SOIL INVERTEBRATE INTERACTIONS WITH MICROPLASTIC POLLUTION

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ABSTRACT

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Microplastics are an unfortunate byproduct of human society's increasing reliance on synthetic plastics for packaging, clothing, and other products. Microplastics have long been known to pollute the world's oceans, but recent work has shown them to be just as prevalent, if not more so, in soil. Early findings indicate similar potential for harm to soil organisms as has been seen for marine microplastics. Yet aside from microplastics' direct physical and toxicological effects on soil organisms, one must also consider their interactions with these organisms, the ways in which organisms may influence microplastics' formation, occurrence, and distribution in soil as well as mediate their effects on the rest of the soil community. My research is focused on soil invertebrates' ability to create microplastics by fragmenting large plastic debris. To advance this goal, I first developed a novel fluorescent counterstaining technique, adding a blend of Calcofluor white and Evans blue to the traditional Nile red staining approach. The counterstain allowed microplastics to be visually distinguished from chitin, cellulose, and other biological materials that may survive chemical digestion along with the plastics, making it possible to detect plastics in samples of soil invertebrate fecal material and biomass. I then investigated four soil invertebrates' ability to generate microplastic from polystyrene (PS) foam debris. Individuals of the beetle larva *Zophobas morio*, the cricket *Gryllodes sigillatus*, the isopod *Oniscus asellus*, and the snail *Cornu aspersum* were placed in glass arenas with pieces of pristine or weathered PS foam for 24 h, after which I counted microplastic particles in the invertebrates' fecal material, cadaver biomass, and the sand substrate

of their arenas. *Z. morio* fragmented all plastics and produced the most detectable microplastic, *C. aspersum* produced almost none, and *G. sigillatus* and *O. asellus* fragmented only the weathered plastics. In a follow-up experiment with *O. asellus*, identical pieces of pristine PS foam were subjected to ultraviolet light, immersion in a soil suspension, and combination treatments to assess the effects of exposure to the elements on fragmentation by the isopods. Plastics immersed in the soil suspension were fragmented to a significantly greater degree than other treatments. Together, these results suggest that large plastic debris could represent a source of microplastics into soil environments, and that laboratory experiments investigating fragmentation of pristine plastics may risk underestimating the phenomenon. My further investigations focused on fragmentation of weathered PS foam by the isopods *O. asellus* and *Trachelipus rathkii*, examining fragmentation over different spans of time and the effects of natural materials as alternate substrates for the isopods. Neither species appreciably fragmented the PS foam until after 48 h, an interesting contrast to the previous experience, and *O. asellus* produced more fragments than *T. rathkii*. The presence of wood as an alternate substrate did not significantly affect fragmentation. More broadly, these results indicate that laboratory experiments should be conducted over short timescales and do not necessarily need to include alternate or supplementary food for the study organisms.

In summary, the potential of soil invertebrates to affect microplastic dynamics, complicating their effects on other organisms compared to what would be seen in a standard ecotoxicological assay, should be considered when assessing this novel pollutant's impact on soil ecosystems.

To Eva

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CHAPTER ONE: TOWARDS AN ECOLOGY OF SOIL MICROPLASTICS

Abstract

Microplastic pollution is a topic of increasing concern for the world's oceans, fresh waters, and most recently, soils. Microplastics have been found in soils across the globe. Like other anthropogenic pollutants, they can negatively affect a range of soil organisms through several mechanisms, though often dependent on particle size, shape, and polymer type. However, microplastics are unique among pollutants due to the diversity of ways in which soil organisms may themselves be able to affect their occurrence and distribution and mediate their effects on the rest of the soil food web. In this review, we argue for a more explicitly ecological framing of this novel issue for the soil environment and discuss their potential interactions with soil communities, including microplastic formation via microbial and faunal fragmentation of large plastic debris and organisms such as earthworms placing microplastic particles in unique pedological contexts they could not otherwise reach. Ecological interactions may be crucial for dictating microplastics' ultimate fate and effect on terrestrial ecosystems.

Introduction

Concern over microplastics in the world's soils has grown as recent studies have found them both prevalent and potentially harmful to living organisms (Chae and An 2018, He et al. 2018, Ng et al. 2018) just as they are in marine environments (Ivar do Sul and Costa 2014). Microplastics are defined as plastic particles with diameters under 5 mm (Hidalgo-Ruz et al. 2012), with particles under 0.1 to 1 μm in diameter often called nanoplastics (Gigault et al. 2018). Microplastics occur in many forms, with high physical and chemical diversity (Figure 1.1A, 1B). They originate from many sources (Bläsing and Amelung 2018) and are almost certainly ubiquitous around the globe, being carried by wind to even the remotest places (Allen et al. 2019). However, urban (Fuller and Gautam 2016), riparian (Scheurer and Bigalke 2018), and agricultural soils (Liu et al. 2018, Piehl et al. 2018, Zhang and Liu 2018, Corradini et al. 2019) have so far received the bulk of research focus (Figure 1.1C). Soils are the basis of virtually all terrestrial ecosystems and one of human society's most important natural resources. They provide ecosystem services such as sequestering atmospheric carbon, removing pathogens and pollutants from water, recycling organic wastes, and of course, providing the crops and native plants humans depend on with the water and nutrients they need to grow (Robinson et al. 2012, Nielsen et al. 2015, Wall et al. 2015). Soils also contain enormous biodiversity of microbes and animals (Giller 1996, Origiazzi et al. 2016), which play crucial roles in the aforementioned ecosystem services (Barrios 2007). Though microplastics have been known to exist in the oceans since the early 1970's (Colton et al. 1974), the earliest publication discussing microplastics in soil is less than a decade old (Rillig 2012), and the bulk of primary research has only been published since 2016 (Figure 1.2).

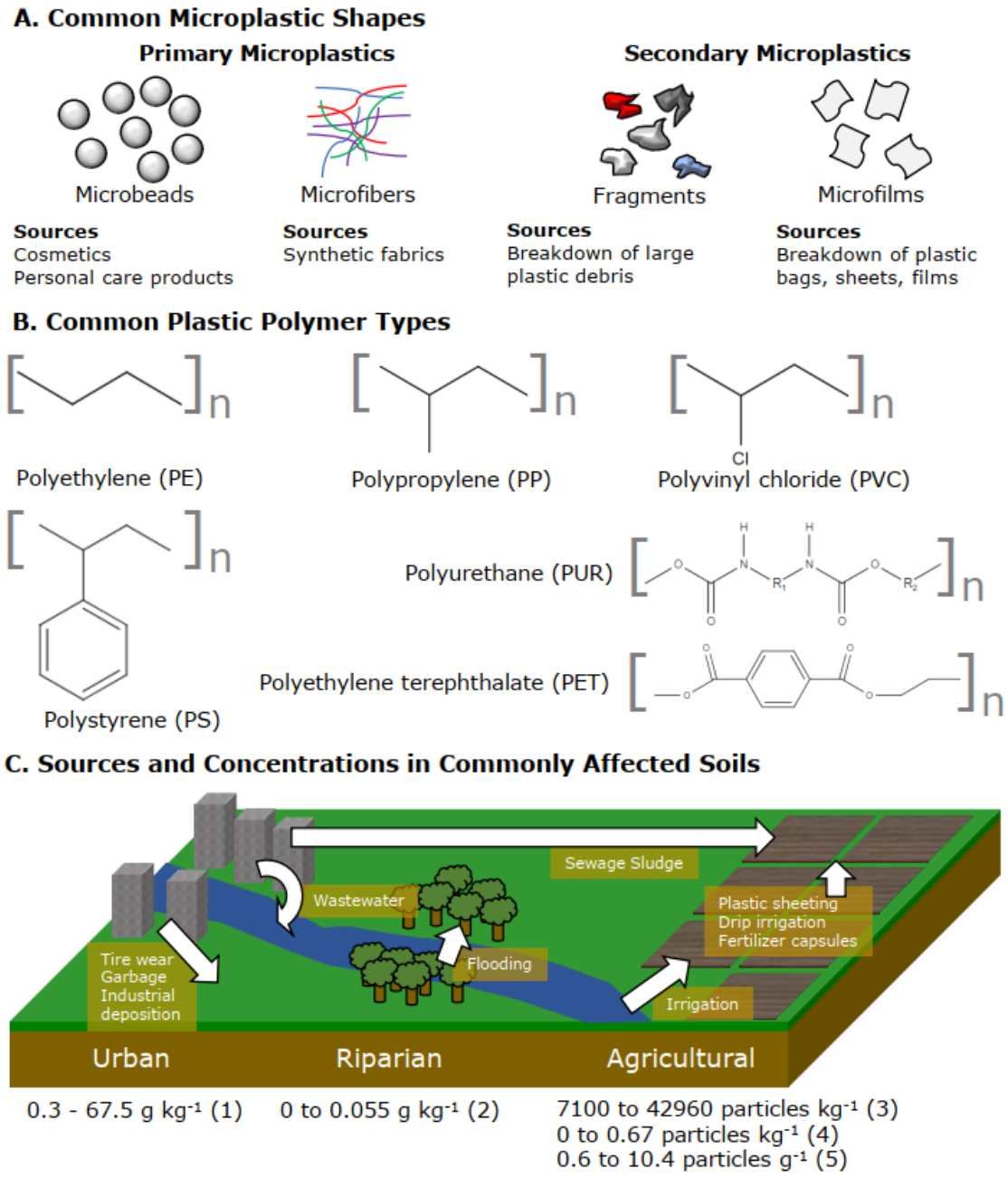

Figure 1.1: Diagram showing common microplastic shapes, six of the most common plastic polymers (Geyer et al. 2017), and sources and observed concentrations of microplastics in urban (1: (Fuller and Gautam 2016)), riparian (2: (Scheurer and Bigalke 2018)), and agricultural soils (3: (Zhang and Liu 2018), 4: (Piehl et al. 2018), 5: (Corradini et al. 2019)). Note that microplastic concentration in (5) is given in particles g^{-1}, not particles kg^{-1}.

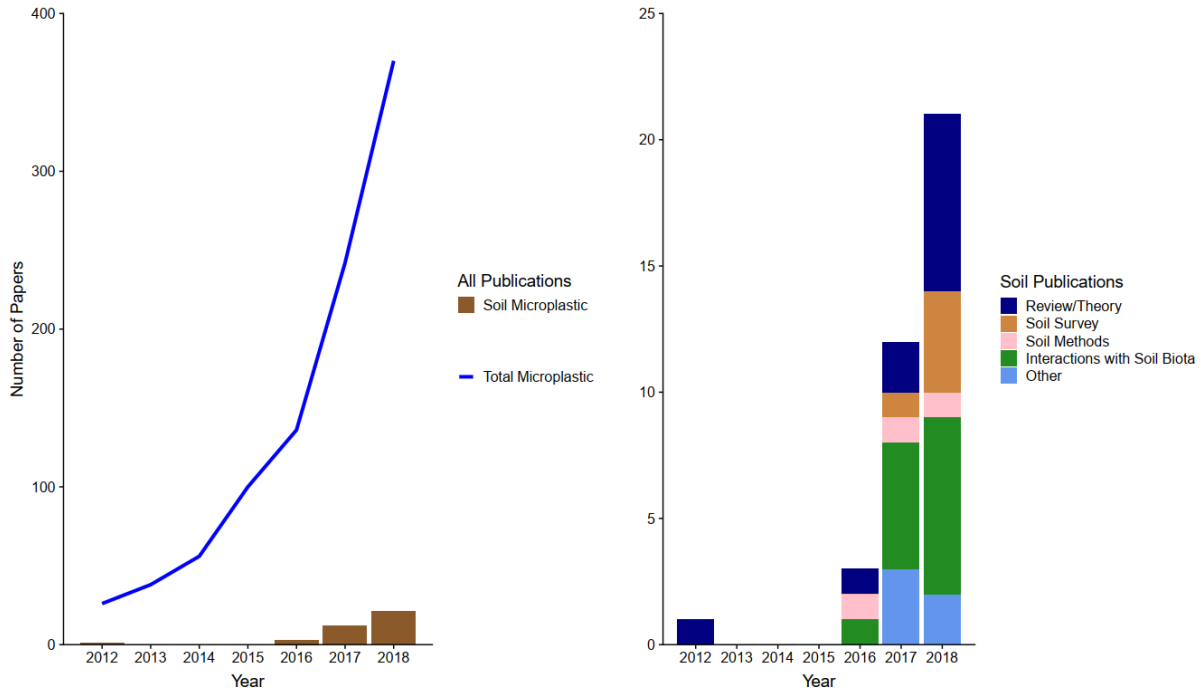

Figure 1.2: Left: The number of soil microplastic publications per year from 2012-2018 (brown bars) compared with total microplastic publications (blue line). Right: Soil microplastic papers broken down into the following categories: Review/Theory, papers primarily reviewing past work or proposing new hypotheses; Soil Survey, papers primarily assessing the occurrence of microplastics in field soils; Soil Methods, papers primarily detailing new methodologies for extracting or quantifying microplastics in soil; Interactions with Soil Biota, papers primarily examining microplastics' effects on and/or interactions with living soil organisms; and Other, papers falling outside of or combining these categories. Both graphs were produced from February 18, 2019 searches of the ISI Web of Science database for the following combinations of terms within a date range of 1990 to 2019 (though the graph only displays 2012 through 2018, as no soil microplastic papers were found before 2012): microplastic + soil, microplastic + terrestrial, microplastic + agricultur*, and nanoplastic + soil (and is not exhaustive). The "Total Microplastic" line in the left graph was produced by a similar search for the single term: microplastic.

Despite their recent entry into the microplastic literature, soils may be even more prone to such pollution than oceans, due to greater estimated release of plastic to terrestrial systems (Horton et al. 2017). Indeed, microplastics are often more abundant in soils than in ocean waters on a mass/mass or particles/mass basis (He et al. 2018, Rezania et al. 2018); the world's agricultural soils alone could hold more microplastic mass than oceanic surface waters (Nizzetto et al. 2016). That said, to assume a given w/w microplastic concentration in soil has the same ecological effect or indicates the same level of environmental harm as that concentration in seawater is likely unwise.

The ecotoxicological effects on soil animals ingesting or otherwise exposed to microplastics have been reviewed extensively (Chae and An 2018, Ng et al. 2018, Zhu et al. 2019) and so are only briefly summarized in this review. Most research has so far focused on earthworms (Huerta Lwanga et al. 2016, Cao et al. 2017, Rodríguez-Seijo et al. 2018, Judy et al. 2019), though has included a fair assemblage of other animal taxa, such as springtails (Zhu, Chen, et al. 2018, Ju et al. 2019), enchytraeid worms (Zhu, Fang, et al. 2018, Lahive et al. 2019, Selonen et al. 2019), isopods (Jemec Kokalj et al. 2018, Selonen et al. 2019), oribatid mites (Selonen et al. 2019), and snails (Song et al. 2019). The nematode *Caenorhabditis elegans* has also been shown to ingest microplastics, which accumulate in the gut and may cause a slew of negative physiological effects (Lei et al. 2018). *C. elegans*, despite being mostly found residing in decaying plant matter rather than bulk soil (Schulenburg and Félix 2017), is still similar in its feeding method to other soil bacterivores as well as aquatic species also known to ingest microplastics (Fueser et al. 2019). Microplastics may also have non-toxicological effects, such as particles immobilizing soil microarthropods by filling pore spaces (Kim and An 2019).

Though it is still hard to draw definitive conclusions from this literature base on the relative threat microplastics pose to soil biota, these early studies concur with the wider base of marine microplastic toxicology in that there is worrying potential (Anbumani and Kakkar 2018, Rezania et al. 2018). However, experiments showing significant harm sometimes do so only with microplastic concentrations beyond what is common in the current environment (Lenz et al. 2016, Phuong et al. 2016, Cunningham and Sigwart 2019). Trophic transfer of microplastics to higher levels of the food web, commonplace in marine systems (Setälä et al. 2014, Carbery et al. 2018, Nelms et al. 2018), has so far been only indirectly shown in terrestrial environments (Huerta Lwanga, Vega, et al. 2017). If microplastics can be transferred from prey to predators in soil, it could result in microplastics escaping the confines of soil and spreading throughout the aboveground food web, via the many terrestrial animals (including vertebrates) that consume soil invertebrates, but requires further investigation.

Microplastics can also influence soil physical characteristics including bulk density and water dynamics (Liu et al. 2017, de Souza Machado, Lau, et al. 2018, de Souza Machado et al. 2019), interestingly decreasing overall soil bulk density but increasing density of the rhizosphere (de Souza Machado et al. 2019). This "ecosystem engineering" may be partly responsible for microplastics' effects on soil microbial communities (de Souza Machado, Lau, et al. 2018, Qian et al. 2018) and plants (Qi et al. 2018, de Souza Machado et al. 2019, Jiang et al. 2019, Rillig et al. 2019). Microplastics also affect these organisms directly. Plant roots can take up very small plastic particles (nanoplastics) and their roots may suffer physical harm by adsorbing microplastics (Kalčíková et al. 2017, Jiang et al. 2019). Plastics may serve as substrates for soil microbes' growth (Shah et al. 2008, Kale et al. 2015, Yoshida et al. 2016), and extensive colonization of microplastic particles by unique communities has been observed in aquatic

systems (McCormick et al. 2014, Kettner et al. 2017) and more recently in soil (Zhang et al. 2019).

Though microplastics are considered pollutants, they may be more complex in their occurrence and their interactions with biota than other commonly recognized pollutant classes (e.g., persistent organic pollutants, pesticides, heavy metals, etc.). Individual pollutants within any of these classes typically differ in their toxicological effects (Gao et al. 2016, Gan and Wickings 2017, Yang et al. 2018), but they still occur mostly in molecular or ionic forms. In contrast, microplastics exist in a vast diversity of sizes, ranging from nanometers to millimeters in diameter; shapes, including fibers, spherical beads, thin films, and irregular fragments (Figure 1.1); and polymer types (Figure 1.1). These are critical factors governing their interactions with biota. For example, (Lehtiniemi et al. 2018) found shrimp and juvenile fish to be more likely to ingest microplastic particles of similar size to their normal prey rather than smaller or larger particles. Irregularly-shaped secondary microplastics were shown to have greater negative effects than uniform primary microplastics on the freshwater crustacean *Daphnia magna* (Ogonowski et al. 2016) and the floating plant *Lemma minor* (Kalčíková et al. 2017). Among several plastic polymers investigated by (de Souza Machado et al. 2019), polyamide (PA) was the only one shown to increase the nitrogen content of spring onion leaves, possibly because it contains nitrogen in its chemical structure and may have thus leached nitrogenous compounds into the soil.

Microplastics' physicochemical diversity means it may be a mistake to consider them as just another anthropogenic pollutant, no different from a pesticide, heavy metal, or organic toxin (Rochman et al. 2019). In addition, their interactions with the soil ecosystem are more complex than those of a chemical pollutant that harms some soil organisms through direct toxicity, while

perhaps being metabolized or otherwise rendered inert by others (Nannipieri and Bollag 1991, Díaz 2004). Even heavy metals, for which interactions with organic matter have implications for their mobility in soil (Kalbitz and Wennrich 1998), or which, in the case of mercury, can be microbially altered into a more bioavailable form (Trevors 1986), do not interact with the soil ecosystem in ways as diverse as microplastics can or with as diverse an array of organisms (Huerta Lwanga, Gertsen, et al. 2017, Maaß et al. 2017, Huerta Lwanga et al. 2018). Microplastics are in some ways more reminiscent of invasive species than inanimate chemical toxins, with effects on soil biota that can be mediated through effects on the physicochemical environment (Eisenhauer et al. 2007, Alerding and Hunter 2019). Also, as with invaders (Shea and Chesson 2002, Levine et al. 2004), organisms already present at a site may affect their occurrence and distribution and mediate their effects on the rest of the food web (Huerta Lwanga, Gertsen, et al. 2017, Maaß et al. 2017). Hence, our use of the word *interact* and our argument for the value of a more explicitly ecological framing of microplastics in soil.

Past reviews on microplastics in soil (Bläsing and Amelung 2018, Chae and An 2018, de Souza Machado, Kloas, et al. 2018, He et al. 2018, Ng et al. 2018, Zhu et al. 2019), of which there have been many for such a recent field (Figure 1.2), have focused primarily on microplastic occurrence and abundance in terrestrial environments, sources of soil microplastics, methods for their extraction and quantification, and their ecotoxicological effects on soil organisms and communities. We chose to focus on the role of ecology in governing the occurrence, distribution, and ultimate impacts of microplastics in soil: in other words, on ecological effects *on* microplastics in addition to those *of* microplastics. We propose that understanding microplastic dynamics in soils requires that knowledge of soil physics, soil and polymer chemistry, and toxicology be placed within an ecological context recognizing interactions between microplastics

and the biotic components of soil ecosystems. To this end, we describe interactions with soilborne plastics first of microbes, then of fauna, and then of both in tandem.

Microbial effects: Colonization and degradation

Plastic is stereotyped as an inert material that does not biodegrade. To be sure, the carbon-carbon backbones of many common plastic polymers make them more resistant to enzymatic breakdown than many biological materials (Wei and Zimmermann 2017) and some types of plastic may be virtually unchanged even after decades of burial (Otake et al. 1995). However, exposure to the elements, particularly ultraviolet radiation, may sensitize some plastics to microbial colonization and biodegradation (Vimala and Mathew 2016, Wei and Zimmermann 2017, Gong et al. 2019), and Otake et al. (1995) reported that polyethylene films were heavily degraded after years of burial. Numerous soil microbes, both bacteria and fungi, have been implicated in plastic degradation (Shah et al. 2008, Kale et al. 2015, Yoshida et al. 2016). This has ramifications both for the fate of *allochthonous* microplastics introduced to a soil via floodwaters (Scheurer and Bigalke 2018), sewage sludge (Corradini et al. 2019), or other means, as well as for the potential formation of *autochthonous* microplastics from larger plastic debris (Figure 1.3).

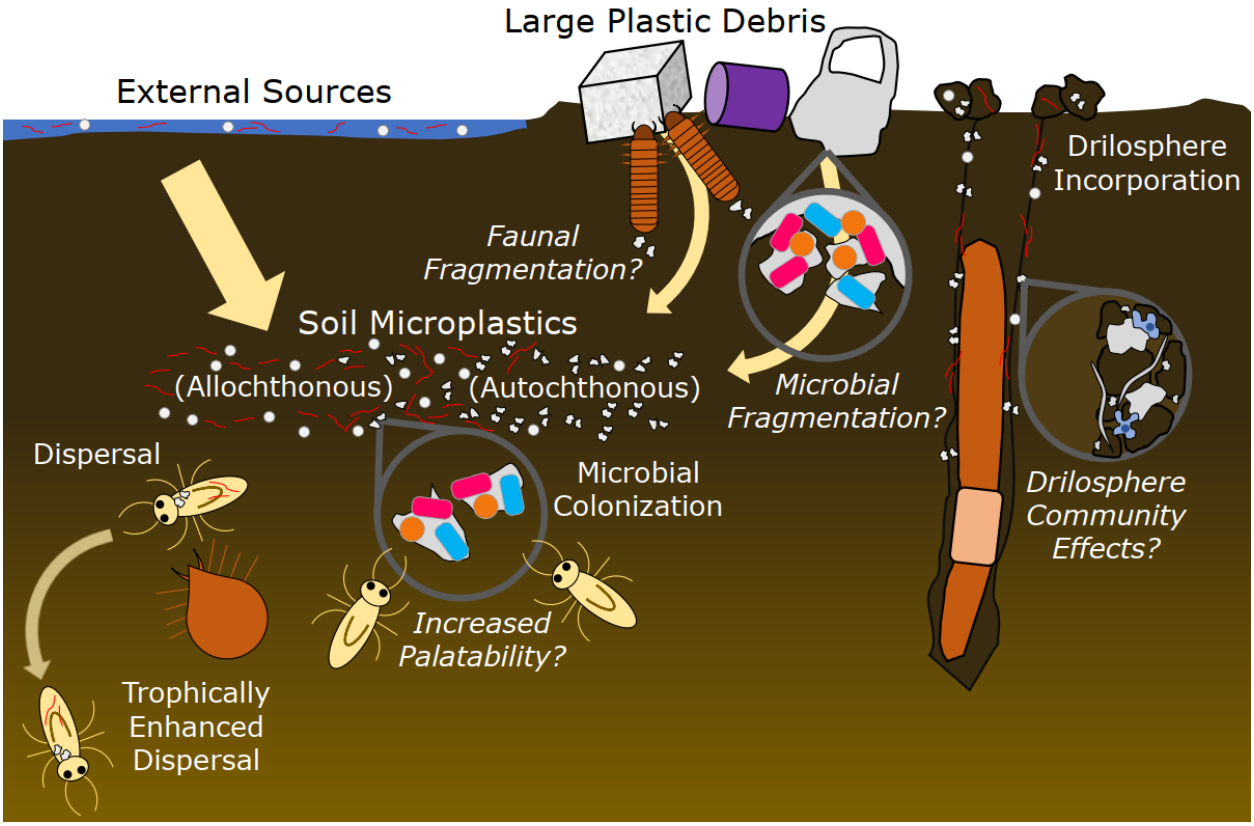

Figure 1.3: Diagram of possible ways soil organisms can affect microplastic occurrence, distribution, and potential effects on other soil organisms. Items in italics are speculated to occur based on current knowledge, but have not yet been explicitly tested.

Allochthonous microplastics introduced to a soil may well be colonized by microbes, as is common in aquatic and sediment systems (Harrison et al. 2014, McCormick et al. 2014, Phuong et al. 2016, Kettner et al. 2017, Hossain 2019). Polyethylene microfilms from the breakdown of agricultural film were found to host microbial communities unique from those of soil, litter, and macroplastic surfaces (Zhang et al. 2019), though the extent of this across other soils and microplastic types has yet to be determined. Degradation of colonized particles could result in reduction of microplastic particle size as well as their eventual disappearance. Bacteria isolated from earthworm guts caused a 60% reduction in total microplastic mass in a sterilized

soil in just 4 weeks and shifted the particle size distribution downward (Huerta Lwanga et al. 2018), indicating that biodegradation and fragmentation may be co-occurring processes. However, microbes may not always colonize and degrade plastics in natural soils with other food resources available (Ng et al. 2018). Microbes may prefer less energetically-expensive carbon sources, and cometabolic degradation of plastic is unlikely to occur to any great extent in field soils (Ng et al. 2018). Still, Liu et al. (2017) observed increases in microbial enzyme activity, including phenol oxidase, which degrades recalcitrant organic matter, following microplastic addition (albeit at high concentrations, 7 to 28% w/w) to a laboratory-incubated carbon-poor soil. This indicates that at least some resident soil microbes can respond to the presence of microplastics.

Macroplastic debris such as agricultural film or garbage may also face microbial colonization, with potential for autochthonous microplastic formation. Some bacteria have been shown to cause significant weight loss from agricultural film, though other studies report little to no degradation (Vimala and Mathew 2016, Wei and Zimmermann 2017). Microbial action can physically weaken plastic (Lucas et al. 2008), and as (Kyrikou and Briassoulis 2007) point out, weight loss observed in any study may indicate physical fragmentation of plastic in addition to degradation and mineralization or assimilation by microbes.

Faunal effects: Dispersal and positioning

Once transported to or generated within a soil, microplastics can be dispersed via both abiotic and biotic mechanisms. Wind can move microplastics (Razaei et al. 2019) distances of at least 95 km through the air (Allen et al. 2019), but presumably also over smaller spatial scales

along the ground. Gravity and flowing water can also transport microplastic particles through the soil matrix. Indeed, use of plastic microbeads to study particle movement through soil actually predates microplastic pollution of soil as a research topic itself (Rillig, Ingraffia, et al. 2017). Abiotic movement of microplastics through the soil matrix may be altered through biological activities. Earthworm burrowing increases soil porosity and water infiltration rates (Lavelle et al. 1997, Capowiez et al. 2009, Bottinelli et al. 2015), which may enable deeper water-assisted spread of microplastics into the soil profile (Rillig, Ingraffia, et al. 2017, Yu et al. 2017). Plant roots can also move soil organisms as they grow and create channels for additional movement (Dighton et al. 1997, Demarta et al. 2014) and thus may also facilitate passive microplastic dispersal deeper into the soil profile.

Moving organisms may themselves disperse microplastics as well. Dispersal of soil animals and microbes on and inside one another is a common process (Norton 1980, Koehler 1999, Türke et al. 2018), one with consequences for organism distribution in space (Dighton et al. 1997), spread of parasitoids and pathogens (Campos-Herrera et al. 2012, Shapiro-Ilan and Brown 2013), and even community succession (Terwilliger and Pastor 1999). Though plastics obviously cannot actively hitchhike, they can still be passively dispersed on or inside soil organisms (Maaß et al. 2017, Rillig, Ziersch, et al. 2017, Zhu, Bi, et al. 2018). Although dispersal by springtails and mites has so far only been demonstrated in Petri dish arenas (Maaß et al. 2017, Rillig, Ziersch, et al. 2017, Zhu, Bi, et al. 2018), microplastics have been found in field-collected earthworm casts (Huerta Lwanga, Vega, et al. 2017). Within soil pores, springtail movement may also facilitate influxes of small microplastic particles (Kim and An 2019). This could alter microplastics' distribution in soil beyond what abiotic forces can accomplish alone, including horizontal transport from a point source such as a piece of fragmenting plastic debris.

In addition to simply moving microplastics, biotic activities may also place them in pedological contexts they would rarely if ever otherwise occupy. Earthworms incorporate microplastics into their casts and burrow walls (Huerta Lwanga et al. 2016, Huerta Lwanga, Gertsen, et al. 2017, Huerta Lwanga, Vega, et al. 2017, Rillig, Ziersch, et al. 2017, Prendergast-Miller et al. 2019). These casts and burrows comprise a unique microhabitat that soil ecologists term the *drilosphere* (Andriuzzi et al. 2013, Sharma et al. 2018). Drilosphere habitats have unique physicochemical properties (Görres et al. 2001, Görres and Amador 2010) and are hotspots of microbial and faunal activity (Andriuzzi et al. 2016, Nuutinen et al. 2017). By bringing these communities into close contact with microplastics, earthworms could enable microplastic effects on other soil organisms that would not otherwise occur, or alter the severity of microplastic exposure. The fecal material of any other organism that ingests and excretes significant amounts of soil may similarly position microplastics for unique ecological interactions, though the ecology of fecal material from isopods, millipedes, scarab grubs, and other such organisms has not been as well described.

Microbes and fauna: Increased palatability and further fragmentation

Microbial colonization of plastics in soil may lead to broader ecological consequences than just those of their own ability to degrade plastic. The "peanut butter on the cracker" hypothesis (Cummins 1974) posits that aquatic detritivores derive most of their nutrition from microbial biofilms covering the organic matter they consume rather than the organic matter itself. Other studies in aquatic systems have lent credulity to this idea (McGoldrick et al. 2008, France 2011), and it is thought to also apply in soil (Digel et al. 2014, Potapov et al. 2019). For

those detritivorous soil animals that consume organic matter simply as a means of obtaining the microbial "peanut butter," microbial colonization could make microplastics (or larger plastic debris) more palatable than the typically virgin particles used in many laboratory studies (Figure 1.3). Some earthworm species are known to prefer colonized and degraded leaf litter to fresh litter (Curry and Schmidt 2007, Ashwood et al. 2017), and Wright (1972) found that coating strips of polyester foam with bacterial cells led to their consumption by the earthworm *Lumbricus terrestris* (and harm to the worms as the foam lodged in their gizzards), strongly supporting this hypothesis. In marine systems, the amphipod *Orchestia gammarellus* ingested and shredded more plastic film material when it was colonized by microbial biofilms than when not colonized (Hodgson et al. 2018). Galloway et al. (2017) suggest the ubiquity of microplastic ingestion by marine biota could be due to microbial colonization of floating particles.

Animals consuming colonized particles would face all the attendant toxicological effects of microplastics and their chemical additives. Yet the particles themselves could also be subject to further fragmentation and/or biodegradation by the animals and their gut microbiota, to the end of altering the size and shape of existing microplastics or generating new particles from larger plastic debris. So far, true *in vivo* biodegradation has only been found in a few insect species, namely tenebrionid beetle larvae (Yang et al. 2015, Peng et al. 2019) and pyralid moth larvae (Yang et al. 2014, Bombelli et al. 2017, Kundungal et al. 2019), the latter of which are not soil-dwellers. Yellow mealworm *Tenebrio molitor* larvae ingested polystyrene; approximately half the mass was mineralized as CO_2, a small percentage was incorporated into biomass, and the rest degraded into depolymerized molecules or smaller plastic fragments and excreted in feces (Yang et al., 2015a, 2015b). This finding was replicated with *T. molitor* populations from several regions of the world (Yang, Wu, et al. 2018) as well as its congener *T. obscurus* (Peng et al.

2019). *T. molitor* was also found to be able to biodegrade polyethylene in much the same manner (Brandon et al. 2018). Kundungal et al. (2019) fed polyethylene film alone or with beeswax to larvae of the lesser wax moth *Achroia grisella* and observed partial biodegradation, with some of the plastic excreted in feces. As with microbes, the studies demonstrating plastic biodegradation in insects have been primarily laboratory-based, yet these species still consume plastic even when also given more natural foods (Kundungal et al. 2019), as has been done in efforts to optimize insect-based biodegradation technologies by improving insect health (Yang, Christopher, et al. 2018). Given insects' staggering taxonomic diversity (Stork et al. 2015), this ability likely exists in additional insect species, and perhaps other soil arthropods and invertebrates, such as earthworms (Huerta Lwanga et al. 2018).

Even if biodegradation *sensu stricto* is rare, both macro- and microplastics may also suffer physical damage and fragmentation as they pass through animals' guts, as was observed for polyethylene terephthalate microfibers ingested by land snails (Song et al. 2019) and bag films shredded by marine amphipods (Hodgson et al. 2018). Solitary bee species have been shown to use fragments torn from large plastic debris as nesting materials, without ingesting them at all (MacIvor and Moore 2013, Allasino et al. 2019). Much harder materials like quartz and feldspar grains can be ground to smaller sizes by passage through earthworm and scarab beetle grub guts (Suzuki et al. 2003), so it is possible that plastic particles could be likewise altered by these and similar organisms that consume large amounts of soil. Reducing microplastic particle size makes ingestion by smaller organisms more likely (Lehtiniemi et al. 2018), and so breakdown by larger organisms could increase microplastics' availability to smaller ones.

Synthesizing ecology's role

Microplastics are unique among anthropogenic pollutants in that their effects on organismal and ecosystem function may strongly depend upon the organisms themselves as they affect microplastic occurrence, form, and distribution (Figure 1.3). In other words, understanding microplastic dynamics in a given soil will likely require some knowledge of that soil's biodiversity and ecology. (Lehmann et al. 2019) demonstrated that microplastic effects on abiotic soil properties were partially mediated by soil microbial communities, and their effects on biotic communities may be likewise dependent on those communities themselves. For example, microplastics could have radically different effects in a European forest soil with a diverse assemblage of native earthworms than in a similar North American soil in a remote area where introduced earthworms have yet to reach, given what earthworms and their gut microbiota have been shown to do to microplastics, dispersing them through soil and potentially reducing their size through partial biodegradation (Huerta Lwanga, Gertsen, et al. 2017, Yu et al. 2017, Huerta Lwanga et al. 2018). We find the following questions raised by the current state of knowledge:

1. What organisms can facilitate microplastic formation from larger plastic debris, to what extent, and under what circumstances?
2. Once microplastics are present in a soil (regardless of source), what organisms contribute to their dispersal throughout the soil profile, either vertically (deeper into the ground) or horizontally (away from a point source such as a piece of large plastic debris)?

3. To what extent are the shapes and sizes of microplastic particles influenced by microbial or animal biodegradation or physical damage due to passage through animal guts?
4. Can organisms besides earthworms create circumstances of unique microplastic positioning in soil that could not arise through abiotic processes, and what are the consequences of this for other organisms?
5. To what extent can one organism's effect on microplastic size, shape, or distribution in soil alter their effect on other organisms?

Answering these questions will require inventive experimental designs, often involving multiple soil organisms simultaneously, as well as further refinement of methods to extract and identify microplastics in soil and invertebrate samples. Research into them could provide initial insight into organismal and community functional traits predictive of specific microplastic dynamic of interest. For example, heavily setaceous arthropods like pincushion millipedes (Polyxenida) and some entomobryid springtails may be more likely to disperse microplastics through the soil due to their setae more readily catching and retaining particles. Functional trait indices have been proposed by soil ecologists to assess ecosystem responses to disturbance and land use change (Vandewalle et al. 2010, Pey et al. 2014) and organisms' contribution to ecosystem services (de Bello et al. 2010, Wood et al. 2015), and one could possibly be developed to predict how much a given soil community is likely to alter microplastic dynamics in that soil. Also, any given microbial or faunal behavior that affects microplastics may itself be affected by other community processes more well-known to soil ecologists. In laboratory assays, dispersal of microplastics by springtails and predatory mites increased when both species were present

compared to single species (Zhu, Bi, et al. 2018), likely due to altered movement behavior of the two arthropods in each other's presence.

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CHAPTER TWO: COUNTERSTAINING TO SEPARATE NILE RED-STAINED MICROPLASTIC PARTICLES FROM TERRESTRIAL INVERTEBRATE BIOMASS

Abstract

The emerging threat microplastic pollution poses to soil and its biota necessitates development of methods to detect microplastic ingestion by soil animals. Fluorescent staining with Nile red dye has proven effective at distinguishing microplastics from inorganic and some biological material, but is not suitable for separating them from invertebrate remains. Here we report on the development and validation of a novel fluorescent counterstaining technique for detection of microplastics within terrestrial invertebrate biomass and fecal material. After staining with a blend of Calcofluor white and Evans blue dyes in addition to Nile red, ground arthropod biomass appeared blueish-purple, whereas different plastic polymers appeared red, green, and yellow when viewed under laser scanning confocal microscopy. Non-arthropod invertebrate biomass and fecal material were also distinguishable from plastic, though to a lesser extent. Our results highlight the value of this method for detecting microplastic ingestion by terrestrial invertebrates.

Introduction

Soilborne microplastics have exploded into recent prominence with research showing their ubiquity in soils potentially matching or exceeding that of marine systems (Nizzetto et al. 2016, Bläsing and Amelung 2018, Chae and An 2018), with concerning but not yet fully characterized effects on soil biota and ecosystem function (Huerta Lwanga et al. 2016, Chae and An 2018, He et al. 2018, Zhu et al. 2018, Helmberger et al. 2020). Microplastics have been found in urban (Fuller and Gautam 2016), riparian (Scheurer and Bigalke 2018), and agricultural soils (Liu et al. 2018, Zhang and Liu 2018, Corradini et al. 2019), and wind may disperse them to even the remotest regions of the world (Allen et al. 2019). Determining which animals readily consume microplastics, whether plastics are passed through animals' guts, and how different plastic types are affected by animal digestive processes are critical for understanding how microplastics interact with soil communities and ecosystems.

Numerous methods have been developed for detecting microplastics in environmental samples and biota (Hidalgo-Ruz et al. 2012, Cole et al. 2014, Avio et al. 2015, Karlsson et al. 2017, Bläsing and Amelung 2018, Windsor et al. 2019). The hydrophobic fluorochrome dye Nile red binds to microplastics, allowing them to be detected via fluorescence microscopy (Shim et al. 2016), either visually or via an automated system (Erni-Cassola et al. 2017). This technique has been used to find microplastics in seawater (Erni-Cassola et al. 2017), marine sediment and beach sand (Erni-Cassola et al. 2017, Maes et al. 2017), fresh water and sediment (Fischer et al. 2016, Wang et al. 2018), mussel tissue (Catarino et al. 2018), and earthworm casts (Wang et al. 2019), among other matrices, and has also been used to pre-stain plastic microfibers so they could be detected in terrestrial snail guts (Song et al. 2019). However, Nile red stains numerous

biological materials (Shim et al. 2016, Ruggero et al. 2020). Therefore, care must be taken when identifying plastics from animal remains or feces (Erni-Cassola et al. 2017). Chemical digestion is one solution to this problem. Hydrogen peroxide or enzymes that leave plastic unharmed are commonly used to remove biological material from samples (Bläsing and Amelung 2018). However, some biological material can survive this treatment and then be stained by the Nile red (Stanton et al. 2019), making plastic differentiation difficult. 4′,6-diamidino-2-phenylindole (DAPI) has been used as a counterstain along with Nile red to detect microplastics in water samples (Stanton et al. 2019), as DAPI stains biological material with high specificity and fidelity and so any particles exhibiting DAPI fluorescence could be discounted as biological in origin, regardless of whether or not they were also stained by Nile red. The advantage of a counterstain is thus the ability to further subdivide any fluorescing particles and reduce the number of particles that need to be manipulated in further tests to confirm their identity as plastics. The goal of our study was to develop an additional counterstaining technique capable of distinguishing microplastics from the biomass of terrestrial invertebrates, evaluating a blend of two fluorochrome dyes, Calcofluor white and Evans blue.

Methods

We developed and evaluated our counterstaining method through the course of several trials assessing 1) the method's ability to separate plastics from invertebrate biomass and fecal material, 2) its ability to separate plastic microfibers from potentially contaminating cotton fibers, 3) its recovery efficiency, 4) its ability to detect microplastics actually ingested by a living invertebrate, and 5) its ability to detect microplastics in field-collected invertebrates.

General trial methodology – sample preparation

All tests of the counterstaining method involved fluorescence microscopy of mixed samples of plastic and biological material caught on 42.5 mm Whatman glass microfiber filters (pore size 1.5 μm). We fixed all invertebrate specimens in filtered 70% ethanol, dried them at 65 °C for 48 to 72 h, then ground them in a mortar and pestle. The ground biomass was placed in glass jars containing 15 mL of 30% hydrogen peroxide for 48 h. If the trial called for plastics to be mixed into the sample, that was done at this step, so that plastics and biomass would be stained together at the same time.

We then vacuum filtered the digested mixtures, let the filters dry, and pipetted sufficient Calcofluor white/Evans blue solution (1.0 g L^{-1} Calcofluor white, 0.5 g L^{-1} Evans blue, Sigma-Aldrich) to cover the filter surface. We washed it with filtered DI water, and allowed it to dry before staining with Nile red (0.05g L^{-1} in acetone, diluted 10 times in *n*-hexane, Santa Cruz Biotechnology) and washing with filtered *n*-hexane. A solution of Calcofluor white alone was also tested (1.0 g L^{-1}, Santa Cruz Biotechnology). During staining, filters were placed atop a square of paper towel to wick away excess moisture and reduce drying time. Calcofluor white (ex. 355 nm, em. 300 to 440 nm) is a selective dye, chemically binding to the polysaccharides chitin and cellulose (Elorza et al. 1983). Evans blue (ex. 470 and 540 nm, em. 680 nm), on the other hand, penetrates dead cells via their degraded membranes and thus is a more general stain of biological material (Baker and Mock 1994). We could not configure our confocal microscope to detect its fluorescence at the same time as that of the Nile red and Calcofluor white, but anticipated it would quench at least some Nile red-induced fluorescence of biological material.

General trial methodology – fluorescence microscopy

For most trials, we viewed the stained filters under a Nikon Eclipse Ni-U upright microscope operating with a 10X air objective (Plan Fluor, numerical aperture 0.30) attached to a Nikon C2+ confocal laser scanning microscope running three simultaneous diode lasers; 405 nm to excite Calcofluor white and 488 and 561 nm to excite Nile Red. We detected the emissions in three channels through 445/35 nm bandpass, 525/50 nm bandpass, and 600/50 nm bandpass filter cubes. We collected each image as a Z series maximum intensity projection through the thickness of the sample with distances between steps ranging from 5 to 15 µm, depending on the sample. When necessary due to suboptimal image acquisition parameters, Look Up Tables (LUTs) for green and red intensities were increased to provide better visibility of the microplastics.

For other trials, notably the field trial, we viewed them through a Leica S8 APO stereomicroscope fitted with a Nightsea fluorescence adapter (440 to 460 nm excitation light to excite the Nile red, 500 nm longpass emission filter) instead of the confocal. We used an AmScope LED UV light (395 nm) to excite the Calcofluor white. This was done both to evaluate a considerably more inexpensive microscopy setup and to enable necessary access to particles during the field trial, which was difficult when using the confocal due to its small field of view.

Initial invertebrate and fecal material imaging trials

We evaluated the counterstaining method with species representing all four arthropod subphyla; larvae and adults of the darkling beetle *Zophobas morio* (Coleoptera: Tenebrionidae),

the woodlouse *Oniscus asellus* (Isopoda: Oniscidae), an unidentified harvestman of the family Sclerosomatidae, and an unidentified millipede of the family Julidae. *Z. morio* larval fragments were used in the majority of imaging trials, the rest being included simply to confirm that biomass stained similarly across multiple arthropod taxa and thus would always be visually distinct from microplastics. *Z. morio* larval fragments were mixed with white high-density polyethylene (PE), polypropylene (PP), and/or polyvinyl chloride (PVC) microplastics ground in a ball mill (Retsch) and sieved to specific size ranges, or expanded polystyrene (EPS) microplastics cut from a piece of white Styrofoam. PE and PP fragments had maximum diameters of approximately 500-1000 μm, PVC fragments approximately 100-500 μm, and EPS fragments approximately 2 mm. These polymers are among the most common waste plastics found in the environment (Geyer et al. 2017). Other arthropod fragments were mixed with PE and PVC together.

Arthropods are far from the only terrestrial invertebrates that may consume microplastics; both annelids (Huerta Lwanga et al. 2016, Cao et al. 2017, Rodríguez-Seijo et al. 2018) and gastropods (Panebianco et al. 2019, Song et al. 2019) are known to do so. These organisms contain some chitin, earthworms in the gizzard (Peters and Walldorf 1986) and gastropods in the radula and shell, including the internal shell of slugs (South 2012). That said, chitin is not as widespread throughout their anatomy as it is in arthropods, and so Calcofluor white may not stain all of their ground and filtered biomass. We prepared additional filters of PE and PVC fragments mixed with ground biomass of the earthworm *Lumbricus terrestris* and a slug of the *Arion subfuscus* group.

Analyzing an organism's fecal material for microplastics can provide evidence of microplastic ingestion from the environment as well as physical breakdown or biodegradation of

plastic particles in the gut and can even do so without killing the organism. However, fecal material can also contain a variety of other biological material from the organism's diet. Much like plastic, this material could also be stained by Nile red but not Calcofluor white. The presence of Evans blue in the counterstain blend, however, may still allow plastics to be distinguished from fecal material and non-arthropod biomass via penetration of dead cells in the biological material. For this trial, filters contained PE and PVC fragments and fecal material from the *A. subfuscus* group slug or from *Z. morio* larvae (fed on dry oatmeal).

Comparison of cotton and plastic microfibers

Calcofluor white stains cellulose as well as chitin (Herth and Schnepf 1980), a cause for concern if airborne cotton microfibers land on a sample and get stained. Working in a laminar flow hood may reduce the likelihood of such contamination, but even so, it is necessary to determine if contaminating cotton fibers (or other cellulosic debris that may be present in a sample) can also be distinguished from similarly shaped microplastics, especially since many microplastic protocols specifically call for wearing a cotton lab coat to prevent deposition of synthetic clothing fibers onto samples (Griet et al. 2015, Murphy et al. 2017, Nelms et al. 2018, Corradini et al. 2019). To test this, we prepared and viewed filters containing cotton fibers (pulled from the end of a cotton swab) alone or in combination with three additional plastic polymers commonly found in fibrous form; green polyamide (PA) fibers cut from a length of nylon paracord, gray polyethylene terephthalate (PET) fibers pulled from a blanket, and acrylic fibers of three different colors cut (red, pink, and blue) from lengths of yarn.

Recovery trials

To test the percentage of microplastic particles within a sample recoverable by this method, we conducted two trials in which we mixed a known quantity of microplastic particles with the dried and crushed biomass of a single soil invertebrate. Trials used 10 PE fragments and 20 red acrylic fibers (slightly varying between replicates) and biomass from the isopod *O. asellus* (mean dry biomass approximately 18 ± 1.4 mg, n = 4) in the first trial and the earthworm *Eisenia fetida* (mean dry mass approximately 29 ± 4.7 mg, n = 5) in the second. We kept the earthworms on moist paper towel in plastic cups for 96 h to allow them to void their guts of soil before fixing and drying them. For both trials, we digested, filtered, and stained the samples as previously described, and then counted the visible fragments and fibers. When grinding the dried invertebrates, we took special care to reduce them to as fine a powder as possible so as to reduce the likelihood of plastic particles being obscured underneath large pieces of digested biomass, a precaution not strictly necessary in the initial imaging trials.

In the first trial, we viewed the filters through the Nikon Eclipse Ni-U microscope oculars using conventional green fluorescence as opposed to the laser scanning confocal microscopy. Upon finding a particle we suspected to be one of the added microplastics, we switched from green to blue fluorescence and then to red. If it fluoresced under blue light, we discounted the particle as biological in origin. Particles appearing at the upper or lower edge of the ocular view were counted only at the upper edge, or else they would have been counted twice as we proceeded from the bottom of the filter upward. For the second trial, we used the fluorescence-adapted stereomicroscope. Viewing the filters, we could turn the two excitation lights on and off

in turn to identify which particles were stained by Calcofluor white alone, Nile red alone, or both dyes at once.

Microplastic ingestion trial

To determine the method's viability for counting microplastics that are actually ingested by an organism and not just mixed among ground fragments, we placed twelve *Z. morio* larvae into individual feeding arenas made from 120 mL plastic cups with 1 cm plaster bottoms and 0.5 g of diet. Four individuals had normal diet with no microplastics (control treatment) and the rest were given microplastic-spiked diet. The diet used was a *Drosophila suzukii* colony diet consisting of 11.25 g agar, 31.25 g cornmeal, 50 g sugar, 17.5 g nutritional yeast, 0.85 L DI water, 4.425 mL propionic acid, 0.825g methyl paraben, and 9.825 mL 100% ethanol, chosen for its liquid consistency enabling us to homogenously mix in microplastics before the diet cooled. We mixed in a combination of PE, PP, and PVC microplastics (40% PE, 40% PP, 20% PVC) into freshly-made diet at a 5% w/w ratio. We allowed the larvae to feed for 48 h before removing them from the feeding arenas. We immediately fixed in 70% ethanol all arthropods from control arenas and half of those from the microplastic-spiked arenas, placing the other half in empty arenas for another 48 h to allow for defecation and gut clearance before also fixing them in ethanol.

Two of the fixed larvae from each treatment were washed in filtered DI water, then dried and ground as described above. For the other two, we dissected out the entire length of their gut and cut it into 5 mm sections with a pair of iris scissors. Both types of remains we processed as above and imaged to look for microplastics. To prevent contamination of the samples with

airborne microplastics, all post-fixing processing (with the exception of gut dissection but including staining) was done in a laminar flow hood while wearing nitrile gloves and a pure cotton lab coat. We also produced a procedural blank every fourth sample to assess any persisting contamination. These consisted of an empty jar filled with H_2O_2 poured from the mortar that was filtered and stained like the other samples.

Field trial

Finally, we used the method to assess the presence of microplastics in field-collected invertebrates and their fecal material. We sampled from a trash-filled wooded area along the edge of a stream in East Lansing, MI, which we speculated would have appreciable microplastic abundance due to presence of plastic debris and proximity to an urban waterway. We hand-sorted invertebrates from leaf litter and from beneath rocks and logs on the day of July 17, 2019. We collected a total of 10 arthropods and 8 non-arthropod invertebrates. All were initially washed in filtered DI water, placed in glass jars, and given 48 h to defecate (though not all survived to do so). Afterwards, the invertebrates were fixed and processed as normal. We also digested and filtered the contents of each jar containing feces. All steps, including staining of biomass and feces filters, employed the same anti-contamination protocols as the ingestion trials.

We used the following criteria to identify potential microplastics on the stained filters.

1. Particles fluoresced under 440-460 nm excitation (Nile red emission).
2. Particles did not fluoresce under UV excitation (Calcofluor white emission).
3. Fluorescence characteristics did not vary strongly across the particle's surface.

4. Particles conformed to all criteria from Mohamed Nor and Obbard (2014) applicable to fluorescence microscopy, i.e., no cellular or organic structures visible and fibers were of equal thickness throughout their lengths, lacked tapered ends, were not segmented, and did not appear as flat, twisted ribbons.

For all suspected microplastic particles, we brought close a heated metal probe made from the finely filed tip of a 30 W soldering iron (Chicago Electric) with a specified operating temperature of 390 °C. If they melted or otherwise deformed in response to the heat, they were identified as plastic. This "hot needle test" approach cannot identify specific polymer types, but is still accepted as a means of distinguishing larger-sized plastic particles from non-plastic organic and mineral material unaffected by the heat (Griet et al. 2015, Silva et al. 2018, Keisling et al. 2020). Chemical analyses such as Fourier transform infrared spectroscopy and Raman spectroscopy would be required to accurately assess smaller particles (Shim et al. 2017), and we did not employ these methods as we were not attempting to fully characterize all microplastics present. Particles were then classified as fragments or fibers based on initial physical appearance. During this process, we also placed two moistened 70 mm filter papers in Petri dishes on either side of the microscope and examined them afterward to account for deposition of airborne microplastics, despite the low likelihood of such contaminants appearing similar to stained microplastics on the filter under fluorescence microscopy.

Results and Discussion

Efficacy of the counterstain – initial imaging trials

Counterstaining with Calcofluor white/Evans blue in addition to Nile red was sufficient for separating microplastic fragments of four polymer types (PE, PP, PVC, and EPS) from arthropod exoskeleton fragments (Figure 2.1). When using a confocal microscope equipped with 405 nm, 488 nm, and 561 nm excitation lasers, exoskeleton fragments appeared in shades of blue. This was true for exoskeleton fragments from all four extant arthropod subphyla; Hexapoda, Myriapoda, Crustacea, and Chelicerata. In contrast, plastics appeared red (PVC), green (PE and PP), or yellow (EPS), depending on the relative intensities of their green and red emissions. These differences were likely due to differences in surface hydrophobicity between the polymers (Karakolis et al. 2019), which may enable a rough estimation of at least some polymer types via this method, although more thorough testing of additional polymers and additional representatives of the tested polymers (e.g., different colors and ages) would be required to confirm this, as hydrophobicity may change with wear of particles by the environment (Karakolis et al. 2019). The Calcofluor white/Evans blue blend alone did not stain our most commonly tested plastics, though some, such as PVC and some colors of acrylic fiber, exhibited at least a modest degree of autofluorescence. We also tested the performance of pure Calcofluor white (1 g L^{-1} in filtered DI water, Santa Cruz Biotechnology) rather than the Calcofluor white and Evans blue blend. Pure Calcofluor white was no less effective at staining chitin, however we found the Evans blue blend more useful. This was because even though we could not readily detect fluorescence from the Evans blue, the dye still turned many pieces of

arthropod and earthworm biomass on the filters dark blue to the naked eye, which was advantageous when analyzing filters under the stereomicroscope. Still, the difference is not so great that we would strongly recommend one Calcofluor white formulation over the other.

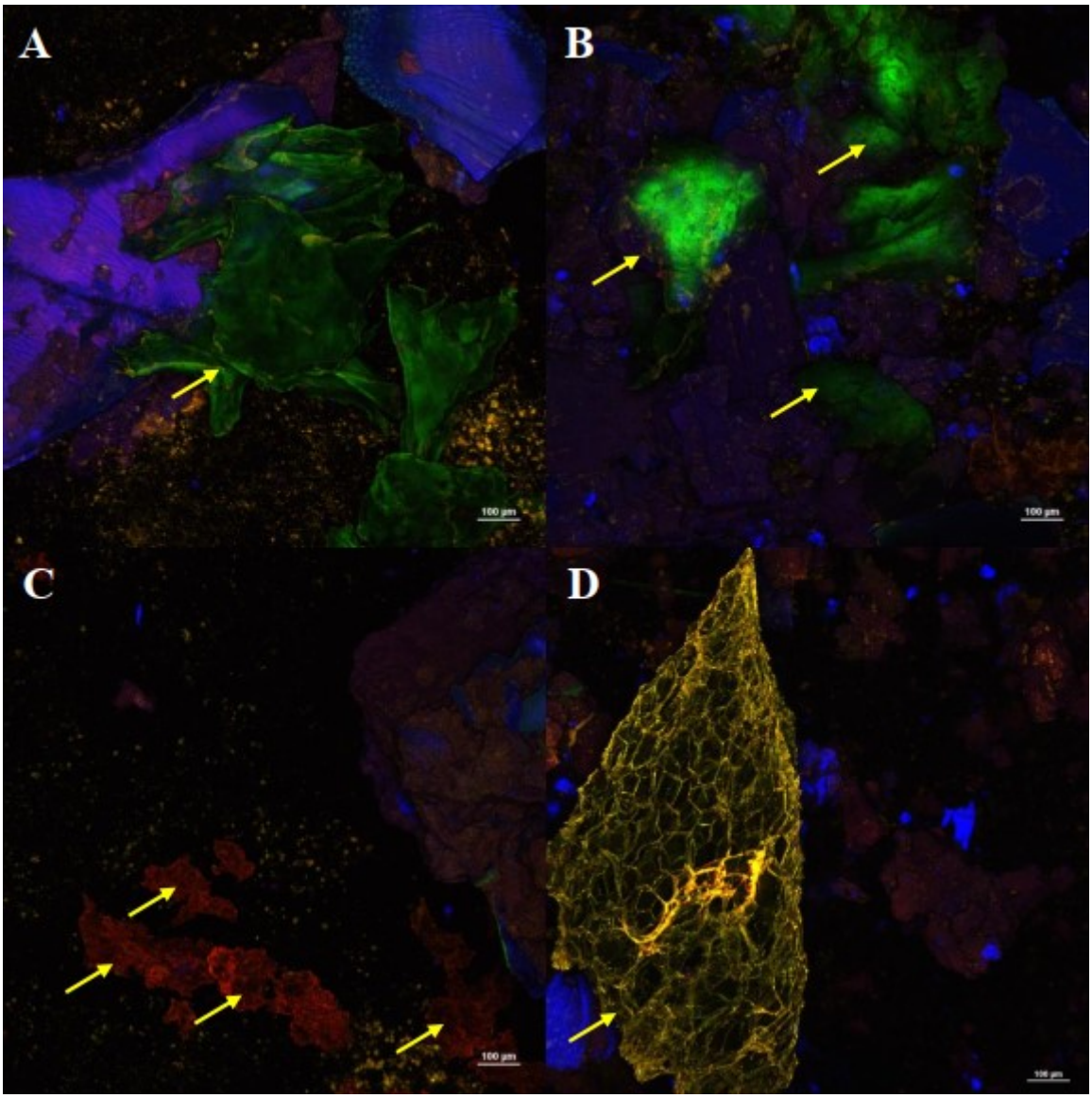

Figure 2.1: Maximum intensity projections of four plastic polymers in mixed samples with ground and chemically-digested *Zophobas morio* larval exoskeleton fragments. A: Polyethylene (PE), B: Polypropylene (PP), C: Polyvinyl chloride (PVC), D: Expanded polystyrene (EPS).

Figure 2.1 (cont'd):

Plastics are stained with Nile red (ex. 488 nm, em. 525 nm) and appear green, red, or yellow depending on polymer type. Exoskeleton fragments are stained with Nile red as well as Calcofluor white (ex. 405 nm, em. 445, blended with Evans blue) and appear blue or purple. Yellow arrows indicate plastic particles. Scale bar is 100 μm. See Methods for more detailed information on confocal microscope configuration.

For non-arthropod invertebrates, the counterstain was slightly less effective. Earthworm biomass appeared blue with a reddish-orange tint and PE and PVC particles were readily distinguishable (Figure 2.2A). Slug biomass, when digested, formed a translucent, blueish-red paste on the filters that at times seemed to occlude smaller plastic particles. Our PE particles were still visible (Figure 2.2B), though our smaller PVC particles were distinctly more difficult to make out (Figure 2.2C), also due to the reddish coloration of the slug biomass. However, when using the stereomicroscope, we observed autofluorescence of both earthworm and gastropod biomass under UV light, which appeared bright green through the 500 nm longpass emission filter, similar to Calcofluor white emission from chitin. Thus, Calcofluor white would actually be unnecessary for these samples, as the biological material can already be distinguished from Nile red-stained plastics by the fact that it also fluoresces under UV. That said, Calcofluor white would still stain any chitinous or cellulosic debris present in such samples and thus would still be useful. DAPI is another option for these samples, as it stains a broader range of biological material than just chitin and cellulose (Stanton et al. 2019), although we found it to be ineffective when using the fluorescence-adapted stereomicroscope, as we could not detect DAPI's UV-induced emission through the emission filter.

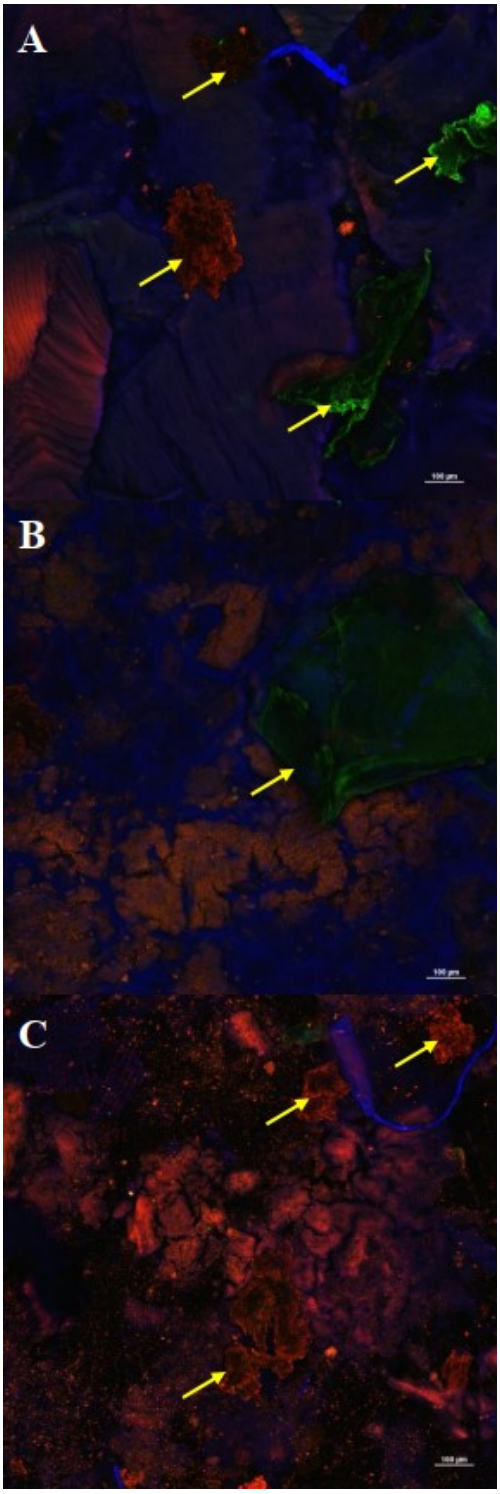

Figure 2.2: Maximum intensity projections of polyethylene (PE) and polyvinyl chloride (PVC) microplastics in mixed samples with ground and chemically-digested biomass of (A) the

Figure 2.2 (cont'd):

earthworm *Lumbricus terrestris* or (B,C) a slug of the *Arion subfuscus* complex. Plastics are stained with Nile red (ex. 488 nm, em. 525 nm) and appear green (PE) or red (PVC). Biomass fragments are stained with Nile red and/or Calcofluor white (ex. 405 nm, em. 445, blended with Evans blue) and appear blue, red, orange, or purple, less well distinguished from microplastics than arthropod exoskeleton fragments. Yellow arrows indicate plastic particles. Scale bar is 100 μm. See Methods for more detailed information on confocal microscope configuration.

The method was also well suited for microplastic detection in invertebrate fecal material such as that of *Z. morio* larvae (Figure 2.3A) and slugs (Figure 2.3B). Slug feces nevertheless contained some potentially confusing particles (Figure 2.3C, 2.3D), objects we could clearly discount as any of the plastics we added that nevertheless appeared red or orange, similar to PVC. It thus remains necessary to heed published plastic particle identification criteria (Mohamed Nor and Obbard 2014, Horton et al. 2017), and not rely solely on fluorescence when examining these samples, as we did in the field trial with the use of a heated probe.

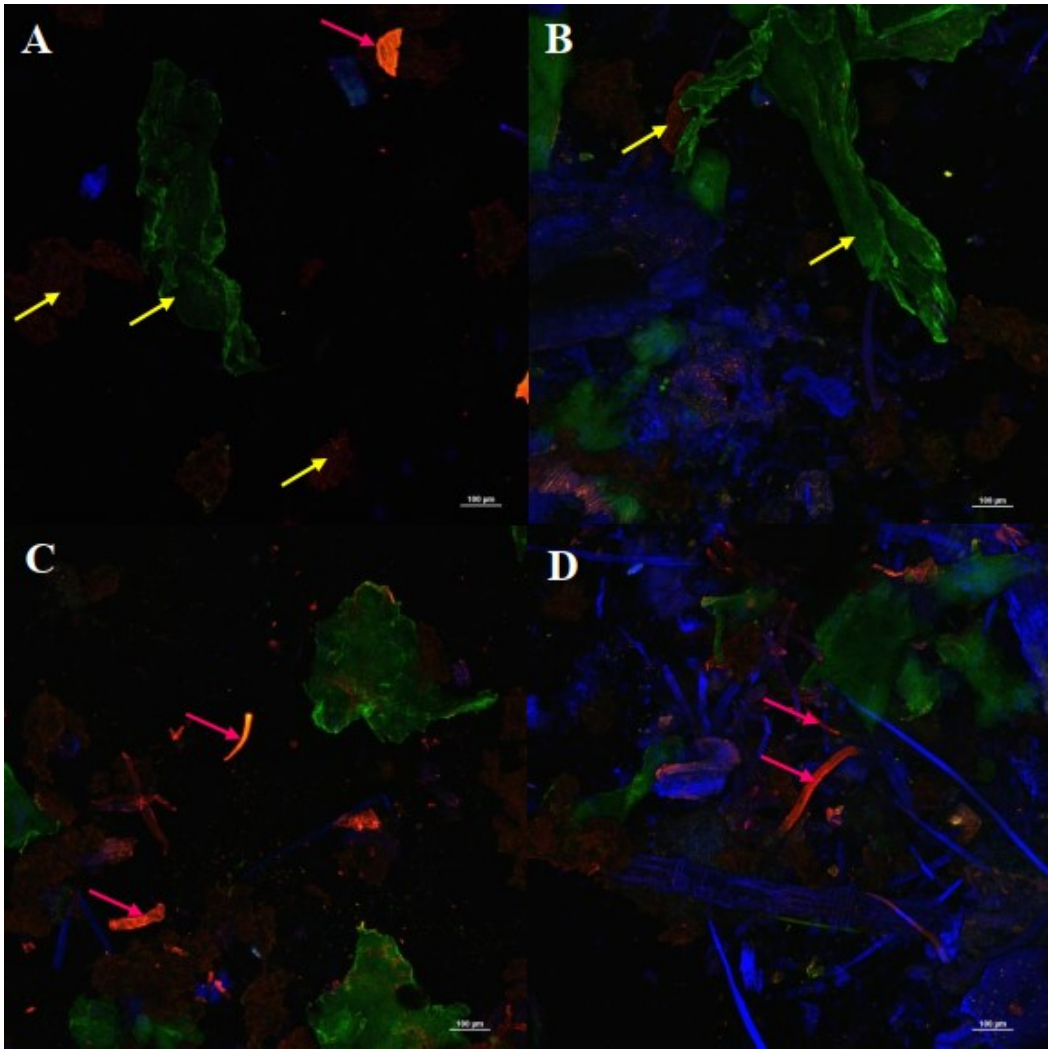

Figure 2.3: Maximum intensity projections polyethylene (PE) and polyvinyl chloride (PVC) microplastics in mixed samples with chemically-digested fecal material from *Zophobas morio* larvae (A) or a slug of the *Arion subfuscus* complex (B,C,D). Plastics are stained with Nile red (ex. 488 nm, em. 525 nm) and appear green (PE) or red (PVC). Biomass fragments are stained with Nile red and/or Calcofluor white (ex. 405 nm, em. 445, blended with Evans blue) and appear blue, purple, red, or orange, in some cases being difficult to distinguish from plastics based on fluorescence alone. Yellow arrows indicate plastic particles and pink arrows indicate potentially confusing natural particles. Scale bar is 100 µm. See Methods for more detailed information on confocal microscope configuration.

Comparison of cotton and plastic microfibers

Cotton fibers were stained only by the Calcofluor white and thus appeared blue as well, easily distinguishable from the various plastic fibers, although blue acrylic fibers were not strongly stained by the Nile red. When examining filters for the recovery and field trials, we exploited this fact for detecting microplastic particles, as any particle that fluoresced under blue excitation wavelengths could be discounted as non-plastic, and indeed, most plastics were barely if at all visible under blue light, or else appeared black.

Recovery trials

Recovery trials found the method to be highly efficient whether counting particles through a conventional fluorescence microscope or a fluorescence-adapted stereomicroscope. In the first test (isopod biomass), we recovered a mean of 98.8 ± 1.3% of added PE fragments and 100 ± 0% of added acrylic fibers, with a processing time of approximately 25 minutes per filter. In the second test (earthworm biomass), we recovered a mean of 87.5 ± 1.1% of added PE fragments and 93.9 ± % of added acrylic fibers, with a faster processing time of approximately 15 minutes per filter.

Ingestion trial

When *Z. morio* larvae were fed microplastic-spiked diet, we were able to identify microplastic particles in ground gut and whole-body samples, regardless of whether or not the

larvae were given time to defecate before fixation in ethanol (Table 2.1, Figure 2.4). We observed no microplastic particles in the control samples, though given the small number of minute particles found on the procedural blanks (mean 2 particles per filter), they may have been present but obscured by pieces of biomass. The microplastic particles, especially the green-fluorescing ones (either PE or PP) seemed to be smaller than their counterparts in the initial imaging trials, a possible indication that *Z. morio*, like other tenebrionids, is capable of partially biodegrading plastics in its gut (Brandon et al. 2018).

Treatment	Whole Body	Gut
Control – Fed no microplastics	0 of 2	0 of 2
Fed microplastic – Fixed immediately	1 of 2	2 of 2
Fed microplastic – Fixed after defecating	1 of 2	2 of 2

Table 2.1: Numbers of samples of each type in which microplastics were detectable within 10 min of searching.

Whereas the initial imaging trials used only small amounts of ground *Z. morio* to demonstrate the counterstain's efficacy, these trials used entire bodies, as would also be the case with any field application of this method. The amount of ground biomass produced from the entire body of an arthropod as large as a *Z. morio* larva seemed likely to cover and block the view of many microplastic particles contained within, even after chemical digestion, making it harder to locate microplastics quickly in these samples (Table 2.1). Thus, for organisms near or exceeding this size and/or possessing thick, highly sclerotized exoskeletons, we recommend the gut or other tissues of interest be dissected out. Regardless of organism size, care should also be

taken to grind the dried specimen as finely as possible to avoid large pieces of biomass persisting through the digestion process and potentially covering plastic particles on the filter.

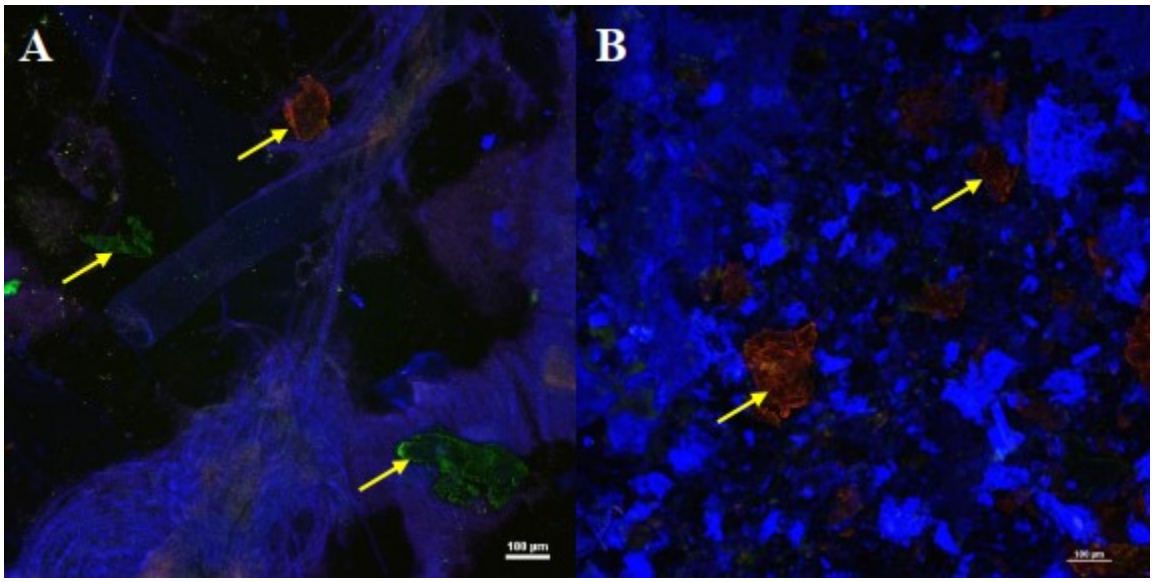

Figure 2.4: Maximum intensity projections of microplastic particles found in *Zophobas morio* gut tissue following ingestion of diet spiked with polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC). Plastics are stained with Nile red (ex. 488 nm, em. 525 nm) and appear green (PE and PP) or red (PVC). Biomass fragments are stained with Nile red as well as Calcofluor white (ex. 405 nm, em. 445, blended with Evans blue) and appear blue. Yellow arrows indicate plastic particles. Scale bar is 100 µm. See Methods for more detailed information on confocal microscope configuration.

Field trial

Emission from Calcofluor white-stained particles was visible through the stereomicroscope adapter's 500 nm longpass emission filter, although appeared green instead of

blue due to the lower wavelengths being blocked. Nile red emission appeared green, yellow, or orange for different particles, similar to what we observed with the confocal microscope.

Recovery of microplastic particles from field-collected organisms was low. Via the hot needle test, we found particles in fecal samples from earthworms (in 1 of 4 collected) and millipedes (in 1 of 3 collected) and cadaver samples from millipedes (1 of 3). All were fragments (Figure 2.5). Isopods, snails, slugs, centipedes, and staphylinid beetles contained no microplastics, though this is not surprising given the small number of total organisms collected. Blanks contained no microplastic particles and few contaminants in general, with the only particles present being stained by the Calcofluor white but not by the Nile red (mean of 3.25 ± 0.75 particles per filter).

Microplastics have so far been found in earthworm casts (Huerta Lwanga et al. 2017) and snails (Panebianco et al. 2019) from natural terrestrial environments, but the full extent of microplastic ingestion by and presence in soil invertebrates and terrestrial animals more broadly is poorly understood. In particular, the question of how much microplastic actually accumulates in the bodies of soil invertebrates as opposed to merely passing through the gut remains unanswered. Yet even when microplastics are simply ingested and excreted, they may be reduced in size through physical fragmentation and/or partial biodegradation (Yang et al. 2018, Kundungal et al. 2019). Microplastic particle size affects their toxicity to biota (Lehtiniemi et al. 2018, Kim et al. 2020), so it is important to determine how ingestion by soil invertebrates may facilitate the severity of microplastic exposure to smaller organisms.

Figure 2.5: Images of microplastic particles detected in samples of earthworm and millipede fecal material, prior to melting with the filed tip of a soldering iron. Particles are fluorescing under Nile red, excited by a 460-480 nm blue LED light and observed through a 500 nm longpass emission filter (Nightsea).

Applicability of this method for terrestrial microplastic research

In sum, this method shows considerable promise for detecting microplastics in samples of terrestrial invertebrate biomass and fecal material. Analysis of counterstained filters via confocal microscopy was straightforward and may even enable crude differentiation between different plastic polymers, although the constrained working space and field of view of these microscopes makes it difficult to perform the hot needle test or collect suspected microplastic particles for further chemical analysis. Confocal microscopy may thus be most suitable for analyzing samples from laboratory experiments involving artificially-generated microplastics more recognizable to researchers than those that would be collected in a field sample. Fluorescent stereomicroscopy, on the other hand, required more finesse to operate but granted greater access to suspected microplastics, making it more suitable for general use.

Counterstaining has recently emerged as a potentially important step in microplastic identification via fluorescence microscopy (Stanton et al. 2019). It is increasingly recognized that when using Nile red alone to stain a sample, one risks falsely identifying non-plastic survivors of chemical digestion procedures as plastic. In the case of terrestrial invertebrate samples, barring the development and implementation of a substantially improved chemical digestion method, counterstaining is likely necessary to have any hope of detecting plastic. Fluorescent counterstaining with dyes such as Calcofluor white or DAPI should be explored further as a complement to existing visual methods for identifying microplastics in biota, water, soil, and other environmental samples. Also, future research should more rigorously assess the ability of this and other fluorescent staining methods to distinguish between different plastic polymers, as ours was able to do to a limited extent, validating with environmental sampling and chemical analysis of the obtained particles.

APPENDIX

FORM 1
RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2022-05

Author and Title of thesis:
Maxwell S. Helmberger
SOIL INVERTEBRATE INTERACTIONS WITH MICROPLASTIC POLLUTION

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	**Genus-Species**	**Life Stage**	**Quantity**	**Preservation**
Tenebrionidae	*Zophobas morio*	larva	7	alcohol
Gryllidae	*Gryllodes sigillatus*	adult	7	pinned
Oniscidae	*Oniscus asellus*	adult	12	alcohol
Trachelipodidae	*Trachelipus rathkii*	adult	12	alcohol

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CHAPTER THREE: SOIL INVERTEBRATES GENERATE MICROPLASTIC FROM POLYSTYRENE FOAM DEBRIS

Abstract

To fully understand microplastics' impact on soil ecosystems, one must recognize soil organisms as not just passively enduring their negative effects, but potentially contributing to microplastics' formation, distribution, and dynamics in soil. We investigated the ability of four soil invertebrates, the cricket *Gryllodes sigillatus* L. (Orthoptera: Gryllidae), the isopod *Oniscus asellus* L. (Isopoda: Oniscidae), larvae of the beetle *Zophobas morio* Fabricius (Coleoptera: Tenebrionidae), and the snail *Cornu aspersum* Müller (Stylommatophora: Helicidae) to fragment macroscopic pieces of weathered or pristine polystyrene (PS) foam. We placed invertebrates into arenas with single PS foam pieces for 24 h, then collected and assessed the microplastic content of each invertebrate's fecal material, its cadaver, and the sand substrate of its arena via hydrogen peroxide digestion, filtration, and fluorescent staining. All taxa excreted PS particles, though snails only to a tiny extent. Beetle larvae produced significantly more microplastics than snails, and crickets and isopods fragmented the weathered PS foam pieces more than the pristine pieces, which they left untouched. A follow-up experiment with pristine PS foam assessed the effect of different treatments mimicking exposure to the elements on fragmentation by isopods. PS foam pieces soaked in a soil suspension were significantly more fragmented than untreated pieces or pieces exposed to UV light alone. These findings indicate that soil invertebrates may represent a source of microplastics to the environment in places polluted with PS foam trash, and that the condition of macroplastic debris likely affects its palatability to these organisms.

Introduction

Though primarily studied in aquatic systems, microplastics (plastic particles <5 mm in diameter) are now known to contaminate soils across the globe (Bläsing and Amelung 2018, He et al. 2018, Zhang, Wang, et al. 2020, Jacques and Prosser 2021). Significant effort has been devoted to unearthing microplastics' effects on soil organisms and broader ecology (Accinelli et al. 2020, Barreto et al. 2020, Lozano et al. 2020, Mueller et al. 2020, Yan et al. 2020) as well as the overall risk they pose to soil biota (Jacques and Prosser 2021). The reverse, effects of soil organisms on microplastics, is also receiving increased attention (Ng et al. 2018, Helmberger, Tiemann, et al. 2020, Song et al. 2020, Kwak and An 2021). Earthworms and springtails have been shown to disperse microplastics (Maaß et al. 2017, Rillig et al. 2017, Zhu et al. 2018) and may thus influence their distribution in soil. However, the potential of soil animals to create microplastics by fragmenting larger pieces of plastic debris, as has been observed in aquatic systems (Hodgson et al. 2018, Mateos-Cárdenas et al. 2020), has so far received less attention.

Microplastics were found in the guts of *Cryptopygus antarcticus* springtails collected on a piece of polystyrene (PS) foam flotsam on King George Island in the Antarctic (Bergami et al. 2020), suggesting fragmentation by even these minute soil animals. The land snail *Achatina fulica* was also found to fragment PS foam (Song et al. 2020). Also, terrestrial invertebrates' inclination and ability to feed on plastic has been studied in waste management contexts. Some insects, alone or in combination with their gut microbiota, have proven capable of ingesting and possibly biodegrading plastics (Brandon et al. 2018, Yang, Wu, et al. 2018, Kundungal et al. 2019, Peng et al. 2019), including polyethylene and PS foam, mostly via mineralization into carbon dioxide. However, fragments of undegraded or partially-degraded plastic can persist in

the insects' fecal material (Yang et al. 2015, Kundungal et al. 2019). Thus, microplastics are formed even if digestion reduces the total mass of plastic. To be sure, not all of these studied taxa live in soil. Two of the pyralid moth larvae shown to fragment plastics (Yang et al. 2014, Kundungal et al. 2019, Zhang, Gao, et al. 2020), are parasites of bee nests and the third is a graminivorous stored products pest. Tenebrionid beetle larvae, the other major group of known plastic-degrading insects, live in soil, leaf litter, and rotting wood (Lawrence, 1991), though they are not especially abundant members of the soil fauna. Nevertheless, insects possess great species diversity (Stork et al. 2015) and many insects spend at least part of their active life in soil, so the ability likely exists in other soil insects and invertebrates more broadly. For example, bacteria from earthworm guts have been found to biodegrade polyethylene (Huerta Lwanga et al. 2018), though to our knowledge this ability has not been tested *in vivo*. Also, even if biodegradation in the strict sense is rare, physical fragmentation by the mouthparts and/or gut may still occur, even if the plastic is only chewed, not ingested.

In this study, we tested the ability of four soil-dwelling invertebrates, the cricket *Gryllodes sigillatus* (L.) (Orthoptera: Gryllidae), the isopod *Oniscus asellus* (L.) (Isopoda: Oniscidae), larvae of the beetle *Zophobas morio* (F.) (Coleoptera: Tenebrionidae), and the snail *Cornu aspersum* Müller (Stylommatophora: Helicidae) to contribute microplastics to terrestrial food chains by fragmenting larger debris, in our case, macroscopic PS foam sourced from a commercial supplier or collected from natural environments. We also conducted a more targeted experiment on *O. asellus* to evaluate how exposure to ultraviolet (UV) light and/or soil solutions might sensitize macroscopic PS foam to faunal fragmentation.

Methods

Both experiments reported in this study used a common set of procedures. Thus, we will first detail this common protocol, then describe where the two experiments differ.

Experimental arenas

Experimental arenas consisted of cylindrical glass jars with an inner diameter of 7 cm and height of 5 cm. We filled the bottom of each arena with mixed and hardened Plaster of Paris to a depth of 10 mm to retain moisture, followed by an additional 5 mm of sand. The sand had been heated in a muffle furnace at 500 °C for 24 h to burn away organic matter and any contaminating plastic (Liu et al. 2019). To provide additional sustenance for the invertebrates, we placed a single oat flake into each arena (Quaker Oats Company, Chicago, IL). Providing non-plastic food does not necessarily prevent organisms from consuming plastic, and may even increase plastic consumption (Yang, Brandon, et al. 2018, Yang et al. 2021). The plaster bottoms of the arenas were then moistened to saturation.

Animal exposure to plastics

We placed each invertebrate into a 60 mL glass jar for 24 h to starve them and allow them to defecate before placing each into its experimental arena with a piece of PS foam (see experiment-specific sections for the treatments present in each). After 24 h at room temperature

in the arenas, we placed the invertebrates into clean jars for 48 h to let them defecate again before removing and freezing them.

Sample processing – feces, sand, and cadavers

We assessed the microplastic content of the invertebrates' fecal material, the sand in the arenas, and the frozen invertebrate cadavers themselves, to obtain as complete as possible metric of the microplastic generated by the animal.

To assess fecal material, we added 10 mL of filtered 30% hydrogen peroxide solution to the defecation jars to digest the contents at room temperature for 48 h. Following the peroxide digestion, jar contents were vacuum filtered onto 1.5 µm glass fiber filters (Whatman) for staining and counting.

For the sand, we washed the contents of each arena into 100 mL glass beakers using 25 mL of hydrogen peroxide, then washed the PS foam piece into the beaker as well to ensure any fecal or microplastic material on it would be dislodged into the sand. After 48 h of peroxide digestion, we then filled the beakers to a depth of 50 mL with filtered DI water (water and hydrogen peroxide being sufficient to separate PS foam and sand by density) and agitated their contents by stirring with a glass rod. We let the beakers stand for 2 min and carefully filtered the supernatant as described above, though we re-filled and re-agitated each beaker with DI water two additional times to ensure as much floating material as possible was transferred onto the filters.

Cadavers were dried at 65 °C for 72 h and finely ground with a glass rod within a glass jar, digested for 48 h in 10 mL of 30% hydrogen peroxide solution, and filtered like the other samples.

Anti-contamination protocols

Because contamination is a significant problem in microplastic research (Prata et al. 2021), we used the following protocols to mitigate it. Forceps, funnels, and other equipment were triple-rinsed with filtered DI water in between each sample. To further assess any contamination due to particles persisting on the equipment, we placed procedural blanks between every five to seven samples, filtering 10 mL of clean 30% hydrogen peroxide from a glass jar as if it contained digested material. Finally, we placed four moistened glass fiber filters throughout the work area during the vacuum filtration process to assess deposition of airborne microplastics (air blanks).

Microplastic staining, visualization, and counting

We stained all filters (including controls and blanks) with a combination of Calcofluor White/Evans Blue (Sigma-Aldrich) and Nile Red (Santa Cruz Biological) fluorescent dyes following Helmberger et al. (2020). We stained each filter with 10-15 drops of Calcofluor White/Evans Blue blend, washed with a similar quantity of filtered DI water, stained again with Nile Red, and washed again with filtered *n*-hexane. Once dry, we observed the filters under a Leica S8 APO stereomicroscope fitted with a Nightsea fluorescence adapter (440 to 460 nm

excitation light, 500 nm long-pass emission filter, and an additional AmScope LED UV excitation light, 395 nm) to locate and count PS foam fragments. By turning the 440-460 nm excitation light on and off, we could determine which particles fluoresced under Nile Red *only*, as opposed to Calcofluor White only or under both dyes' excitation wavelengths. Any particles fluorescing under Nile Red but not Calcofluor White/Evans Blue were considered potential microplastics, although fibers, films, and any other obviously non-PS-foam particles were ignored. Particles resembling PS foam were prodded with the finely filed tip of a soldering iron (Chicago Electric, operating temperature 390 °C) to confirm their identity as plastic if they melted deformed in response to the heat. Visual detection methods, however, are limited in their ability to detect very small plastic particles (Lv et al. 2021), and though fluorescent staining can facilitate identification of microplastics with diameters in the tens of microns (Sfriso et al. 2020), particles smaller than that could escape observation and go uncounted.

To account for sample contamination, we then subtracted the highest number of suspected PS foam particles found on any of that experiment's negative control, procedural blank, or airborne blank filters from each sample's count. Thus, for each replicate, since we counted microplastics in fecal material, arena sand, and cadaver biomass separately, we applied this correction to each count and summed the corrected counts to produce a total microplastic value.

Experiment 1: Four-species

This experiment compared fragmentation of PS foam into microplastics by four invertebrate species; the cricket *G. sigillatus* L. (Orthoptera: Gryllidae), the isopod *O. asellus* L.

(Isopoda: Oniscidae), larvae of the beetle *Z. morio* Fabricius (Coleoptera: Tenebrionidae), and the snail *C. aspersum* Müller (Stylommatophora: Helicidae). All four are to some extent decomposers, feeding on decaying plant and/or animal material. We collected the isopods from a wooded area in East Lansing, MI (42°44'16" N, 84°27'04" W), obtained the crickets and beetle larvae from a pet supply store (Preuss Pets, Lansing, MI), and obtained the snails from a private hobbyist. Taxonomy for *Z. morio*, *G. sigillatus*, and *C. aspersum* were confirmed by the suppliers and the isopods were identified as *O. asellus* following (Shultz 2018).

We prepared three treatments of PS foam out of pieces cut from larger items, two "weathered" and one "pristine." For the two weathered treatments, we collected two items of PS foam trash, a shard of a drink cup and an irregular lump of indeterminate origin, from the wooded bank of the Red Cedar River in East Lansing, MI, USA (42°44'01" N, 84°29'28" W). We specifically sought pieces showing wear, discoloration, or other visible signs of age and prolonged exposure to the elements. The pristine treatment used freshly-purchased PS foam cup lids (Dart Container Corporation, Mason, MI) stored indoors. We cut all smaller pieces in such a way that original outer surface area was equally distributed between pieces, since the surfaces exposed by cutting may have had different properties than the outer surfaces exposed directly to the elements. We triple-rinsed each piece in filtered DI water to remove any clinging microplastic fragments potentially created via cutting, then wrapped the pieces in aluminum foil and stored them at room temperature until needed.

Prior to use, we confirmed our field collected pieces as PS via attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), conducted on a FTIR spectrometer (Vertex 70, Bruker) equipped with an A225/Q Platinum ATR Diamond accessory operating in the 4500-400 cm^{-1} mid-IR region, using a 4 cm^{-1} resolution with 60 scans/sample and medium

Norton-Beer apodization. We took background spectra of an empty and cleaned system before each sample, as recommended by Andrade *et al.* (2020). We then identified the background-subtracted spectra with the online Open Specy tool (Cowger et al. 2021), without processing. Our plastics, cut pieces, and identification spectra are shown in Figure 3.1.

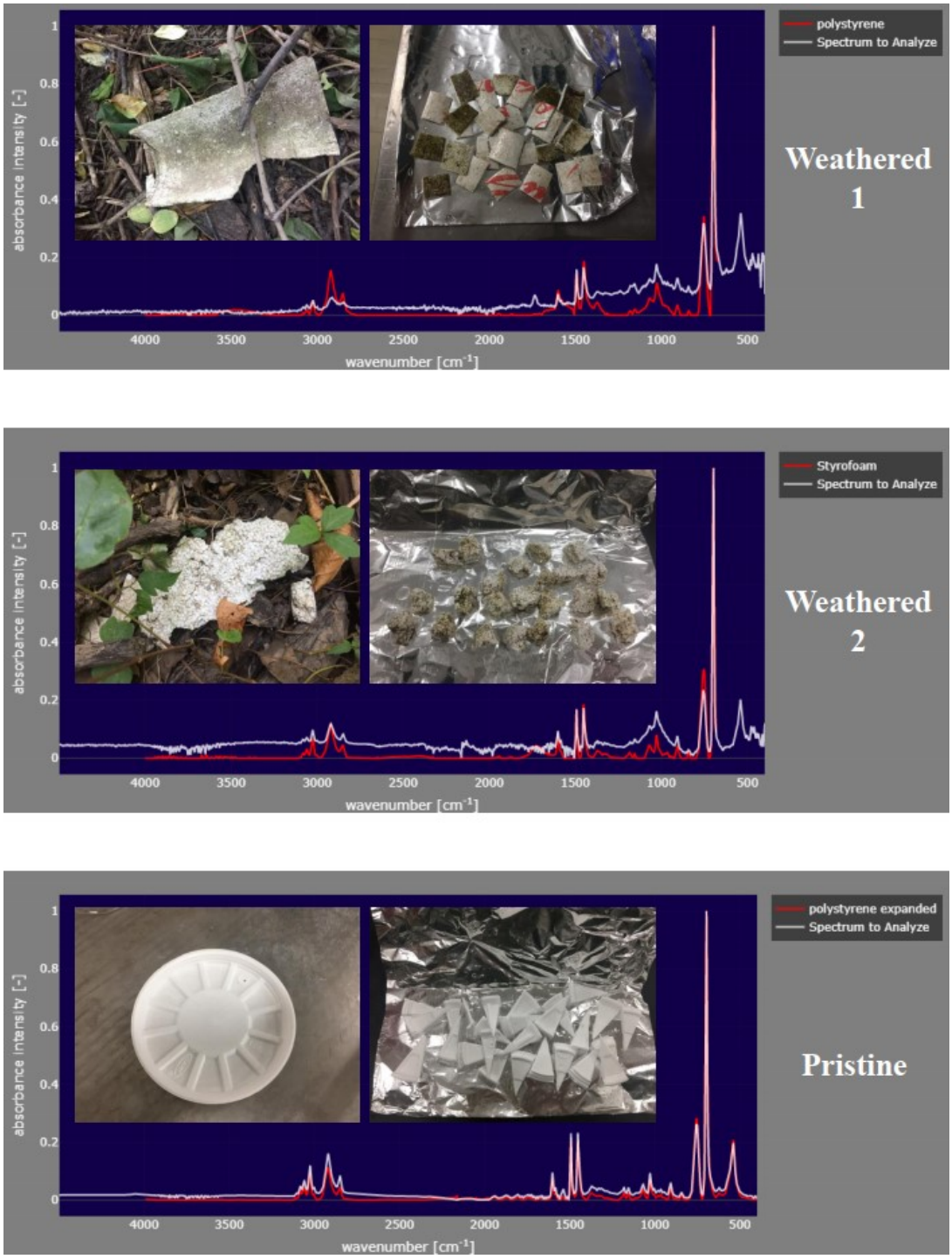

Figure 3.1: Images of the original and cut pieces of the PS foam items comprising the three experimental treatments, with confirmatory ATR-FTIR spectra. Spectra in white represent our samples, spectra in red represent the closest spectral match obtained via OpenSpecy.

This experiment used 5 replicates of each invertebrate x plastic combination. We also included three types of negative control; arenas containing a piece of PS foam and an oat flake but no invertebrates (n = 3 for each PS foam type), arenas containing invertebrates and an oat but no PS foam (n = 3 for each fauna type), and arenas containing only an oat atop the sand (n = 3). These allowed us to account for; any shedding of microplastics from the PS foam via physical, chemical, or microbial processes; any microplastic present on or in the invertebrates before being placed in the arenas; and any microplastic present in the arena substrate itself, respectively.

Positive control samples consisted of fecal material from each of the four invertebrates (n = 2 for *Z. morio,* n = 3 for all others) mixed with a known 50 PS foam fragments counted out for each sample (fragments were grated from the cup lids used for the pristine treatment, in a room separate from the one in which most other protocols took place) and subject to the same processing as fecal material from the arenas (see next section). This method, however, did not simulate any potential loss of particles due to biodegradation or apparent addition of particles via further physical fragmentation in the gut, and was restricted to larger-sized particles that could be reliably counted and transferred. We also created positive controls for the sand samples, in which the same numbers of PS foam fragments were added to 10 g of sand, along with fecal material, as some would be present in the arena sand after invertebrates were removed.

Experiment 2: Isopod-only

This experiment compared fragmentation by the isopod *O. asellus* between initially identical PS pieces subject to different treatments. All of the PS foam pieces placed into the arenas were cut from the cup lids used for the previous experiment's pristine treatment and were

subjected to different treatments mimicking exposure to the elements. These treatments were; 1) immersion in sterile DI water for 48 h, 2) exposure to 405 nm UV light (Comgrow, Shenzhen, China) for 24 h per side, then immersion in sterile DI water, 3) immersion in an aqueous suspension of agricultural field soil collected from East Lansing, MI, and 4) exposure to UV light, then immersion in the soil suspension, as well as 5) an untreated control. We then air-dried all plastics under aluminum foil for 24 h at room temperature.

For the experiment, we placed lone, starved isopods into arenas with either no plastic or a piece of untreated plastic, water-treated plastic, UV-and-water-treated plastic, soil-suspension-treated plastic, or UV-and-soil-suspension-treated plastic (n = 10 for all treatments). Negative controls consisted of arenas with an isopod but no plastic (n = 10), with plastic but no isopod (n = 5), and neither plastic nor isopod (n = 5).

Statistical analysis

For the four-species experiment, we tested the effect of invertebrate species and plastic treatment on the number of total PS foam particles with Kruskal-Wallis tests (Zar 1999), then used a post-hoc Kruskal Nemenyi test with a Chi-square distribution to correct ties (Zar 1999), via the R package PMCMR (Pohlert, 2014), to determine which species produced more fragments across all three plastic types. We used the same procedure in the isopod-only experiment to compare the number of fragments produced between the five plastic treatments.

Results

Four-species experiment

The beetle larva *Zophobas morio* was most inclined to fragment the PS foam, producing hundreds to thousands of particles from all tested PS types. The isopod *Oniscus asellus* and cricket *Gryllodes sigillatus* fragmented both types of weathered PS foam, but not the pristine PS. The snail *Cornu aspersum* did not appreciably fragment anything. Examples of produced particles are shown in Figure 3.2. In general, *Z. morio* produced the largest particles, some exceeding 1 mm in size. Particles produced by *G. sigillatus* and (rarely) *C. aspersum* reached up to 500 µm in size, and *O. asellus* produced the smallest particles, rarely exceeding 250 µm in size.

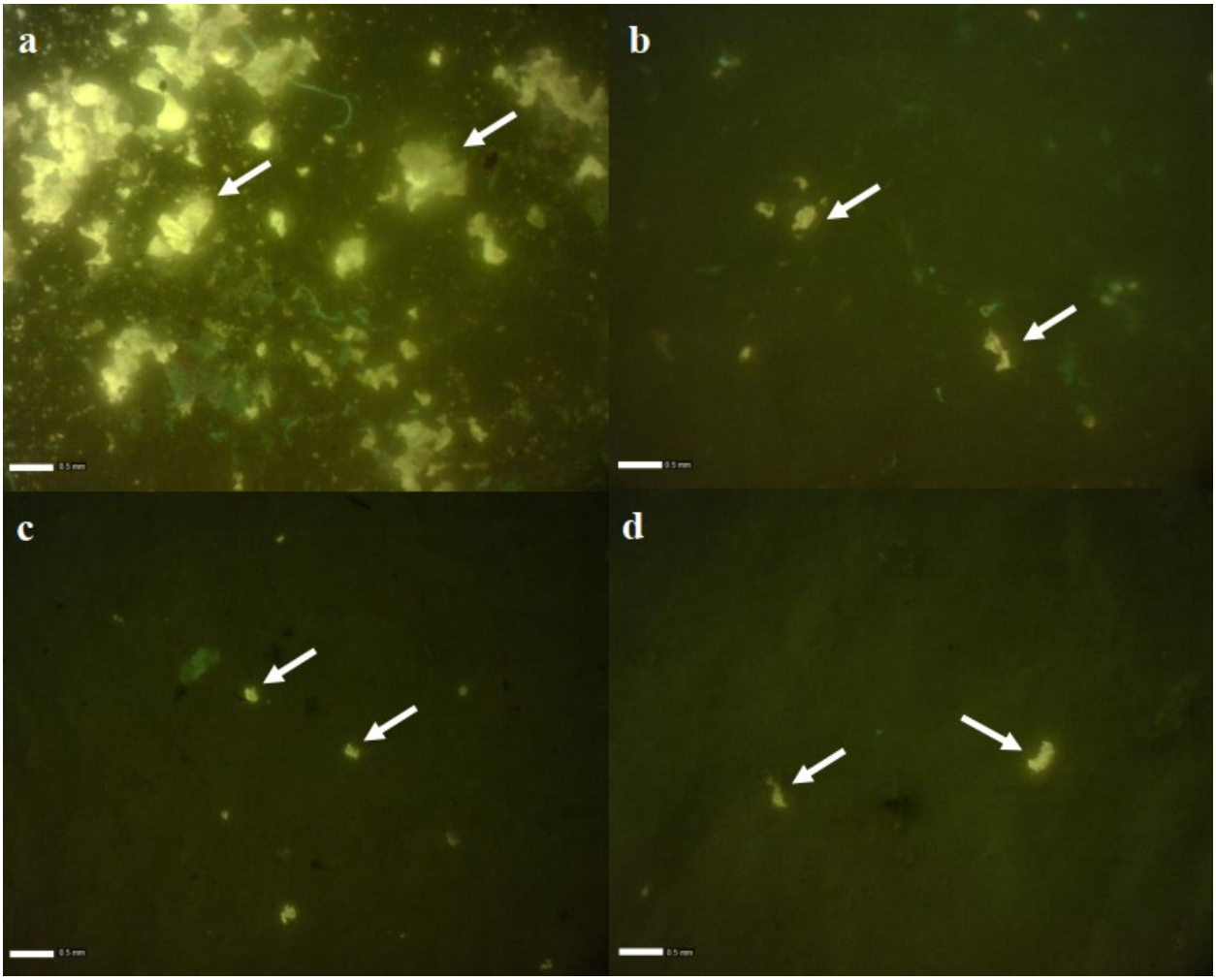

Figure 3.2: Photographs of PS foam fragments produced by; a) the beetle larva *Zophobas morio*, b) the cricket *Gryllodes sigillatus*, c), the isopod *Oniscus asellus*, and d) the snail *Cornu aspersum*. Examples of produced particles are indicated with arrows. Scale bar is 500 µm. All photographs were taken with a DinoEye microscope eyepiece camera (Dunwell Tech, Inc., Torrance, CA) and we determined scale using DinoCapture 2.0 software.

Invertebrate species had a significant effect on the total number of PS foam fragments found in fecal material, arena sand, and cadavers (Kruskal-Wallis rank sum test, $X^2 = 17.168$, df = 3, p = 0.0007), but plastic treatment did not (Kruskal-Wallis rank sum test, $X^2 = 3.176$, df = 2, p = 0.2043). Due to high variance in the data, only the difference between beetle larvae and snails was statistically significant (post-hoc Kruskal Nemenyi test, p = 0.0007) (Figure 3.3).

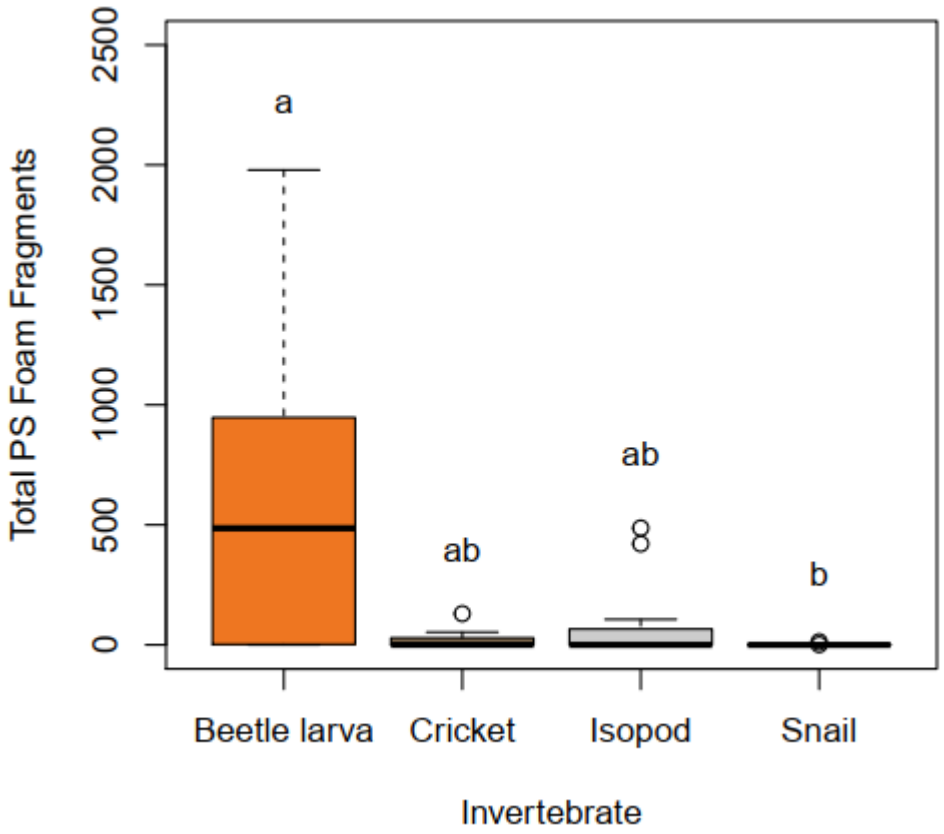

Figure 3.3: Total numbers of PS foam microplastic particles found in invertebrate fecal material, arena sand, and invertebrate cadaver biomass, averaged across all three PS foam treatments (two weathered and one pristine) for the four invertebrate species. Different letters denote species producing significantly different amounts of microplastic. Lower and upper whiskers represent dataset minimums and maximums, respectively, excluding outliers.

Within individual species, plastic treatment had a significant effect on fragmentation by crickets (Kruskal-Wallis rank sum test, $X^2 = 7.983$, df = 2, p = 0.0185) and isopods (Kruskal-Wallis rank sum test, $X^2 = 6.477$, df = 2, p = 0.0392), but not by beetle larvae (Kruskal-Wallis rank sum test, $X^2 = 1.207$, df = 2, p = 0.547) or snails (Kruskal-Wallis rank sum test, $X^2 = 1.086$, df = 2, p = 0.5811). Crickets fragmented one of the weathered plastics (Treatment 1) significantly more than the pristine plastic (post-hoc Kruskal Nemenyi test, p = 0.018) and isopods fragmented the other weathered plastic (Treatment 2) significantly more than the pristine plastic. (post-hoc Kruskal Nemenyi test, p = 0.041) (Figure 3.4).

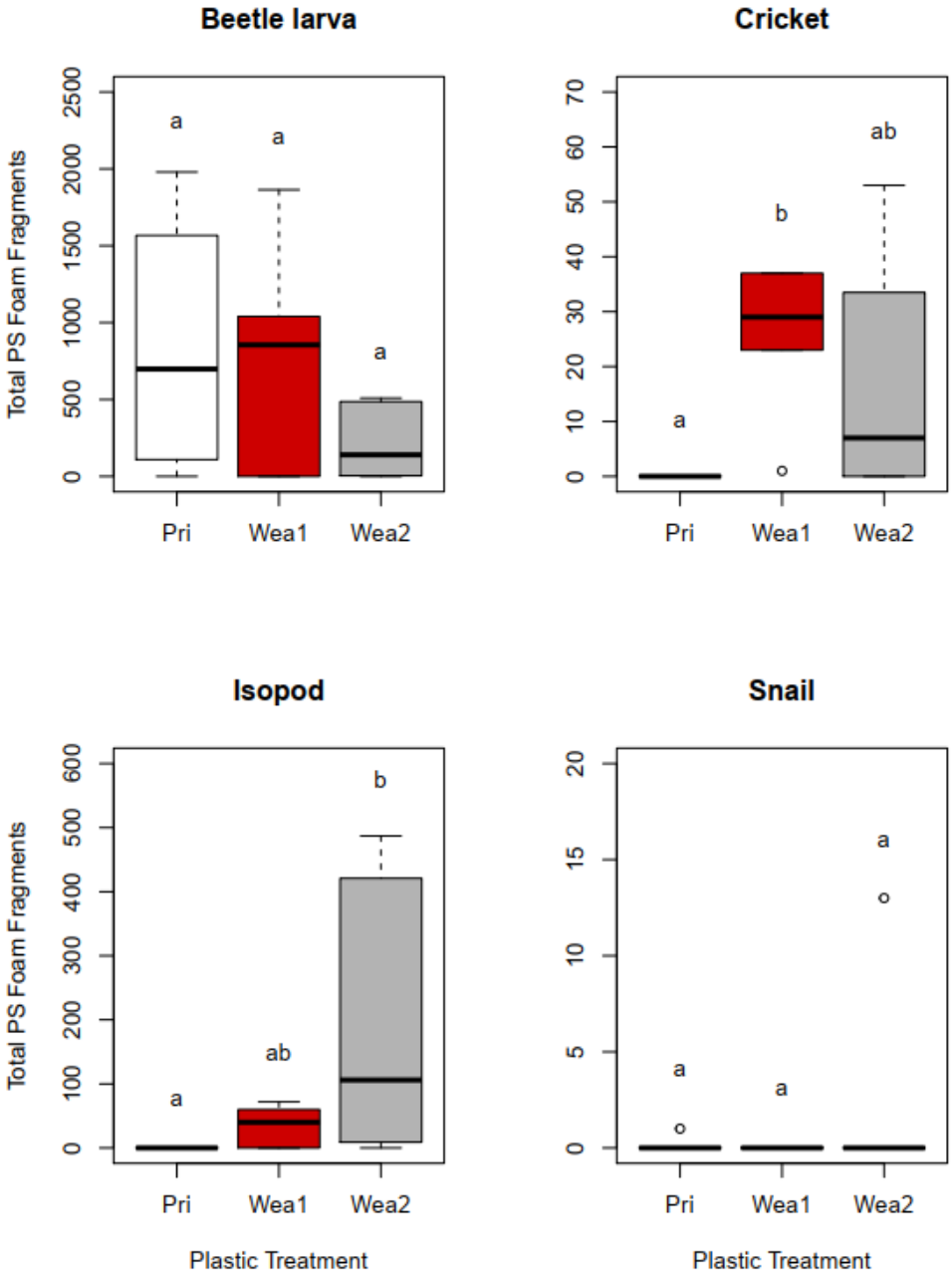

Figure 3.4: Total numbers of PS foam microplastic particles found in invertebrate fecal material, arena sand, and invertebrate cadaver biomass of each species across the three PS foam treatments (Pri = Pristine, Wea1 = Weathered 1, Wea2 = Weathered 2). Different letters denote PS foam treatments from which the invertebrates produced significantly different particle numbers. Lower and upper whiskers represent dataset minimums and maximums, respectively, excluding outliers.

We found very few suspected PS foam particles on our negative control, procedural blank, and air blank filters (1 particle each on 1 procedural blank and 1 air blank out of 82 total), though air blanks contained microfibers consistent with an indoor environment. Some fluoresced under Calcofluor White excitation and were most likely cotton; others did not and could have been plastic, but were not mistakable for the PS foam particles we were counting. In addition, the air blank filters were exposed to the lab environment for 1-3 h, depending on the number of samples being filtered, whereas most individual samples were exposed for no more than 10 min each throughout filtration, staining, and particle counting.

Recovery from positive control samples was high, with a mean ± SE across all four species of 91.4 ± 3.2% (n = 11) added fragments counted in spiked feces samples. Recovery from spiked feces + sand samples was nearly identical, with 92.5 ± 2.4% of added fragments counted (n = 11). Some individual positive control samples yielded slightly more PS foam fragments than the originally added 50, which we attribute to abiotic fragmentation, perhaps due to abrasion by sand, or disentangling of particles initially counted as single fragments.

Isopod-only experiment

Plastic treatment had a significant effect on the number of fragments the isopods produced (Kruskal-Wallis rank sum test, $X^2 = 22.077$, df = 4, p = 0.0002). Though isopods fragmented all types of plastic to at least a slight extent, plastics treated with only the soil suspension were fragmented significantly more than untreated plastics (post-hoc Kruskal Nemenyi test, p = 0.012) or plastics treated with UV and water (post-hoc Kruskal Nemenyi test,

p = 0.022). Plastics treated with only water or with the soil suspension and UV were not significantly different from any others (Figure 3.5).

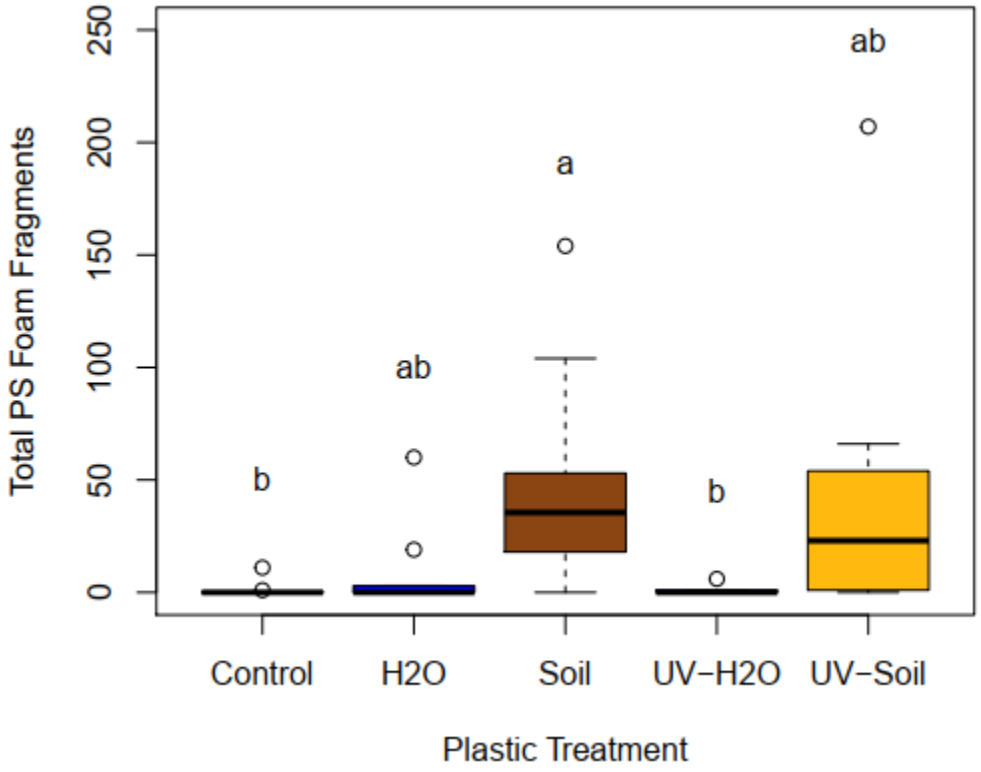

Figure 3.5: Total numbers of PS foam microplastic particles found in isopod *Oniscus asellus* fecal material, arena sand, and isopod cadaver biomass, across all five PS foam treatments (untreated control, water only, soil suspension only, UV light and water, and UV light and soil). Different letters denote PS foam treatments from which the isopods produced significantly different particle numbers. Lower and upper whiskers represent dataset minimums and maximums, respectively, excluding outliers.

We again found very limited contamination by PS foam particles, detecting only 1 particle on a single isopod-only negative control and 1 particle on a single air bank, out of 68 total negative control, procedural blank, and air blank filters.

Discussion

This study demonstrates the potential for soil invertebrates to produce PS foam microplastics by fragmenting larger debris after only brief contact and provides further evidence that the environmental history of plastic affects its relative "palatability" to decomposer organisms. *Zophobas morio*, like other tenebrionid beetle larvae (Yang, Brandon, et al. 2018, Peng et al. 2019), was already known to fragment and consume PS foam (Zielińska et al. 2020), so it was no surprise to find it doing so here. Of the other three taxa investigated, isopods fragmented PS foam the most consistently, though only weathered pieces. Wood & Zimmer (2014) observed the *Porcellio scaber* ingesting starch- and cellulose-based biodegradable plastics, though this is to our knowledge the first record of terrestrial isopods fragmenting conventional plastics. Crickets have previously been shown to consume polyurethane foam (Khan et al. 2021); our study demonstrates their ability to fragment PS as well. The lack of fragmentation by snails was surprising, as Song et al. (2020), one of few other papers investigating plastic fragmentation by soil animals, observed significant microplastic production by the snail *Achatina fulica*. Our conflicting results could be due to our choice of a different snail species and/or the fact we gave the snails much less time in contact with the plastic, 24 h rather than 4 wks. Interestingly, both isopods and crickets fragmented a different one of the two weathered plastics significantly more than the pristine plastic, but not the other. The cause of this

is unclear and a more thorough investigation of individual species' preferences within plastics of the same type is likely warranted.

Though UV exposure did not lead to higher plastic fragmentation in our isopod-only experiment, it is known to sensitize plastic to microbial colonization (Vimala and Mathew 2016, Wei and Zimmermann 2017). Many common soil microbes are known to colonize plastics (Kale et al. 2015), which may lead to increased palatability of plastic as it does for dead plant material (Cummins 1974, Digel et al. 2014, Potapov et al. 2019). That said, we did not directly quantify microbial biomass on the plastic pieces in either experiment, so this remains a question for future research, both with respect to macroplastic debris and microplastic particles. Also, our plastics' immersion in the soil suspension may have resulted in microbially-colonized soil particles clinging to the plastic rather than colonization of the plastic itself, but how much this distinction matters is debatable, since rain spatter off the soil surface could replicate the effect in natural settings.

As discussed above, visual detection methods, even augmented by fluorescent staining, cannot locate microplastic particles below a certain size threshold. Our microplastic counts may thus be underestimations, if large numbers of particles smaller than 10 μm were produced. Fully characterizing the size distribution of microplastic particles produced by biotic fragmentation is an important step for future research, as size is known to affect microplastics' bioavailability and toxicity to other organisms (Lehtiniemi et al. 2018, Fueser et al. 2019). Slight overestimation may have come from us grinding the invertebrates' cadavers prior to digestion, which could have caused additional fragmentation of PS foam particles present within. However, the proportion of microplastics recovered from the cadavers as opposed to feces or arena sand was low in most

cases, so production of additional fragments is unlikely to have significantly altered our total microplastic counts.

Microplastics' interactions with soil organisms are a potentially important piece to understanding the true impact microplastics have on the soil ecosystem (Helmberger, Tiemann, et al. 2020). Fragmentation of large plastic debris could facilitate uptake of microplastics by other organisms, perhaps especially if the newly created microplastics are passed through the gut of the fragmenting animal and chemically altered or coated with organic material. We did not conduct chemical analyses of excreted microplastics in our study, though studies with snails (Song et al. 2020) and tenebrionid larvae similar to *Z. morio* (Yang et al. 2015) demonstrated chemical changes, including depolymerization of PS molecules, following fragmentation. Along with incorporation into fecal material itself, these chemical changes could increase the microplastics' bioavailability.

In conclusion, our results show that the propensity of soil macroinvertebrates to fragment PS foam debris and create microplastics varies by taxa, and within certain taxa such as isopods, depends on the condition of the plastic. These results add to our understanding of potential biotic sources of soil microplastics and conditions leading to their formation. Future work should assess the field prevalence of biologically-fragmented or biologically-altered microplastics as well as any unique ecological properties of these particles compared to microplastics produced via physical weathering. These properties could mediate microplastic uptake by and effects on terrestrial organisms.

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CHAPTER FOUR: INTERACTIONS OF ISOPODS *ONISCUS ASELLUS* AND *TRACHELIPUS RATHKII* WITH POLYSTYRENE FOAM DEBRIS: TEMPORAL DYNAMICS AND ALTERNATE SUBSTRATE EFFECTS

Abstract

Microplastics present a novel and potentially unique threat to soil ecosystems, one whose effects may be mediated by soil organisms themselves. We investigated fragmentation of polystyrene (PS) foam into microplastic particles by two isopods, *Oniscus asellus* L. and *Trachelipus rathkii* Brandt, in laboratory arena experiments. First, we examined the temporal dynamics of fragmentation across a time span of 96 h. *O. asellus* produced more fragments than *T. rathkii*, and neither species significantly fragmented the PS foam until 48 h had passed. Second, we asked whether *O. asellus* would still fragment PS foam in the presence of an alternate, more natural substrate like wood. Wood did not significantly affect fragmentation rates, in line with the few other studies examining the effect of alternate food on soil invertebrates' propensity to consume and/or fragment plastics. Our results provide additional characterization of PS foam fragmentation by isopods and indicate that laboratory experiments involving soil invertebrates and plastic debris should take place over relatively short timespans, but do not necessarily need to provide alternate food.

Introduction

Interactions between biota and plastic pollution have gained attention as a potential complication of plastic's ecological effects. Plastics, particularly microplastics (defined as particles or fibers with a diameter below 5 mm), may negatively affect aquatic and terrestrial organisms in a variety of ways, including direct mortality (Huerta Lwanga et al. 2016, Piccardo et al. 2021), decreased growth and reproduction (Huerta Lwanga et al. 2016, Lahive et al. 2019, Schöpfer et al. 2020, Ji et al. 2021, Kwak and An 2021), and oxidative stress (Rodríguez-Seijo et al. 2018, Piccardo et al. 2021). However, these effects may be mediated by the actions of other organisms (Helmberger, Tiemann, et al. 2020). Organisms may generate microplastics directly by fragmenting larger plastic debris (Brandon et al. 2018, Hodgson et al. 2018, Immerschitt and Martens 2020, Song et al. 2020), transport microplastics through solid media like soil (Maaß et al. 2017, Rillig et al. 2017, Zhu et al. 2018), and potentially alter their bioavailability by reducing their size and/or incorporating them into casts and fecal matter (Huerta Lwanga et al. 2017, Dawson et al. 2018, Mateos-Cárdenas et al. 2020, Kwak and An 2021). As one example of this phenomenon, we previously showed that terrestrial isopods can fragment polystyrene (PS) foam in laboratory settings, preferring plastic collected from outdoors or immersed in a soil suspension to mimic exposure to the elements (Helmberger et al. 2022).

Here, we sought to further characterize isopods' fragmentation ability in two ways, using the species *Oniscus asellus* L. (Isopoda: Oniscidae) and *Trachelipus rathkii* Brandt (Isopoda: Trachelipodidae), two widespread species commonly found in or near urban areas (Vilisics et al. 2012). First, we determined how much fragmentation occurs at different time points following contact with PS foam. Second, we asked whether the presence of an alternate, more natural

substrate for the isopods affects their fragmentation behavior. More broadly, these experiments were also an effort to test certain assumptions of some laboratory experiments investigating biotic fragmentation of macroplastic debris. Some such experiments assess fragmentation plastic over prolonged periods of time and/or do not provide organisms with anything to eat except the plastic (Hodgson et al. 2018, Song et al. 2020). Though laboratory experiments can never fully mimic field conditions, it is still worth assessing whether or not steps toward realism are worthwhile or necessary.

Methods

Arena construction, isopod maintenance, and plastic preparation

We built experimental arenas out of cylindrical glass jars following Helmberger et al. (2022). Briefly, we filled the jars with 10 mm of mixed and hardened Plaster of Paris, moistened the bottoms to saturation after they cured, and then covered the plaster with an additional 5 mm of sand, which had been heated at 500 °C in a muffle furnace for 24 h to burn away contaminating plastic (Liu et al. 2019).

We collected the isopods *Oniscus asellus* and *Trachelipus rathkii* from a woodlot in a suburban area of East Lansing, Michigan, U.S.A. and maintained them on wood mulch with organic carrots as additional food until they were needed for experiments. We made the taxonomic identifications following Shultz (2018).

We prepared pieces of PS foam from unused Styrofoam cup lids (Dart Container Corporation, Mason, MI). For the first experiment (temporal dynamics), we cut the circular lids

into wedges, as this was the most material-efficient shape. For the second experiment (alternate substrate), we cut rectangular pieces from the lids' centers to better match of the shape of the wood pieces used as alternate substrates (described below in greater detail). In both experiments, we treated the PS foam pieces by soaking them in a suspension of forest soil for 48 h to mimic exposure to the outdoors, then air-dried them under aluminum foil for 24 h prior to placing them in the arenas.

Temporal dynamics experiment

To determine how quickly isopods fragment PS foam debris after exposure, we placed single *O. asellus* or *T. rathkii* isopods in arenas containing a piece of treated plastic after starving them in glass jars for 24 h. Isopods remained in the arenas, at room temperature, for 6 h, 24 h, 48 h, or 96 h (n = 10 per time treatment) before we removed them, froze them, and dried them at 65 °C for 48 h. We also included 15 negative control arenas, five with an *O. asellus* isopod but no plastic, five with a *T. rathkii* isopod but no plastic, and five with a piece of treated plastic but no isopods, to account for any PS foam fragments present on or in the isopods or produced from the plastic pieces via abiotic processes. The isopods in these arenas were removed at 96 h as well. Arenas in the 96-h treatment and negative controls were remoistened halfway through the experiment with 3 mL of filtered DI water.

Alternate substrate experiment

We conducted a second experiment to determine how the presence of an alternate substrate affects isopod fragmentation of PS foam. For the alternate substrate, we used 1-2 g rectangular pieces of birch wood cut from paint stirring sticks. *O. asellus* is known to consume wood as well as decaying leaves, as well as decomposer fungi present on these substrates (Hartenstein 1964, Gunnarsson and Tunlid 1986, Potapov et al. 2022). We soaked the wood pieces for 48 h in a forest soil suspension just as we did the PS foam pieces. Since in the previous experiment *O. asellus* produced markedly more fragments than *T. rathkii*, here we used only *O. asellus*. We placed single isopods into arenas containing a piece of PS foam only or PS foam and wood (n = 20 per treatment). We also included two types of negative control; arenas with an isopod and wood but no PS foam (n =10), and arenas with wood and PS foam but no isopods (n =10). Isopods remained in the arenas for 48 h before we removed them and placed them into clean, 60 mL glass jars for an additional 48 h to let them defecate. Afterward, we froze and dried the isopods as in the previous experiment.

Isopod and arena sand processing

In the temporal dynamics experiment, we processed the arena sand and the cadavers of the isopods, which had not been given time to defecate outside of the arenas and would thus still retain their fecal material. In the alternate substrate experiment, we processed isopod feces and cadavers separately to more closely adhere to established methods (Helmberger et al. 2022). This

did not present a problem, however, as results from the two experiments were never intended to be compared.

We added 10 mL of filtered 30% hydrogen peroxide to each 60 mL jar containing feces or cadaver remains, the latter of which had been finely ground with a glass rod prior to digestion. After 48 h at room temperature, we vacuum filtered the contents of each jar onto Whatman 1.5 µm glass fiber filters (42.5 mm diameter). The contents of each arena were washed into identical jars with hydrogen peroxide, with any PS foam or wood in the arenas being rinsed off over the jars as well. These jars were also left to digest for 48 h, after which we filtered the supernatant. We rinsed the vacuum funnel and forceps used to manipulate the filters with filtered DI water between each sample. Samples were exposed to the laboratory air for no more than 1 min between opening their jars and sealing the filters back inside them. To account for potential sample contamination from PS foam particles in the air and/or persisting on the equipment between samples, we placed four moistened filters in the vicinity of the vacuum pump during the filtration process and also incorporated procedural blanks, clean jars of hydrogen peroxide, between every fifth or sixth sample.

Microplastic counting

To visualize the PS foam fragments on the filters, we stained each filter with a mix with Calcofluor White and Nile Red fluorescent dyes (Helmberger, Frame, et al. 2020). Once dry, we viewed the stained filters through a Lecia S8 APO stereomicroscope equipped with a Nightsea fluorescence adapter with a 440 to 460 nm excitation light, a 500 nm long-pass emission filter, and an additional AmScope LED UV excitation light, (395 nm). The former excited the Nile Red

while the latter excited the Calcofluor White dye. By turning the two lights on and off, we could determine which particles fluoresced under Nile Red but not Calcofluor White, an indicator of potential plastics. PS foam particles exhibited a unique yellow fluorescence color under the scope (Figure 4.1), and any such particles we confirmed as plastic by prodding with the finely filed tip of a soldering iron (Chicago Electric, operating temperature 390 °C).

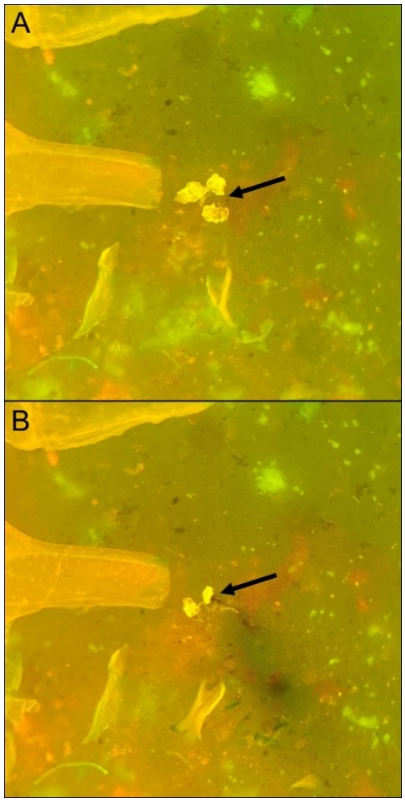

Figure 4.1: Fluorescence image of PS foam particles before heat-testing (A) and after (B). Note the deformation of the upper two particles. The lowest of the three melted completely and was lost, though was still counted in the sample.

Statistical analysis

We summed all PS foam particle counts within each replicate (arena sand, cadavers, and feces if processed separately) to obtain a total number of fragments produced by the isopod. We observed no PS foam contamination in the negative controls and blanks and so did not adjust our counts. We also did not include our negative controls or blanks in our statistical models.

For the temporal dynamics experiment, we used Wilcoxon rank sum tests to assess differences in the numbers of PS foam fragments between the two isopod species (*O. asellus* and *T. rathkii*) at each time point (6 h, 24 h, 48 h, and 96 h). Within each species individually, we tested for differences between time points using Kruskal-Wallis tests, then post-hoc Kruskal Nemenyi tests with a Chi-square distribution to correct ties, via the R package PMCMRplus (Pohlert 2021).

For the alternate substrate experiment, we used a Wilcoxon rank sum test to look for a difference between the two treatments (PS foam only and PS foam + wood).

Results

Temporal dynamics experiment

Oniscus asellus produced significantly more PS foam fragments than *Trachelipus rathkii* after 48 h (Wilcoxon rank sum test, $W = 78.5$, $p = 0.029$) and 96 h (Wilcoxon rank sum test, $W = 79$, $p = 0.031$), but not after 6 h (Wilcoxon rank sum test, $W = 45$, $p = 0.368$) or 24 h (Wilcoxon

rank sum test, W = 65, p = 0.150), as neither species appreciably fragmented the plastic until after 48 h.

Time had a significant effect on PS foam fragmentation by both *O. asellus* (Kruskal-Wallis test, $X^2 = 22.135$, df = 3, $p < 0.001$) and *T. rathkii* (Kruskal-Wallis test, $X^2 = 16.599$, df = 3, $p < 0.001$). *O. asellus* produced significantly more fragments at 48 h than at 6 h (posthoc Kruskal Nemenyi test, p = 0.011), and significantly more at 96 h than at 6 h ($p < 0.001$) or 24 h (p = 0.034) (Figure 4.2A). *T. rathkii* produced significantly more fragments at 96 h than at 6 h (p = 0.006) or 24 h (p = 0.006) (Figure 4.2B).

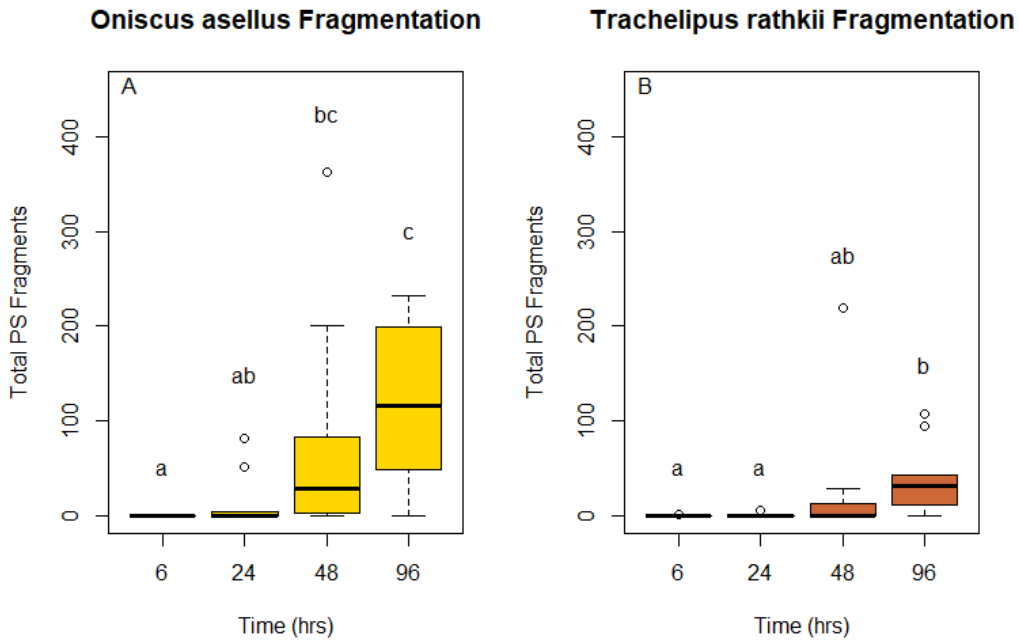

Figure 4.2: Total numbers of PS foam microplastic particles found in isopod *O. asellus* (A) and *T. rathkii* (B) cadaver biomass and arena sand across the four exposure times. Different letters denote times at which isopods produced significantly different amounts of microplastic. Lower and upper whiskers represent dataset minimums and maximums, respectively, excluding outliers.

Alternate substrate experiment

The presence of wood as an alternate substrate had no significant effect on *O. asellus* fragmentation of PS foam (Wilcoxon rank sum test, W = 233.5, p = 0.359), though the isopods produced slightly more fragments when exposed to PS foam alone (Figure 4.3).

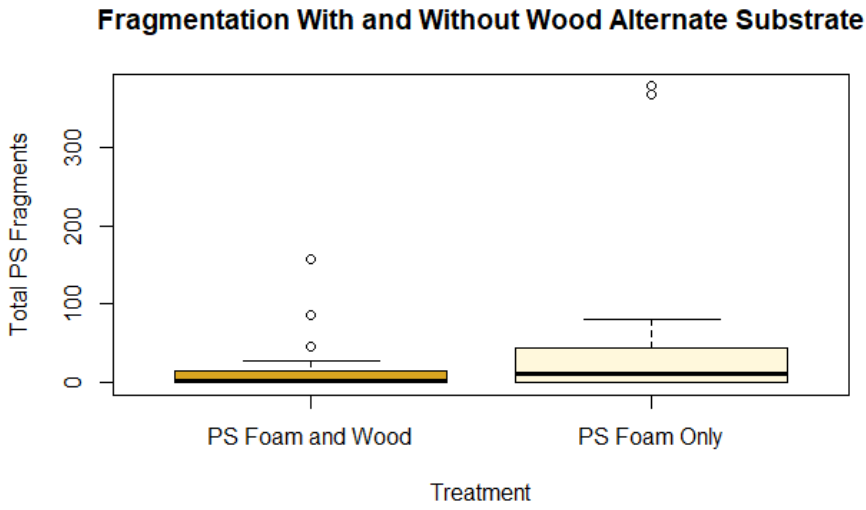

Figure 4.3: Total numbers of PS foam microplastic particles found in isopod *O. asellus* fecal matter, cadaver biomass, and arena sand when exposed to large PS foam degree with and without the additional presence of wood as a natural, alternate substrate. Lower and upper whiskers represent dataset minimums and maximums, respectively, excluding outliers.

Discussion

Both isopod species fragmented PS foam, though not at an appreciable level until the 48-h mark. At and after that point, *Oniscus asellus* produced significantly more fragments than *Trachelipus rathkii*. This difference is interesting in light of those two isopod species having

similar feeding preferences and consumption rates when given relatively unpalatable oak and beech litter (Gerlach et al. 2014). It also raises the possibility that other isopod species, those more inclined toward phenolic-rich or otherwise unpalatable litter, might consume and fragment PS foam even more readily.

Microplastics have so far not been shown to cause significant harm to terrestrial isopods (Jemec Kokalj et al. 2018, 2021), so plastic consumption could be a benign behavior for them but make PS foam microplastics available to other members of the soil food web. To our knowledge, although several studies have assessed the effect of PS microplastics on soil organisms such as earthworms (Cao et al. 2017, Jiang et al. 2020) and nematodes (Kim et al. 2020), all of them used beads of unexpanded PS as opposed to PS foam. The specific effects of PS foam microplastics on soil organisms have so far not been assessed, though they can leach chemical additives and lead to bioaccumulation in earthworms (Li et al. 2019). If terrestrial isopods are found to fragment PS foam in the field, as marine boring isopods are known to do (Davidson 2012), then characterizing the effects of these microplastics will be important.

Most previous studies on invertebrate consumption and fragmentation of PS foam and other plastics took place over a longer span of time (Yang et al. 2015, 2021, Kundungal et al. 2019, Song et al. 2020). Even outside the waste management context of many of those studies, a prolonged experiment is valuable for determining if organisms will consume plastic continuously. However, shorter experiments such as Bombelli et al. (2017) and Helmberger et al. (2022) assess how quickly organisms can begin consuming plastic, which in ecological contexts is equally if not more relevant.

Of course, the perennial critique of laboratory studies is that they do not effectively replicate organisms' physiological, behavioral, or ecological responses to pollutants (Underwood

1995, Carpenter 1996). Some microplastic studies have been criticized for using environmentally unrealistic particle shapes and concentrations (Lenz et al. 2016, Rozman and Kalčíková 2022). Even in a natural environment heavily polluted with PS foam or other plastic trash, isopods would still have plant litter and decaying wood available for food. In our study, the presence of wood did not significantly reduce fragmentation of the PS foam, but other alternate food source could have still done so.

Few studies have explicitly assessed the effect of alternate substrates on plastic fragmentation. Our previous study included oat flakes as alternate food for isopods and other invertebrates and observed fragmentation, though did not compare fragmentation rates between arenas with and without alternate food (Helmberger et al. 2022). Other than that, tenebrionid beetle larvae are known to increase consumption (and resultant fragmentation/partial biodegradation) of PS and polypropylene when given supplemental food (Yang et al. 2018, 2021), though these are perhaps exceptional among invertebrates in their propensity to consume plastic. They are also not especially ubiquitous members of the soil fauna. Thus, examining plastic fragmentation by a wider range of more representative taxa as well as confirming this phenomenon in the field remain two critical priorities for soil microplastic research.

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SYNTHESIS: FUTURE DIRECTIONS FOR SOIL MICROPLASTIC RESEARCH

In this dissertation, I investigated how soil invertebrates interact with plastic pollution, specifically characterizing their ability to produce microplastic particles by fragmenting macroplastic debris. My findings help highlight the importance of considering organisms not just as passive victims of this novel anthropogenic contaminant, but potential mediators of its effects on soil ecosystems.

Reviewing the literature, I found a focus on ecotoxicological studies investigating microplastic effects on soil organisms, primarily in the laboratory (Ju et al. 2019, Lahive et al. 2019, Song et al. 2019, Jiang et al. 2020). Others have noted how this approach alone is not sufficient to estimate the true scale and nature of the threat posed by microplastics to soil ecosystems because of microplastic's great diversity as an environmental contaminant. Microplastics vary considerably in terms of size, shape, color, polymer composition, age and weathering status, and additive chemistry (Rochman et al. 2019), all of which can affect their bioavailability and toxicity to organisms (Lehtiniemi et al. 2018, Fueser et al. 2019, Lozano et al. 2021). Most laboratory studies, however, deal with only one type of microplastic particle or particles varying along only a single axis, and even then, different sizes or plastic polymers are typically used in separate treatments. Organisms are exposed to only one type of plastic at a time, whereas in natural environments, they would encounter multiple types simultaneously. Another limitation of these studies has gotten considerably less attention, which is that, with few exceptions, they only include one type of organism. Organisms can generate microplastics (Yang et al. 2015, Song et al. 2020), transport microplastics (Huerta Lwanga et al. 2017, Maaß et al. 2017, Rillig et al. 2017, Yu et al. 2019), incorporating them into burrows or casts (Huerta

Lwanga et al. 2017, Yu et al. 2019) or dispersing them from a point source like a piece of fragmenting trash. In addition, organisms can physically and chemically alter microplastics through ingestion and excretion (Yang et al. 2015, Song et al. 2020, Kwak and An 2021), which may change how other organisms interact with them. Understanding how organisms may mediate microplastic effects on other organisms or act as entry points into the soil food web is something I see as a second critical complication for the study of microplastics in soil.

To begin my investigations, I first needed a reliable method to detect microplastics in samples of soil invertebrate feces and biomass. The fluorescent dye Nile red has been extensively used to detect microplastics in environmental samples (Shim et al. 2016, Erni-Cassola et al. 2017, Hengstmann and Fischer 2019), though its tendency to stain chitin can make it, on its own, insufficient for work with terrestrial arthropods. I investigated another dye, Calcofluor white, for use as a counterstain. Its chitin-staining ability (Elorza et al. 1983) and blue fluorescence proved effective for distinguishing Nile red-stained microplastic particles in invertebrate samples, both under laser scanning confocal microscopy and fluorescence stereomicroscopy. The visual nature of the method means it cannot detect particles below a certain size threshold, but when using a fluorescence-adapted stereomicroscope, it has a significant cost advantage over chemical detection methods such as infrared or Raman spectroscopy for situations wherein polymer identification is not necessary.

Using this method (Helmberger et al. 2020), I found variation in which of a diverse swath of soil invertebrates fragmented polystyrene (PS) foam in laboratory arenas. The snail *Cornu asperum* did not fragment; the cricket *Gryllodes sigillatus* and isopod *Oniscus asellus* did, but not to the extent of the beetle larva *Zophobas morio*, previously well-known to consume and fragment plastic (Zielińska et al. 2020, Yang et al. 2021). They did so after just 24 h exposure to

plastic, sometimes producing hundreds of PS foam particles. Also, whereas *Z. morio* fragmented both pristine PS foam and weathered PS foam collected from outside, *G. sigillatus* and *O. asellus* fragmented only the weathered PS. This finding was further supported when, in a follow-up experiment, *O. asellus* fragmented pristine PS foam only after soaking it in a soil suspension to mimic exposure to the elements. This again speaks to the diversity of plastic pollution, and even though this study investigated fragmentation of macroplastic debris, still suggests that laboratory studies using pristine microplastic particles may underestimate their bioavailability.

Further work muddied the estimate of exactly how much time *O. asellus* and its fellow isopod *Trachelipus rathkii* need to significantly fragment plastic. I examined fragmentation rates at 6, 24, 48, and 96 h and only found appreciable fragmentation at the latter two time points. Methodological differences between this and the previous study could explain the differences, such as the second study's processing of isopod cadavers and arena sand only as opposed to letting the isopods defecate and counting microplastics separately in the fecal material. The soil suspension used to treat the pristine PS foam was sourced differently as well, from an agricultural field in the first experiment and a forest in the second. An additional experiment showed that the presence of wood as an alternate, more natural substrate for the isopods did not significantly affect plastic fragmentation rates. Few laboratory studies provide organisms with non-plastic food, though those doing so do not necessarily find that it prevents feeding on plastic (Yang et al. 2021, Helmberger et al. 2022). That said, it remains to be determined whether or not isopods or other soil invertebrates will fragment plastic in field settings.

All of this work has only scratched the surface. As mentioned previously, numerous other invertebrate-plastic interactions exist. Also, the diversity of plastic pollution in the environment must still be considered. For example, earthworms and isopods have been shown to consume

biodegradable plastic films (Wood and Zimmer 2014, Zhang et al. 2018), but whether they will consume the polyethylene commonly used in agricultural mulching film remains to be seen. Although work has continued to characterize invertebrate-plastic interactions, what I note as the missing next step is examining their downstream effects on soil ecosystems. Determining whether microplastics formed, placed, or altered by one organism become more or less bioavailable and/or damaging to the rest of the soil food web remains a key priority for soil microplastic ecology research.

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