a combination of mixed effect models and correlation analysis. Linear mixed models were built with gap location and watershed as fixed effects and stream as a random effect to determine their effect on aquatic leaf litter richness, and ash (*Fraxinus* spp.) and oak (*Quercus* spp.) leaf relative mass in leaf packs. For those models that did not meet the assumption of normality, mixed effects logistic regression with proportional outcomes was used. In the case that the mixed effects logistic regression model had singular fit, due to little variability attributed to the stream random effect, a logistic regression for proportion data outcomes model was used without the random effect included. To determine if there was a relationship between riparian live tree basal area of the adjacent reach (upstream, gap, and downstream) and aquatic leaf litter, Pearson correlation analysis was conducted. For those variables that were not normally distributed (Aquatic leaf litter richness, ash relative dry mass, and oak relative dry mass), Spearman correlation analysis was performed.

Variation in bacterial community composition was visualized using nonmetric multidimensional scaling (NMDS) constructed from a weighted UniFrac dissimilarity matrix. Clustering was statistically evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations in R using the “vegan” package (Anderson, 2001; Smith, 2019). Indicator species analysis (ISA) was performed with Indicator Value (IndVal) Index and its significance using 99,999 permutations in the “signassoc” function in the R package indicspecies to identify taxa representative of watershed and gap location bacterial communities, with Sidak’s $p<0.05$ indicating a significant indicator taxon (Cáceres et al., 2010). Estimates of bacterial community α-diversity (Faith’s phylogenetic diversity and Chao 1 richness) were calculated in QIIME2 version 2019.10 (Chao, 1984; Faith, 1992; Kuczynski et al., 2012). Linear mixed models were built using gap location, watershed and leaf source (live leaves, terrestrial leaf litter, and aquatic leaf litter) as fixed effects and stream as a random effect to determine effects on bacterial community α-diversity.

Because leaf source (live leaves, terrestrial leaf litter, and aquatic leaf litter) significantly influenced bacterial community diversity and composition (see Tables S3.5-S3.6), additional individual tests (linear mixed models, PERMANOVA, NMDS, and ISA) were performed for each leaf source. To determine if unique amplicon sequence variants (ASVs) were introduced via riparian leaf litter subsidies to streams at sites in EAB-related gap or forested areas, Venn diagrams were built using the "VennDiagram" r package to visualize ASVs unique to forested or gap habitats among the three leaf source types: live leaves, terrestrial leaf litter, and aquatic leaf litter (Chen, 2018). More specifically, Venn diagrams were used to display ASVs shared among live leaves, terrestrial leaf litter, and aquatic leaf litter in forested sites; but not found in live leaves, terrestrial leaf litter, or aquatic leaf litter samples at gap sites; and vice versa for those found in gaps but not in forested sites.

Variation in macroinvertebrate community composition (family level and functional feeding groups) was visualized using nonmetric multidimensional scaling (NMDS) constructed from a zero-adjusted Bray-Curtis (bray0) dissimilarity matrix. Permutational multivariate analysis of variance (PERMANOVA) was used to test for effects of gap location, watershed, and stream on macroinvertebrate community structure (family level and functional feeding groups) with 9999 permutations in R using the "vegan" package (Anderson, 2001; Smith, 2019). Indicator species analysis was performed with Indicator Value (IndVal) Index and its significance using 99,999 permutations in the "signassoc" function in the R package indicspecies to determine families and functional feeding groups that represented watershed and gap location communities, with Sidak's $p<0.05$ indicating a significant indicator taxon (Cáceres et al., 2010). Macroinvertebrate community estimates of α-diversity (family richness and Simpson's diversity) were calculated in R (Oksanen et al., 2019). Linear mixed models were built with gap location and watershed as fixed effects and stream as a random effect to determine effects on macroinvertebrate community α-diversity, significant indicator families, and functional feeding groups found in all sampled streams. Because each sampling date served as a replicate for statistical tests on aquatic leaf litter, leaf litter microbes, and macroinvertebrates; there is an issue with pseudoreplication and non-independence of samples. Therefore, results should be interpreted cautiously, as the statistical power may be inflated.

Results

Aquatic habitat

A total of 354 aquatic CWD pieces were tallied in the streams in all six sites, ranging from 0.17 m to 24.6 m in length (Table 3.2). Ash CWD were found within all surveyed streams, except for Frayer Creek in the Grand River watershed. The only aquatic CWD variable that was affected by watershed abundance of ash relative to all CWD pieces (ANOVA, $F_{2,3}$=12.04, p=0.04). Streams in the Clinton watershed in southeast Michigan which had the earliest EAB-impact (2006-2008), had significantly greater abundance of ash relative to all CWD pieces than the Grand River watershed, which was impacted by EAB later in the chronosequence (Tukey HSD: p=0.03). Standing dead tree frequency (number per ha) and aquatic CWD frequency (number per ha) were positively correlated (Pearson: t(4)=3.55, p=0.02, r=0.87, Figure 3.3), but standing dead tree frequency (number per ha) was negatively correlated with abundance of logjam associated CWD pieces relative to all CWD pieces (Pearson: t(4)=-3.49, p=0.03, r=-0.87, Figure S3.2). Riparian CWD frequency (number of pieces per ha) was negatively correlated with logjam frequency (number of logjams per ha; Spearman: S=66, p=0.03, ρ=-0.89, Figure S3.3). Riparian CWD density (volume per ha) was positively correlated with the volume of aquatic *in situ* CWD relative to all aquatic CWD volume (Pearson: t(4)=2.81, p=0.05, r=0.82). Frequency of standing dead ash trees (number per ha) positively correlated with the aquatic ash CWD frequency (number per ha; Pearson: t(4)=3.68, *p*=0.02, r=0.88, Figure 3.4).

Table 3.2. Mean (± standard error) and results of one-way ANOVAs testing the effects of watershed on aquatic coarse woody debris (CWD) variables. Numerator degrees of freedom=2, Denominator degrees of freedom=3. Values in Clinton, Grand River, and Kalamazoo watershed columns represent means and standard error. Significant *p*-values indicated with an asterisk.

Aquatic CWD Variable	**Clinton**	**Grand River**	**Kalamazoo**	**F-value**	***p*-value**
Number of logs per stream area	59.60 (25.40)	52.50 (2.77)	63.40 (25.80)	0.07	0.94
Volume CWD per area	5.74 (0.95)	2.99 (0.10)	8.00 (3.04)	1.86	0.30
Number of logjams per stream area	2.00 (0.29)	3.86 (1.49)	2.84 (0.30)	1.08	0.44
Number of logjam associated CWD pieces per stream area	6.28 (3.98)	18.10 (2.04)	6.73 (1.63)	5.94	0.09
Logjam pieces relative abundance	0.16 (0.14)	0.35 (0.06)	0.14 (0.08)	1.35	0.38
Logjam volume per stream area	0.65 (0.59)	1.22 (0.23)	0.79 (0.46)	0.43	0.68
Logjam relative volume	0.13 (0.13)	0.41 (0.07)	0.14 (0.11)	2.24	0.25
Number *in situ* per stream area	12.0 (1.76)	10.10 (7.74)	9.97 (7.88)	0.03	0.97
In situ pieces relative abundance	0.23 (0.07)	0.19 (0.14)	0.13 (0.07)	0.28	0.78
Volume *in situ* per stream area	4.01 (1.83)	0.50 (0.36)	1.65 (1.38)	1.79	0.31
In situ relative volume	0.67 (0.21)	0.16 (0.11)	0.17 (0.11)	3.65	0.16
Number of ash per stream area	24.40 (12.40)	3.57 (3.57)	18.70 (9.32)	1.37	0.38
Ash pieces relative abundance	0.39 (0.04)	0.07 (0.07)	0.28 (0.03)	12.04	0.04*
Volume ash CWD per stream area	2.37 (0.03)	0.38 (0.38)	2.66 (1.44)	2.03	0.28
Ash relative volume	0.43 (0.12)	0.12 (0.12)	0.31 (0.06)	2.21	0.26

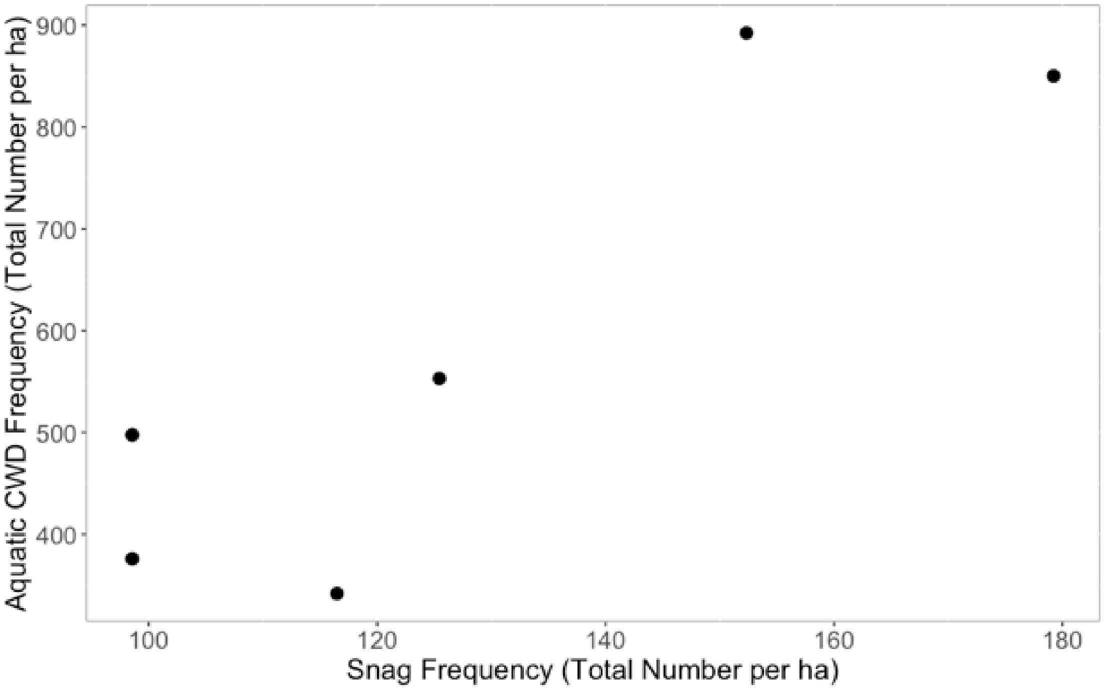


Figure 3.3. Aquatic CWD frequency (total number of CWD pieces per stream ha) and standing dead tree frequency (total number of standing dead trees per ha) were significantly, positively correlated (Pearson: t(4)=3.55, *p*=0.02, r=0.87). n=6.

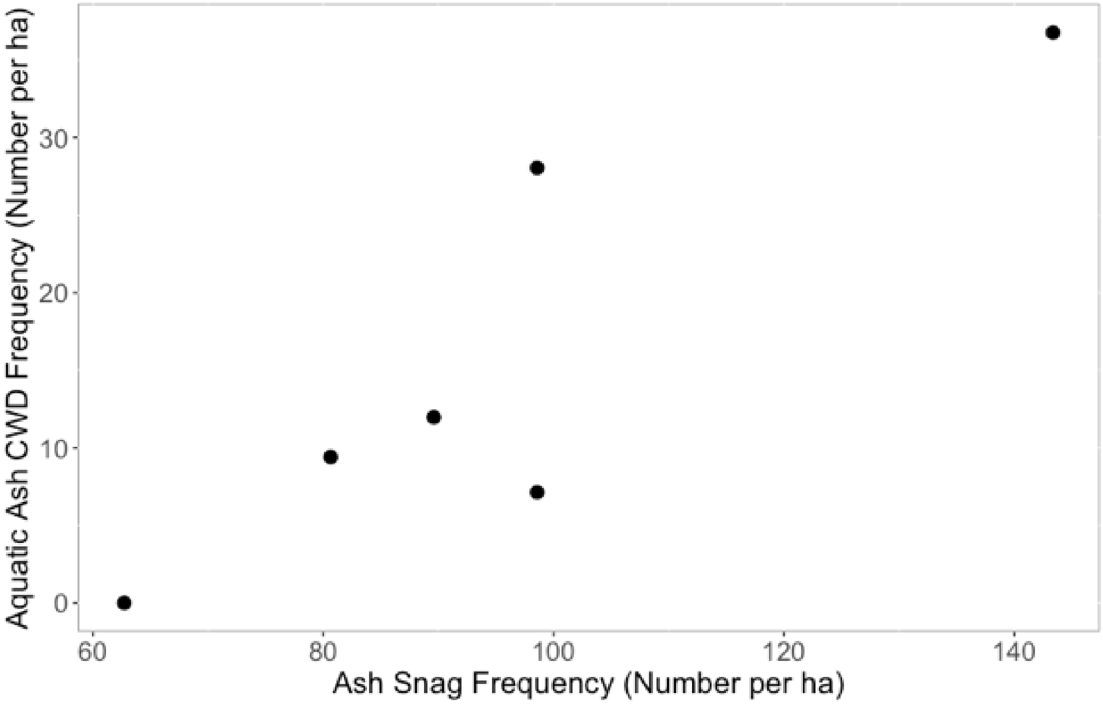


Figure 3.4. Aquatic ash CWD frequency (number of ash CWD pieces per stream ha) and standing dead ash trees frequency (number of standing dead ash trees per ha) are significantly, positively correlated (Pearson: t(4)=3.68, *p*=0.02, r=0.88). n=6.

Total live basal area of the adjacent riparian forest (measured at the reach scale of upstream, gap, and downstream) did not influence any water chemistry parameters (*p*>0.05). Water temperature, conductivity, and turbidity did not differ among watersheds or gap location (*p*>0.05, Table 3.3). Percent dissolved oxygen in downstream locations, however, was 14% (±7%) lower than in the locations upstream of gaps (t(42)=-2.14, *p*=0.04, Figure 3.5, Table S3.2). In the Grand River watershed, acidity was 0.21 (±0.05) pH units higher than in the Clinton watershed (t(3)=4.7, *p*=0.02) and was 0.10 (±0.05) higher in downstream compared to upstream locations (t(38)=2.06, *p*=0.05, Figure S3.4).

Table 3.3. Mean (± standard error) water chemistry parameters from streams sampled in watersheds across a chronosequence of EAB-impact (Clinton=early, Grand River=mid, Kalamazoo=late).

Water Chemistry Variable	Clinton	Grand River	Kalamazoo
Temperature (°C)	8.61 (1.84)	8.86 (1.60)	7.48 (1.80)
Conductivity (mS/cm)	0.67 (0.05)	0.48 (0.03)	0.38 (0.03)
Dissolved Oxygen (% Saturation)	96.50 (4.73)	88.70 (3.61)	83.10 (3.97)
Acidity (pH)	8.16 (0.04)	8.37 (0.03)	8.17 (0.03)
Turbidity (Nephelometric Turbidity Units)	12.80 (2.89)	6.96 (1.62)	23.20 (7.51)

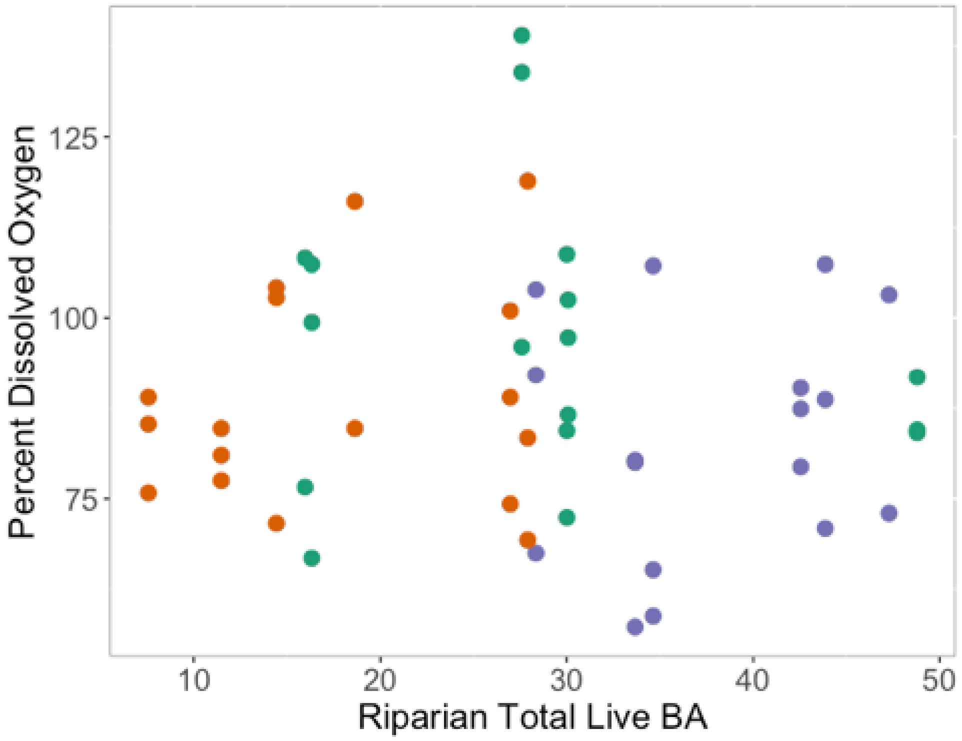


Figure 3.5. Percent dissolved oxygen saturation over riparian total live tree basal area (BA) in upstream (green), gap (orange), and downstream (purple) locations (n=51). Percent dissolved oxygen in downstream locations was 14% (±7%) lower than in upstream locations (t(42)=-2.14, *p*=0.04).

A total of 13 leaf genera represented the leaf litter of the study streams, with a median richness of 3 genera per gap location (Table 3.4). The most abundant genera in leaf packs was oak, representing an average of 28% (±3%) dry mass of leaf litter samples. Watershed and gap location did not significantly influence aquatic leaf litter richness, ash foliage dry mass relative to entire leaf pack, or oak foliage dry mass relative to entire leaf pack (*p*>0.05, Tables S3.3 and S3.4). Correlations were not significant for aquatic leaf litter richness and live tree richness (Spearman: S=19913, *p*=0.08, ρ=0.24), ash foliage dry mass relative to entire leaf pack and live ash relative basal area (Spearman: S=22282, *p*=0.28, ρ=0.15), or oak foliage dry mass relative to entire leaf pack and live oak relative basal area (Spearman: S=26063, *p*=0.96, ρ=0.01).

Table 3.4. Mean (± standard error) aquatic leaf litter variables from streams sampled in watersheds across a chronosequence of EAB-impact (Clinton=early, Grand River=mid, Kalamazoo=late). Relative mass is the taxon proportion of total leaf pack dry mass.

Variable	**Clinton**	**Grand River**	**Kalamazoo**
Leaf Genus Richness	2.560 (0.294)	2.940 (0.357)	3.440 (0.364)
Ash (*Fraxinus* spp.) Relative Mass	0.055 (0.034)	0.001 (0.001)	0.004 (0.004)
Aspen (*Populus* spp.) Relative Mass	0.016 (0.012)	0.001 (0.001)	0.028 (0.020)
Basswood (*Tilia* spp.) Relative Mass	0.046 (0.028)	0.037 (0.024)	0.055 (0.030)
Beech (*Fagus* spp.) Relative Mass	0.004 (0.004)	0.019 (0.009)	0.059 (0.023)
Hophornbeam (*Ostrya* spp.) Relative Mass	0.064 (0.030)	0.007 (0.005)	0.016 (0.013)
Cherry (*Prunus* spp.) Relative Mass	0.006 (0.003)	0.019 (0.008)	0.008 (0.004)
Walnut (*Juglans* spp.) Relative Mass	0 (0)	0.003 (0.002)	0 (0)
Buckthorn (*Rhamnus* spp.) Relative Mass	0 (0)	0.002 (0.002)	0.002 (0.002)
Dogwood (*Cornus* spp.) Relative Mass	0 (0)	0.004 (0.004)	0.001 (0.001)
Elm (*Ulmus* spp.) Relative Mass	0.003 (0.003)	0.042 (0.014)	0.041 (0.020)
Grass (Poales) Relative Mass	0.017 (0.011)	0.001 (0.001)	0.001 (0.001)
Maple (*Acer* spp.) Relative Mass	0.021 (0.009)	0.282 (0.083)	0.107 (0.036)
Oak (*Quercus* spp.) Relative Mass	0.249 (0.039)	0.278 (0.063)	0.321 (0.064)
Unknown Origin Relative Mass	0.467 (0.052)	0.298 (0.060)	0.311 (0.053)

Leaf Bacterial Communities

A total of 3183 ASVs represented leaf litter bacterial communities pooled from all sources (live leaves, terrestrial leaf litter, and aquatic leaf litter). Bacillaceae was the most abundant family, representing a mean (SE) of 20% (±2%) of the communities. Community composition was influenced by leaf source (live leaves, terrestrial leaf litter, and aquatic leaf litter) and a source by watershed interaction (PERMANOVA, p=0.02, Table S3.5). Compared to aquatic leaf litter, live leaves had 2.8 (±1.6, t(60)=2.4, *p*=0.02) greater and terrestrial leaf litter had 8.5 (±1.5, t(60)=5.4, *p*<0.01) greater phylogenetic diversity (Table S3.6, Figure S3.5). Terrestrial leaf litter communities had 164 (±31) greater Chao 1 richness compared to aquatic leaf litter (t(60)=4.7, *p*<0.01, Figure S3.6).

To examine whether EAB-related canopy gaps were influencing certain microbial taxa, venn diagrams were used to visualize ASVs unique to gap or forested habitats among the three leaf types (live leaves, terrestrial leaf litter, and aquatic leaf litter). Many ASVs were unique to gap or forest leaf bacterial communities, but very few of these unique ASVs were shared among all leaf sources in a given location. For live leaves and terrestrial leaf litter bacterial communities there were 268 and 734 ASVs, respectively, found only in intact forest but absent in gaps (Figure S3.7-S3.8). In aquatic leaf litter, 65 ASVs were found in both upstream and downstream gap locations, but absent in gaps (Figure S3.9). Among samples collected at the gap and adjacent, intact forests; 29 and 61 ASVs, respectively, were shared in live leaves, terrestrial leaf litter, and aquatic leaf litter (Figures S3.10-S3.11). In samples collected in gaps, there were 1109 ASVs present in samples collected in gaps, but absent from forest samples. Only 1 ASV, an unknown species in the family Methylobacteriaceae, was collected in all leaf types (live leaves, terrestrial leaf litter and aquatic leaf litter) in gaps, but never found in intact forest samples. There were 1388 ASVs found in intact forest leaf bacterial communities, but absent from gap communities. Similar to gaps, only 1 ASV, belonging to genus *Novosphingobium*, was found in all sample types in intact forests, but were absent from any samples in the gap.

Since bacterial communities varied among leaf sources, separate analyses were performed for each source: live leaves, terrestrial leaf litter, and aquatic leaf litter. The most abundant family in both live leaves and terrestrial litter was Sphingomonadaceae which comprised an average of 26% (±4%) and 19% (±3%) of those communities, respectively. There were 568 ASVs detected in live leaves. Watershed significantly influenced the bacterial community structure of live leaf communities (PERMANOVA: p=0.03, Figure S3.9), and two indicator families (Fimbriimonadaceae and Bacillaceae) had significantly greater abundance in the Kalamazoo watershed in the late stage of EAB invasion in the chronosequence (ISA: Sidak's p=0.05).

Terrestrial leaf litter bacterial communities included 1520 ASVs, and a watershed by gap location interaction significantly influenced the bacterial community structure of terrestrial leaf litter communities (PERMANOVA: p=0.02, Figure S3.10). The two significant indicator families (Patulibacteraceae and Caulobacteraceae) were absent from Grand River watershed, but present in low relative abundance (<1%) in Clinton and Kalamazoo watersheds (ISA: Sidak's p=0.04). Aquatic leaf litter bacterial community structure was significantly altered by aquatic leaf litter leaf richness (genera) and its interaction with gap location (PERMANOVA: p=0.04). Gemmataceae, the indicator family of communities found on aquatic leaf litter in downstream locations, was only present in downstream gap locations [0.4% (±0.2%) relative abundance; ISA: Sidak's p=0.01] Bacterial phylogenetic diversity and richness were not significantly altered by watershed or gap location in live leaves, terrestrial leaf litter, or aquatic leaf litter communities (p>0.05).

Macroinvertebrate Communities

A total of 1935 specimens representing 47 macroinvertebrate families were captured in the streams. Elmidae (collector-gatherer) was the most abundant family [24% (±3%) relative abundance, Table S3.7]. Watershed significantly influenced macroinvertebrate communities (PERMANOVA: p<0.01, Table S3.8, Figure 3.6), with 4, 1, and 5 indicator families representing Clinton, Grand River, and Kalamazoo watershed macroinvertebrate communities, respectively (ISA: Sidak's p<0.05, Table S3.7). Overall, mean family richness averaged 7 (±1). Downstream reaches averaged 2 (±1) fewer families than upstream reaches (t(46)=-2.3, p=0.3, Table S3.9). Similarly, inverse Simpson's diversity was decreased by 1.2 (±0.5) units at downstream locations compared to upstream (t(46)=-2.3, p=0.3). Elmidae, Gammaridae, and Hydropsychidae were the only indicator taxa captured in all streams, and Elmidae and Hydropsychidae relative abundance was not significantly affected watershed or gap location (p>0.05). Gammaridae relative abundance was significantly lower in the Kalamazoo watershed [late, 6% (±5%)] compared to Clinton watershed [early, 20% (±7%) $p<0.01$].

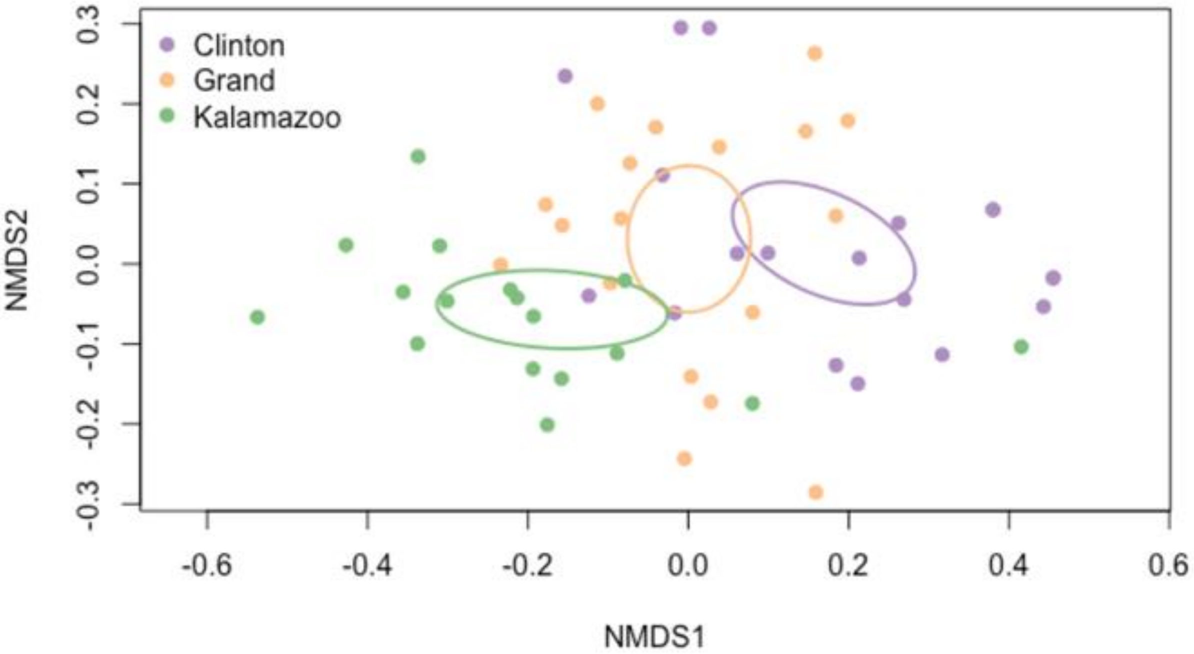


Figure 3.6. Nonmetric multidimensional scaling based on the zero-adjusted Bray-Curtis matrix for β-diversity of macroinvertebrate community family level taxonomy. Purple represents Clinton watershed (early), orange represents Grand River watershed (mid), and green represents Kalamazoo watershed (late) across the chronosequence. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. Stress = 0.15.

Collector-gatherers were the most abundant functional feeding group [53% (±4%) relative abundance]. Watershed significantly affected macroinvertebrate functional feeding group communities (PERMANOVA: $p<0.01$, Table S3.7, Figure 3.7), with collector-filterers, grazers and predators representing Kalamazoo watershed and collector-gatherers and shredders representing Clinton watershed (ISA: Sidak's $p<0.05$, Table S3.10). The relative abundance of collector-filterers increased across the EAB-related canopy gap chronosequence, with significantly lower relative abundance in the Clinton watershed [early, 6% (±1%)] compared to Kalamazoo watershed [late, 9% (±2%), $p=0.02$, Table S3.11]. Shredders exhibited the opposite pattern, with significantly higher relative abundance in Clinton watershed [early, 24% (±7%)] compared to Kalamazoo watershed [late, 12% (±5%), $p<0.01$]. Grazers had significantly lower relative abundance at gap locations [6% (±2%)] than upstream [9% (±33%), $p=0.02$].

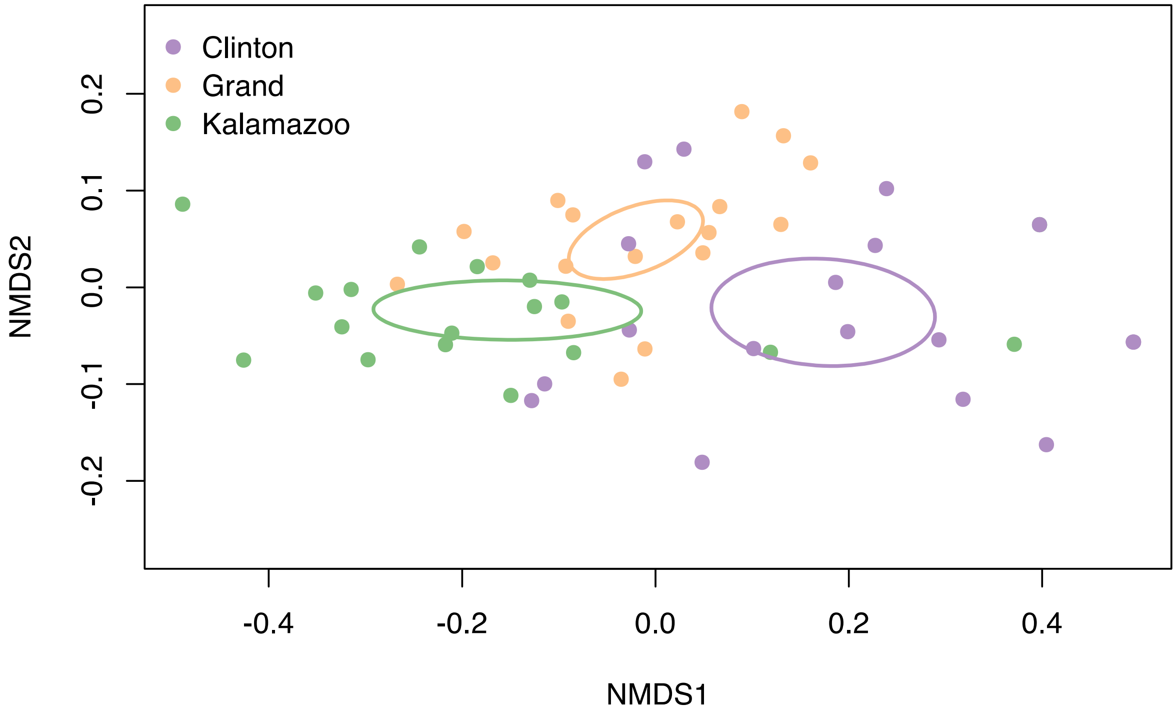


Figure 3.7. Nonmetric multidimensional scaling based on the zero-adjusted Bray-Curtis matrix for β-diversity of macroinvertebrate communities functional feeding groups. Purple represents Clinton watershed (early), orange represents Grand River watershed (mid), and green represents Kalamazoo watershed (late) across the chronosequence. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. Stress = 0.09.

Discussion

Aquatic CWD

The objective of this study was to evaluate habitat, resource, and consumer relationships in southern Michigan streams following EAB-related ash mortality in the adjacent riparian forests. We hypothesized that ash CWD in streams would increase, particularly in the Clinton watershed that was invaded early on by EAB. Our data supported this hypothesis. The proportion of CWD pieces that were ash was higher in the Clinton watershed than in the Grand River watershed, which was invaded more recently, although no difference was detected in the Kalamazoo watershed, which was invaded latest. It is interesting that we did not find other CWD variables (volume, *in situ*, and logjams) to significantly vary by watershed, but Engelken et al. (2020) also did not find a significant independent influence of watershed on standing dead trees or CWD, although there was a significant interaction between watershed and gap location, with the Clinton watershed having greater total CWD volume in the gap compared to forest location. Engelken et al. (2020) did find more total and standing dead ash tree basal area and ash CWD volume in gap locations compared to adjacent forest. Due to low sample size we could not analyze stream CWD at the reach scale to determine if there were differences between upstream, gap, and downstream sites. Given stream flow and ephemeral or periodic flooding, we would not anticipate a change in stream CWD dynamics at such a fine spatial scale (Gregory et al., 2003).

Total and ash standing dead tree frequency positively correlated with total and ash aquatic CWD frequency, respectively. Standing dead trees are the most direct source of CWD to aquatic habitats because standing dead trees are more likely to fall into the stream than terrestrial CWD is to be transported on the ground (Gregory et al., 2003). Because Engleken et al. (2020) found significantly greater total and standing dead ash tree basal area in gaps compared to the adjacent forest, we can speculate that there is greater recruitment to the stream at those sites as well.

Riparian CWD volume was positively correlated with the aquatic *in situ* relative CWD volume, demonstrating the continuum of CWD recruitment from riparian to aquatic habitats, reported in other studies of riparian forest and stream CWD linkages (Keeton et al., 2007; Warren et al., 2009; Wohl et al., 2010). Riparian CWD volume per ha positively correlated with the relative amount of stream CWD that was directly recruited from the riparian zone relative to the total stream CWD volume. It is possible that some of these CWD pieces were measured in both surveys, as some logs that crossed the riparian survey transect were also contained in the stream. This pattern is also important, because there was a negative linear relationship found by Engelken et al. (2020) between the year of gap formation and volume CWD. We can speculate that this relationship also exists for aquatic *in situ* relative volume, due to its positive correlation with riparian CWD volume.

Water chemistry

It is notable that percent dissolved oxygen was lower in downstream locations compared to those upstream, because dissolved oxygen is an indicator of stream health (Rosenberg and Resh, 1993). Certain aquatic organisms can be sensitive to low levels of oxygen (Allan and Castillo, 2007). Typically, the driver of lowered oxygen is water temperature or eutrophic conditions, but we did not see a significant change in water temperature at downstream locations. This shift in dissolved oxygen downstream, may be a result of increased algae growth in the gap, due to increased light. Although there is greater production at gap sites, when these organisms die they drift downstream, and the subsequent decomposition results in higher biological oxygen demand (Reichert et al., 2009), as in eutrophic conditions reported for other aquatic ecosystems (Dodds, 2007). Although there were significant changes in pH across watershed and gap location, these changes were <0.3 units, which are unlikely to affect aquatic biota (Courtney and Clements, 1998).

The scale of riparian influence on water chemistry parameters may be far greater than the reach scale and undetectable at the scale we sampled. For example, Pan et al. (2004) found a significant correlation between land use and stream conductivity and total phosphorous at the reach (150 – 320 m), network (entire stream network flowing to reach), and catchment (whole land area of watershed) scales; but dissolved organic carbon was only correlated with land use at the network and catchment scales, indetectable at the reach scale. This demonstrates that water chemistry changes may be occurring at larger scales than we sampled, or water chemistry is resilient to EAB-related canopy gap disturbance in Michigan streams. Further studies across watersheds whole watersheds with multiple EAB-related canopy gaps are necessary to determine how stream water chemistry is responding.

Aquatic Leaf Litter

Contrary to our hypothesis, we did not detect watershed and gap location effects on aquatic leaf litter variables. Previous studies of leaf litter dynamics in riparian EAB-related canopy gaps have documented significant shifts in leaf litter composition over time since EAB invasion, with a reduction of overall leaf subsidies after invasion (Kreutzweiser et al., 2020). It is notable that although we sampled at the scale of one EAB-related canopy gap per stream, there are many gaps impacting streams upstream and downstream our sampling locations For example, Engelken and McCullough (2020) quantified EAB-related canopy gaps along a 3-5km stretch of three rivers in northern Michigan. Within these rivers, there were 3.6-7.1 canopy gaps per km, suggesting multiple canopy gaps may compound effects downstream. It may be that although we are not detecting a reach-scale difference in leaf litter composition, there may be larger, watershed-wide shifts in response to multiple gaps related to EAB invasion and subsequent riparian forest changes. Further study in the patchy gap systems of southern Michigan streams, compared to wide-scale mortality in northern Michigan streams, would further elucidate aquatic leaf litter dynamics in response to EAB-related riparian disturbance.

Additionally, we did not find a correlation between aquatic leaf litter and adjacent riparian trees. Previous work on riparian forest harvesting found that riparian forest composition did not alter leaf litter accumulations in streams, attributing this to continuous input from upstream sources (Kreutzweiser et al., 2004). Downstream transport of leaf litter can outweigh direct inputs from adjacent riparian zones, especially as stream order increases along a watershed continuum (Bilby and Likens, 1980; Vannote et al., 1980; Bretschko, 1990). Therefore, our findings that there was no correlation between aquatic leaf litter and adjacent riparian trees may simply reflect greater impact of downstream transport of leaf litter.

Leaf Bacterial Communities

We hypothesized leaf bacterial community composition would differ across the watersheds given the chronosequence of EAB invasion, due to the shift in leaf resources at canopy gaps. We found evidence for this influence in pooled leaf samples, live leaves and terrestrial leaf litter, with specific indicators in higher abundance in the watersheds. There was only a single ASV shared among gaps or forested sites between habitats, and this may be due to a low sample size, as we were not able to replicate gap/forested conditions among each stream location. Methylobacteriaceae (phylum Proteobacteria), was collected in all leaf litter types in gaps (live leaves, terrestrial litter and aquatic litter), but never found in intact forest samples. This group of bacteria fix nitrogen, a critical ecosystem service to forests (Dworkin et al., 2006; Singh et al., 2010; Mirza et al., 2014; Moyes et al., 2016). Stone and Jackson (2016) found a spatial difference in Methylobacteriaceae relative abundance on magnolia tree (Magnoliaceae) leaves related to canopy cover ; therefore, our result of greater Methylobacteriaceae collected in gaps can be attributed to greater light in the gap.

For forest leaf litter communities, *Novosphingobium* (Proteobacteria) was found in all sample types but was absent from any leaf litter bacterial communities collected from gaps. *Novosphingobium* can degrade certain aromatic compounds, having implications for environmental remediation (Garrity, 2005). Therefore, the reduction of these taxa in gaps may have functional impacts on the forest and stream ecosystems. Tlaskal et al. (2016) found

Novosphingobium as a common bacterial group detected during forest leaf litter decomposition; therefore, our detection of this taxa in forest samples is in line with other forest bacterial communities. Sequencing of additional terrestrial leaf samples will be an important component to investigate whether there is indeed a terrestrial-aquatic (Ruiz-González et al. 2015) or allochthonous resource-aquatic environment (Larson et al. 2020) bacterial connection at the ASV level, as has been found in past studies. This study is the first to characterize bacterial communities across a terrestrial to aquatic gradient in response to forest disturbance, representing an important contribution to understanding ecosystem level effects of gap formation in watersheds.

Across leaf types, terrestrial leaf litter had greater diversity and richness compared to live leaves and aquatic leaf litter. This result of highest diversity during terrestrial decomposition corresponds to previous work done in terrestrial or aquatic environments, which is attributed to differences in leaf structure and chemistry (carbon to nitrogen ratio and lignin concentration). In the terrestrial environment, microbial diversity and richness increased over decomposition time from live leaves to terrestrial decomposed leaf litter of European beech (Purahong et al., 2016) and oak (Voříšková and Baldrian, 2013) leaves. Abelho and Descals (2019) found that fungal diversity decreased with additional time decomposing in aquatic environments in the terrestrial leaf litter to aquatic leaf litter gradient. To our knowledge, this is the first study that examines bacterial communities along the continuum of leaf decomposition from live leaves, terrestrial leaf litter, and aquatic leaf litter, providing a more complete view of microbial community structuring across decomposition time and ecosystem boundaries.

Aquatic leaf litter bacterial community structure was significantly altered by aquatic leaf litter leaf genera richness and its interaction with gap location, supporting our hypothesis that bacterial community structure would be altered by gap location. Gemmataceae, the indicator family for gap location, is an abundant aquatic bacterial family that is positively correlated with Tryptophan-like C2 dissolved organic carbon (Zhang et al., 2019). Tryptophan-like C2 is a leachate component of leaf litter, especially abundant in oak leaf leachate, which was the most abundant leaf type found in aquatic leaf litter samples (Franklin et al., 2020). Experimental leaf litter decomposition studies that incorporate microbial community assessment across riparian disturbance gradients are needed to further elucidate the mechanisms behind this pattern in Gemmataceae abundance.

This microbial dataset is limited to bacteria, and, therefore, does not include the largest microbial component of leaf litter decomposition: fungi (Gulis and Bärlocher, 2017). We can speculate that we would find similar trends with the fungal community in our samples, based on previous literature. Similar to our trend in bacteria communities, Koivusaari et al. (2019) found higher richness in their terrestrial litter fungal communities, compared to stream submerged litter. Yet, contrary to our findings in which <1% of the bacterial ASVs were shared among live leaves, terrestrial leaf litter, and aquatic leaf litter; Koivusaari et al. (2019) found 65% of the fungal OTUs were shared among terrestrial leaf litter and aquatic leaf litter. It would be interesting to see if this trend of a large component of the fungal community being shared between terrestrial and aquatic environments holds true in a disturbed, canopy gap environment, where additional light may alter the fungal community.

Macroinvertebrate communities

We hypothesized that benthic macroinvertebrates would shift structurally and functionally in gap locations. Specifically, we anticipated greater abundance of grazer macroinvertebrate taxa at gap locations, due to increase in light availability and primary production. We found grazers had significantly lower relative abundance at gap locations, refuting our hypothesis. This is contrary to previous literature on canopy opening in the riparian zone either increasing grazer abundance (Feminella et al., 1989; Houghton and Wasson, 2013) or having no effect (Hawkins et al., 1982; Cole et al., 2003; Alberts et al., 2018). Although primary production typically increases with increased light, there reaches a point in naturally forested streams (where algae are adapted to low light levels) that increasing light will decrease photosynthesis because of photooxidation of enzymes and chlorophyll inactivation (Naiman and Bilby, 2001). Perhaps because the streams are not adapted to the high light levels, there is a decrease in primary production at canopy gap locations and subsequent decrease in grazer populations.

Although there was no difference in community structure in response to gap location, we found that richness and Simpson's diversity was significantly lower downstream of gaps, compared to upstream sites. This is particularly interesting, considering we also found significantly lowered dissolved oxygen in downstream sites. There is an abundance of literature describing the influence lowered dissolved oxygen has on lowering stream macroinvertebrate diversity (Allan and Castillo, 2007; Kaller and Kelso, 2007; Ngodhe et al., 2014). Further research will be critical to determine the mechanism behind lowered dissolved oxygen downstream of riparian disturbances and how this is impacting stream biota.

There were significant differences in the macroinvertebrate community structure and function across the chronosequence of EAB-related canopy gap formation. Specifically, the relative abundance of shredders decreased from the Clinton watershed (early) to Kalamazoo watershed (late), driven by Gammaridae population dynamics, which were significantly lower in the Kalamazoo river watershed. Although Gammaridae primarily act as shredders in Michigan streams, they have a generalist feeding strategy and are generally regarded as tolerant to disturbance (Bouchard, 2004). Gammaridae had greater macroinvertebrate community dominance in streams adjacent to young forest stands post clear-cutting compared to those streams adjacent to older stands, which was attributed to their tolerance to disturbance (Cole et al., 2003). Streams in Clinton watershed are the most disturbed because, as Engelken et al. (2020) found, dead ash trees have fallen, while in the Grand River and Kalamazoo watersheds, dead ash are still standing. Therefore, we can also attribute this difference to Gammaridae being tolerant to forest disturbance.

Conclusions

Overall, these findings reveal negative impacts to sites downstream of EAB-related canopy gaps, through decreases in dissolved oxygen and macroinvertebrate diversity. Yet, there is resilience across the reach scale in organic matter dynamics of leaf litter and coarse woody debris. It is important to document these reach scale impacts over time and over additional gaps in streams to understand long term response of streams to riparian disturbance. To date, this study is the only one that connects EAB invasion, the most destructive insect invasion to forests in US history (McCullough, 2019), directly to stream ecosystem structure and function. The work presented here highlights the need to further investigate EAB impacts, as well as other terrestrial invaders as they continue to spread, on other cross-ecosystem subsidies and community interactions in a diversity of geographic areas.

CHAPTER 4: NATIVE AND INVASIVE LEAF LITTER INVERTEBRATE COLONIZATION MEDIATED BY RIPARIAN CANOPY GAPS

Abstract

Leaf litter subsidies are critical to stream ecosystem functioning and can be altered by riparian invasive species, which influences macroinvertebrate community structure and function. Emerald ash borer (EAB) *Agrilus planipennis* is an invasive insect that has been killing native ash trees, potentially resulting in indirect impacts on stream ecosystems through altered leaf litter subsidies and subsequent changes in macroinvertebrate communities. The aim of this study was to determine how forest disturbance due to an invasive insect affects stream macroinvertebrates and ecosystem function. Leaf packs of four species (ash, oak, buckthorn, and cotton control) were introduced upstream, downstream, and at the center of a canopy gap from EAB-related ash mortality in Augusta Creek, MI, and collected monthly from December 2018-March 2019 to quantify decomposition and assess colonizing macroinvertebrate communities. There was no gap effect on leaf litter decomposition rates, despite there being significantly less canopy cover in the gap. Buckthorn [-k=0.061 (±0.009)] decomposed significantly faster than ash [-k=0.041 (±0.001)] and oak [-k=0.034 (±0.009)] leaves (t(125)=-2.205, *p*=0.029). *Gammarus*, *Nemoura*, and *Taeniopteryx* (shredders) were more abundant on ash compared to buckthorn leaves, and macroinvertebrate richness, diversity, and indicator taxa abundance were significantly higher (ANOVA $p<0.03$) in gap leaf packs, compared to upstream and downstream stream reaches, implying recovery from gap conditions. The only functional group that differed significantly based on leaf type was collector-gatherers, and this was driven by greater Chironomidae abundance in buckthorn leaves (ANOVA *p*=0.04). Our findings suggest a structural (e.g. macroinvertebrate diversity highest in gaps) and functional (e.g., collector-gatherers most abundant on buckthorn

leaves) shift in macroinvertebrate communities in response to EAB by indirect routes of leaf litter subsidies and light availability. These results provide an important connection between the indirect impacts of invasive species on altered leaf litter subsidies (ash, buckthorn, and oak) and altered habitat in riparian zones (canopy gap creation) that further the field of stream ecology.

Introduction

Aquatic ecosystems are highly impacted by adjacent riparian forest habitat. The surrounding canopy can impact stream condition, including temperature and light availability, as well as leaf litter subsidies (Gregory et al., 1991; Polis and Strong, 1996; Kiffney et al., 2004; Richardson et al., 2010; Roth et al., 2010). Leaf litter serves as the base of the food web for most low order streams (Vannote et al., 1980). Changes in leaf litter content can have functional impacts on streams, such as altering leaf litter decomposition rates (Young et al., 2008; Tank et al., 2010; Kominoski et al., 2011). These changes influence the macroinvertebrate communities that use leaf litter for habitat or food resources, with increases in more palatable leaf litter increasing shredder macroinvertebrate abundances (Cummins et al., 1973, 1989; Petersen and Cummins, 1974; Wallace et al., 1997).

Leaf nutritional value is determined by the structure of the leaves themselves (i.e. physical and chemical deterrents and nitrogen and protein content) as well as the amount and community structure of microbes that colonize the leaves (Cummins and Klug, 1979; Strong et al., 1984; Suberkropp, 1992). This leaf nutritional value shifts over decomposition. When leaves enter a stream they undergo rapid leaching of soluble organic compounds and microbial (bacteria, fungi, and eukaryotic microbes) colonization, which makes them more palatable to shredders (Cummins et al., 1989). For example, the invertebrate shredder *Paratya australiensis* (Decapoda: Atyidae) preferred native *Eucalyputus camaldulensis* that had been microbially colonized to unconditioned

E. camadlulensis or exotic *Salix babylonica* leaves, and this is attributed to greater bacteria quantities residing on conditioned *E. camaldulensis* (Schulze and Walker, 1997).

Several foundational studies on leaf litter-macroinvertebrate interactions were performed in Augusta Creek, Michigan; including Petersen and Cummins' 1974 study that found a continuum from low (e.g., oak) to high (e.g., ash) leaf litter processing rates, which related to higher nitrogen availability in fast decomposing leaves. Macroinvertebrate shredders selectively feed on the most labile leaves available to them temporally, and over time there may be stepwise consumption of leaves, with slower conditioning leaf species consumed later once they become functionally available after microbial processing (Cummins, 1974). For example, in Augusta Creek, it took two weeks longer for aspen, a slow decomposing leaf type, to be colonized by shredder macroinvertebrates compared to ash, a fast decomposing leaf type (Petersen and Cummins 1974). A shift in leaf litter taxa alters this stepwise consumption by macroinvertebrates if decomposition timing is changed; therefore, riparian forest conditions are important in shaping macroinvertebrate community structure (Cummins et al., 1989; Minshall and Rugenski, 2007; Pereira and Ferreira, 2020).

Invasive species can have significant negative impacts on riparian forest integrity, altering tree composition and quantities (Greene, 2014). For example, invasive plant leaf litter were reported to decompose at different rates, most often faster due to lower carbon to nitrogen ratios in leaves, than native plants, and supports less abundant and diverse macroinvertebrate communities (Richardson et al., 2007; Going and Dudley, 2008; Davies and Boulton, 2009; McNeish et al., 2012; Mineau et al., 2012). Terrestrial animal invasions have direct and indirect impacts on the riparian zone, as some kill native vegetation, eliminating subsequent leaf litter subsidies to streams and create a canopy gap primed for invasive plant species colonization

(Stohlgren et al., 1998; Daehler, 2003; Warren et al., 2016). For example, the terrestrial insect invader hemlock woolly adelgid *Adelges tsugae* Annand (Hemiptera: Adelgidae) indirectly led to an increase in herbivorous stream invertebrates at sites of severe infestation (>75% canopy loss), due to habitat changes caused by hemlock decline (Diesburg et al., 2018). With forest pests continuing to invade riparian forest ecosystems, it is important to understand how these invasive species have indirect impacts on adjacent ecosystems.

Emerald ash borer (EAB) *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive insect pest that has killed millions of ash trees since its North America discovery in 2002 (Herms and McCullough, 2014). Loss of ash trees following EAB invasion may negatively impact the trophic structuring of stream communities via changes in leaf litter subsidies and habitat structure (Nisbet et al., 2015). When ash trees die after EAB invasion, canopy gaps form thus increasing light in the stream (Engelken et al., 2020), which potentially increases primary production and causes bottom-up impacts on the trophic structuring of the stream (Albariño et al., 2008).

Canopy disturbance also increased aquatic oak (*Quercus robur*) and alder (*Alnus glutinosa*) litter decomposition rates, due to increased light and primary productivity (Mckie and Malmqvist, 2009). Primary producer algae on leaf litter increased with additional light, and algae are higher quality food resources, compared to fungi and bacteria, for shredders (Franken et al., 2005). Increased shredder activity led to faster decomposition rates of stream leaf litter (Barnes et al., 1986). Additionally, algae residing on leaf litter prime heterotrophic microbial decomposition, increasing decomposition rates, because they fix inorganic carbon which can then be transferred to bacteria and fungi (Kuehn et al., 2014). Leaf litter decomposition rates are an important metric of ecosystem functioning, because faster decomposition results in faster transfer of nutrients and energy through the stream ecosystem (Benfield et al., 2017).

The aim of this study was to determine how forest disturbance due to an invasive insect (EAB) affects stream macroinvertebrates and leaf litter decomposition. It is hypothesized that: 1) leaf litter decomposition rates will differ among taxa of recalcitrant oak leaves will have slower decomposition rate than the presumed more liable ash and buckthorn leaves; 2) leaf litter packs in light gaps will decompose faster than those upstream or downstream of gaps, due to increased light from the canopy gap; 3) leaf packs of differing taxa will support different macroinvertebrate communities, due to differences in leaf structure. Specifically, ash leaves support higher macroinvertebrate densities than oak due to lower C:N ratio in deciduous forests (Lecerf et al., 2007). Buckthorn leaves support the lowest density of macroinvertebrates, due to its fast decomposition rate and potentially toxic leachates, as found in a Wisconsin study (Lewis et al., 2017a). Thus, ash will have a greater proportion of large particle shredders, while oak and buckthorn will have a greater proportion of small particle collectors; and 4) The macroinvertebrate community upstream of the gap will have a greater proportion of shredders, while the community in gaps will have more grazers. Shredders respond more positively to adjacent riparian sites with intact forests (Irons et al., 1988), and grazers increase in abundance with increased primary productivity due to canopy gap light (Feminella et al., 1989).

Materials and Methods

Experimental Design

This study was conducted in Augusta Creek on the property of Michigan State University's W. K. Kellogg Experimental Forest in Augusta, Michigan, USA (42.36, -85.36). Augusta Creek is a groundwater fed, third order stream in the Kalamazoo River watershed, and was the site of leaf litter decomposition studies undertaken well before EAB invasion of Michigan (Petersen and Cummins, 1974; Martin et al., 1981; Merritt and Lawson, 1981, 1992; Hanson et al., 1984; Lawson et al., 1984; Merritt et al., 1984; Knollenberg et al., 1985).

A leaf litter decomposition experiment was performed using three reaches of Augusta Creek: 20 m upstream, at an EAB-related canopy gap center, and 20 m downstream of the gap (hereafter as upstream, gap, and downstream reaches, respectively (Figure 4.1). The gap size was 1.7 ha as determined via aerial images by Engelken et al. (2020). Four pack types were used in this experiment: green ash (*Fraxinus pennsylvanicus*), swamp white oak (*Quercus bicolor*), common buckthorn (*Rhamnus cathartica*), and sterile cotton strips (control) (Tiegs et al., 2013). The three leaf species were chosen based on the results of previous riparian and aquatic surveys of the area (Engelken et al. 2020, Larson unpublished data). Green ash was originally a dominant overstory species in the canopy gap area but has since been killed by EAB infestation when the gap formed in 2014, as saplings of green ash remain in the area (124 in the gap), but no live green ash overstory trees persisted at the time of this study (Engelken et al. 2020). Swamp white oak is a dominant overstory species in the riparian areas surrounding the gap, comprising 27% of total basal area (Engelken et al. 2020). Ash (*Fraxinus* spp.) and Oak (*Quercus* spp.) were both previously used in aquatic leaf litter decomposition studies in Augusta Creek, and therefore serve as a model of comparison to pre-EAB invasion studies (Petersen and Cummins, 1974; Hanson et al., 1984). Common buckthorn (*Rhamnus cathartica*) was an invasive shrub in the area and comprised 7% of the live tree basal area in the EAB-related canopy gap of Augusta Creek used in this study (Engelken et al., 2020).

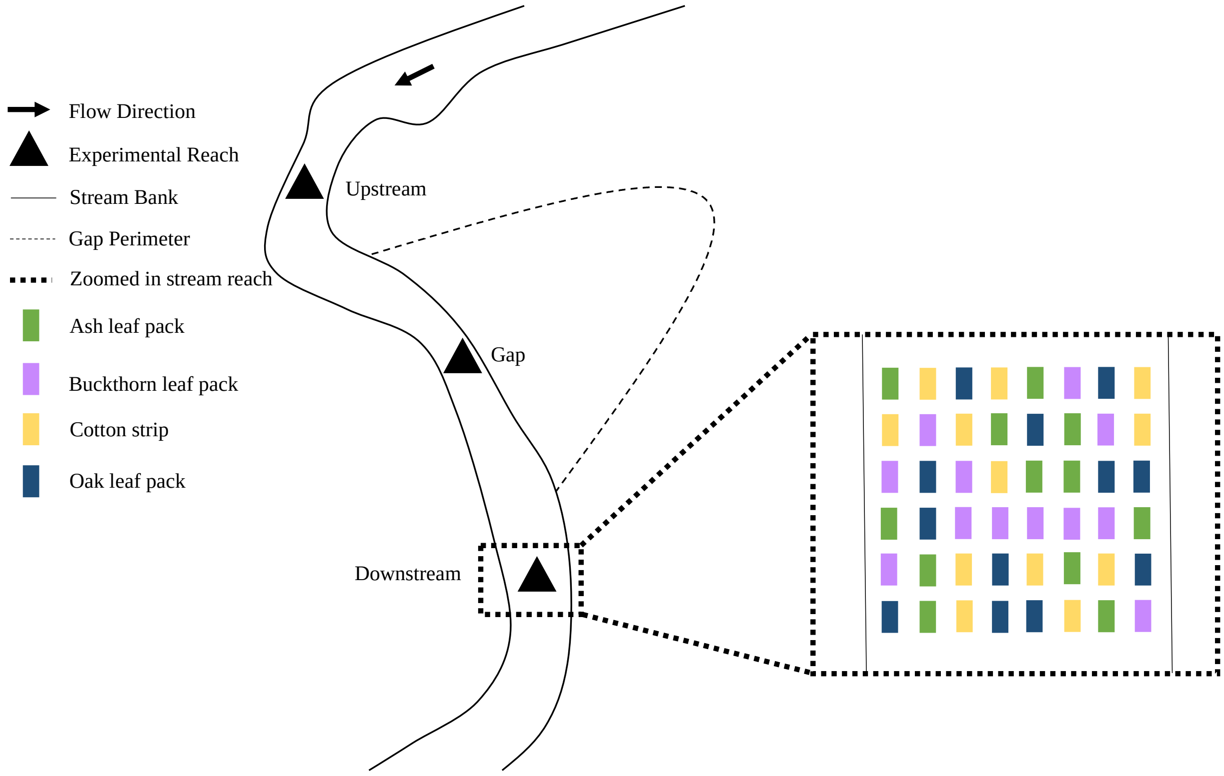


Figure 4.1. Experimental design of Augusta Creek in Kellogg Experimental Forest during December 2018-March 2019. Solid lines represent the stream bank. The solid arrow represents the direction of stream flow. The EAB-related canopy gap perimeter is represented by a dashed line. The experimental reach locations (upstream, gap, and downstream) are represented by a black triangle. Forest surrounds the gap. The dotted line around the downstream experimental reach zooms in on the arrangement of leaf packs. In green are ash leaf packs, in purple are buckthorn leaf packs, in yellow are cotton strips, and in blue are oak leaf packs. The leaf packs attached to bricks were arranged in a 6x8, randomly determined design, spanning from stream bank to stream bank (stream width range: 10.3-12.6m, stream depth range: 0.5-0.8 m).

All three leaf species were collected from living trees (oak), shrubs (buckthorn), or saplings (ash) prior to abscission (leaves haphazardly chosen) along a 500 m transect along Augusta Creek 10-20m from the bank in October 2018 using sterile cutters and placed in sterile bags. Leaves were air dried to a constant weight over 3 weeks in a sterile plastic box with 1 cm air vents. Single species leaf packs (2 grams dry weight) were constructed using the methods of Petersen and Cummins (1974) with sterile supplies. Whole leaves were sewn together manually with sterile fishing line at one point ~1 cm from the base of the leaf, and the fishing line was attached to a sterile metal clip. Sterile cotton strips were constructed using the methods of Tiegs et al. (2007) with sterile supplies, dried, and massed prior to deployment in the stream. Cotton strips' initial mass ranged from 0.80-0.95 grams dry weight, rather than 2 grams as the leaf packs were constructed, to maintain methods of Tiegs et al. (2007).

Field sample collections

At each experimental reach, 12 leaf packs of each type (ash, oak, buckthorn, and control) were anchored to bricks with a metal clip in a randomly generated 8x6 block, with 8 brick columns spanning from bank-to-bank (stream width range: 10.3-12.6 m, stream depth range: 0.5-0.8 m), and 6 rows down a ~6m length of the stream. Bricks were placed ~1 m apart, and one leaf pack was attached to each brick. Additionally, on the day of leaf pack deployment (December 2018), 3 packs of each leaf type and 1 cotton strip (due to anticipated minimal mass loss in cotton controls) per reach were transported to the site, then placed in a 188 mL sterile Whirlpak© to account for handling loss. A HOBO™ temperature logger (Onset Computer Corporation, Bourne, MA, USA) placed in the middle of each stream reach recorded the water temperature every 15 minutes at each experimental reach during the length of the experiment. Three randomly selected (via random number generation within each species) packs of each leaf type at each experimental reach were collected during 4 time points (December 16th, 2018; January 18th, 2019; February 14th, 2019; and March 24th, 2019) over 98 days of decomposition in the stream. Leaf packs were individually placed in 188 mL sterile Whirlpaks©, kept on ice during transport, and stored at -20 °C until laboratory processing. During each sampling event, water chemistry parameters of dissolved oxygen (mg/L), pH, conductivity (mS/cm), and temperature (°C) were collected using a YSI 6-

Series multiparameter water quality 6600 V2-4 sonde and canopy cover measured using a densiometer (Hill, 2017).

In the laboratory, leaf packs were rinsed with sterile water over a sterile 250 um sieve to collect macroinvertebrates, weighed for wet mass, then divided into two subsamples: one for ash free dry mass (AFDM) determination and one for bacterial community assessment (*not addressed in this manuscript due to COVID-19 delays in research activities including MSU core facilities cessation of sequencing services on March 16th, 2020*). AFDM and leaf pack decay coefficients (-k) were determined using methods described in Benfield et al. (2017). The mass of leaves used for mass loss calculations were standardized to represent 100% mass. The difference between this mass and mass recorded from experimental leaf packs is designated "mass lost.", which is log transformed and regressed over time to determine the decay coefficients (-k), which are the slopes in the regression for each leaf species/gap location combination. Macroinvertebrates were preserved in 90% molecular grade ethanol and identified to the lowest taxonomic level possible (genus) and matched with a functional feeding group (FFG) (Merritt et al., 2008; Bright, 2016). Voucher specimens were deposited in the Albert J. Cook Arthropod Research Collection, Michigan State University (Appendix S1). Taxon richness was determined by the number of unique genera present in a leaf pack sample.

Statistical analyses

All statistical analyses were performed using R version 3.6.3 (R Core Team, 2018), and code can be found at https://github.com/BenbowLab/AugustaLittR. Mean ± standard error (MSE) was calculated for each individual macroinvertebrate taxon and FFG. Estimates of α-diversity in macroinvertebrate communities (genus richness, inverse Simpson's diversity index) were calculated using the vegan 2.5-2 library diversity function in R (Oksanen et al., 2019). Variation in macroinvertebrate community composition (genus and FFG beta diversity) was visualized using non-metric multidimensional scaling (NMDS) and statistically tested among factors time, gap location (upstream, gap, and downstream), and leaf species (ash, buckthorn, cotton strip, and oak) with PERMANOVA, using a Bray-0 dissimilarity matrix and 999 permutations in the vegan 2.5-2 library adonis function in R (Anderson, 2001). To identify genera and FFG representative of leaf type macroinvertebrate communities and changes over time, we used indicator species analysis (ISA) with Indicator Value (IndVal) Index and its significance using 999 permutations in the "signassoc" function in the R package indicspecies, with p-value adjustments for multiple comparisons using the Sidak method (Dufrêne and Legendre, 1997; Cáceres and Legendre, 2009; Cáceres et al., 2010). A two-way ANOVA with repeated measures was used to test the effects of reach, leaf type, time, and their interactions on macroinvertebrate community richness, inverse Simpson's diversity, and taxon abundances (individuals per pack). The assumption of normality was tested using Shapiro-Wilk tests, and those variables that did not meet the assumption were $\log_{10}$ transformed. The assumption of homoscedasticity was determined by residuals versus fits plots. Pairwise contrasts of modelled treatment means were performed using the "emmeans" function with Bonferroni adjustments used for multiple comparisons (Kassambara, 2020). Results of all other statistical tests were considered significant at $\alpha=0.05$.

Results

Leaf litter decomposition

There was 40% less canopy cover in the gap reach compared to upstream and downstream reaches, but no statistical difference in water temperature (ANOVA, F(2)=2.55, $p>0.05$, Table S4.1). The upstream reach had significantly higher pH [8.41 (±0.03)] compared to the downstream reach [8.00 (±0.06), ANOVA, F(2)=33.40, $p<0.01$]. Leaf type (i.e., plant species) was the only significant effect on leaf litter decomposition; buckthorn decomposed significantly faster while cotton decomposed significantly slower than all other leaf types (ANOVA, *p*<0.01, Table S4.2-S4.3, Figures S4.1-S4.2). Surprisingly, cotton strips had a negative decomposition rate, meaning that they on average gained mass, rather than lost mass, over time. Because cotton was an artificial substrate that confounded results, further analyses were run with and without cotton included. Results without cotton strips are reported below, but results where cotton was included can be found in Appendix S2. Without cotton included in analyses, leaf litter decomposition was significantly influenced by leaf type; buckthorn was significantly faster than ash and oak leaves (ANOVA, *p*<0.01, Table S4.4-S4.5, Figure 4.2-S4.3).

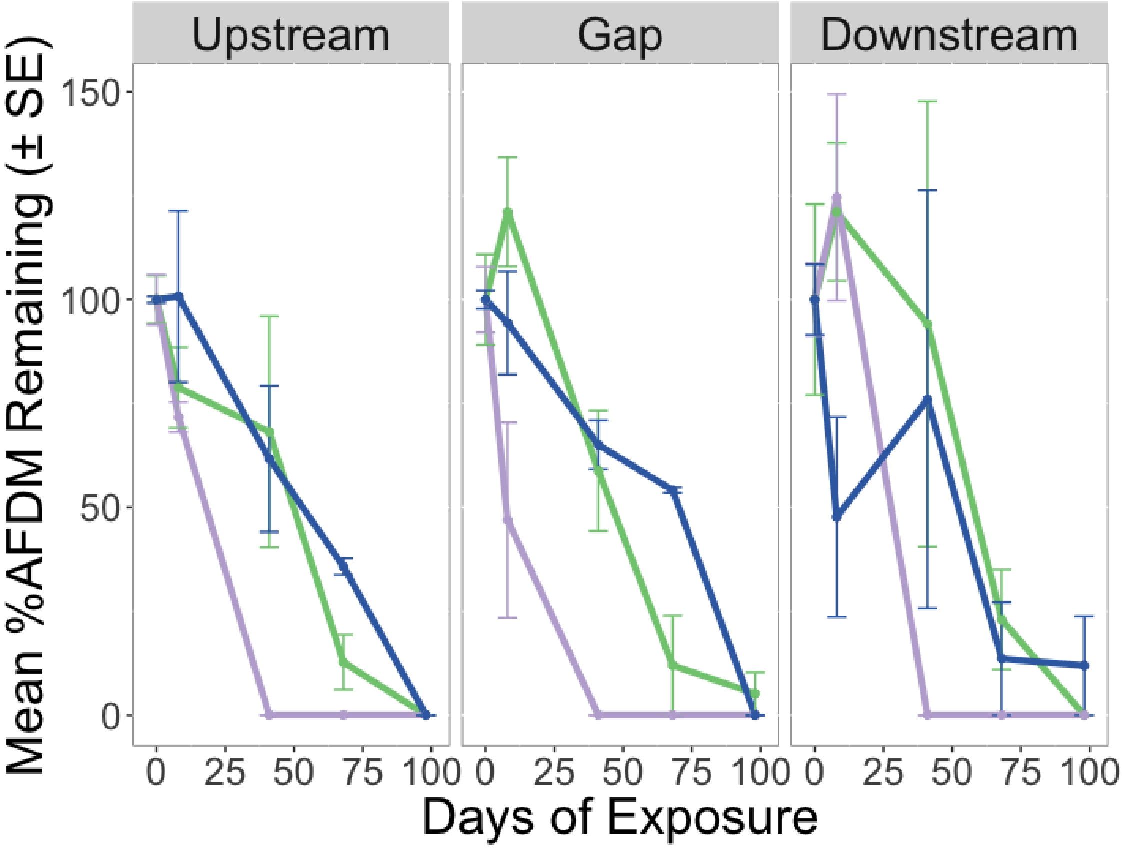


Figure 4.2. Mean (± standard error) percent ash free dry mass remaining over time in upstream, gap, and downstream reaches for ash (green) buckthorn (purple), and oak (blue) leaf packs. n=135. Day 0=December 8th, 2018. There was significant fragmentation to an oak leaf pack (an entire leaf broke off the pack) that was collected on day 8 of exposure at the downstream site.

Macroinvertebrate Community Structure

A total of 548 aquatic macroinvertebrates were identified, comprising 20 taxa with *Taeniopteryx* sp. as the most abundant with a mean relative abundance of 20% (± 3%) of the total community. Reach, leaf type, and time significantly affected the macroinvertebrate community (PERMANOVA, $p<0.01$, Table S4.6, Figures 4.3-4.4) and genus richness (ANOVA, $p<0.05$, Table S4.7, Figure S4.4). Macroinvertebrate communities in the gap had significantly increased richness, with 2.0 (±0.2) more genera, compared to those upstream and downstream ($p<0.01$). Oak leaf packs had significantly less richness than ash, while buckthorn had significantly fewer genera than oak or ash, and ash having 2.0 (±0.3) more genera than buckthorn ($p<0.01$). Similar to richness, Inverse Simpson's diversity was two times higher in the gap, compared to upstream and downstream (ANOVA, $p<0.03$, Figure S4.3). Ash had the highest diversity, with oak being significantly lower than ash and buckthorn being significantly lower than both ash and oak (ANOVA, $p<0.01$).

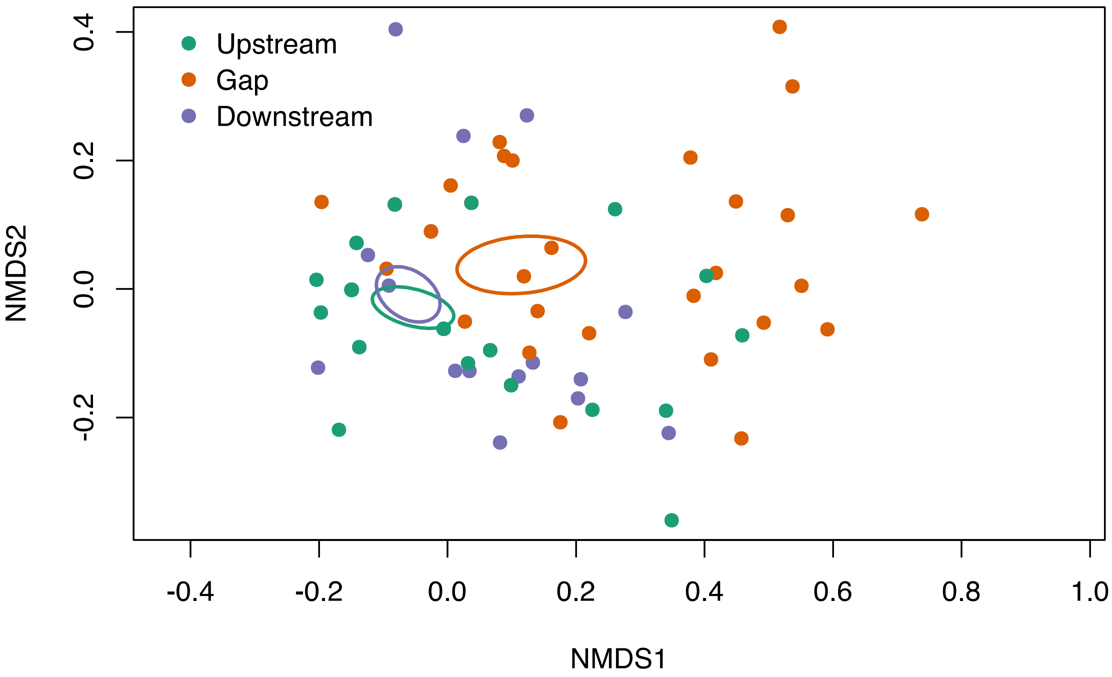


Figure 4.3. Nonmetric multidimensional scaling (NMDS) of macroinvertebrate (genus) communities based on the bray0 matrix for β-diversity. Communities from upstream sites are in green, gap sites are in orange, and downstream sites are in purple. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. n=108. Stress=0.12.

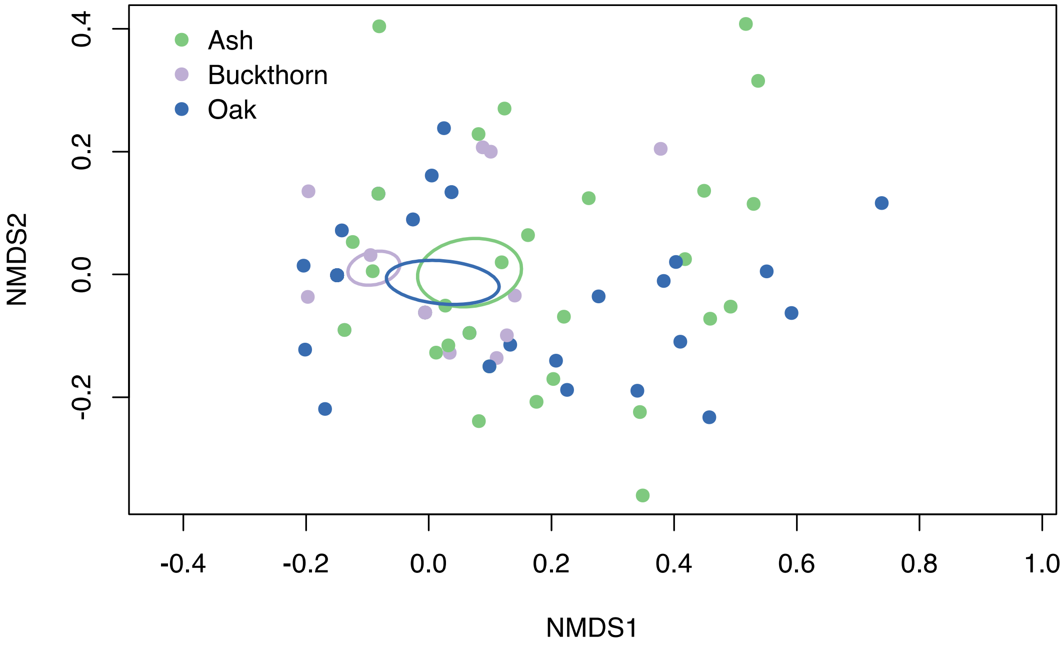


Figure 4.4. Nonmetric multidimensional scaling of macroinvertebrate (genus) communities based on the bray0 matrix for β-diversity. Communities from ash are in green, buckthorn are in purple, and oak are in blue. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. n=108. Stress=0.12.

Indicator species analysis showed Chironomidae, *Ephemerella* (Ephemerellidae, collector-gatherer), *Nemoura*, *Prosimulium*, and *Taeniopteryx* best indicated communities in the gap (Sidak's $p<0.02$, Table S4.6, Figures S4.4 -S4.7, 4.6-4.7) and had significantly greater abundance in the gap compared to upstream and downstream (ANOVA, $p<0.01$, Table S4.7). *Gammarus* (Gammaridae, shredder) and *Nemoura* (Nemouridae, shredder) indicated ash and *Taeniopteryx* (Taeniopterygidae, shredder) indicated buckthorn leaf packs (Sidak's $p<0.05$, Table S4.8). *Gammarus* was significantly more abundant in ash [0.69 (±0.36)] compared to buckthorn [0 (±0)] and oak [0.03 (±0.03)] (ANOVA, $p<0.01$, Figure 4.5, Table S4.9). Ash leaves had significantly greater abundance of *Nemoura* [1.9 (±0.7)] compared to buckthorn leaves [0.2 (±0.1), $p<0.01$, Figure 4.6). *Taeniopteryx* was more abundant in ash [1.8 (±0.4)] and oak [2.0 (±0.6)] leaves compared to buckthorn [0.3 (±0.1), ANOVA $p<0.01$].

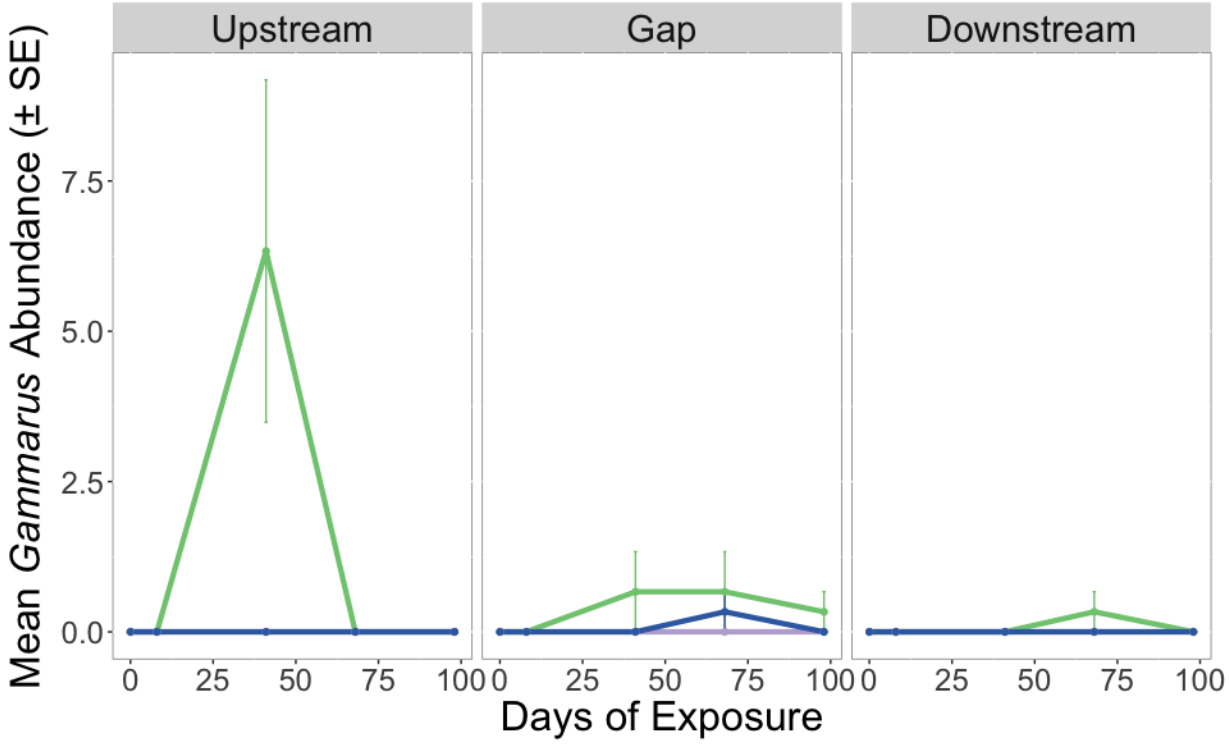


Figure 4.5. Mean (± standard error) *Gammarus* abundance in each leaf pack over time in upstream, gap, and downstream reaches for ash (green) buckthorn (purple), and oak (blue) leaf packs. n=108. Day 0=December 8th, 2018.

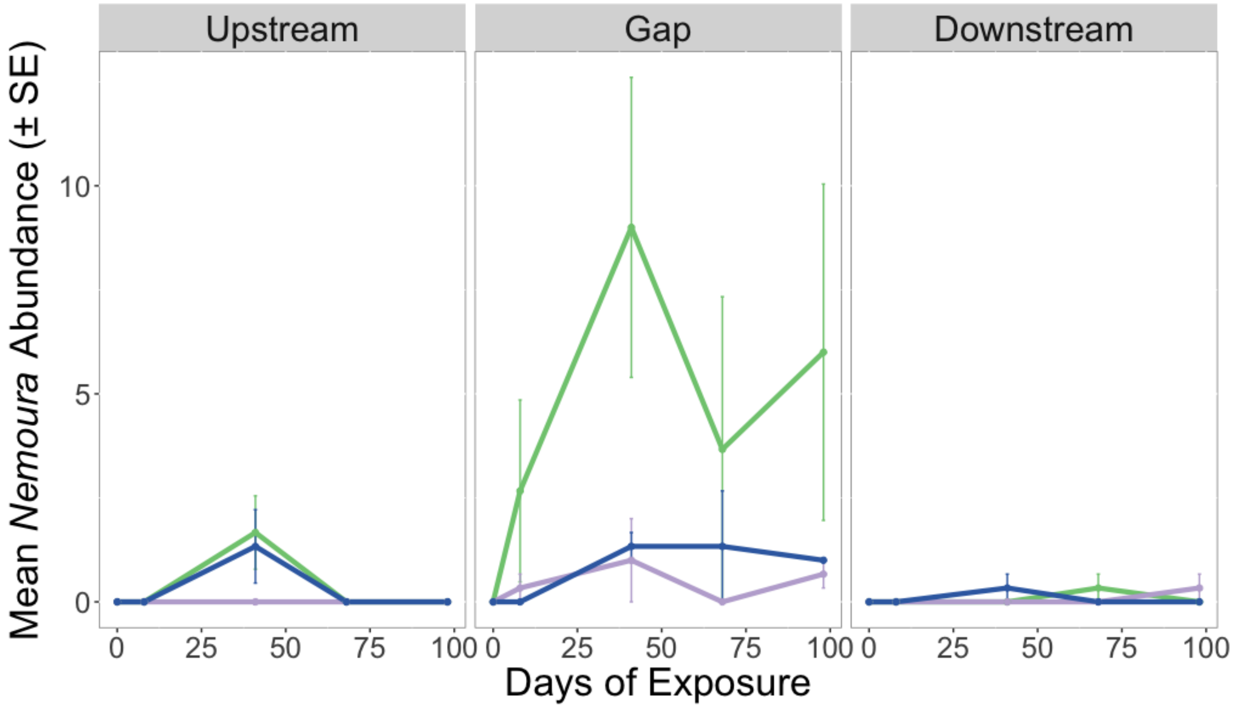


Figure 4.6. Mean (± standard error) *Nemoura* abundance over time in upstream, gap, and downstream reaches for ash (green) buckthorn (purple), and oak (blue) leaf packs. n=108. Day 0=December 8th, 2018.

Macroinvertebrate Community Function

Reach and leaf type significantly altered the functional feeding group structure of macroinvertebrate communities (PERMANOVA, $p<0.03$, Table S4.4, Figures 4.7-4.8, S4.11). Shredders were most representative of buckthorn leaf communities, whereas collector-filterers, collector-gatherers, and shredders indicated gap communities (Sidak's $p<0.01$, Table S4.6). Collector-gatherer, shredder, and grazer relative abundances did not vary by reach or leaf type (ANOVA, $p>0.05$, Table S4.8). Collector-gatherers were significantly more abundant in buckthorn leaves compared to ash at the downstream site on the second sampling day ($p=0.04$).

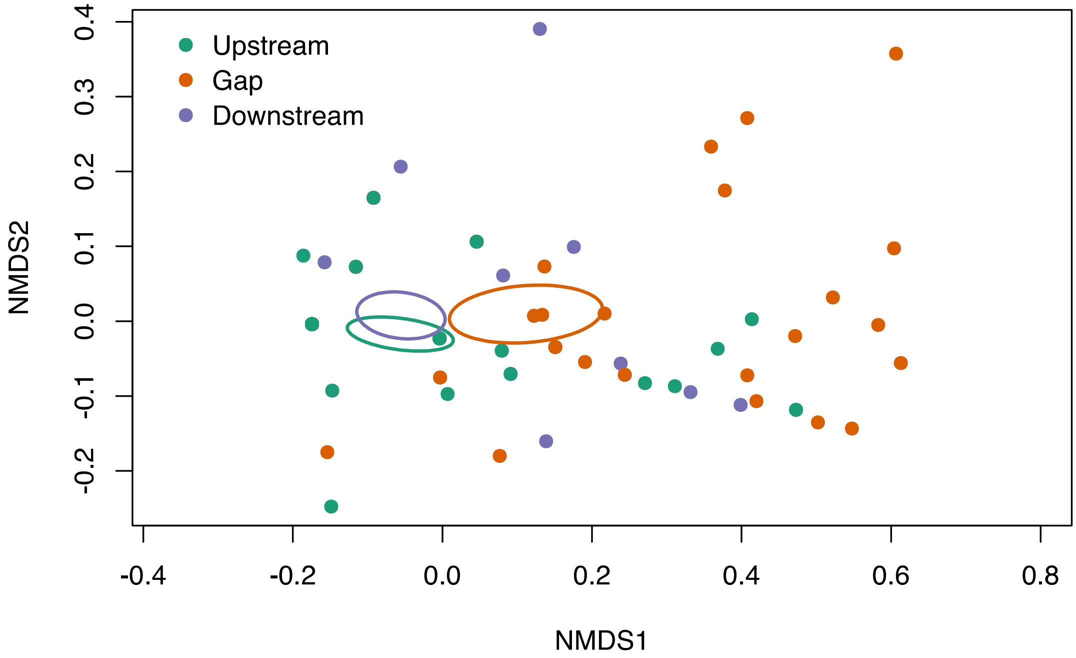


Figure 4.7. Nonmetric multidimensional scaling of macroinvertebrate functional feeding group communities based on the bray0 matrix for β-diversity. Communities from upstream sites are in green, gap sites are in orange, and downstream sites are in purple. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. Stress=0.08. n=108.

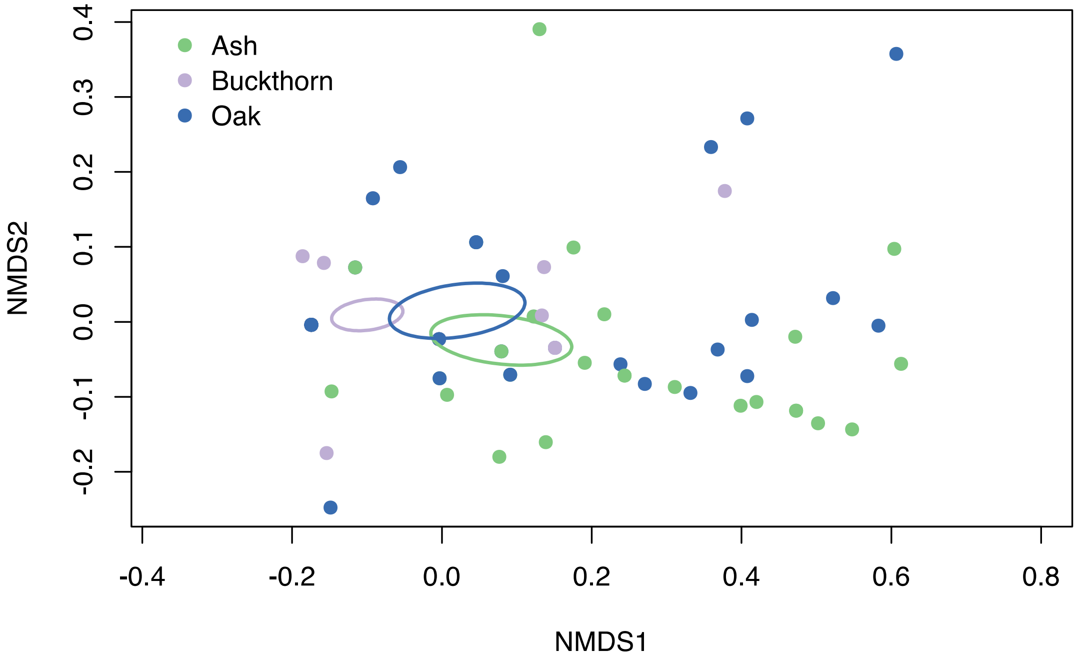


Figure 4.8. Nonmetric multidimensional scaling of macroinvertebrate functional feeding group communities based on the bray0 matrix for β-diversity. Communities from ash are in green, buckthorn are in purple, and oak are in blue. Ellipses represent 95% confidence interval. Points in greater proximity correspond to similar community composition. Stress=0.08. n=108.

Discussion

The aim of this study was to determine how forest disturbance due to an invasive insect (EAB) affected stream macroinvertebrate communities and ecosystem function through leaf litter decomposition. A confounding, but interesting, result was found with cotton strips used as an initial form of organic matter control, which decomposed much slower than the leaves. In fact, cotton strips gained mass over the course of the experiment in the upstream reach, rather than losing mass, as the other leaf types did. Based on personal observations, this was likely due to appreciable colonization and abundant biofilm growth. Tensile strength is a better measure of decomposition than mass loss (Tiegs et al., 2013); however, we needed to maintain sterile conditions to determine associated bacterial communities in a future associated manuscript evaluating leaf litter bacterial communities, which tensile strength equipment would not allow.

Chironomidae was an indicator taxon for cotton strips and was significantly more abundant in cotton strips compared to buckthorn leaves (Supplementary Appendix S1). We predict this was due to the physical structure of the cotton strips, which had ~1mm mesh weave that Chironomidae could use as habitat (we observed Chironomidae individuals in the weave). Yet previous publications on the cotton strip assay have not reported any significant invertebrate colonization or washed macroinvertebrates off prior to analysis (Tiegs et al., 2007; Goodman et al., 2010). Fritz et al. (2011) found higher macroinvertebrate richness and density in oak leaves compared to cellulose filters, which are similar material to cotton strips, but do not have a woven mesh structure. Although the cotton strip communities were less dense, rich, and diverse than those of leaf litter, there were macroinvertebrates present on the cotton strips that differed by gap location (upstream, gap, or downstream). Future studies using the cotton strip assay method may be able to use them as a macroinvertebrate or Chironomidae community standard, in addition to standardizing decomposition rates, because it is a standard habitat that can easily be compared among different stream types.

After cotton strip controls were removed from analyses, buckthorn was found to decompose significantly faster than oak and ash, supporting our hypothesis. Results similar to previous research on invasive leaf litter decomposition in streams; McNeish et al. (2012) found invasive *Lonicera maackii* had increased decomposition rates compared to native ash (*Fraxinus* spp.) leaves. Yet, our study did not find a difference in ash and oak leaf decomposition rates, which has been reported in many previous studies, including those done in Augusta Creek (Petersen and Cummins, 1974; Hanson et al., 1984). Oak and ash leaves have different lignin content (ash = 6%, oak = 24%); which is associated with slower breakdown rates (Royer and Minshall, 2001; Alonso et al., 2010; Hobbie et al., 2014). We had much greater variability in our leaf litter mass lost measurements, which may be driving our inability to detect differences. Our leaf packs spanned the stream from bank to bank, rather than staying in a particular depth, and this may have increased our variability. We also had flood pulses in December that fragmented several leaf samples. Using a leaf pack design that has leaf material contained in a litter bag would have avoided this problem, but we wanted to replicate the design of Petersen and Cummins 1974 in order to best make comparisons to pre-EAB invasion conditions. Cummins did not span Augusta Creek bank to bank, but rather placed leaf packs in a smaller area (personal communication), resulting in less error in their study. This experimental design may be why they had less overall variability and lower decomposition rates. Petersen and Cummins' (1974) experiment was conducted from November 20th 1960 to March 19th 1961, one month longer than the current study, which may also be why we did not find differences in oak and ash decomposition rates. Future analysis in a separate manuscript will compare abiotic results from the 1974 study to the current study.

We did not find evidence that leaf packs in gaps decomposed faster than those upstream or downstream. Again, this may be due to variability in leaf pack mass lost measurements, but also seasonality. Because this study was conducted after autumnal leaf fall (same season as when the Peterson and Cummins 1974 study was conducted), the differences in canopy cover between upstream, gap, and downstream sites were minimal. Replicating this study with pack introduction during full canopy would be an important step to evaluate how seasonality may be affecting our decomposition rates, or confirm no canopy gap effect of on decomposition rates (Robinson and Minshall, 1986). The evidence of this chapter and Chapter 3 that gap location did not have an effect on aquatic leaf litter decomposition rate, richness, and oak and ash relative mass in leaf packs suggests that leaf litter organic matter subsidies are not influenced by EAB-related canopy gaps. Although individual leaf taxa may have different decomposition rates (as expected, due to different leaf structure), this does not scale up towards changes in natural leaf pack composition in streams. Although previous work has speculated impactful shifts in aquatic leaf litter subsidies in response to EAB invasion (Nisbet et al., 2015; Kreutzweiser et al., 2018, 2020), our work in Chapters 3 and 4 demonstrates streams are resilient to this potential shift.

The macroinvertebrate communities residing on buckthorn were significantly less rich and diverse than ash, supporting our hypothesis that ash would have the greatest diversity and buckthorn would have the lowest. This pattern of greater diversity in ash leaves is particularly relevant to assessing EAB-impact on stream ecosystems, given the conservation impacts that ash leaves are no longer subsidized into the stream, with EAB-invasion and subsequent ash tree death. Without these leaves, our work shows there will be less diversity of macroinvertebrates supported in Augusta Creek, reinforcing previous conclusions when a leaf litter decomposition experiment in two Ontario streams, which demonstrated that macroinvertebrate richness and diversity were lower in leaf packs that did not contain ash (*Fraxinus* sp.) (Kreutzweiser et al., 2018). In combination, this is evidence that the pattern of higher diversity and richness in ash leaf litter is consistent among Great Lakes area streams, but further research in other areas of North America are needed to determine if this trend can be expanded to other stream types. We also postulated ash would have a greater proportion of large particle shredders, while oak and buckthorn would have a greater proportion of small particle collectors. The only functional group that differed significantly based on leaf type was collector-gatherers, and this was driven by Chironomidae abundance. This suggests the difference in functional feeding groups is driven by habitat structure, rather than food quality, because collector-gatherers would not be consuming the leaf material directly (Cummins and Klug, 1979).

Although collector-gatherers were the only FFG that responded as a whole to reach or leaf type, specific taxa exhibited significant responses. *Gammarus*, *Nemoura*, and *Taeniopteryx* (shredders) were more abundant on ash leaves compared buckthorn, supporting our hypothesis that ash leaves would have the most shredders, compared to invasive buckthorn leaves. This trend was particularly apparent in *Gammarus,* where no individuals were collected within buckthorn leaves. This reinforces the findings of Lewis et al.'s mesocosm experiment, where they found *Gammarus pseudolimnaeus* Bousfield (Amphipoda: Gammaridae) had higher mortality and lower body mass when fed a diet of common buckthorn leaves compared to green ash (2017b). In the current study and Lewis et al (2017) lower Gammarus on buckthorn can be attributed to its fast decomposition rate and potentially detrimental secondary compounds such as emodin (Seltzner, 2003). Peterson and Cummins (1974) in their Augusta Creek study also found the greatest abundance of shredders on ash leaves, compared to aspen, which they attributed to its faster decomposition rate. Aspen is considered a slow decomposing leaf, similar to oak (Peterson and

Cummins 1974). Other studies such as Alonso et al. (2010) did not find any difference in macroinvertebrate shredders in native oak and invasive [e.g. *Robinia pseudoacacia* (black locust)] leaves, suggesting this trend is taxa and context dependent (Sampaio et al., 2001). Interactions with the microbial (bacteria, fungi, microeukaryotes) community growing on the leaf litter may be important in mediating their fitness and consumption of the leaf material. For example, invasive *Hovenia dulcis* leaf packs had a faster decomposition rate and lower fungal beta diversity compared to a native leaf mixture in three streams in Brazil (Biasi et al., 2020). Future studies on the leaf litter microbial communities may give insight to this pattern.

We hypothesized the macroinvertebrate community upstream of the gap would have a greater proportion of shredders, while the community in gaps would have more grazers, and downstream would have more collectors. We did not find evidence that shredders or grazers were influenced by reach, but collector-gatherers were influenced by a reach by leaf type by time interaction. Yet, there was a consistent trend of gap having more abundance than upstream and downstream, and was exhibited by many macroinvertebrate response variables, including richness, diversity, and Chironomidae, *Ephemerella*, *Nemoura*, *Prosimulium*, and *Taeniopteryx* abundances. This response can be attributed to an increase in primary productivity, driven by increased light availability at the gap, as seen in streams experiencing riparian deforestation (Hawkins et al., 1982; Noel et al., 1986; Eveleens et al., 2019). We hypothesized that grazer abundance would be highest in this location, because of increased primary productivity and biofilm growth, but because we were not sampling benthic stones, we were not targeting that functional group. Shredders may have been consuming biofilm rather than leaf material; therefore, an increase in biofilm growth on leaves would increase shredder fitness and abundance (Franken et al., 2005). This may be why all indicator taxa indicated the gap reach; they were all positively impacted by the increase in light and productivity. This increase in abundance and diversity at the gap may only be temporary, as the canopy will re-form, decreasing light in the stream and productivity, and resulting in bottom up trophic restructuring (Stone and Wallace, 1998).

It is notable that this result of increased richness and diversity at the gap location is contrary to what was found in Chapter 3's survey of EAB-related canopy gaps throughout southern Michigan. In this survey, there was significantly lower macroinvertebrate richness in downstream sites, compared to upstream of the gap. We attributed this to lower dissolved oxygen levels in downstream sites. In the current experiment, we did not find significant differences in dissolved oxygen among the three gap locations. There are two conclusions that can be made from this difference in macroinvertebrate diversity results: 1. Stream responses to EAB-related canopy gaps are context dependent, and streams with different underlying chemistries respond differently to the riparian disturbance; and 2. Although the trend overall in southern Michigan streams was lowered diversity of macroinvertebrates downstream of EAB-related canopy gaps, certain streams, such as Augusta Creek, may be resilient to this disturbance and see no difference in upstream vs. downstream sites.

It is important to note the limitations of this study in both size and scope. Because our study was limited to one EAB-related canopy gap in one stream, it is difficult to expand results to a wider range of streams and across watersheds, where multiple gaps are occurring. To have confidence in the statistical power of the results, we would have liked to replicate the study across multiple gaps and multiple streams in the watershed. Another limitation of this study was the applicability to natural systems, where leaf litter species are naturally mixed in packs, rather than single species. Mixing leaf species has interacting effects on decomposition rates and macroinvertebrate colonization (Leroy and Marks, 2006; Lecerf et al., 2007), so mixed leaf species packs would be a better representation of natural systems.

Conclusions

Our findings suggest a functional and structural shift in communities in response to EAB by indirect routes of leaf litter subsidies and light availability. Without ash leaf litter in streams, less biodiversity was supported. A temporary increase in diversity at gap locations can be attributed to the increase in light, but over time this is anticipated to decrease as canopy growth occurs in areas of ash tree death. Because there were no downstream impacts detected, this suggests resilience in streams, which is especially important considering there are many gaps along stream lengths. Measuring long-term, successional changes in riparian and stream ecosystems in response to invasive species is necessary to understand full extent of effects on stream structure and function.

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