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The Effect of Plastic on Leaf Litter Breakdown in Urban Streams

Lisa Haneul Kim

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................... iii

LIST OF TABLES .................................................................................................................... viii

LIST OF FIGURES .................................................................................................................. ix

ABSTRACT ............................................................................................................................... xi

CHAPTER I: INTRODUCTION ................................................................................................ 1
Growing prevalence of global plastic use ................................................................. 1
Plastic pollution in marine environments ................................................................. 2
Emerging research on plastic pollution in streams ............................................... 3
Stream ecosystems transport and retain plastic litter ........................................ 4
Stream retain leaf litter: a key source of nutrients for food webs ..................... 8
Interactions between leaf and plastic litter in urban streams.............................. 9

CHAPTER II: THE EFFECT OF PLASTIC ON LEAF LITTER BREAKDOWN IN URBAN
STREAMS .................................................................................................................................. 12
Introduction ............................................................................................................................ 12
Methods ..................................................................................................................................... 15
Study sites ............................................................................................................................... 15
Leaf litter breakdown and macroinvertebrate community assessment ................ 15
Microbial community across treatments ............................................................... 17
Microbial community respiration rates ................................................................. 19
Physical and chemical measurements ................................................................. 20
Data analyses ....................................................................................................................... 21
Results ..................................................................................................................................... 23
Study site characteristics ............................................................................................... 23
Leaf litter breakdown ........................................................................................................... 23
Richness and diversity of bacteria, fungi, and algae ............................................. 28
Community composition of bacteria, fungi, and algae .......................................... 29
Macroinvertebrate community composition ......................................................... 39
Discussion ............................................................................................................................... 45
Plastic did not affect leaf litter breakdown rates ..................................................... 46
Microbial communities on plastic and leaf litter surfaces showed no interaction .. 45
Microbial degradation of plastic ............................................................................... 51
Macroinvertebrate communities were not distinct across treatment type, but varied over
time ......................................................................................................................................... 53
Study design and environmental plastic consideration for experiments ............ 55
Other common types of plastic litter may affect organisms and ecosystem processes .... 56
Conclusion .............................................................................................................................. 56

REFERENCE LIST .................................................................................................................. 58
LIST OF TABLES

Table 1. Site characteristics for 3 study sites in Chicago: 1) North Branch Chicago River, 2) Salt Creek, 3) McDonald Creek. Q=discharge, Cond=specific conductivity, Temp=temperature, NO₃⁻=nitrate, NH₄⁺=ammonium, and SRP=soluble reactive phosphorus........23

Table 2. Breakdown rate for leaf litter in litterbags with leaves alone or leaves mixed with plastic. The slope of the line is the breakdown rate (k; d⁻¹) of P. deltoides leaves measured as ash-free dry mass (AFDM) at each of the 3 sites. N. Br.=North Branch ............27

Table 3. Analysis of covariance (ANCOVA) to compare leaf litter (P. deltoides) breakdown rates measured as ash-free dry mass (AFDM) and dry mass from litterbags with leaves only and mixed treatments. We also compared change in dry mass of plastic litter from plastic alone and mixed litterbags. Site was the covariate. Significant values are listed in bold (p<0.05) ..........................................................25

Table 4. Two-way analysis of variance (ANOVA) to compare Shannon-diversity indices (H’) and number of operational taxonomic units (OTUs) across treatment and number of days of incubation. Significant values are listed in bold (p<0.05) ..........................................................32

Table 5. Results from linear mixed effects model (LME) analyses for macroinvertebrates treatment and number of days of incubation at all 3 study sites. Macroinvertebrates were characterized as the total number of macroinvertebrates per gram of dry mass (DM) and total number per bag. ‘Adjusted’ macroinvertebrates excludes hydrozoans and oligochaetes. Significant values were listed in bold (p<0.05). N. Br. Chi. R=North Branch Chicago River, Cr=creek ..........................................................42
LIST OF FIGURES

Figure 1. Relative composition of AL and net accumulation rates in the riparian zone of the North Branch of the Chicago River in Miami Woods (N=8). Continuous stream discharge is shown by the grey line (McCormick and Hoellein 2016) ............................................................... 5

Figure 2. A) A debris dam structure at an urban stream in Baltimore, MD. Plastic litter (yellow arrows) is mixed with organic material (orange arrows) on B) a stream bank and C) within a debris dam ........................................................................................................ 7

Figure 3. Illustration of our predictions where the macroinvertebrate and microbial community in experimental leaf packs. We expect A) the most diverse and highest in abundance in leaf only treatments, B) reduced in leaf and plastic mixed treatments, and C) the least diverse and lowest in abundance in plastic only treatments ................................................................. 10

Figure 4. Breakdown of P. deltoides leaves in leaf only and mixed treatments and plastic and plastic only and mixed treatments over a period of 164 days at each of the 3 sites measured by dry mass for: A) McDonald Creek (MC), B) North Branch Chicago River (NBC), and C) Salt Creek (SC). Change in ash-free dry mass (AFDM) of P. deltoides leaves over a period of 164 days at D) MC, E) NBC, and F) SC ........................................................................................................ 24

Figure 5. Mean (±SE) Shannon diversity index (H’) illustrated by (A) 16S rRNA gene abundance (bacteria), (B) 23S rRNA gene abundance (algae) communities, and (C) ITS region (fungi) by substrate type according to day of incubation. Mean (±SE) observed operational taxonomic units (OTUs) for (D) bacterial assemblages, (E) algal communities, and (F) fungal communities from all study sites. Small letters indicate significant differences among treatments on incubation date as shown by Tukey’s multiple comparison test, which was completed following a significant interaction between date and treatment ........................................................................................................... 31

Figure 6. Non-metric multi-dimensional scaling (nMDS) ordination of 16s sequencing data (Bray-Curtis similarity index) comparing bacterial assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek ......................................................................................................................... 33

Figure 7. A stacked bar graph of relative abundance of observed OTUs in bacterial communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom) ................................................................................................................................. 34

Figure 8. Non-metric multi-dimensional scaling (nMDS) ordination of 23s sequencing data (Bray-Curtis similarity index) comparing algal assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek.... 35
Figure 9. A stacked bar graph of relative abundance of observed OTUs in algal communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom)..........................36

Figure 10. Non-metric multi-dimensional scaling (nMDS) ordination of ITS sequencing data (Bray-Curtis similarity index) comparing fungal assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek..................................................................................................................37

Figure 11. A stacked bar graph of relative abundance of observed OTUs in fungal communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom).................................................................38

Figure 12. Mean (±SE) total number of macroinvertebrates per gram of dry mass during incubation in A) McDonald Creek (MC), B) North Branch Chicago River (NBC), and C) Salt Creek (SC). Hydrozoan and oligochaetes observations were omitted for 'adjusted' total number of macroinvertebrates at D) MC, E) NBC, and F) SC ......................................................43

Figure 13. Mean (±SE) total number of macroinvertebrates per bag during incubation in A) McDonald Creek (MC), B) North Branch Chicago River (NBC), and C) Salt Creek (SC). Hydrozoan and oligochaetes observations were omitted for 'adjusted' total number of macroinvertebrates at D) MC, E) NBC, and F) SC ......................................................43

Figure 14. Non-metric multi-dimensional scaling (nMDS) ordination of total macroinvertebrates (Bray-Curtis similarity index) comparing macroinvertebrate assemblages across 3 different treatments and days of incubation at all study streams: McDonald Creek, North Branch Chicago River, and Salt Creek.................................................................44

Figure 15. Mean (±SE) biofilm respiration on P.deltoides leaves from the leaf only treatment and mixed treatment, and plastic substrates from mixed treatment and plastic only treatments. Small letters indicate differences among substrates as shown by Tukey's multiple comparison test, done following significant 1-way ANOVA........................................45
ABSTRACT

The plastic component of anthropogenic litter (AL) is an emerging ecological concern and has been a focus of research, as it is long-lived, mobile, interacts with physical and chemical components of aquatic ecosystems, and breaks down into smaller pieces (i.e., microplastic, <5mm particles). Rivers are considered a major source of plastic to oceans, but little is known about plastic’s abundance, distribution, and effects on ecological processes in urban streams. Previous studies report plastic is abundant in freshwater systems, especially in naturally occurring debris dam structures and overhanging vegetation which accumulate high quantities of AL, especially plastic items (i.e. bags, wrappers, packaging material), along with leaf litter and other coarse particulate organic matter. In temperate, forested streams, leaf litter is a critical food source and plays an essential role in the stream food web. The rate at which leaves break down is affected by many variables such as hydrology, water chemistry, and macroinvertebrate and microbial communities. We predicted that plastic accumulation along with leaf litter can slow leaf breakdown by reducing diversity and abundance of microbial decomposers, as well as macroinvertebrate consumers. We measured leaf breakdown, and characterized macroinvertebrate and microbial (i.e., bacterial, fungal, algal) communities in 3 litter bag treatments: leaves alone, plastic alone, and leaves mixed with plastic. Although plastic did not reduce leaf breakdown rates or have a significant effect on macroinvertebrate consumer communities, it showed distinct microbial communities compared to leaf substrates.
CHAPTER I
INTRODUCTION

Growing prevalence of global plastic use

Since the invention of the first synthetic polymers in the early 1900s, plastic has become one of the most widely used synthetic materials in the world (Thompson et al., 2009). There is a diversity of synthetic polymers currently in production, facilitated by their versatility and adaptation to materials across the economic spectrum (Galgani et al., 2015). Plastic is integrated into almost all aspects of modern human life (Andrady and Neal, 2009). This includes personal items such as hygiene, food, and clothing, as well as critical global industries like electronics, construction, fisheries, and shipping. Geyer et al. (2017) estimated that since its initial industrialization in 1950, approximately 8300 million metric tons (Mt) of virgin plastics were produced through 2015. The abundance of plastic waste generated was 6300 Mt during this time interval, and estimates suggest that every person disposes an average of 52 kg of plastic every year (Worm et al., 2017).

Of all the plastic waste that has been generated since 2015, about 9% has been recycled and 79% has accumulated in landfills or the natural environment (Geyer, et al., 2017). Once in the environment, plastic polymers can persist for long time periods. Plastic is broken down by microbial activity, UV radiation, or physical abrasion (Wilcox et al., 2015). Some portion of plastic waste may remain permanently in the sediment record, however, suggesting plastic litter can serve as an effective stratigraphic marker for the Anthropocene Era (Zalasiewicz, et al.,
2019). The high rates of plastic disposal, the difficulty in removing it from the environment, and its slow degradation has created new challenges in waste management, and pushed the scientific, government, and private sectors to find ways to manage its negative impacts on aquatic environments and organisms (Rochman et al., 2013; Gregory 2009; Thompson et al., 2009).

**Plastic pollution in marine environments**

Much of the research on plastic pollution has been done in marine environments with an emphasis on quantifying its abundance and studying its distribution around the world. Recent efforts to quantify the global plastic budget have focused on measuring the movement of plastic from land into oceans. For example, there are nearly 200 million metric tons of plastic produced globally each year, and an estimated 10% of this plastic enters marine ecosystems (about 20 million Mt; Vannela 2012). Estimates from Jambeck et al. (2015) are similar, suggesting that of 275 million metric tons of plastic generated from 192 coastal countries, 4.8-12.7 million metric tons entered the ocean in 2010. While rivers are an important conduit of plastic to oceans, some amount of plastic is retained within rivers, and the plastic within rivers is an important part of the global plastic budget. To date, no studies have estimated the retention of plastic litter in freshwaters at a global scale.

Once in marine ecosystems, plastic can accumulate in ocean gyres, remain stranded on beaches, settle to the benthic zone, and interact with biota (Moore et al., 2001). Organisms that encounter plastic can be negatively impacted through entanglement and ingestion. For example, seabirds ingest plastic in high frequency, and it can cause gut blockages or organ damage from the transmission of toxic chemicals that leach out of plastic material (Wilcox et al., 2015). Microplastic (i.e., plastic particles <5 mm), are consumed by filter feeders, which can be
transferred to higher trophic levels (Eriksson and Burton, 2003; Moore, 2008). Negative impacts of plastic are not unique to marine habitats, and the same types of dynamics likely occur in freshwater ecosystems, although this is less widely studied (McNeish et al., 2018).

Emerging research on plastic pollution in streams

Rivers are considered a major source of plastic litter to marine environments, which originates from a variety of point and nonpoint sources on the landscape. Illegal dumping and littering are major sources of plastic litter into freshwater ecosystems (Williams and Simmons, 1999; McCormick and Hoellein, 2016). Plastic manufacturing facilities are a source of litter in the form of so-called ‘virgin’ plastics and other pre-production material which can enter the environment (Lechner and Ramler, 2015). Stormwater runoff, combined sewer overflows, and wastewater treatment plants also add plastic litter to the environment (Horton et al., 2017; Windsor et al., 2018). Finally, atmospheric deposition is a source of plastic to aquatic ecosystems. Dris et al. (2015) found that 90% of the microplastic found in atmospheric fallout samples collected in France were fibers, which are also common in soils and rivers (Rillig et al., 2017; Barrows et al., 2018). It is likely that industrial laundering facilities expel microfibers into the atmosphere, which ends up in our waters (GESAMP 2016).

Plastic litter interacts with living organisms in freshwaters, such as microbial biofilms, macroinvertebrates, and fish. McCormick et al. (2014) found significantly higher colonization of wastewater-associated bacteria on microplastic (<5mm) surfaces than non-plastic samples, which suggests plastic could act as novel a vector for transporting disease-causing bacteria in rivers. Aquatic macroinvertebrates may consume plastic or use it for habitat (Windsor et al., 2018). Caddisflies (Trichoptera), that build cases from stream substrates such as organic matter, sand,
and rocks, have been found to incorporate plastic into their cases (Ehlers et al., 2019). Fish also consume plastic, and microplastic abundance may increase with trophic position of fish. For example, zoobenthivores, such as round gobies, were found to have a higher abundance of microplastic in their gut contents than omnivores and detritivores (McNeish et al., 2018). In two agricultural reservoirs in Illinois, largemouth bass, a predatory fish, had 3.2-fold higher concentrations of microplastic than gizzard shad, a filter feeder and a food source to many sportfish (Perry et al., 2020). Understanding the interactions between plastic and riverine biota is a key component of documenting the ecosystem effects for plastic pollution. More research is needed to quantify its effect on organisms’ physiology, food webs, and ecosystem processes (Singh et al., 2018).

Stream ecosystems transport and retain plastic litter

While some of the microplastic and macroplastic that enters streams can be transported to marine environments, some portion will be retained in sediment, riparian vegetation, or naturally occurring debris dams along the river continuum (Hoellein et al., 2019; Williams and Simmons, 1997; Williams and Simmons, 1999). Retention and transport dynamics are well studied for naturally occurring allochthonous material such as wood, leaf litter, and fine particulate organic matter (Webster et al., 1999; Tank et al., 2008). Variability in water velocity and discharge (i.e., flooding and drying periods) redistributes coarse and fine particulates to different habitats in streams. For example, storms and high floods can dislodge leaf litter and sticks entrapped in riparian vegetation, the particles are moved, and are eventually retained further out from the water's edge or some distance downstream (McCormick and Hoellein, 2016). This process of particle transport is referred to as ‘spiraling’ (Newbold et al., 1982; Griffiths et al., 2012).
Macro- and microplastic litter most likely moves downstream in the same fashion as naturally occurring allochthonous particles, but this is not yet well studied (Hoellein et al., 2019).

Measurements of macroscopic plastic distribution in streams suggest that it co-accumulates with leaf litter. McCormick and Hoellein (2016) quantified density of anthropogenic litter (AL; trash) in urban streams in the Chicago region. The authors assessed the relative abundance of plastic, the spatial distribution of litter, and the dynamic nature of litter composition. First, McCormick and Hoellein (2016) found plastic made up a large proportion of total AL in the benthic and riparian habitats of urban streams. Plastic and styrofoam made up 44-48.7% and 2.6-5.6%, respectively, of the total litter abundance in the North Branch Chicago River over a period of several months (Figure 1). The authors observed that the plastic litter was
trapped in vegetation and debris dams. Finally, McCormick and Hoellein (2016) found that litter composition was highly variable through time in the same stream reach. Lighter litter (i.e., plastic food wrappers, paper) remained in the riparian habitat during flood periods while heavier material accumulated in benthic habitats. These patterns are most likely due to the complexity of the physical structure of the material (McCormick and Hoellein 2016). The lighter plastic material was more pliable and more likely to be retained in riparian vegetation. These patterns were corroborated by a study on riverine litter in South Wales, UK, where litter movement was mainly controlled by vegetation (i.e. overhanging branches) producing a ‘Christmas tree effect’ of litter entanglement and obstructions along the watercourse (Williams, 1997; Williams and Simmons, 1999). Similarly, Williams and Simmons (1997) noted the various fates of riverine litter as burial or stranding on river bank vegetation. Together, these measurements and observations suggest that plastic litter is retained and moved in streams in a similar fashion as coarse benthic organic matter such as leaf litter. However, interactions between the two substrates have not yet been measured.
Figure 2. A) A debris dam structure at an urban stream in Baltimore, MD. Plastic litter (yellow arrows) is mixed with organic material (orange arrows) on B) a stream bank and C) within a debris dam.
Streams retain leaf litter: a key source of nutrients for food webs

Organic matter inputs from the terrestrial environment (i.e., allochthonous material) represent critical food resources for stream biota (Webster et al., 1999). Leaf litter from surrounding trees and riparian vegetation that is retained in streams is broken down and consumed by macroinvertebrates and microbial communities. In relatively pristine streams, specialized shredding invertebrates break leaf litter into smaller pieces. For example, *Lepidostoma* sp., a common shredder, break down coarse benthic organic matter and use stable substrates as pupation sites (Tank et al., 2008). The family Capniidae, which are winter stoneflies, also relies on organic matter, such as leaves, as their main food resource in forested streams of the Upper Midwest (Entrekin et al., 2008; Reice, 1980). In urban streams with lower invertebrate taxonomic diversity, generalists such as isopods and amphipods increased leaf breakdown rates (Cook and Hoellein, 2016). The microbial organisms (i.e., fungi and bacteria) which contribute to leaf breakdown rates are affected by physical and chemical factors including water chemistry and the structure of the organic matter compounds within the leaf tissue (Tank et al., 2010; Cook and Hoellein, 2016). For example, Robinson and Gessner (2000) found that increasing concentrations of phosphorus and nitrogen in leaf packs of an alpine spring significantly increased leaf breakdown rates. Other factors, such as high litter species richness can increase breakdown rates as well (Kominoski et al., 2007). The energy and nutrients consumed by microbes and macroinvertebrates during leaf litter breakdown are a foundational component of stream food webs.

The rate of breakdown for leaf litter is an important process in streams, and has been used as a management tool to assess the health of stream ecosystems. Because leaf breakdown
integrates many environmental factors simultaneously (i.e., action of microbes, invertebrates, and physio-chemical conditions), it can be used to compare environmental conditions among streams. For example, Young et al. (2008) noted the advantages of using leaf breakdown rates as a way to compare river health in reference and impacted ecosystems since it links characteristics of riparian vegetation, activities of macroinvertebrate and microbial communities, and is affected by natural and anthropogenic stressors. Ferreira et al. (2017) found that leaf breakdown rates were significantly inhibited and remained consistent across leaf species and litter bag mesh sizes in acidified streams, which indicated that it was an effective assessment tool to measure acidification effects on stream ecosystem health. Although studies show that natural and anthropogenic stressors affect leaf breakdown rates, it is possible that plastic litter could affect litter breakdown as well. However, the interaction between plastic litter and leaf litter in streams, and the potential effects on breakdown rates, microbial biofilms, and macroinvertebrates, have not previously been measured.

*Interactions between leaf and plastic litter in urban streams*

The retention of plastic and leaf litter in debris dams in urban streams suggests that plastic may affect the organisms and ecosystem processes that occur in leaf packs. Debris dams with woody debris and leaf litter are hot spots of biological activity (Bilby and Likens, 1980), so interruptions in naturally occurring dynamics could have important implications for urban streams in general. The goal of this research is to measure how plastic litter may affect leaf litter breakdown by quantifying 1) breakdown rates, 2) macroinvertebrates, and 3) microbial communities. To do this, we used the traditional ‘litter bag’ method with 3 treatments: leaves
alone, plastic alone, and leaves and plastic mixed. We deployed the experiment at 3 streams in the Chicago metropolitan area: North Branch Chicago River, McDonald Creek, and Salt Creek.

Overall, we anticipated that the close interaction of leaf and plastic litter in the mixed treatment, which is frequently observed in urban streams, would cause a 'smothering' effect on leaf biofilms that would reduce oxygen and water flow within the leaf pack. Therefore, we expected slower breakdown rates, reduced microbial respiration, and lower abundance and diversity of macroinvertebrate and microbial communities in the leaf-plastic mixed treatments compared to the leaf alone treatment. In addition, we expected the plastic only treatment would show the lowest macroinvertebrate and microbial abundance and diversity compared to the leaf and leaf-plastic mixed treatments, as the plastic treatment had an absence of leaves as a food resource.

Figure 3. Illustration of our predictions for the macroinvertebrate and microbial communities in experimental leaf packs. We expect A) the most diverse and highest in abundance in leaf only treatments, B) reduced in leaf and plastic mixed treatments, and C) the least diverse and lowest in abundance in plastic only treatments.

This research will quantify the capacity of plastic litter to alter the critical ecosystem process of leaf breakdown rates in urban streams. Results will provide new insight into basic structure and function of urban streams, and will inform strategies for urban stream management. While rivers are a key source of plastic to marine ecosystems (Ryan et al., 2009), they are also
important sites of plastic retention and transformation, and thereby plastic litter is likely to have an impact on biological processes in urban streams. This work will contribute a novel and robust assessment of plastic ecology within urban rivers to inform a greater understanding of plastic fate at a global scale.
CHAPTER II

THE EFFECT OF PLASTIC ON LEAF LITTER BREAKDOWN IN URBAN STREAMS

Introduction

The abundance, fate, and ecological impacts of anthropogenic litter (AL; trash such as plastic, metal, and other refuse) have been well documented in marine ecosystems worldwide (Thompson, 2009). The plastic component of AL has been a focus of research, as plastic is persistent, mobile, breaks down into smaller pieces (i.e., microplastic, <5mm), and interacts with physical, chemical, and biological components of aquatic ecosystems (Arthur et al., 2009). Plastic, in macroscopic or microscopic form, can create a novel risk to aquatic invertebrates and fish through ingestion and entanglement (Moore, 2008, McNeish et al., 2018). It also has the potential to act as a vector for the transport of pathogenic microorganisms (McCormick et al., 2014). In addition, leaching of chemicals such as phthalates from plastic may impact fish development and reproduction (Oehlmann et al., 2009).

Despite a rapidly growing field of research in marine AL, studies on AL abundance, movement, and biological interactions in streams are less common. Rivers are often considered a dominant source of plastic to marine ecosystems (Ryan et al., 2009). Thus, robust assessments of the ecology of plastic within urban rivers is needed to inform a greater understanding of its fate at a global scale (Hoellein et al., 2014, McCormick and Hoellein, 2016). Recent research on AL in urban streams has documented its overall density and composition, but has not yet examined the impact of plastic AL on basic stream ecosystem processes (i.e., nutrient and carbon cycling)
or biological communities. For example, plastic was more abundant than other AL material types, such as glass, rubber, and ceramic, in benthic and riparian habitats of urban streams (McCormick and Hoellein, 2016), especially in debris dams where leaf litter and fine sediment also accumulate (L. Kim, unpublished data). In streams with forested riparian zones, leaf litter is a critical food source for many organisms and plays an essential role in the stream food web (Tank et al., 2010, Paul et al., 2006, Meyer et al., 1998). The rate at which leaves break down is affected by many variables such as hydrology, water chemistry, and animal consumers (Tank et al., 2010). While leaf breakdown in urban streams can be affected by elevated nutrient concentrations and reduced diversity and abundance of macroinvertebrates and biofilm taxa (i.e., bacteria, algae, and fungi) (Walsh et al., 2005, Paul and Meyer, 2001, Wenger et al., 2009), breakdown rates may also be affected by intermixing of plastic with leaf litter. However, the effect of plastic and leaf mixtures on leaf breakdown rates and the influence on macroinvertebrates and microbial biofilms that carry out leaf breakdown have not previously been measured.

Microbial biofilms are able to form on any submerged surface in aquatic environments (Battin et al., 2016). Factors such as the chemistry of the substrate surface and the physical conditions of the stream affect the growth and succession of microbial biofilms (Battin et al., 2016, Lyautey et al., 2005, Bridier et al., 2017). This includes organic substrates (i.e., fine sediments, leaf litter, and wood), which have unique microbial community composition compared to plastic litter. Plastic substrates provide a novel form of recalcitrant allochthonous carbon to streams and support distinct microbial communities (McCormick et al., 2014, Hoellein et al., 2014). Leaf litter and plastic litter accumulate together in urban streams at debris dams and
backwaters, so co-mingling of plastic and leaf surfaces may influence microbial biofilm community composition or activity on either material through changes in redox conditions and water flow, but this has not been measured.

Macroinvertebrates, along with microbial biofilms, play a crucial role in leaf litter breakdown through fragmentation and consumption. Previous work in the urbanized North Branch of the Chicago River showed that abundance of macroinvertebrates, such as isopods and amphipods, were positively correlated with faster leaf breakdown rates (Cook and Hoellein, 2016). Leaf litter is a critical food resource for macroinvertebrates that digest both the leaf tissue and the associated microbial biofilms (France, 2011; Cummins, 1974). In contrast, while plastic litter supports biofilm growth on its surface which could be consumed by invertebrates, the plastic itself is unpalatable and indigestible. Because plastic is found with leaf litter and other coarse particulate organic matter in urban streams (McCormick and Hoellein, 2016), it may slow leaf breakdown by reducing access to macroinvertebrate consumers. However, no previous research has studied the effect of plastic on leaf litter breakdown rates or pathways.

The goal of this research is to determine whether plastic litter may affect leaf litter decomposition rates, and associated microbial and macroinvertebrate communities in urban streams. To measure the effect of plastic on leaf litter breakdown, we used the litter bag method with 3 treatments at each study site: leaves alone, plastic alone, and leaves and plastic mixed. Overall, we anticipated that the close interaction of leaf and plastic litter in the mixed treatment, which is frequently observed in urban streams, would cause a 'smothering' effect on leaf biofilms that would reduce oxygen and water flow within the leaf pack. Therefore, we expected slower breakdown rates, reduced microbial respiration, and lower abundance and diversity of
macroinvertebrate and microbial communities in the leaf-plastic mixed treatments compared to the leaf alone treatment. In addition, we expected mixed litter bags would show higher macroinvertebrate and microbial abundance and diversity compared to the plastic only treatment, as the latter has an absence of leaves as a food resource.

**Methods**

*Study Sites*

We measured the effects of plastic on leaf litter breakdown in 3 streams in northeastern Illinois, USA: North Branch Chicago River, Salt Creek, and McDonald Creek. All three sites are located in areas of high population density and are characterized by high nutrient levels and low macroinvertebrate densities, which are typical of urban streams (Turek and Hoellein, 2015, Cook and Hoellein, 2015, McCormick and Hoellein, 2016). We obtained research permits from the Forest Preserve of Cook County.

*Leaf litter breakdown and macroinvertebrate community assessment*

Three treatments were generated for this experiment: leaf only, plastic only, and leaves and plastic mixed. We selected eastern cottonwood (*Populus deltoides*) leaves to measure leaf litter breakdown rates as it is a common riparian species at our field sites (Friends of the Forest Preserves & Friends of the Parks, 2002). We used low-density polyethylene for the plastic treatment as it was commonly encountered in urban streams in the region (McCormick and Hoellein, 2016). In October 2016, we collected naturally senesced *P. deltoides* leaves and air-dried them for ~9 days in a greenhouse. We purchased a blue 2.8 x 3.7 m polyethylene plastic sheet (i.e., a drop cloth for painting; thickness ~ 0.15 mm), and cut triangular plastic shapes that were similar in surface area as the *P. deltoides* leaves (55 cm²). For the leaf only treatments, we
placed 8 g of *P. deltoides* leaves (~11 individual leaves) into each litter bag. For plastic only treatment, we placed 11 of the plastic ‘leaves’ into each bag. We considered these treatments directly comparable as they contained approximately equal surface area, which was the relevant factor for comparing the microbial and macroinvertebrate communities, rather than including the same mass of material in the leaf and plastic alone litter bags (i.e., due to different densities of plastic and leaf litter). The mixed treatment bags had 8 g of *P. deltoides* leaves alternately stacked with 11 plastic ‘leaves’. We acknowledge that the mixed leaf bag had more total surface area than either of the individual treatment bags. However, our objective was to directly compare the leaf breakdown rate of leaves in the leaf alone and mixed litter bags, so we considered it imperative that the starting leaf litter mass was identical between treatments. At each stream, we arranged 99 litter bags (N=33/treatment) randomly in strings of 6 (N=2/treatment) with each bag attached together by plastic cable ties. The strings were placed in areas with unobstructed water flow and secured to the streambed using rebar at each of the 3 sites on the following dates: October 31, 2016 (Salt Creek), November 6, 2016 (North Branch of Chicago River), November 11, 2016 (McDonald Creek). We transported 6 additional bags (N=2/treatment) into the field when all litter bags were deployed at each site and returned them directly to the laboratory to account for handling loss (Benfield, 2006; Cook and Hoellein, 2015).

After deployment of the litter bags, 18 bags (N=6/treatment) were removed from each site on each of 5 collection dates over autumn, winter, and spring of 2016-2017 (Table 1). Upon its removal from the stream, each litter bag was immediately placed into a plastic zip-top bag and stored in the laboratory at 4°C. We processed 9 of the 18 bags collected for leaf litter breakdown (i.e., mass remaining) and macroinvertebrate community assessment and the other 9 bags were
processed for microbial community composition. Litter bags designated for macroinvertebrate community assessment were refrigerated and processed within 3-4 days of collection and those used for microbial community assessment was processed within 24 hours.

To measure mass remaining and macroinvertebrates, we removed the leaves and gently rinsed each leaf of debris and macroinvertebrates with deionized water into white collection trays. All macroinvertebrates were sorted and preserved in 70% ethanol. Preserved macroinvertebrates were later counted and identified to family level using a dissecting microscope. Each leaf from each treatment, along with all visible leaf particles from inside the litter bag and collection trays, was placed in brown paper bags and into a drying oven at 70°C. After 3-4 days drying, we transferred leaves into pre-ashed and weighed pans and measured dry mass of the leaf tissue. Then, we moved *P. deltoides* leaves into a stone mortar and ground the leaves to facilitate combustion for ash-free dry mass (AFDM). All leaf particles were transferred from the brown paper bags to the pre-ashed and weighed aluminum pans and placed in a muffle furnace at 550 °C for 5 hours. They were then removed and cooled in a desiccator for at least 2 hours before weighing and measuring AFDM. Plastic ‘leaves’ were transferred to pre-ashed and weighed aluminum pans (Thermo Fisher Scientific Inc., Millville, NJ, U.S.A.) to measure dry mass only (i.e., the plastic was not combusted). To calculate the breakdown rate (k), we used linear regression between the relative amount of dry mass or AFDM remaining in each treatment (ln transformed) over time (days) (Benfield, 2006; Cook and Hoellein 2015). We also examined the temporal pattern for the dry mass of plastic ‘leaves’.
Microbial communities across treatments

We processed the remaining 9 of the 18 litter bags within 24 hours for bacterial, fungal, and algal community composition on the *P. deltoides* leaves and plastic ‘leaves’, on the 3rd and 5th collection date at each stream. The contents of each litter bag were gently removed, rinsed of debris and macroinvertebrates with deionized water, and placed into a sterilized collection tray. One *P. deltoides* leaf was randomly chosen from each of the 3 leaf only and 3 mixed bags, and a 4 x 4 cm square was cut using a sterilized plastic square stencil. This step was repeated for plastic ‘leaves’ from 3 plastic only and 3 mixed treatment litter bags. All samples were placed in separate sterile 2 mL screwcap tubes and frozen until processing. For DNA extraction, each 4 x 4 cm sample was removed and separated into 4 quadrants. Using a random number generator, we chose one quadrant to cut with a sterilized blade before being placed into 2 mL microcentrifuge tubes. DNA was extracted using a DNeasy PowerSoil kit (Qiagen, Venlo, Netherlands). PCR amplification was performed using primers 515F and 806R to amplify the V4 hypervariable region of bacterial and archael 16s rRNA genes (Caporaso et al., 2011), primers 23SrV_f1 and 23SrV_r2 to amplify plastid 23s rRNA genes for algae (Sherwood and Presting, 2006; Steven et al., 2012), and primers ITS1f-ITS2 to amplify ITS1 and ITS2 regions of fungal ribosomal DNA (Gardes and Bruns, 1993; White et al., 1990). We used agarose gel electrophoresis to confirm successful DNA isolation and amplification for all samples.

We sent amplified samples to be sequenced by the DNA Services Facility, University of Illinois at Chicago using the Illumina MiSeq Platform (Caporaso et al., 2012). MOTHUR v.1.40.4 was used as explained by Schloss and Westcott (2011) and Kozich et al. (2013) to process sequences. We assembled and demultiplexed paired reads and removed any sequences
with ambiguities or homopolymers. Bacterial sequences were aligned using the SILVA small subunit alignment database (updated 2017-12-13), algal sequences were aligned using SILVA large subunit database (updated 2017-12-13), while fungal sequences were aligned using the UNITE ITS reference (updated 2017-12-01). Bacterial sequences were trimmed to a uniform length of 310 base pairs, algal sequences were trimmed to 410 base pairs, and fungal sequences trimmed to 314 base pairs. We removed chimeric sequences using Uchime (Edgar et al., 2011). We then classified bacterial sequences using the MOTHUR-formatted version of the 16s rRNA reference training set (v.9), the SILVA 23s large subunit reference database (v.132) for algal sequences, and the UNITE ITS reference database for fungal sequences. Any sequences not identified as bacterial, algal, or fungal respectively were removed. We clustered all sequences into operational taxonomic units (OTUs) based on 97% sequence identity. The bacterial dataset was randomly subsampled to 10,901 sequences per sample, the algal dataset was randomly subsampled to 6,927 sequences per sample, and the fungal dataset was randomly subsampled to 1,948 sequences per sample. This was done to avoid biases associated with uneven numbers of sequences across samples.

Microbial community respiration rates

We assessed community respiration on *P. deltoides* leaves from the leaf only and mixed treatments, and on the plastic ‘leaves’ from the plastic only and mixed treatments on the last collection date (~135-164 days) for each stream. Similar to the method for DNA processing, each sample was processed within 24 hours of collection and a 4 x 4 cm square was cut from each sample using a sterilized plastic square stencil. We used chamber incubation in the light and dark to measure net ecosystem production (NEP) and community respiration (CR) (Hoellein et
al., 2014). We equilibrated water collected from the study streams to lab conditions for ~1 hour in a 20 L bucket and measured dissolved oxygen (DO) concentration using a portable DO meter (DO HQ40d portable meter, Hach). We then placed each 4 x 4 cm square sample into individual 160 mL specimen containers (N=12) and carefully submerged and capped each container in the 20 L bucket to prevent air bubbles. We filled another set of 160 mL specimen containers (N=12) using the same method and wrapped them in foil to eliminate any penetration of light. All samples were placed in an environmental chamber set at 20°C and incubated for 3 hours with constant illumination. Each specimen container was then individually uncapped and each sample was carefully removed with forceps. We noted the start and end time of incubation for each sample and measured DO. We calculated community respiration by using the net change in DO concentration for the samples covered in foil. Net ecosystem production (NEP), which were the illuminated samples, and gross primary production (GPP) was calculated by difference (Bott, 2007).

**Physical and chemical measurements**

We measured several physiochemical parameters in each stream on each collection date, including the day we deployed all litter bags. We measured discharge by suspending a meter tape across the stream and measuring depth and velocity at 1-2 m intervals along the tape (Marsh-McBirney Flo-Mate 2000® Portable Velocity Flow Meter (Hach Company, Loveland, CO, U.S.A.). Discharge was calculated for each interval and summed across intervals. We measured conductivity and temperature at a single location in the thalweg of each stream (YSI Model 30 conductivity probe, YSI Incorporated, Yellow Springs, OH, U.S.A.). We collected and filtered 3 water samples using a glass microfiber filter (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) into
separate 20 mL, acid-washed Wheaton plastic scintillation vials (Thermo Fisher Scientific Inc., Millville, NJ, U.S.A.). All water samples were frozen until analyzed for inorganic nutrient concentrations using an AutoAnalyzer 3 (Seal Analytical, Inc., Mequon, WI, U.S.A.). We used the antimonyl tartrate technique (Murphy and Riley, 1962) to measure soluble reactive phosphorus (SRP), the phenol hypochlorite technique to measure ammonium (NH$_4^+$) (Solorazano 1969), and the cadmium reduction technique to measure nitrate (NO$_3^-$) (APHA1998).

Data analysis

We used analysis of covariance (ANCOVA) to compare the breakdown rate measured as AFDM of *P. deltoides* leaves between leaf only and mixed treatments where sites were the covariate. The same analysis was used to compare breakdown rate by dry mass of *P. deltoides* between treatments. We completed a final ANCOVA to determine if dry mass of plastic ‘leaves’ was significantly different between plastic alone and mixed treatments. A natural log transformation was applied to the leaf litter breakdown data measured as AFDM and dry mass to meet the normality assumptions.

We assessed biofilm diversity by calculating the observed number of OTUs (i.e., 'species' richness) and Shannon-Weiner (H’) indices. A 2-way ANOVA was used to assess the effects of treatment type and days of incubation on the H’ index and the number of OTUs. If there was a significant interaction of the 2-way ANOVA between treatment type and time, we conducted a 1-way ANOVA on each collection day alone (day 3, or a mean of 43 days of incubation across the 3 streams, and day 5, or a mean of 150 days of incubation across the 3 streams) using a Bonferroni-corrected p-value of 0.05/2=0.025. Then, we used Tukey’s multiple comparison test to compare among treatments. Data were assessed for normality and equal variance of the
residuals assumptions of the 2-way and 1-way ANOVA, and no transformations were required. We compared the assemblage of bacteria, algae, and fungi on *P. deltoides* leaves and plastic ‘leaves’ across all treatments and days of incubation by calculating the Bray-Curtis similarity index. The resulting distance matrix was visualized using a non-metric multidimensional scaling (nMDS) ordination that was run on R Studio. We used permutational multivariate analysis of variance based on Bray-Curtis indices. Finally, we generated stacked bar graphs using the relative abundance of OTUs across treatment type and days of incubation for all microbial data. This allowed us to visualize relative abundances of taxonomic groups of bacteria, algae, or fungi by substrate type at different time points.

We used a linear mixed effects model (LME) to quantify patterns in total macroinvertebrate abundance per g of DM and per litter bag across treatments and number of days of incubation. We applied a natural log transformation to meet the assumptions of LME. The same statistical analysis was used to assess for differences in macroinvertebrate abundance excluding hydrozoans and oligochaetes (i.e., adjusted macroinvertebrates). These macroinvertebrates were removed because of their high abundance compared to other macroinvertebrates that were identified. To compare differences across macroinvertebrate communities in treatment type over the number of incubation days at all 3 study sites, we used 2-way ANOVA, Bray-Curtis similarity index, nMDS, and PERMANOVA as described above for the biofilm communities. When the variables were found to be significant (p<0.05), they were plotted on the nMDS as vectors. The length of the vector was scaled to the strength of correlation.
Last, we used 1-way ANOVA to assess for statistical differences in respiration rates among substrate types across treatments on the final day of incubation (~150 days) at each site. A Tukey’s multiple comparison test was completed to determine differences among substrates. No transformations were used as data met the normality and equal variance of the residuals assumptions.

All statistics were completed using the ‘base’ package in the R version 1.1.447 unless otherwise stated. ANCOVA, 2-way ANOVA, 1-way ANOVA analyses were run on the R statistical program using aov(). The Tukey’s multiple comparison test, LME, and PERMANOVA were run using the same program with the following: tukeyHSD(), aov(lme()), and adonis(), respectively. The Shapiro-Wilk test and Levene’s Test were used to check for normality and equal variance of the residuals assumptions, respectively, using shapiro.test and leveneTest(). Line plots and stack bar graphs were generated using ggplot(). We used the envfit function found in vegan package of R Studio to assess for correlations between macroinvertebrate taxa and community distance matrices.

Results

Study site characteristics

Physical and chemical characteristics of the study streams were typical of urban conditions, but showed differences among sites. Nutrient concentrations and conductivity were generally elevated, although variable across all three study sites and time points. In general, nutrients and conductivity were lower at McDonald Creek than the other two sites (Table 1), where conductivity was $\geq 747$ $\mu$S/cm and generally increased during the study period. Discharge was lowest in McDonald Creek, which was the smallest of the 3 streams (Table 1).
Leaf litter breakdown

We used ANCOVA to compare change in mass over time for leaves and plastic across the 3 streams. The breakdown rate of leaves measured as AFDM in leaf only and mixed treatments were not significantly different from each other (ANCOVA p=0.097), but were different among the 3 sites (ANCOVA p=0.005; Table 3; Figure 4A, 4B, and 4C). North Branch Chicago River and Salt Creek had a faster breakdown rate than McDonald Creek (Tukey’s multiple comparison test p<0.001 and p=0.019, respectively), but breakdown rates in the Chicago River and Salt Creek were not significantly different (Tukey’s multiple comparison test, p=0.443). We also compared change in dry mass over time for leaves and plastic from mixed and single material bags (Figure 4D, 4E, and 4F). The rate of change in dry mass of the leaves in leaf only and mixed bags were not significantly different (ANCOVA p=0.494; Table 3) and there was no difference among sites (ANCOVA p=0.299). Similarly, the rate of change in dry mass of the plastic in plastic only and mixed bags were not significantly different (ANCOVA p=0.695; Table 3) and there was no difference among sites (ANCOVA p=0.202; Table 3; Figure 4D, 4E, and 4F).
Table 1. Site characteristics for the 3 study sites in Chicago: 1) North Branch Chicago River, 2) Salt Creek, 3) McDonald Creek. Q=discharge, Cond = specific conductivity, Temp=temperature, NO$_3^-$ = nitrate, NH$_4^+$ = ammonium, and SRP = soluble reactive phosphorus.

<table>
<thead>
<tr>
<th>Collection Period</th>
<th>Date</th>
<th>Q (m$^3$/s)</th>
<th>Cond. (µS/cm)</th>
<th>Temp (°C)</th>
<th>NO$_3^-$ (µg N/L)</th>
<th>NH$_4^+$ (µg N/L)</th>
<th>SRP (µg P/L)</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>11/6/16</td>
<td>11.4</td>
<td>788</td>
<td>13.2</td>
<td>2,288</td>
<td>134</td>
<td>99</td>
</tr>
<tr>
<td>1</td>
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<td>4.3</td>
<td>935</td>
<td>12.2</td>
<td>6,783</td>
<td>123</td>
<td>507</td>
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<tr>
<td>2</td>
<td>11/27/16</td>
<td>2.8</td>
<td>890</td>
<td>6.9</td>
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<td>90</td>
<td>569</td>
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<td>3</td>
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<td>538</td>
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<td>4</td>
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<td>7,735</td>
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<td>303</td>
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<td>2.4</td>
<td>4,368</td>
<td>161</td>
<td>209</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>11/7/16</td>
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<td>819</td>
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<tr>
<td>2</td>
<td>11/21/16</td>
<td>11.7</td>
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<td>711</td>
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<td>4.9</td>
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<td>10,640</td>
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<td>1,432</td>
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<tr>
<td><strong>McDonald Creek</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11/11/16</td>
<td>1.2</td>
<td>1,027</td>
<td>9.4</td>
<td>404</td>
<td>98</td>
<td>34</td>
</tr>
<tr>
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<td>11/17/16</td>
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<td>1,169</td>
<td>12</td>
<td>357</td>
<td>83</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>12/5/16</td>
<td>1.5</td>
<td>1,943</td>
<td>3.4</td>
<td>438</td>
<td>138</td>
<td>6</td>
</tr>
<tr>
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<td>2.0</td>
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<td>1.3</td>
<td>773</td>
<td>171</td>
<td>7</td>
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<tr>
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<td>4</td>
<td>377</td>
<td>46</td>
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<tr>
<td>5</td>
<td>4/24/17</td>
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<td>1,577</td>
<td>11.2</td>
<td>414</td>
<td>51</td>
<td>&lt;2</td>
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Figure 4. Breakdown of *P. deltoides* leaves in leaf only and mixed treatments and plastic and plastic only and mixed treatments over a period of 164 days at each of the 3 sites as measured by dry mass for: A) McDonald Creek (MC), B) North Branch Chicago River (NBC), and C) Salt Creek (SC). Change in ash-free dry mass (AFDM) of *P. deltoides* leaves over a period of 164 days at D) MC, E) NBC, and F) SC.
Table 2. Breakdown rate for leaf litter in litterbags with leaves alone or leaves mixed with plastic. The slope of the line is the breakdown rate (k; d^{-1}) of *P. deltoides* leaves measured as ash-free dry mass (AFDM) at each of the 3 sites. N. Br. = North Branch.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Site</th>
<th>Mass loss equation</th>
<th>r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf only</td>
<td>N. Br. Chicago R</td>
<td>y = -0.0067x + 4.53</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Salt Creek</td>
<td>y = -0.0030x + 4.46</td>
<td>0.60</td>
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<tr>
<td></td>
<td>McDonald Creek</td>
<td>y = -0.0032x + 4.61</td>
<td>0.90</td>
</tr>
<tr>
<td>Leaf mixed</td>
<td>N. Br. Chicago R</td>
<td>y = -0.0073x + 4.49</td>
<td>0.85</td>
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<tr>
<td></td>
<td>Salt Creek</td>
<td>y = -0.0034x + 4.49</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>McDonald Creek</td>
<td>y = -0.0023x + 4.51</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Table 3. Analysis of covariance (ANCOVA) to compare leaf litter (*P. deltoides*) breakdown rates measured as ash-free dry mass (AFDM) and dry mass from litterbags with leaves only and mixed treatments. We also compared change in dry mass of plastic litter from plastic alone and mixed litterbags. Site was the covariate. Significant values are listed in bold (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>ANCOVA</th>
<th>df</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
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<td>5.60</td>
<td><strong>0.005</strong></td>
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<tr>
<td></td>
<td>Treatment</td>
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<td>2.81</td>
<td>0.097</td>
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<td></td>
<td>Residuals</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Leaf litter: Dry mass</td>
<td>Site</td>
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<td>1.22</td>
<td>0.299</td>
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<td></td>
<td>Treatment</td>
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<td>0.47</td>
<td>0.494</td>
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<tr>
<td></td>
<td>Residuals</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plastic litter: Dry Mass</td>
<td>Site</td>
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<td>0.36</td>
<td>0.695</td>
</tr>
<tr>
<td></td>
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<td>1.65</td>
<td>0.202</td>
</tr>
<tr>
<td></td>
<td>Residuals</td>
<td>104</td>
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<td></td>
</tr>
</tbody>
</table>
Richness and diversity of bacteria, fungi, and algae

We compared the Shannon-diversity and number of OTUs (i.e., ‘species’ richness) for bacterial, algal, and fungal assemblages across four substrate types: 1) *P. deltoides* leaves from the leaf only bags, 2) *P. deltoides* leaves from the mixed bags, 3) plastic from the plastic only bags, and 4) plastic from the mixed bags, after 43 days and 150 days of incubation using 2-way ANOVA (Table 4). For 16s rRNA genes, there was a significant interaction between treatment and days of incubation for Shannon-Diversity (2-way ANOVA p<0.001) and the number of OTUs (2-way ANOVA p=0.027; Table 4; Figure 5A, 5D). Thus, we used a 1-way ANOVA to compare among the 4 substrates (i.e., treatments) on each day of incubation using a Bonferroni correction p-value of 0.05/2=0.025. There was no significant difference among treatments on the after 150 days of incubation for Shannon-diversity (1-way ANOVA p=0.524) and number of OTUs (1-way ANOVA p=0.053). However, after 43 days of incubation, there was a significant difference among treatments for Shannon-diversity (2-way ANOVA p<0.001) and for the number of OTUs (1-way ANOVA p=0.009). Comparing among treatments, there was greater diversity of 16S rRNA genes on the leaves from the leaf only and mixed bags compared to the plastic in the plastic only and mixed bags (Figure 5A). Similarly, we found a greater number of OTUs on leaves from the leaf only and mixed bags and the lowest number of OTUs on the plastic from the mixed bag (Figure 5D).

Patterns for 23s rRNA and ITS region showed some similarities with the results for bacteria. For example, Shannon-diversity of the 23s rRNA and ITS region showed significantly higher diversity on leaves (from the leaf only and mixed treatments) compared to plastic (from plastic only and mixed treatments; 2-way ANOVA p=0.022, p<0.001, respectively). We also
noted significant differences between incubation dates (2-way ANOVA p=0.030, p=0.023, respectively; Table 4, Figure 5B, 5C), although the patterns were different for each gene. Diversity of algae tended to increase during the incubation, except with communities on plastic only which had a slight decline (Figure 5B, 5C). However, we observed a decline in fungi diversity on all substrates during incubation (Figure 5B, 5C). For the number of OTUs (i.e., species richness), the patterns were different between the algae and fungal gene markers. For 23S rRNA genes, there were no differences by treatment type (2-way ANOVA p=0.495), across incubation dates (2-way ANOVA p=0.116), and no significant interaction (2-way ANOVA p=0.884; Table 4). For the ITS region, the number of fungal OTU’s was significantly higher on leaves compared to plastic, e (2-way ANOVA p=0.004), with no change over time (2-way ANOVA, p=0.602; Table 4, Fig. 5E, 5F).

*Community composition of bacteria, fungi, algae*

We used PERMANOVA and nMDS to compare assemblages of bacteria, algae, and fungi among 4 substrate types and two collection dates for each stream. For bacteria, Bray-Curtis indices showed significant difference among 4 substrates (PERMANOVA p-value <0.001) and when comparing days of incubation (Figure 6; PERMANOVA, p-value<0.001). One phyla of bacteria in particular, Proteobacteria, appeared in higher relative abundance on plastic surfaces from both plastic only (>75%) and mixed litterbags (>75%), compared to leaves from leaf only (>50%) and mixed bags (>50%) (Figure 7). In contrast, Bacteroidetes appeared in higher relative abundance on leaves from both leaf only (>25%) and mixed litterbags (>25%) compared to the plastic from plastic only and mixed litterbags (Figure 7).
For algae, Bray-Curtis indices showed significant difference among the 4 substrates (PERMANOVA p-value <0.001) and among incubation dates (PERMANOVA p-value <0.001; Figure 8). Leaves from the leaf only and leaf mixed treatments showed >75% relative abundance of ‘unclassified Eukaryotes’, which decreased slightly after 135-164 days of incubation (Figure 9). In contrast, unclassified Eukaryotes were lower on plastic mixed with leaves and on plastic-alone (>25% across sampling dates) (Figure 9). Bacillariophyta (diatoms) were higher on plastic substrates from both plastic only and mixed treatments (>25%) compared to the leaves from leaf only and mixed treatments (Figure 9). Finally, we observed <25% relative abundance of Eustigmatophyceae on plastic substrates after 38-47 days of incubation, but none on leaf substrates (Figure 9).

Patterns for fungal ITS sequence were similar to algae, as Bray-Curtis indices were significantly different when comparing treatments (PERMANOVA p-value <0.001; Figure 10) and when comparing days of incubation (PERMANOVA p-value<0.001; Figure 10). The fungal class Dothideomycetes appeared in higher relative abundance on leaves from the leaf only and mixed treatments (>50%) and decreased slightly after 135-164 days of incubation. We found >20% relative abundance of the same fungal class on plastic from plastic only and mixed treatments, which decreased slightly after 135-164 days (Figure 11). In contrast, the class Sordariomycetes appeared in higher relative abundance after 38-47 days of incubation on plastic from both plastic only and mixed treatments (>30%) compared to the leaves from leaf only and mixed treatments (Figure 11). Unclassified fungi were found in high relative abundance on plastic from both plastic only and mixed treatments (>30% at the mid-point of the experiment), which increased to >50% after 135-164 days of incubation.
Figure 5. Mean (±SE) Shannon diversity index (H’) illustrated by (A) 16S rRNA gene abundance (bacteria), (B) 23S rRNA gene abundance (algae) communities, and (C) ITS region (fungi) by substrate type according to day of incubation. Mean (±SE) observed operational taxonomic units (OTUs) for (D) bacterial assemblages, (E) algal communities, and (F) fungal communities from all study sites. Small letters indicate significant differences among treatments on incubation date as shown by Tukey’s multiple comparison test, which was completed following a significant interaction between date and treatment.
Table 4. Two-way analysis of variance (ANOVA) to compare Shannon-diversity indices (H’) and number of operational taxonomic units (OTUs) across treatment and number of days of incubation. Significant values are listed in bold (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Time</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-ratio</td>
<td>p-value</td>
<td>F-ratio</td>
</tr>
<tr>
<td><strong>Shannon-diversity indices (H’)</strong></td>
<td></td>
<td></td>
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<tr>
<td>16s</td>
<td>22.07</td>
<td>&lt;0.001</td>
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<tr>
<td>23s</td>
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<td>0.022</td>
<td>5.633</td>
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<tr>
<td>ITS</td>
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<tr>
<td><strong>Richness of OTUs</strong></td>
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<td></td>
<td></td>
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<tr>
<td>23s</td>
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<tr>
<td>ITS</td>
<td>6.50</td>
<td>0.004</td>
<td>0.28</td>
</tr>
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</table>
Figure 6. Non-metric multi-dimensional scaling (nMDS) ordination of 16s sequencing data (Bray-Curtis similarity index) comparing bacterial assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek.
Figure 7. A stacked bar graph of relative abundance of observed OTUs in bacterial communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom).
Figure 8. Non-metric multi-dimensional scaling (nMDS) ordination of 23s sequencing data (Bray-Curtis similarity index) comparing bacterial assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek.
Figure 9. A stacked bar graph of relative abundance of observed OTUs in algal communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom).
Figure 10. Non-metric multi-dimensional scaling (nMDS) ordination of ITS sequencing data (Bray-Curtis similarity index) comparing fungal assemblages across 4 different substrates in 3 study streams: A) McDonald Creek, B) North Branch Chicago River, and C) Salt Creek.
Figure 11. A stacked bar graph of relative abundance of observed OTUs in ITS communities after 38-47 days of incubation (top) and 135-164 days of incubation (bottom).
Macroinvertebrate community composition

We compared macroinvertebrate abundance across 3 collection dates and 3 litterbag treatments for each study site, and each site showed a different pattern. At the North Branch Chicago River, the total number of macroinvertebrates/g DM was significantly different across the number of days of incubation (LME p<0.001), but there was no effect of treatment (LME p=0.060; Table 5; Figure 12). When we compare the total number of macroinvertebrates/bag, there was also a significant difference across days of incubation at North Branch Chicago River (LME p<0.001) and there was a significant interaction between treatment and time (LME p=0.050; Table 5; Figure 13). For Salt Creek, we found no significant difference between the number of days of incubation or treatment for total macroinvertebrates, when counted as number/g DM or number/bag (LME p>0.110; Table 5; Figure 12, 13). We note that the data for total number of macroinvertebrates per bag for Salt Creek could not be normalized with a transformation, but the test is more robust to include non-normal data (Butler and Louis, 1992; Verbeke and Lesaffre, 1997). However, in McDonald Creek, we found significant effect of treatment (LME p=0.020), but no differences over time in total macroinvertebrate/g DM, and no effect of treatment or time when considering the number of macroinvertebrates/bag (Table 5; Figure 12, 13).

After adjusting the data to exclude hydrozoans and oligochaetes, we compared macroinvertebrate abundance across 3 collection dates and 3 treatments for each study site, and once again each site showed a different pattern. For the North Branch Chicago River, we found a significant effect of time on the total number of macroinvertebrates/g DM (LME p=0.001) and treatment (LME p=0.030; Table 5; Figure 12). We also found a significant effect of time on the
In Salt Creek, there was a significant effect of time in both adjusted total macroinvertebrates/g DM and number/bag (LME $p=0.020$; Table 5; Figure 12, 13). However, in McDonald Creek there was a significant effect of treatment in adjusted macroinvertebrates/g DM (LME $p=0.040$) and an effect of time in number/bag (LME $p=0.020$; Table 5; Figure 12, 13).

We used PERMANOVA and nMDS to compare assemblages of macroinvertebrates across treatment and number of days of incubations for all study sites combined and for each site individually. With all data combined, the Bray-Curtis indices did not show significant difference among treatment types, but showed significant difference across days of incubation (PERMANOVA $p=0.895$, $p<0.001$). The lengths of the vectors were scaled to indicate the strength of correlation for taxa driving patterns on each date (Figure 14). Gammaridae and Simuliidae showed a higher correlation with the community distance matrices after 38-47 days of incubation, whereas the Chironomidae and Hydropsychidae showed a higher correlation after 130-164 days of incubation (Figure 14). Examining data from each stream individually, the same patterns were repeated (i.e., differences in macroinvertebrate assemblages through time, with no treatment; data not shown).

*Respiration rates of microbial communities*

To assess the respiration rates of the microbial community on the final day of incubation, we ran a 1-way ANOVA to compare measurements across substrate type for the 3 sites individually. We found significant differences among treatments at North Branch Chicago River and McDonald Creek (1-way ANOVA $p=0.003$, $p<0.001$, respectively). At North Branch Chicago River, respiration rates were significantly higher on leaves from leaf only treatments.
and mixed treatments compared to plastic substrates from plastic only and mixed treatments (Figure 15). Results at McDonald Creek were similar, with differences across leaf and plastic substrates, and also higher respiration rates on leaf substrates from the leaf only bag compared to the mixed bag (Figure 15). Salt Creek showed a similar pattern across leaf and plastic substrates, but no significant difference (1-way ANOVA p=0.061; Figure 15).
Table 5. Results from linear mixed effects model (LME) analyses for macroinvertebrates treatment and number of days of incubation at all 3 study sites. Macroinvertebrates were characterized as the total number of macroinvertebrates per g of dry mass (DM) and total number per bag. 'Adjusted' macroinvertebrates excludes hydrozoans and oligochaetes. Significant values are listed in bold (p<0.05). N. Br. Chi. R = North Branch Chicago River, Cr = creek.

<table>
<thead>
<tr>
<th>Site</th>
<th>LME</th>
<th>No./g DM</th>
<th>p-value</th>
<th>No./ bag</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F-value</td>
<td></td>
<td>F-value</td>
<td></td>
</tr>
<tr>
<td>Total Macroinvertebrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.730</td>
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<td>1.55</td>
<td>0.230</td>
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<td>Interaction</td>
<td>0.45</td>
<td>0.640</td>
<td>0.45</td>
<td>0.640</td>
</tr>
</tbody>
</table>

Adjusted Macroinvertebrates

| N. Br. Chi. R | Time  | 108.84   | **<0.001** | 101.96  | **<0.001** |
|               | Treatment | 4.07  | **0.030** | 2.41  | 0.110  |
|               | Interaction | 0.15  | 0.860  | 0.001  | 1.000  |

| Salt Cr.      | Time  | 5.87     | **0.020** | 5.86  | **0.020** |
|               | Treatment | 1.27  | 0.300  | 0.19  | 0.830  |
|               | Interaction | 0.36  | 0.700  | 0.63  | 0.540  |

| McDonald Cr.  | Time  | 4.02     | 0.060  | 5.74  | **0.030** |
|               | Treatment | 3.17  | **0.040** | 0.44 | 0.650  |
|               | Interaction | 0.49  | 0.620  | 0.22  | 0.800  |
Figure 12. Mean (± SE) total number of macroinvertebrates per gram of dry mass during incubation in A) McDonald Creek (MC), B) North Branch Chicago River (NBC) and C) Salt Creek (SC). Hydrozoan and oligochaetes observations were omitted for 'adjusted' total number of macroinvertebrates at D) MC, E) NBC) and E) SC.

Figure 13. Mean (±SE) total number of macroinvertebrates per bag during incubation in A) McDonald Creek (MC), B) North Branch Chicago River (NBC), and C) Salt Creek (SC). Hydrozoan and oligochaetes observations were omitted for 'adjusted' total number of macroinvertebrates at D) MC, E) NBC), and F) SC.
Figure 14. Non-metric multi-dimensional scaling (nMDS) ordination of total macroinvertebrates (Bray-Curtis similarity index) comparing macroinvertebrate assemblages across 3 different treatments and days of incubation at all study streams: McDonald Creek, North Branch Chicago River, and Salt Creek. (coll.= collembola, biva.=bivalvia, hydropti.=hydroptilidae, coen.=coenagrionidae, oligo.=oligochaete, plana.=planariidae, hydropsy.=hydropsychidae).
Figure 15. Mean (±SE) biofilm respiration on *P. deltoides* leaves from the leaf only treatment and mixed treatment, and plastic substrates from mixed treatment and plastic only treatments. Small letters indicate differences among substrates as shown by Tukey's multiple comparison test, done following significant 1-way ANOVA.

**Discussion**

Although research on plastic pollution within marine systems has been well established, it is still an emerging field of study in freshwater environments. Rivers are considered major conduits of plastic to the ocean, but retention of plastic pollution within freshwaters is common. Research is needed to examine factors that drive the accumulation and export of plastic in freshwater ecosystems, as well as its effect on stream biota and ecosystem processes.

*Plastic did not affect leaf litter breakdown rates*

Contrary to our expectations, there was no difference in leaf breakdown rates when leaves were incubated alone or mixed with plastic. We expected slower rates in the mixed bags because of the close physical interaction, where plastic would reduce oxygen availability needed
to maintain the macroinvertebrate community and sustain biofilm growth. Plastic could also reduce the fragmentation of cottonwood leaves by providing a layer of protection from turbulence caused by water flow. The similarity in rates suggest none of these mechanisms of leaf breakdown were affected when plastic was mixed with leaves.

Leaf litter breakdown rates were previously documented for the same leaf species, using the same methods and location, which is valuable for comparison with this study. Cook and Hoellein (2016) found cottonwood leaf breakdown rates were 0.0042-0.0077 d$^{-1}$ across 5 sites in the North Branch Chicago River watershed, measured in fall 2012-spring 2013. At Harms Woods (our study site), Cook and Hoellein (2016) measured leaf breakdown rate of 0.0072 d$^{-1}$, and the rate we measured in fall 2016-spring 2017 at the same site was nearly identical: 0.0067 d$^{-1}$. Cook and Hoellein (2016) concluded discharge and macroinvertebrates communities (i.e., density of isopods and amphipods) drove differences in leaf litter breakdown across the 5 study sites. Our analysis was not designed to test environmental drivers of breakdown in different urban stream sites as was that study, however, our data showed some similar patterns when comparing sites. McDonald Creek had the slowest leaf breakdown rates, and had the lowest mean discharge and macroinvertebrate density relative to the other two sites. Overall, plastic litter, in the form of low density polyethylene sheets, was not a driving factor influencing leaf breakdown rates, which were similar to past estimates and suggest other physical and biological factors were more important to controlling variation in breakdown rates among sites and treatments.
Microbial communities on plastic and leaf litter surfaces showed no interaction.

We expected to find differences in community composition for biofilms on leaves and plastic when incubated alone, however, we expected when leaves and plastic were incubated together, the biofilm communities would be less distinct, as they were incubated in close physical proximity. Our results followed our expectation that biofilm community composition was very different on plastic and leaves. However, our second prediction was not supported, as differences in biofilm communities on leaves and plastic were consistent, regardless if they were incubated alone or together.

The primary factor that drove differences in community composition was likely the capacity for leaves to serve as a food resource for biofilm organisms, whereas the plastic was highly recalcitrant and not readily decomposed by biological enzymes. Leaf litter from terrestrial plants is a primary source of allochthonous carbon that contributes to stream food webs (Paul et al., 2006), so there is a long evolutionary history and a large suite of fungal and bacterial taxa that decompose organic polymers in leaves. In general, after the chemical components of leaf litter leach into the stream, fungi play a major role in the initial conditioning, or ‘softening’, of the leaf substrates, whereas bacterial conditioning is thought to increase over time (Newman et al., 2015). This pattern was evident from the OTU richness analyses among substrates and dates. Taxa richness of fungi was higher on leaves than plastic, and the same patterns in taxa richness were observed on the third collection date and the final collection date. For bacteria, the taxa richness was lower on plastic than leaves on the third collection date, but by the final collection date, the taxa richness was similar on all substrates. These patterns suggest low colonization of plastic surfaces by fungi throughout the study, as polyethylene is a recalcitrant carbon source.
Although bacterial colonization is initially low, increases over time is consistent with the development of a periphyton-like biofilm that colonizes hard surfaces, which is distinct in composition from the bacterial community that colonizes leaf litter for organic carbon degradation.

Community composition on plastic and leaf litter showed distinct groups dominated the bacterial representatives in the biofilm, which is similar to the findings of previous research that showed differences in biofilms on organic substrates (i.e., leaves and cardboard) and hard synthetic substrates, such as plastic or aluminum (Hoellein et al., 2014). The phylum Bacteroides, which is thought to be specialized in the breakdown of complex organic matter in the environment (Thomas et al., 2011), was highest on leaf substrates (>25%) from leaf only and mixed treatments in the first few weeks of incubation and decreased to 0% relative abundance on the last days. In contrast, Proteobacteria was highest in relative abundance on plastic substrates from mixed and plastic only treatments throughout the incubation period (>50%). Debroas et al. (2017) also found Proteobacteria was the dominant phyla of bacteria to colonize poly(ethylene terephthalate) substrates collected from marine environments. McCormick et al. (2016) measured bacterial communities on microplastic (i.e., particles < 5 mm) in an urban stream, as well as seston, and water. The authors reported higher relative abundance of Proteobacteria on microplastic (74.9%), compared to seston (56.9%), and the water column at (33.7%) (McCormick et al., 2016). Proteobacteria has been identified as one of the largest phyla of the bacteria domain and contains a vast array of organisms. For example, Gammaproteobacteria are early biofilm members of artificial surfaces in marine habitats (Lee et al., 2008), and common in streams influenced by wastewater effluent (Marti et al., 2013). Finally, some Proteobacteria are
common human pathogens (e.g., *Salmonella* and *Helicobacter*) (Rizzatti et al., 2017) as well as non-harmful components of human gut microbiota. Overall, a higher abundance of this phylum of bacteria on plastic than organic substrates suggests that plastic could be vectors for unique suite of microbes, with variable sources, pathogenic considerations, and contributions to ecosystem processes.

Similar to bacteria, the fungal communities were distinct on plastic and leaf surfaces. The major groups exhibiting differences between substrates were Dothideomycetes, Sordariomycetes, and ‘unclassified’ fungi. A higher abundance Dothideomycetes, one of the most diverse class of fungi, was noted on leaf substrates from the mixed bag and leaf-only incubations. This class of fungi plays an important role in carbon cycling by colonizing and degrading dead plant biomass, so its enhanced presence on leaf litter is expected (Ohm et al., 2012; Shearer et al., 2009). Sordariomycetes was more abundant on plastic substrates from the plastic only and mixed treatments than on the leaf surfaces. Like Dothideomycetes, Sordariomycetes functions in the decomposition of plant litter. Although most taxa in this group are plant or mammal pathogen, it includes *Chaetomium*, which has been documented to break down synthetic materials such as paper or fabrics (Zhang et al., 2006). Finally, unclassified fungi were more abundant on plastic than leaves, especially on the last day of incubation. Given the lack of taxonomic identification, we can’t offer an explanation for the presence of this group on plastic. However, we suggest that culturing and identification of these groups in future research will be important for documenting the biological interactions and fate of plastic in freshwater ecosystems.

Bacillariophyta (diatoms) are an important phylum of algae, responsible for about 20% of earth’s total primary production, a key food source to microorganisms and macroinvertebrates in
aquatic ecosystems (Malviya et al., 2016), and have been found on plastic litter in ecosystems worldwide. Distribution of diatom species is related to environmental characteristics (e.g., pH, nutrients, and climate), so diatom communities are commonly used in bioassessment (Dixit et al., 2011). In this study, Bacillariophyta only appeared on plastic substrates rather than leaves, and were found in similar abundance on plastic from the mixed and plastic-only treatments. We attribute the presence of diatoms on plastic substrates to the surface properties that support their attachment and growth. Diatoms sustained the same relative abundance throughout the incubation period, supporting our inference that plastic substrates select for diatom growth. We are not aware of other studies that have examined diatom community composition on plastic relative to natural substrates (e.g., leaves, sand, rocks), or assessments of individual taxa via visual identification. However, diatoms have been noted as constituents of plastic biofilms in other ecosystems. For example, Kumar et al. (2017) collected discarded polyethylene from urban ponds and ditches in India, and noted diatoms and other algal taxa may be linked to biodegradation processes. Plastic substrates incubated in the Mediterranean Sea showed diatoms were early colonizers of plastic litter, and their abundance varied by plastic type (i.e., polyethylene and biodegradable plastic) (Eich et al., 2015). Plastic particles from ocean gyres support dense diatom communities, which are enhanced by surface roughness (Carson et al., 2013). Overall, plastic supported diatom growth in our study, and the clear distinction in diatom assemblage between leaves and plastic shown here, regardless of whether plastic was in close contact with leaves, suggest plastic substrates may be an overlooked and potentially significant factor affecting diatom assemblage at the scale of stream substrate and reach.
Aside from the diatoms, the other algal taxa detected using the 23S gene abundance approach did not offer clear insight into community composition or ecosystem processes affected by plastic relative to leaf litter. We observed a relatively low abundance of Eustigmatophyceae only on plastic substrates in the initial incubation period, and not on leaf litter (Figure 6). This phyla of algae is not as well studied or as abundant as diatoms. Eustigmatophyceae are unicellular, common in freshwater, and have been documented on plants (i.e., epiphytic) or on rocks (i.e., epilitic) (Wehr et al., 2018). In our study, plastic surfaces rather than leaf litter selected for growth of Eustigmatophyceae, suggesting a periphytic growth form in our study streams. We are not aware of other studies that have documented this pattern for plastic in streams, and suggest additional research to compare algal communities on multiple stream substrates (e.g., sand, rocks, macrophytes, and plastic) will be valuable to determine the extent to which plastic selects for a novel periphytic community that is well represented by constituents from Eustigmatophyceae.

One taxon of algae that also showed high relative abundance on leaf substrates throughout the study was the 'unclassified Eukaryota' (Figure 8), which represent chloroplast sequences that could not be assigned to a specific algal taxa from our Silva 23s reference database. Any chloroplast that did not match to a specific sequence in our database (i.e., cyanobacteria or a chloroplast) were removed with the remove.lineages command in the MOTHUR processing. Our reference database most likely did not contain the chloroplast 23s sequence we had in our samples. The database for algal taxa is not as well developed as those for bacteria. As molecular tools are more frequently used to address questions about algal community structure, we anticipate the fraction that is unknown will decrease in the future.
Microbial degradation of plastic

Unlike cottonwood leaves, plastic is highly resistant to biological degradation (O'Brine and Thompson, 2010), but will eventually breakdown in the environment through a combination of abiotic and biotic processes. Through exposure to ultraviolet (UV-B) radiation from sunlight and the effects of the pH of the water, plastic polymers fragment into smaller chains, and functional groups exposed during fragmentation may be more susceptible to enzymatic attach from microbes (Gewert et al., 2017). Relatively few studies have measured plastic degradation via microbial activity. Early analyses suggest that some microbes breakdown plastic in laboratory conditions, but do not offer much insight into microbial breakdown of plastic in the environment. For example, Balasubramanian et al. (2010) found that two marine bacterial strains, *Arthrobacter* sp. and *Pseudomonas* sp., degraded high density polyethylene (HDPE) samples collected from plastic waste dump sites along the coast of Gulf of Mannar. The authors observed 12-15% weight loss of HDPE after 30 days of incubation using *in vitro* conditions, which were unlikely to reflect environmental breakdown rates by these bacterial isolates. Lobelle et al. (2011) found that bacteria rapidly colonized polyethylene surfaces submerged in marine environments and changed its physical properties by making it more hydrophobic, but saw no evidence to suggest that any plastic degrading microorganisms were present. However, Yoshida et al. (2016) were able to isolate a new bacterium, *Ideonella sakaiensis 201-F6*, that has the ability to use Poly(ethylene terephthalate) (PET) as its main carbon and energy source. Our *in situ* assessment of microbial communities suggest distinct assemblages on plastic relative to leaves. We also did not observed plastic breakdown (as mass loss) during the course of our study, and do not know the enzymatic capacities of the unique suite of microorganisms which
preferentially colonized the plastic surfaces. We recommend future studies analyze abundance of

target genes or conduct metagenomics for insight into the functional capacity of biofilms on
plastic relative to natural surfaces.

We predicted similarity in microbial respiration rates on plastic and leaf substrates when
incubating together in the mixed bag, compared to respiration rates of biofilms on plastic and
leaves alone. We expected the close physical interaction between the two substrates within the
same bag while experiencing the same environmental conditions (i.e., temperature, nutrient
levels) would mix the biofilm organisms together. In addition, we anticipated hydrologic
turbulence could cause biofilms to intermix. Contrary to our predictions, however, we found that
there was strong, consistent, and unique communities of bacteria, algae, and fungi depending on
substrate rather than method of incubation. Overall, the surface of these substrates behaved as
separate and distinct habitats for community composition and respiration rates, even when mixed
together.

*Macroinvertebrate communities were not distinct across treatment type, but varied over time.*

We predicted to find distinct macroinvertebrate communities in different treatment types.
Cottonwood leaves alone would provide a natural and historically abundant food source and
habitat, and we expected plastic mixed with leaves would be less palatable and show lower
invertebrate density. We expected plastic alone would have the fewest macroinvertebrates.
However, we found no differences in macroinvertebrate communities across treatment types.
Therefore, plastic itself and plastic mixed with leaves was not a deterrent for macroinvertebrate
colonization. The type of macroinvertebrates present may reveal why the organisms were not
sensitive to the presence of plastic. Chironomidae and Oligochaetes were in highest abundance
across all incubation days and treatment types. We predicted to find a high abundance of these taxa in our study because of the high level of pollution in our urban streams. Chironomidae in particular are commonly used to assess the health of aquatic environments because they are known to be in great abundance in highly polluted and anoxic conditions (Rosa et. al, 2014). Oligochaetes also show high relative abundance in polluted streams (Martin et al. 2008). The high abundance of Chironomidae may have also been related to the stable habitat that plastic substrates provide. Some Chironomidae construct loose sediment cases that are attached to various surfaces in benthic habitats. Anecdotally, we observed many of these cases attached to our plastic substrates when pulled out of the streams. It is possible that the resistance of plastic to degradation in urban streams (i.e., to a greater degree than organic substrates such as leaves), attracts macroinvertebrates that need stable surfaces for case attachment. Additional studies of case attachment across natural and synthetic surfaces would reveal the role of plastic in providing habitat for macroinvertebrates. We did not measure plastic ingestion by macroinvertebrates, but suggest a follow-up study could also examine invertebrate gut contents for insight into additional interaction with plastic litter.

Other macroinvertebrate groups including caddisflies (Trichoptera), beetles (Coleoptera), crustaceans (Gammaridae and Asellidae), and damselflies (Odonata) were common across treatments and study sites. For example, we observed the caddisfly families Hydropsychidae, which are collector-filterers, and Hydroptilidae, which are scrapers/predators, were generally high across treatments, rather than indicating any preference for leaf litter. At the North Branch Chicago River, we observed a high abundance of amphipods (Gammaridae) across treatments. Cook and Hoellein (2015) also found high abundance of amphipods in leaf bags at the same site,
and surmised their role in this urban stream was to consume leaf litter (Cummins et al., 1989, Felten et al., 2008; Voshell, 2002). Coenagrionidae (damselflies) appeared in our plastic only and mixed treatment bags at McDonald Creek after 47 days of incubation, and in our mixed treatment bag after 38 days of incubation at Salt Creek. Their presence in plastic only and mixed bag treatments suggest that they may be mostly attracted to the stable and structurally complex habitats that these leaf bags provided.

The macroinvertebrate results suggest that our assumptions about the role of plastic litter on macroinvertebrate habitat selection in urban streams was imprecise and merits revision. Although the relative abundance of specific classes of bacteria, fungi, and algae differed on leaf and plastic substrates, biofilm growth was abundant on plastic and leaf surfaces, thereby each can support feeding opportunities that attract macroinvertebrates. In addition, mixed bags showed the highest abundance of macroinvertebrates compared to leaves or plastic alone. We surmise that the combination of leaves and plastic may have given this treatment a more structurally complex habitat and with more surface area for feeding and biofilm growth, and perhaps greater protection from flowing waters or predators.

*Study design and environmental plastic: considerations for other experiments*

Contrary to our predictions, we did not observe any interactions between plastic and leaf litter as it relates to breakdown rates, biofilms, and macroinvertebrates in the mixed relative to single substrate treatments. One reason may be due to the size and placement of the plastic in the litter bags. In concurrent study, we observed a high abundance of plastic bags tangled and buried in naturally occurring debris dams along the stream (T. Hoellein, unpublished data). We noticed that intact plastic bags (i.e., shopping and garbage bags) trapped debris such as soil and coarse
particulate organic matter and restrict water and gas exchange. Although we used a similar plastic material (low-density polyethylene), the simple shape of our experimental plastic may have prevented any smothering effect on leaf litter, biofilm, and macroinvertebrates. Similarly, at every collection period we observed that the contents inside each treatment bag were pushed to the end of the bag due to stream flow. This meant that the plastic and organic material remained in close physical contact throughout the incubation period of the bags. However, its low density, simple shape, and flexibility might have reduced any of the ‘smothering’ effect we predicted. Based on the outcomes of our study, we predict that the size, placement, and shape of the plastic litter will strongly drive its biological effects. For example, if the plastic pieces we used were more similar in size and shape as plastic bags and were placed in various riparian and benthic habitats, we believe we would have observed different results in leaf litter and plastic interactions.

*Other common types of plastic litter may affect organisms and ecosystem processes.*

Our study examined the effect of low-density polyethylene on stream biota and fundamental ecological processes, but a variety of plastic litter occurs in streams (McCormick and Hoellein, 2016). Low-density polyethylene shopping bags are relatively thin, pliable, and can become stretched and torn *in situ*. Other, more rigid plastic polymers and litter shapes might have a greater effect on ecological processes. Common materials in urban streams (i.e., high-density polyethylene, polystyrene, polyvinyl) could have a more substantial smothering effect, or distinct chemical interactions with stream biota. Therefore, we suggest a variety of plastic litter could be assessed to determine its effects on stream biofilms, macroinvertebrates, and organic matter processing rates.
Conclusion

Macroinvertebrates and microbes interact with plastic litter in urban streams. Contrary to our predictions, however, the presence of plastic litter within leaf packs did not slow down leaf breakdown or alter leaf pack-inhabiting macroinvertebrates communities. We also found that microbial communities were distinct on plastic and leaf surfaces, but did not show any interaction when in close contact in mixed treatments. From the perspective of leaf breakdown, biofilm growth, and urban stream macroinvertebrates, plastic litter provides an additional surface for microbial colonization and macroinvertebrate habitat, and does not appear to alter fundamental ecosystem processes or community structure. Additional research is needed to examine the fate and biological interactions of plastic litter in urban streams, including assessments of plastic consumption, trophic transfer, biofilm community succession, and long-term burial, degradation, and export.
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Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics.


VITA

Lisa H. Kim was born in South Korea and raised in Chicago, Illinois. She graduated Loyola University Chicago in May 2016 with a Bachelor of Science degree in Biology and a minor in Biostatistics. She also worked in Dr. Timothy Hoellein’s Aquatic Ecology lab at Loyola, to study the concentration of microplastic pollution in Lake Michigan. During her time at Loyola as a graduate student, Ms. Kim also worked as an Aquatic Invasive Outreach Assistant at Illinois-Indiana Sea Grant. In 2019, she received the John A. Knauss Marine Policy Fellowship and worked as an Ocean Policy Fellow at the National Oceanic and Atmospheric Administration’s (NOAA) National Ocean Service Headquarters.