12-2022

MICROPLASTIC ACCUMULATION IN TERRESTRIAL GASTROPODS AND SOILS

Gregory B. Bonilla
University of Tennessee, Knoxville, Gbonilla@vols.utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Gregory B. Bonilla entitled "MICROPLASTIC ACCUMULATION IN TERRESTRIAL GASTROPODS AND SOILS." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Geology.

Michael L. McKinney, Major Professor

We have read this thesis and recommend its acceptance:

Benjamin Keck, Andrew Steen, Sean Schaeffer, Jake Benner

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

MICROPLASTIC ACCUMULATION IN TERRESTRIAL GASTROPODS AND SOILS

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Gregory B Bonilla

December 2022

ACKNOWLEDGEMENTS

This project has been one of the greatest challenges and most interesting experiences I have had thus far. The gratitude I have for those that have helped me along the way is immense. I would first like to thank Michael McKinney for giving me the opportunity to work on something that I was passionate about and for giving me sound advice whenever I was faltering or unsure of what to do next. I would have never been able to do this without his guidance. I would also like to thank my committee members, Jake Benner, Ben Keck, Sean Schaeffer and Andrew Steen, for their advice when I didn't know what I didn't know. I never would have learned as much about the complexity of soils in relation to microplastics or how microplastics are counted in fish. Nor would I have the slightest clue on how to analyze data without guidance on using the ever so lovely programming language R in data analysis. I also owe a debt of gratitude for Jakes idea of making my soil device entirely out of metal; I would still be cutting glass to this day! I would like to thank Dr. Chen-fei-chen, as I would have never met some contacts in the data world to help me advance my skills enough to reach this point, and for giving me the chance to publish our research.

To my fellow graduate students, thank you for my random questions about things I should have probably known about, but went right over my head. Specifically, thanks to Allison for the countless times I needed help navigating graduate school. John and Ethan for struggling alongside me in labs and data classes and listening to me anxiously stressing over my degree. To the graduate students in the 7th floor offices, thank you for always welcoming me back after my months long hiatus from staying in the lab and not coming into the office. Dana and Jake, thank you for working alongside me in the lab and offering advice and encouragement on handling the ever-ending issues we seemed to face dealing with microplastics. A special and needed thanks to Michael for being the absolute best undergraduate helper I could have imagined. You played a huge role in this project and crawled through many soaked and dirty bushes to collect snails for me countless times.

Finally, my family for always believing in me and being stern with me when I would make jokes about switching careers. You all always believed I was making a good choice and I cannot thank you all for that enough.

ABSTRACT

Microplastics (MPs) have become an emerging threat to ecosystems across the world. Transport, impacts, and fates are grossly understudied, especially in terrestrial environments. Current research on MP bioaccumulation has focused mainly on aquatic organisms with little study of terrestrial organisms, including snails where data are nearly nonexistent. To address this, we collected and examined land snails and their surrounding soil for MP content in shell and tissue. From September 11, 2020, to October 25, 2021, cover boards were placed (n=30) along relatively undisturbed sites in hardwood, forested areas, and tall grasses in a Wildlife Management Area (WMA) in Oak Ridge Tennessee. A total of 150 individuals were collected and 2.2 lbs of soil per site was taken directly underneath coverboards. For comparison with a more disturbed habitat, 150 snails and soil were also collected on the University of Tennessee campus from March 1, 2022, to July 10, 2022. Snails were placed in 70% ethanol for analysis and digested for 24 hours using a 1:1 ratio of potassium hydroxide pellets and sodium hypochlorite for organic matter removal. Samples were vacuum filtered, air dried for 1 week and then examined via stereomicroscope. MP shape, color, and number were recorded, and samples were examined twice, independent of one another to ensure accurate MP counts. Two tests were performed if MPs could not be determined. The “hot needle test” causes plastics to deform and melt when exposed to heat, and the “spring test” to examine the physical characteristics of the MP. Results for the less disturbed WMA (Oak Ridge) showed total MP abundance at 214 particles in snails and 478 for soils. MP counts ranged from 0 to 6 per snail and 5 to 33 per soil sample. For campus (Knoxville), MP counts were higher, with a total of 270 particles in snails and 540 particles in soils. MP counts ranged from 0 to 8 per snail and 7 to 35 particles per soil sample. This study confirms the accumulation of MPs in land snails and indicates that higher human activity is associated with higher densities of MPs in both snails and soils.

TABLE OF CONTENTS

SECTION 1. INTRODUCTION *1*

SECTION 2. BACKGROUND *4*

MICROPLASTIC ACCUMULATION IN SNAILS *4*
MICROPLASTIC ACCUMULATION IN SOILS AND FRESHWATER *5*
CURRENT MICROPLASTIC IDENTIFICATION METHODS *6*

SECTION 3. MATERIALS AND METHODS *10*

SITE SELECTION AND SNAIL COLLECTION *10*
Wildlife Management Area *10*
UT campus *11*
Snail Subsample Collection *11*
CHEMICAL DIGESTION SELECTION *11*
SAMPLE PREPARATION *15*
SNAIL DIGESTION *20*
SOIL COLLECTION *20*
SOIL ISOLATION UNIT DESIGN *20*
SOIL DENSITY SEPARATION *21*
Validation *25*
FILTER READING PROCEDURE *26*
MICROSCOPE IDENTIFICATION *26*
Identification Rules *28*
Rule 1: Lack of cellular or organic structures *28*
Rule 2: MP structures should generally exhibit homogeneity *29*
Rule 3: Particles should exhibit a similar color pattern *29*
NON-PLASTIC MATERIALS *29*
Calcium Carbonate Shells *29*
Plant material *32*
Contamination *32*

SECTION 4. RESULTS *36*

SNAIL MP ABUNDANCE *36*
SUB-SAMPLES *36*
SOIL SAMPLES *42*
STATISTICAL ANALYSES *43*

SECTION 5. DISCUSSION *51*

MICROPLASTIC ABUNDANCE AND TYPE IN SOILS AND SNAILS VARIED ACCORDING TO DISTURBANCE LEVEL *51*
THEORIZED METHODS OF ACCUMULATION *52*
CHEMICAL SORPTION ONTO PLASTIC SURFACES *53*
POTENTIAL HARMFUL EFFECTS *54*

SECTION 6. SUMMARY *57*

SECTION 7. FUTURE WORK *59*

REFERENCES *60*

VITA *66*

LIST OF TABLES

Table 1 Digestion Efficacy for selected chemicals and damage examination for heat drying and chemical use. No damage recorded (NA); Low damage recorded (L); High damage recorded (H). 19
Table 2 Summary characteristics comparing WMA vs UT campus snails and soils..... 41

LIST OF FIGURES

Figure 1 Potential microplastic classification types of fragments, pellets, films, foams and fibres. Modified from Dr. Alexander Kunz of the Microplastic Research of Taiwan. 2
Figure 2 Transportation mechanisms of microplastics via terrestrial, aquatic, and atmospheric deposition. 3
Figure 3 Microplastic analysis by type, length and particles by hectare in Bavaria Germany. Original image courtesy of Plastic Atlas granted by Creative Commons License. 7
Figure 4 Oak Ridge sampling locations in a secluded, low disturbance area. Bull bluff road (yellow), Solway Bend Road (green) and coverboards (blue). 12
Figure 5 Knoxville sampling locations at the University of Tennessee. Perimeter defined by black lines and sites by red squares. 13
Figure 6 Chemical digestion and efficacy chart. LDPE, HDPE, PP, PA, PS, PET subjected to varying chemical concentrations to examine degradation. Modified from Pleiffer et al. 2020. 16
Figure 7 750-micron examination of heat damage on a piece of polystyrene foam. 17
Figure 8 Chemical bubbling on a piece of Polyethylene plastic at 750 microns. 18
Figure 9 Soil Isolation Unit schematic and unit made from Poly-Vinyl Chloride tubing, and tin foil. Use granted courtesy of Dr. Coppock, Plymouth Marine Laboratory. 22
Figure 10 Improved SIU designed to prevent plastic contamination for use in small-scale density soil experiments 23
Figure 11 (a) Soil isolation unit as previously designed by Dr. Coppock; (b) degradation from wear and tear of unit; (c) Lubrication of unit to prevent degradation; (d) contaminated particles from device, Nel et al. 2019. 24
Figure 12 (a) filter reading procedure on a clean filter moving from top right corner left (b) good digestion low organic matter (c) bad digestion, shifting of shells and large organic matter (d) excessive concentration and digestion. 27
Figure 13 Hot needle test on a suspected MP particle. Confirmation from melting and separation. 30
Figure 14 Undigested calcium carbonate snail shell 31
Figure 15 Filter grid line that has peeled away from chemical digestion 33
Figure 16 White translucent fibers and undigested snail shells 34
Figure 17 Bundles of white and translucent fibers 35
Figure 18 Color distribution of Oak Ridge Snails 37
Figure 19 Jittered graph of MP concentration by site in Oak Ridge 38
Figure 20 Color distribution of Knoxville snails 39
Figure 21 Jittered graph of MP concentration in Knoxville 40
Figure 22 Total MP count of soils by site in Oak Ridge 44
Figure 23 Total MP count in soils in Knoxville 45
Figure 24 Large Blue MP thread found in a Knoxville Mesodon snail sample 46
Figure 25 Red microplastic thread found from Oak Ridge snail sample 47
Figure 26 Blue MP film found in Oak Ridge soils 48
Figure 27 Blue MP flakes found in Knoxville soil samples 49
Figure 28 Red MP thread amongst undigested material found in Oak Ridge snail 50
Figure 29 Kinetic and Isothermic modeling for mechanisms of pollutants on MPs. Use courtesy of Fu et al 2021 under the Creative Commons License. 56

SECTION 1. INTRODUCTION

The first plastics, created in 1862 by Alexander Parkes, led to the eruption of polymers that are found in nearly every product today. In 1907, the first fully synthetic plastic was made and a new era of microplastic (MP) pollution began. Plastic has since become a staple of a vast array of commercial products, and there is increasing evidence that they may persist indefinitely in many environments. Despite a long history of plastics pollution, environmental MP accumulation is a relatively new area of research.

Microplastics were originally defined as small pieces of plastic fragments between 0.1-2mm in size (Carpenter et al., 1972), but MPs now include any plastic particles < 5 mm in diameter (Cole et al., 2011). Plastics such as polypropylene, polyethylene, polystyrene, polyethylene terephthalate, and polyvinylchloride make up the tiny particles, which are now widely transported into many habitats via many pathways (Santos et al., 2015). Research in the past few decades centered on the fate and sources of MPs with a recently emerging focus on both biotic and abiotic systems.

MPs form when larger plastic pieces are exposed to erosion, degradation, photo-thermal oxidation, UV degradation, or friction (Li et al., 2018; Cole et al., 2011). Primary MPs come directly from production of tiny plastic particles, and secondary MPs come from the fragmentation of larger particles (Figure 1). Both are transported via several media (Gola et al., 2021). Freshwater systems carry a significant number of MPs (Figure 2) providing nearly 80% of the total input of plastic into the oceans (Andrady, 2011).

This poses significant concerns about mechanisms of transport and the toxic effects of MPs, including adsorption of many other chemicals or toxic substances (Andrady, 2011). With over 350 million tons of plastic produced worldwide every year, there is enormous potential for plastics to have harmful impacts on ecosystems at many scales (Lim, 2021).

Despite this threat, we are far from a full understanding of the accumulation, distribution, and effects of MPs. Furthermore, MP research has largely focused on oceanic areas, and only recently have high quality studies been performed on freshwater and terrestrial environments (Eriksen et al., 2013; Wright et al., 2013; Sul et al., 2014). It is therefore critical to develop studies on MPs in freshwater and terrestrial ecosystems for a full understanding of MP transport and impacts.

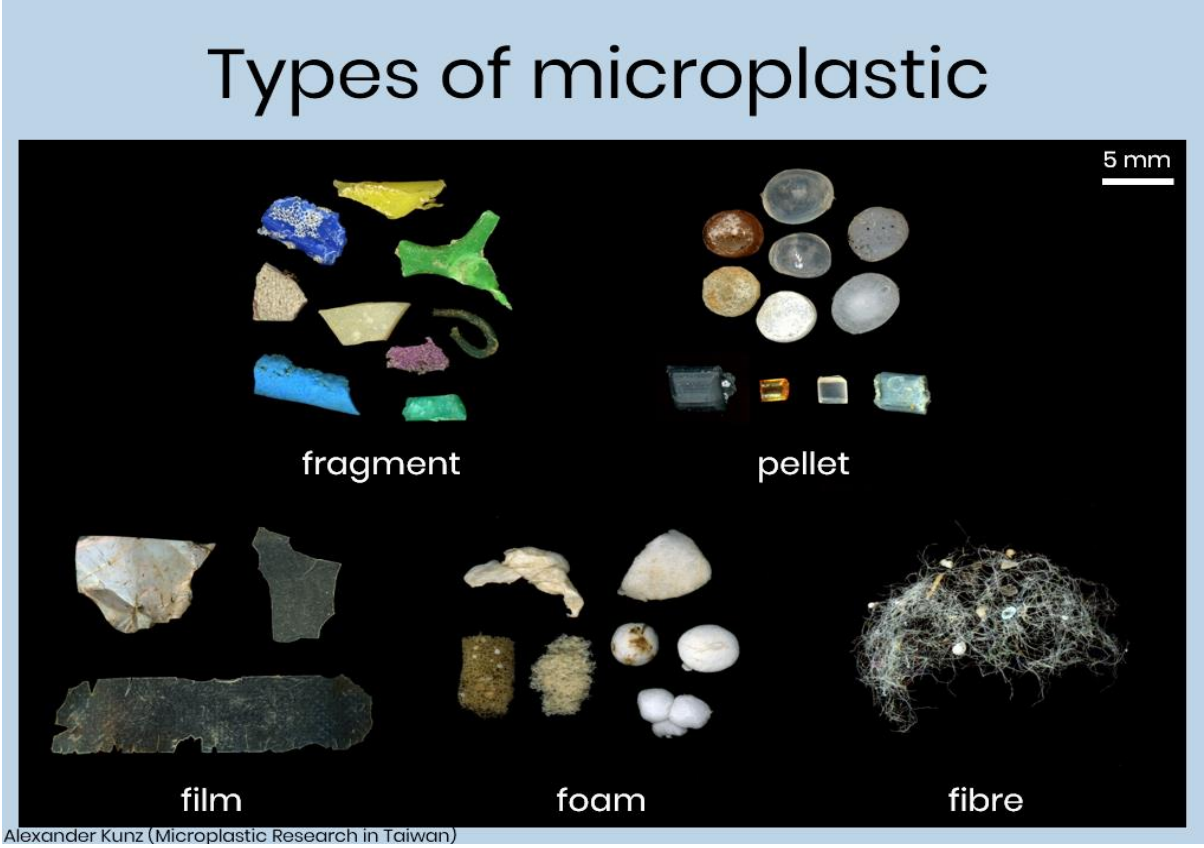

Figure 1 Potential microplastic classification types of fragments, pellets, films, foams and fibers. Modified from Dr. Alexander Kunz of the Microplastic Research of Taiwan.

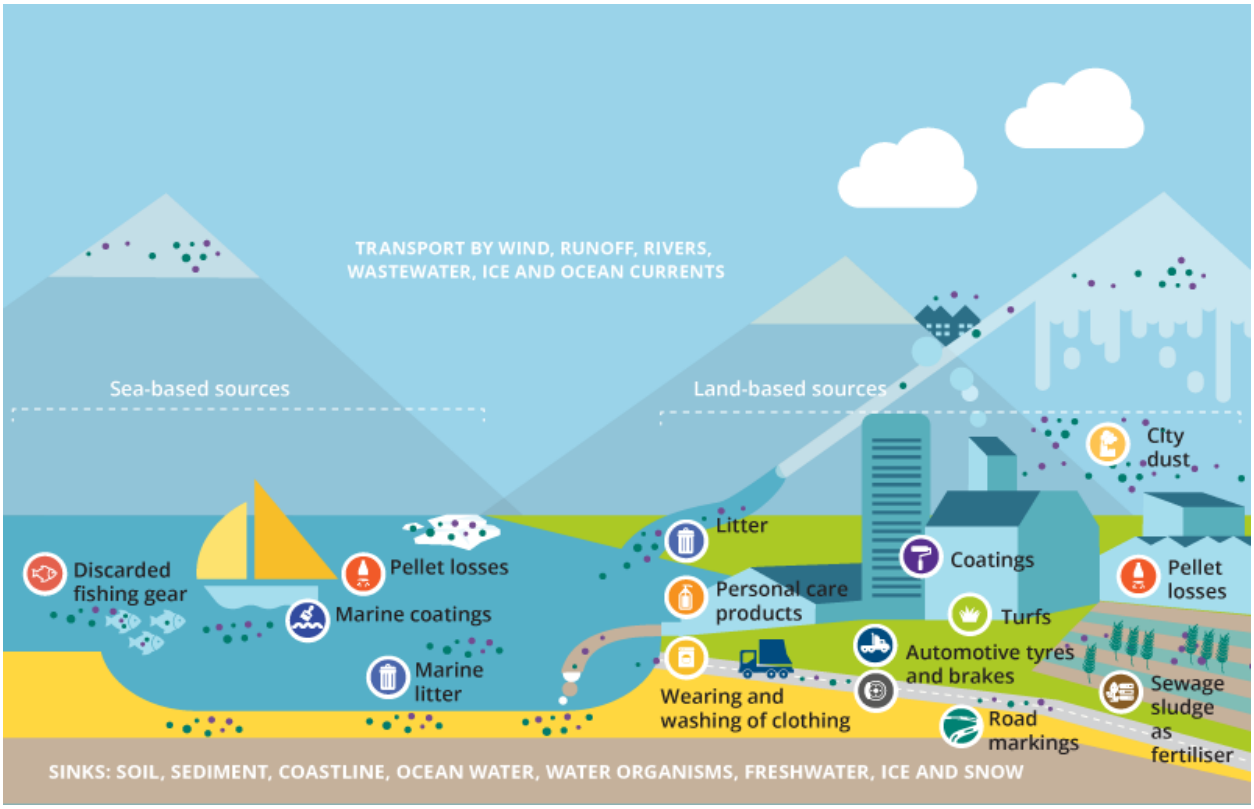

Figure 2 Transportation mechanisms of microplastics via terrestrial, aquatic, and atmospheric deposition.

SECTION 2. BACKGROUND

Microplastic accumulation in snails

Until recently, studies concerning organismal MP uptake and biological effects have been relatively rare. Marine organisms including mollusks, fish, and crustaceans, are commonly examined across multiple species from within the same taxa (Xu et al., 2020). This study expands MP research to a relatively understudied group (gastropods) in an understudied environment (terrestrial). Even though MP research has become prominent in the past few decades, research quantifying MP particles in gastropods has been rare in recent years. Gastropods have previously been used as bioindicators for monitoring heavy metal pollution due to their tendency to accumulate heavy metals and resistance to long term effects from bioaccumulation (Samsi et al.,2017). Reproduction and survival are often used as indicators of stress and nutrient absorption within an ecosystem, and the same techniques used to examine heavy metal pollution have been applied for MPs (Mardsen et al., 2015). When examining gastropods in marine environments Xu et al. (2020), found six types of MPs (cellophane, polyethylene terephthalate, polyamide, polypropylene, polyethylene, and polyacrylonitrile) apparently related to feeding mode and biological criteria. When compared to other organisms there were significantly more MPs found in gastropods.

The primary source of MP uptake in gastropods is not fully understood but is apparently related to contaminated food sources (Weber et al., 2021). Several studies have sought to examine the methods by which MPs are digested, and these often attempt to establish standardized procedures to ensure the highest efficacy. The most common method is to capture live snails and purchase varying shapes, colors, and sizes of MPs to mix with substrate and food (Song et al., 2019; Weber et al., 2021). Live snails are subjected to a 48 hr+ detoxification time to ensure they are clear of MPs and are then fed the MP mix to examine the digestion processes and effects. One important study has found that, given the same amount of MP particles per individual, the concentration between species changed dramatically (Naji et al.,2018). This indicates that the uptake and bioavailability of MPs is species-dependent and creates a poorly understood set of vulnerability factors. When snails were examined in an uncontrolled setting, Zhang et al. (2020) found that within the Beibu Gulf, MPs in the same species of gastropods changed dramatically depending on the location. Even within the same estuaries, the MP difference can be significant (Zaki et al., 2021). This further compounds complexity, as the proximity to urbanization can play a major role on the amount of MP exposure. It has been theorized that an increase in MP concentration in pore water may increase concentration in organs but there have been no conclusive results (Zhang et al., 2020).

One of the first attempts to study how MPs could be transferred among different trophic levels through mucus retention during gastropod locomotion found two main kinds of transfer (Weber et al., 2021). First, MPs were found to be retained at significant levels in pedal mucus. This resulted in small molluscan herbivores such as periwinkles, who feed on the mucus, to ingest the MPs. Second, through predator-prey interactions the MPs could be consumed from contaminated prey. MP size, density, abundance, and color have been theorized to play an important role in how species interactions, whether passive or active, can influence the uptake and transfer (Wright et al., 2013). Gastropods play an active role in food webs across many environments, and this highlights how easily MPs can bioaccumulate across trophic levels. This type of primary and secondary consumption of MPs has rarely been examined, posing a gap in understanding how common species that play a significant role in food webs interact with surrounding biota in areas with substantial plastic input.

Understanding that MPs may be transferred via a series of interactions and areas is important, but analyses that look specifically at the number of particles within individuals and among species is often overlooked, especially in the land snails. The baseline level of MP particles in land snails is unknown, as many snail species have never been examined. Indeed, the first documentation of MPs in land snails occurred in 2019 (Panebianco et al., 2019). Since then, there have been many studies conducted on other invertebrates including marine snails, but none published on land snails.

The importance of gathering baseline MP data in land snails is further illustrated by the known MP variation from marine snail studies. These studies document drastic variations in MP concentrations, from 0-40 MPs per snail to as many as 300 particles per snail (Kleinschmidt et al, 2021; Hamra et al, 2019). This indicates that a large amount of MP abundance data will need to be gathered in order to understand and characterize the wide MP variation that may be present in land snail populations.

Microplastic Accumulation in Soils and Freshwater

There are currently no widely accepted standard methods for extracting MPs from freshwater or terrestrial sediment. Instead, there are a wide variety of methods used that typically combine density fractionation, sieving, and acid digestion (NOAA, 2015). Some studies have attempted to develop cost-effective methods utilizing small-scale PVC devices using gravity and a series of sieves to separate MPs from sediment (Coppock et al.,2017). These separation methods have not been well established however, and the sediment cores themselves represent an additional labor-intensive process of extraction.

One of the first studies of freshwater MPs found that particle counts in large freshwater lakes can reach a maximum of 450,000 per sq km (Eriksen et al., 2013), and can vary by three orders of magnitude, from 450 to 450,000 per sq km. Other studies have sought to examine specific sources such those from distinct agricultural sources, but results are inconclusive about the amount of these plastics relative to other pollutants (Chen et al., 2020). These studies highlight the many challenges of terrestrial MP research, such as the differences between urbanized and non-urbanized areas, proximity to point sources, and distinguishing plastic from non-plastic particles. Because of their concentrations in the medium, MPs in small and urban water bodies may cause outsized impacts when compared to vast oceanic areas (Vaughan et al., 2017). Several factors may influence the deposition of MPs in urban areas. These include: water characteristics involving inflow-outflow patterns, sediment accumulation rates, point and non-point sources, proximity to bodies of water, atmospheric deposition of MPs, and native properties of the MPs (Dris et al, 2016; Simmerman et al, 2019; Darabi et al, 2020).

There have been attempts to quantitively examine the abundance of MP particles in industrial or urbanized areas, and these offer insight on the type and rate of deposition. Farmlands are often targets of MP research as large areas of plastic cover are placed and tilled continuously in the soil. Sediment cores have been examined (Figure 3) in these areas to determine rate of MP accumulation over time and these studies have concluded that most MPs are found within the upper soil layers at concentrations up to 6 times the deeper layers (Willis et al., 2017).

Current microplastic identification methods

There are several ways to characterize MPs into various categories of shapes, textures, and compositions. However, these are often costly, time consuming, damaging, or disregard certain types of plastics. In general, both terrestrial and aquatic samples first undergo pre-treatments including density separation, acidic or alkaline digestions, or visually removing plastics. Organic matter is then often separated by hydraulic pumps through nano or micro sized filters for analysis. Currently, three methods are among the most commonly used for identification of remaining particles: Fourier Transform Infrared Spectroscopy (FTIR), optical stereomicroscopic examination, and dyes/influorescence.

FTIR is widely used to confirm plastic types by examining characteristics of infrared absorption patterns of different materials. In this method, discrete particles can be located and analyzed, or a single IR scan can provide the bulk chemical makeup of all materials within an image. The benefit to using FTIR is the ease with which samples can be prepared via filtration and then analyzed with the assistance of machine learning algorithms. As sample size can often number into

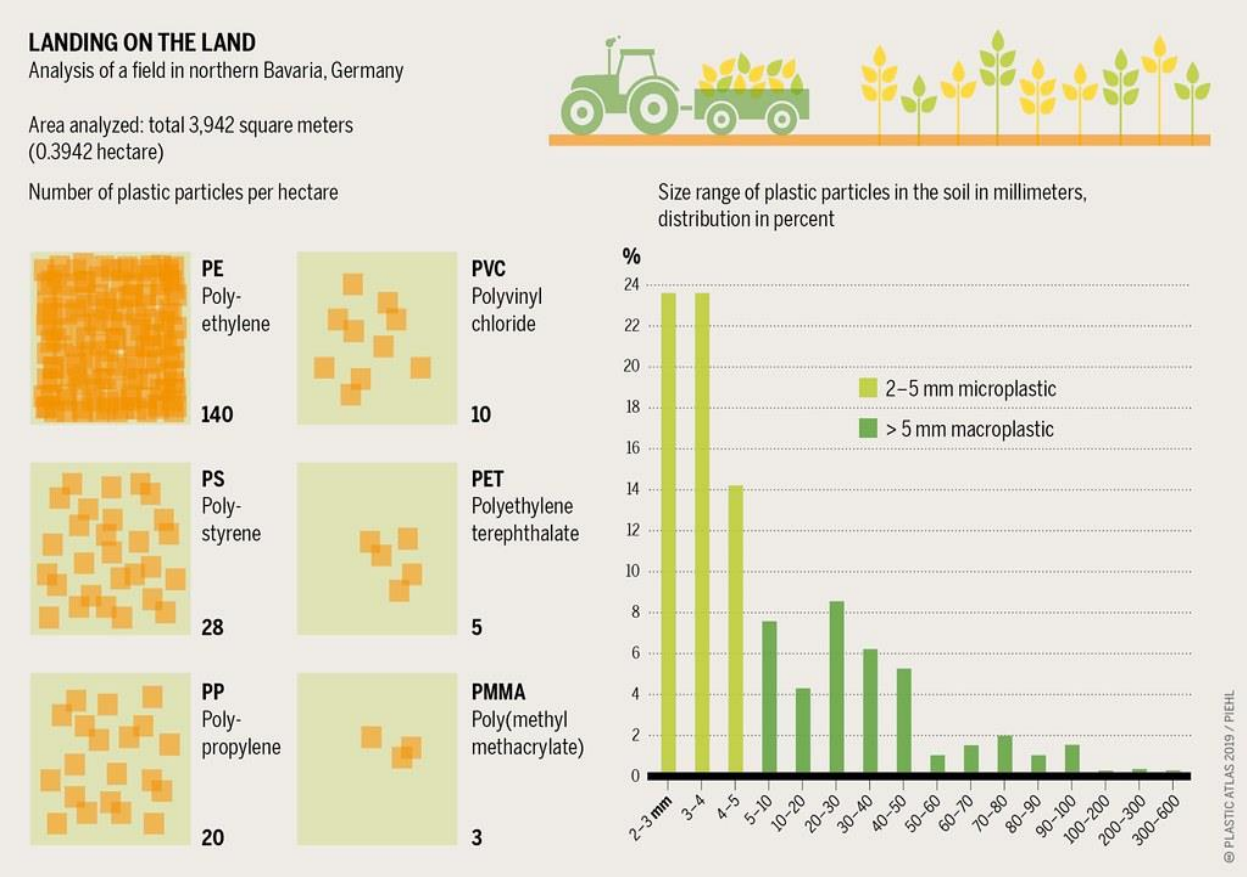

Figure 3 Microplastic analysis by type, length and particles by hectare in Bavaria Germany. Original image courtesy of Plastic Atlas granted by Creative Commons License.

the thousands, this automation is highly efficient. However, FTIR microscopes are costly and machine learning for microplastics is still in development and plastics are commonly miscategorized. In addition, spectra libraries for identification can be limited, which can result in fewer plastics being recognized than are actually present. Despite these obstacles, FTIR remains one of the most useful and growing tools for MP identification.

Stereomicroscope identification, the chosen method in this study, is likely the most widely used form of characterization because of its low cost and simplicity. This method relies on visual depictions and heat testing of particles to determine characteristics of plastics (Blair et al, 2019; Mariano et al, 2021). Magnifications of 30X and higher are often used, and particles above 1mm in their longest dimension can generally be identified confidently. Observing objects this way uses reflected light from illumination sources above and below the sample. Higher magnifications can help show detailed structures of organic material and at 50X+ magnification the minimum visible size is nearly 100 microns. This method is cheap, accessible, and requires minimal training. There are drawbacks, however. This method generally must be combined with highly efficient chemical digestion, as dense sediment that is not digested can interfere with counts. A frequent problem with identification is human-induced bias. Often, observed particles may not appear as plastic to one observer but may be to another. For example, organic fibers can be included in counts as MPs unless additional identification criteria are used. Similarly, there is significant difficulty in counting clear plastics, particularly in areas with sand. Some of these misidentification problems can be minimized by using heat and spring tests on all particles (Reichert et al, 2021; NOAA, 2015), but this is time consuming and may not be suitable for large sample sizes. Therefore, a best practice is to ensure that multiple observers are trained to repeatedly examine the same samples to get the most accurate MP count.

Finally, a narrower but highly accurate process involves the use of fluorescent dyes (Karakolis et al, 2019). A fluorescence microscope examines emissions of fluorophore that occur either naturally or artificially. Organisms naturally produce some fluorophore within their tissues, and this can be examined by exposing the sample to light to excite the fluorophore molecules. This creates a radiating fluorescent light that differs in intensity depending on the abundance of fluorophore molecules. Microplastics largely have an innate ability to reflect inflorescent light, and this is useful when attempting to detect difficult to identify or nano-sized MPs. The response to inflorescence is largely dictated by the chemical makeup of the specific polymer and varies greatly.

New tools have been recently introduced to refine dye methods and show promising results. Nile Red is a lipophilic stain that normally does not fluoresce but can greatly fluoresce when exposed

to lipids. This stain can therefore be used in examination of biological tissues and cell structures. When combined with digestion of organic matter, clear and transparent particles become stained a deep red and can be counted, reducing miscounts of MPs as long as the digestion process is highly efficient. As with other methods, there are drawbacks. The wavelengths of light generated from inflorescence are not contained inside a single area. The inflorescence can occur in areas surrounding the samples resulting in background noise. Additives to MPs also pose a significant drawback in the accuracy of fluorescence measurements through impurities because they can change the level of fluorescence, which can only be remedied by pre-treatment removal.

In summary, despite the lack of definitive standards for examining MPs, significant strides are being made toward more accurate and faster analytical methods. Currently, a combination of methods is likely to provide the highest Efficacy for accurate MP counts.

SECTION 3. MATERIALS AND METHODS

Site Selection and Snail Collection

Sites were not chosen based upon any elevation gradient, habitat type, or biotic factors, but were chosen based on disturbance level: accessibility, visible litter, estimated foot traffic and proximity to urbanized areas were all used as site selection factors. The Wildlife Management Area (WMA) in Oak Ridge, TN and University of Tennessee (UT) campus sites were chosen because of the contrasting amount of human disturbance, with the WMA site being less disturbed. The WMA area located in Oak Ridge is locked behind gates requiring an access key and has nearly nonexistent visible litter in the sampling areas and is considered less disturbed. The UT campus is a constantly disturbed landscape with routine mowing, mulching, foot traffic and dense human activities. The UT campus in Knoxville TN is publicly accessible, contains large amounts of visible litter, and experiences frequent foot traffic and is considered a more disturbed area

To remove sampling bias the ArcGIS randomized points tool was used to select points within a boundary for each site. If the pre-determined randomized points were not accessible (e.g., roadways, waterways, non-permitted areas), the closest area to a surveyed point within a 5 meter buffer was selected.

This study focused only on the gastropod macrofauna (snails larger than 5mm) because microsnails (species less than 5 mm) are less likely to contain as many MPs or the same diversity of MP types as larger snails, and microsnails are more difficult to identify taxonomically. We used non-standardized cardboard pieces as coverboards for gastropod collection along Solway Bend Farm Road and Bull Bluff Road at the WMA and at the (UT) campus in Knoxville, TN.

Wildlife Management Area

Boards (n=30) were placed in accessible areas between 9/11/20 and 10/25/20. Thirteen boards were placed in areas composed mostly of tall grasses or open field edges, and 17 boards within hardwood forested areas as indicated by Figure 4.A total of 9 collections were conducted from 9/19/20 to 12/1/20. All gastropods (macrosnails and slugs) found on boards were collected and placed in a tube of 70% ethanol for laboratory analysis. Data recorded during collection included total number of gastropods collected, number of snails, number of slugs, and environmental data including relative humidity, dew point, wind speed average, temperature range, current weather, and recent weather (within 48 hours). Coverboards were replaced as needed if too weathered or destroyed by animals.

UT campus

Sampling on the UT campus (Figure 5) utilized the same methods noted above, within two different habitat areas. Boards (n=30) were placed 04/01/22 and collected through 08/01/22. Seventeen Boards were placed in light forested areas or in dense shrubs and ornamental vegetation, and 13 Boards were placed in open short grass areas. Both sampling habitats were located near highly disturbed areas such as parks and roadways. Many areas with shrubs were frequently mulched, and due to human activity, the campus sampling sites experienced frequent destruction of coverboards which were replaced 3X more frequently than Oak Ridge sites.

Snail Subsample Collection

The possibility of introducing a sampling bias from contamination of the coverboards was considered so a separate sampling technique was developed. Coverboards were paper based cardboard but could potentially accumulate MPs from open air and during transport. This could increase the MP presence at each site and artificially inflate intake of MPs in snails. Therefore, this sampling was used to compare MP count to ensure no bias was introduced. 35 samples were collected from 6 randomly chosen sites from each area and hand sampled for snails. Samples were collected within a 4-foot buffer of the previous coverboards to ensure the same microhabitats. Snails were collected using metal tweezers, washed with ethanol, and placed into glass tubes. These samples were compared with coverboard samples to determine if there were any significant differences due to contamination as outlined in the Results sections.

Chemical Digestion Selection

The most common methods of MP study rely on acid digestion of organic material to isolate MP particles followed by counting the number of particles under a microscope. However, it is difficult to find a digestion chemical that results in low chemical damage to plastics, and there are many methods that can result in the deformation or distortion of plastic pieces, leading to inaccurate results. Common plastic polymers have varying resistances to different chemical combinations (Figure 6). If the effects of MPs are to be understood, it is essential that high quality methods are developed to successfully identify and analyze plastics without introducing these sources of error.

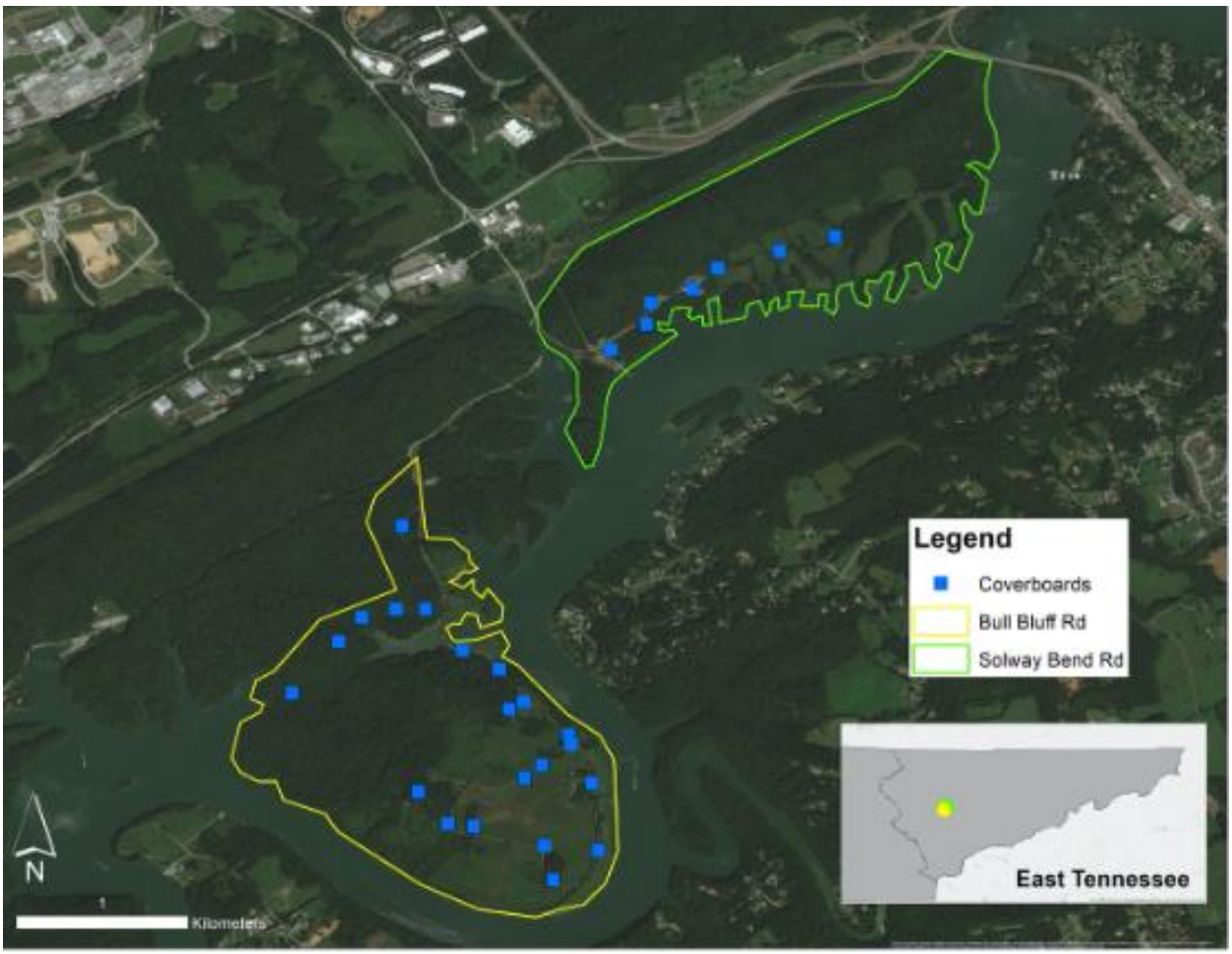

Figure 4. WMA sampling locations in a secluded, low disturbance area. Bull bluff road (yellow), Solway Bend Road (green) and coverboards (blue).

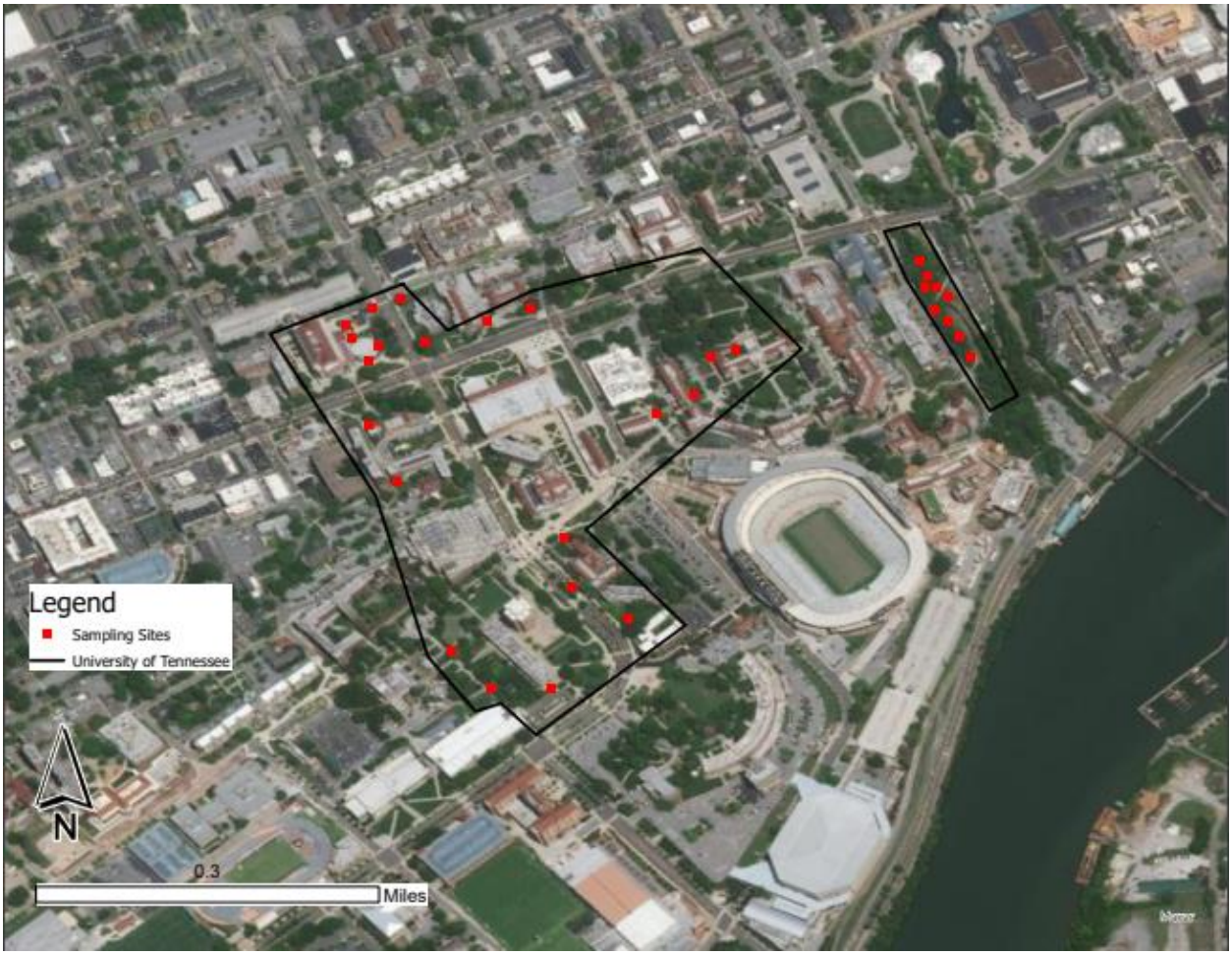

Figure 5. Sampling locations at the University of Tennessee. Perimeter defined by black lines and sites by red squares.

Early methods for quantifying particles involved experimental plans that used combinations of iron sulfate, sodium chloride and wet peroxide (NOAA, 2015). This combination resulted in digestion of organic matter but not at an acceptable rate. A need for more efficient digestion resulted in the use of stronger chemicals such as potassium hydroxide, sodium hypochlorite, and nitric acid (Shim et al., 2017; NOAA 2015). Still, many problems exist with under-digestion, polymer destruction, and significant foaming (Li et al.,2018; Enders et al., 2017; Turner et al., 2019). A variety of different methods need to be utilized to discover the most efficient way to dissolve organic matter and to create an effective plan to both digest sediment and snail tissue.

A protocol was developed based on the work of Enders et al. (2017; ICES Journal of Marine Science) and was adapted and used for acid/base digestion. Acid digestion and heat drying are typical practices when dissolving organic matter to differentiate organic material from MPs (Li Ruilong et al., 2019; Panebianco A. et al.,2019; Song Yang et al., 2018). Specifically, Nitric acid (HNO3) and hydrogen peroxide (H2O2) are frequently used, but each has significant drawbacks in altering the morphology of MP particles (Figures 7 and 8) and failing to completely dissolve organic material (Enders et al. 2017). Recently attempts have been made to use sodium hypochlorite (NaClO) and potassium hydroxide (KOH) but the studies are limited and have not been attempted with calcium carbonate shells. Further, it has been shown that drying samples at temperatures from 40-80℃ have caused destruction across varying types of plastics (CITE THIS).

In this study, digestion Efficacy and potential polymer damage were determined by a subset experiment using H202,HNO3,KOH and NaClO. Polystyrene, and low-density polyethylene were examined due to high abundance in the environment and generally low resistance to chemical degradation. Polyvinyl chloride was chosen for the experiment due to its high chemical resistance and lower abundance in the environment.

To test the effect of different digestion agents, organic matter and plastic were weighed and combined with various chemicals (see below). The resulting weight difference of plastics and organics from pre-digestion to post-digestion was used to determine the efficacy of each chemical, and to see if any damage was present on the plastics. 15 commercially-available pieces of uniform size and shape (5 polystyrene, 5 low density polyethylene, and 5 polyvinyl chloride) were placed in a digesting solution with organic material. Prior to digestion, each piece of plastic was examined via microscope at 50X magnification to ensure prior damage was recorded. Nine grams of plant and snail material were added to 25ml each of h202 (30%), HNO3 (85%), HNO3 (60%), KOH (85%), KOH (85%) +

NaClO (14%) respectively. Five samples were replicated for each chemical group (25 total) and a subset of 5 control groups in water were later tested for efficacy of heat drying.

First, control groups containing water and 15 pieces of plastic were heat dried to determine if damage was present. Second, digestion Efficacy was established to determine high efficacies >75% between groups. Next, high efficacy groups were examined for chemical destruction. Efficient drying after digestion is important, as moisture can cause microplastic identification difficulties during analyses. Five samples per control group were shaken for 24 hours, placed on a petri dish and cotton filter and dried at 40, 50, and 60℃ for 10, 20 and 30 minutes respectively in an incubator oven to determine if oven drying would cause significant heat destruction. Following microscope examination, drying across all samples occurred at 30 minutes but heat damage was significant. Therefore, ambient temperature drying for 7-14 days was used as needed.

Dried samples were weighed and then shaken for 24 hours on an orbital shaker, vacuum filtered via a dried cotton filter, air dried for 7 days and then examined under a stereo microscope for morphological changes or chemical bubbling. Final digestion efficacies were calculated using the following formula**:**

$$\boldsymbol{Digestion\ efficacy}\ (\%) = \mathbf{100} * \frac{\boldsymbol{Initial\ Weight - Final\ Weight}}{\boldsymbol{Initial\ Weight}}.$$

The highest efficacy chemical with the least destruction was KOH (85%) + NaClO (14%) with a high digestion Efficacy (90%) with little to no chemical degradation. Therefore, KOH and NaClO were chosen and data from each experiment was compiled and shown in Table 1.

Sample Preparation

Contamination of samples by MPs from external sources such as airborne particles, lab clothing, and equipment is a frequent problem when examining MP abundance in gastropods (NOAA, 2015). To prevent such contamination, all filters, materials, and tools were cleaned thoroughly using deionized (DI) water that was vacuum filtered through a 0.47 μm pore size nylon membrane. Glass petri dishes, vials, and aluminum tweezers were used to minimize contact with plastics. Cotton lab coats and masks were worn, hands were washed three times between touching materials, and all sampling and cleaning was done in a closed room with minimal foot traffic and movement to prevent stirring of existing particles. A Honeywell HP300 0.3-micron filtration system was used to help further eliminate existing airborne contamination. Background contamination blanks were placed in the lab

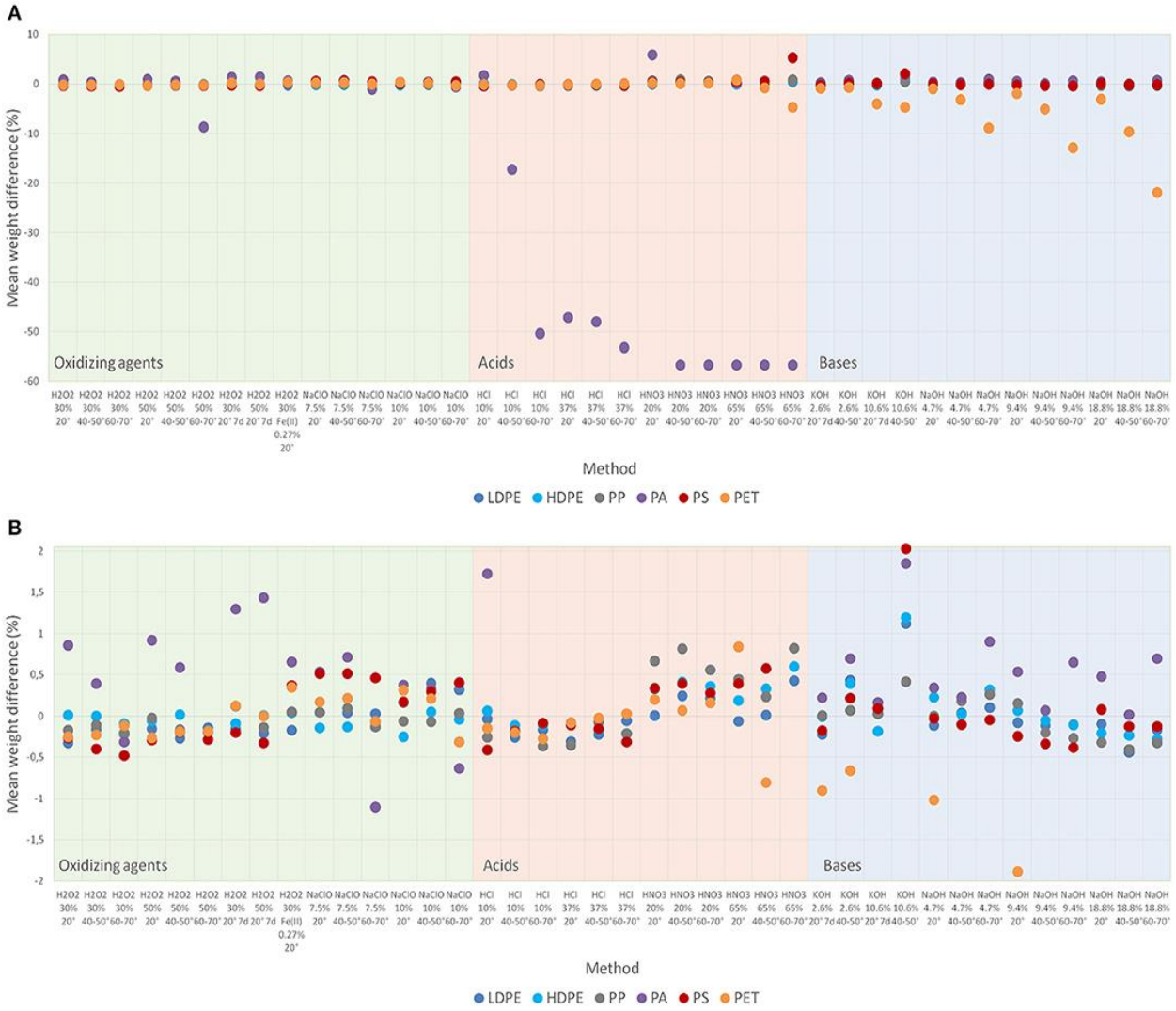

Figure 6 Chemical digestion and efficacy chart. LDPE, HDPE, PP, PA, PS, PET subjected to varying chemical concentrations to examine degradation. Modified from Pleiffer et al. 2020.

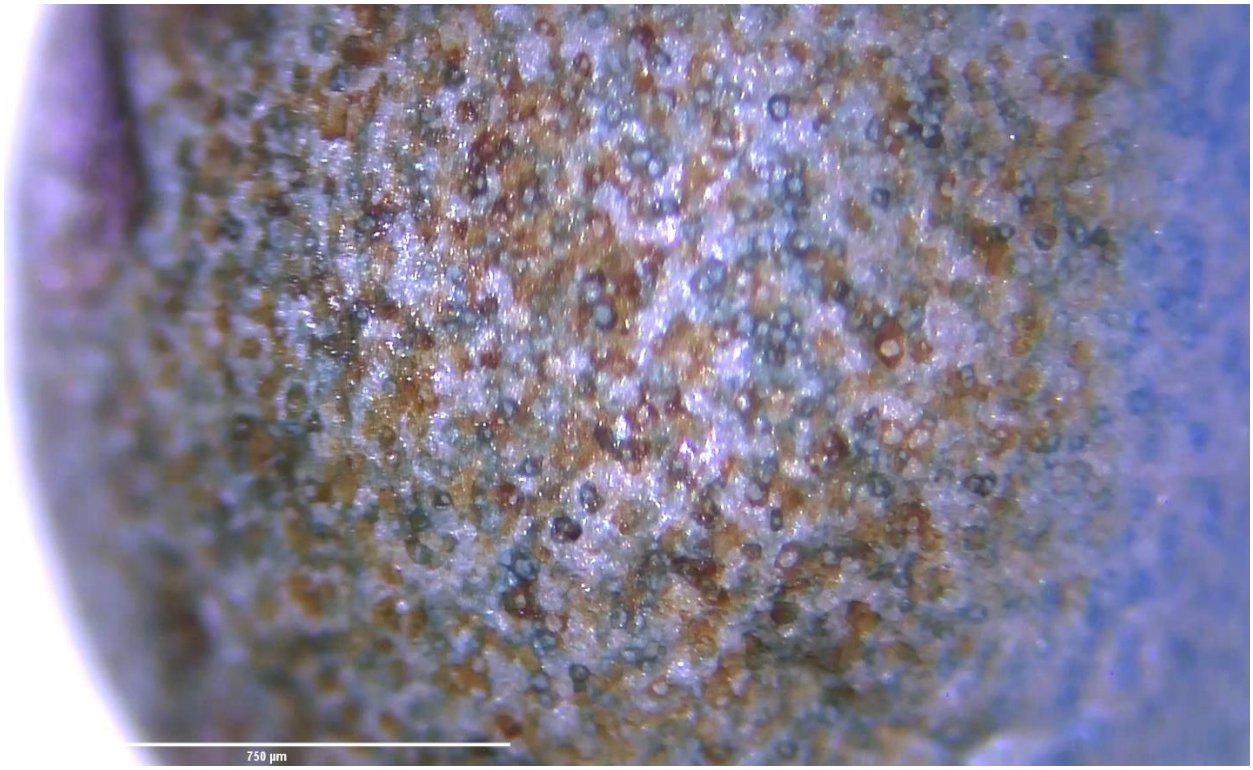

Figure 7. Optical microscopic examination of heat damage on a piece of polystyrene foam. Scale bar is 750 microns.

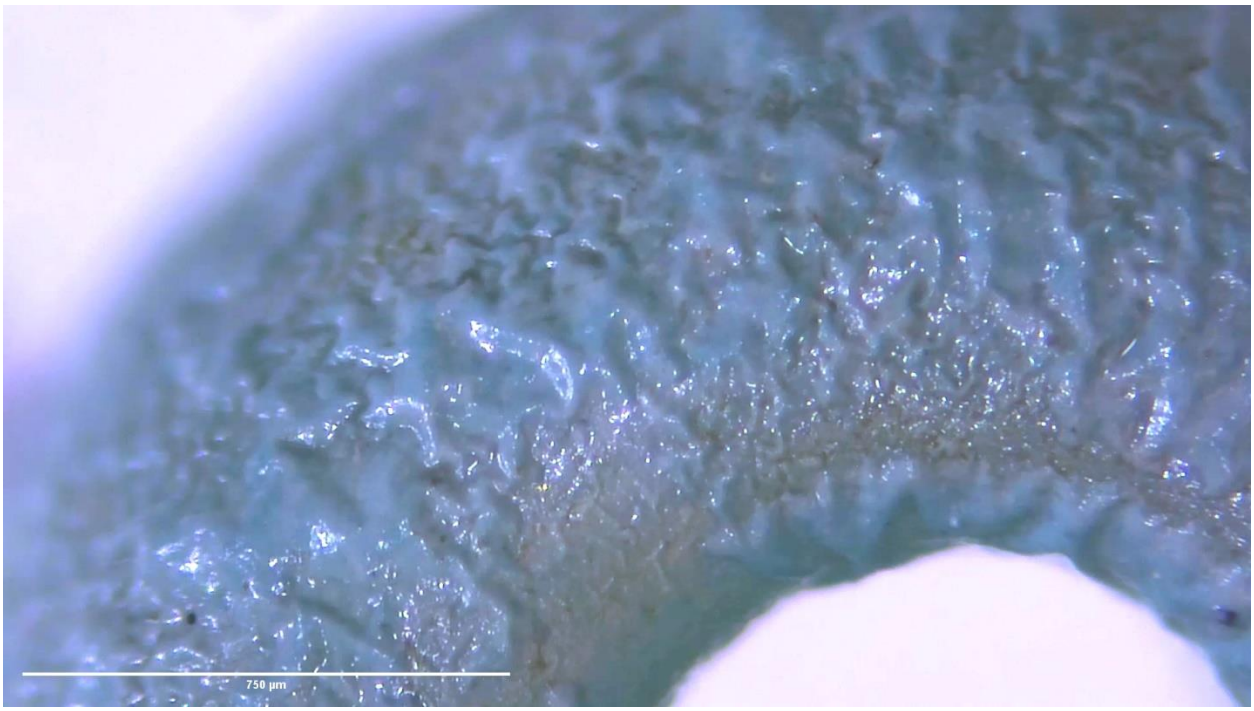

Figure 8 Chemical bubbling on a piece of Polyethylene plastic at 750 microns.

Table 1 Digestion Efficacy for selected chemicals and damage examination for heat drying and chemical use. No damage recorded (NA); Low damage recorded (L); High damage recorded (H).

N	Chemical Name	Organic matter (g)	Mean Microplastic Weight (g)	Mean Total Weight (g)	Mean Final Weight (g)	Digestion Efficacy (%)	Oven damage °C			Chemical damage
							40	50	60	
5	H2O2(60%)	9	1.0579	10.0579	4.02316	60	L	H	H	L
5	HNO3 (85%)	9	1.074	10.074	0.80592	92	L	H	H	H
5	HNO3 (60%)	9	1.033	10.033	2.0066	80	L	H	H	H
5	KOH	9	1.054	10.054	2.0108	80	L	H	H	L
5	KOH/NaCl	9	1.043	10.043	1.0043	90	L	H	H	L
5	H2O	9	1.065	10.065	9.56175	5	L	H	H	NA

for each session to determine background levels. Should airborne MPs be present on the sampling blank, re-evaluation and cleaning would occur on all materials, and samples were reanalyzed accordingly. Control digestion samples were processed for every 12 digestions to ensure contamination was not occurring and were re-examined between samples (Davison & Asch,2011; Foekema et al., 2013). Any contamination that was discovered was recorded and compared to other samples.

Snail Digestion

Snail digestion began by combining 175ml of filtered DI water with 37.5 ml of saturated KOH solution (280g/250ml) and 37.5 ml NaClO solution (14% active chlorine) and stirring for 5 minutes with a glass rod before transferring to a mason-jar container. Individual snails that had been allowed to air dry were weighed, cleaned with DI water to remove ethanol, and transferred to a clean conical tube. 5ml of solution was transferred to the tube with a glass syringe, shaken at 180 RPM and left to digest for 24 hours. Following digestion, the remaining solution was vacuum filtered through a 0.47 μm nylon membrane. During filtration, all open-air containers, regardless of contents, were covered using a glass petri dish to ensure that particles drawn into the lab space via suction or ambient air flow were not landing on samples. Further, all instruments were placed in a previously cleaned aluminum pan to prevent potential contamination from counter surfaces. DI water was run through the filter following each sample to ensure no residual MP particles in the attachment tubing. The filter was then transferred onto a clean glass petri dish to be dried. Secondary drying ensured that any residual moisture did not hinder the ability to identify MP particles. All instruments and filter containers were thoroughly cleaned following each snail digestion.

Soil Collection

Soil samples were collected at each snail coverboard location. Soil MP concentration and variation have been poorly documented, especially in the region of interest. Indeed, while most plastics are found within the top 5 cm,the abundance of MPs within the same area may show heterogeneity.. Therefore, 4 samples were taken at each coverboard corner and combined to be representative of the soil characteristics of that area. In all, 120 samples were taken from each sampling region, given 30 coverboard locations each. Glass mason style jars with aluminum lids and a metal shovel were used to collect these samples. Samples were labeled and placed in a covered cardboard box for transport.

Soil Isolation Unit Design

Accurate and efficient methods for examining MPs in soils using density separation are lacking. Soil Isolation Units (SIUs) have only recently been developed to aid in the extraction of

microplastics, but designs are in early stages and focused on cost Efficacy and wide distribution (Figure 9). An improved prototype SIU was designed and developed for this project to address the flaws in previous studies (Figure 10). Soil isolation units are normally designed utilizing cheap and easily replaceable PVC or polyethene valves and tubing, but the use of plastic materials creates an environment in which contamination may be introduced from the components physically breaking, wearing, or abrading, or through the use of chemicals during separation (Figure 11). Because of this, plastic SIUs require considerably more control blanks to be tested. In fact, with little as 20 uses contamination can occur, and often unknown particles may be introduced (CITE THIS). Avoiding contamination with plastic SIUs requires priming with the chemicals to introduce some lubrication and prevent abrasion from ball valve use (Figure 11), but the process does not entirely prevent contamination from occurring (CITE THIS). The new unit was designed in autocad2D, and a prototype was developed using the same materials as previous studies (PVC, PET) to capture accurate dimensions and for testing (Figure 9). Ultimately, the improved SIU was constructed using iron "tubing" and a metal faucet valve, which provided increased durability and eliminated any potential plastic contamination from the device itself (Figure 10). Inexpensive materials make this SIU an easy and affordable testing device that could be remade should the dimensions or design contain flaws. This was mainly utilized to test the allowable capacity of soil particles to move throughout the device.

Soil Density Separation

After collection and transport of soil samples, each was transferred into triple-cleaned 100ml glass vials to be air dried for 2 weeks. Small holes were created in the aluminum foil covering to allow for evaporation of moisture, and vials were placed inside a closed drawer. Five blank filters on petri dishes were placed within the same area to test for potential background airborne contamination. Following drying, the samples were processed through a series of sieves to break up clumps that could potentially contain microplastics and to aid in density separation. Stainless steel mesh sieves of 2000 µm, 2000 µm – 250 µm, and <250 µm were used, and each size fraction for each site was combined and weighed to extract 100g. The separation method here was adapted from the University of Tennessee environmental and soil sciences. Ludox TM-50 was chosen as the separation medium due to its high density (50% silica solution at 1.4gcm^3). The SIU was triple cleaned and filled with 120 ml of Ludox TM-50 before each use. Fifty grams of each sample was weighed for the procedure, which

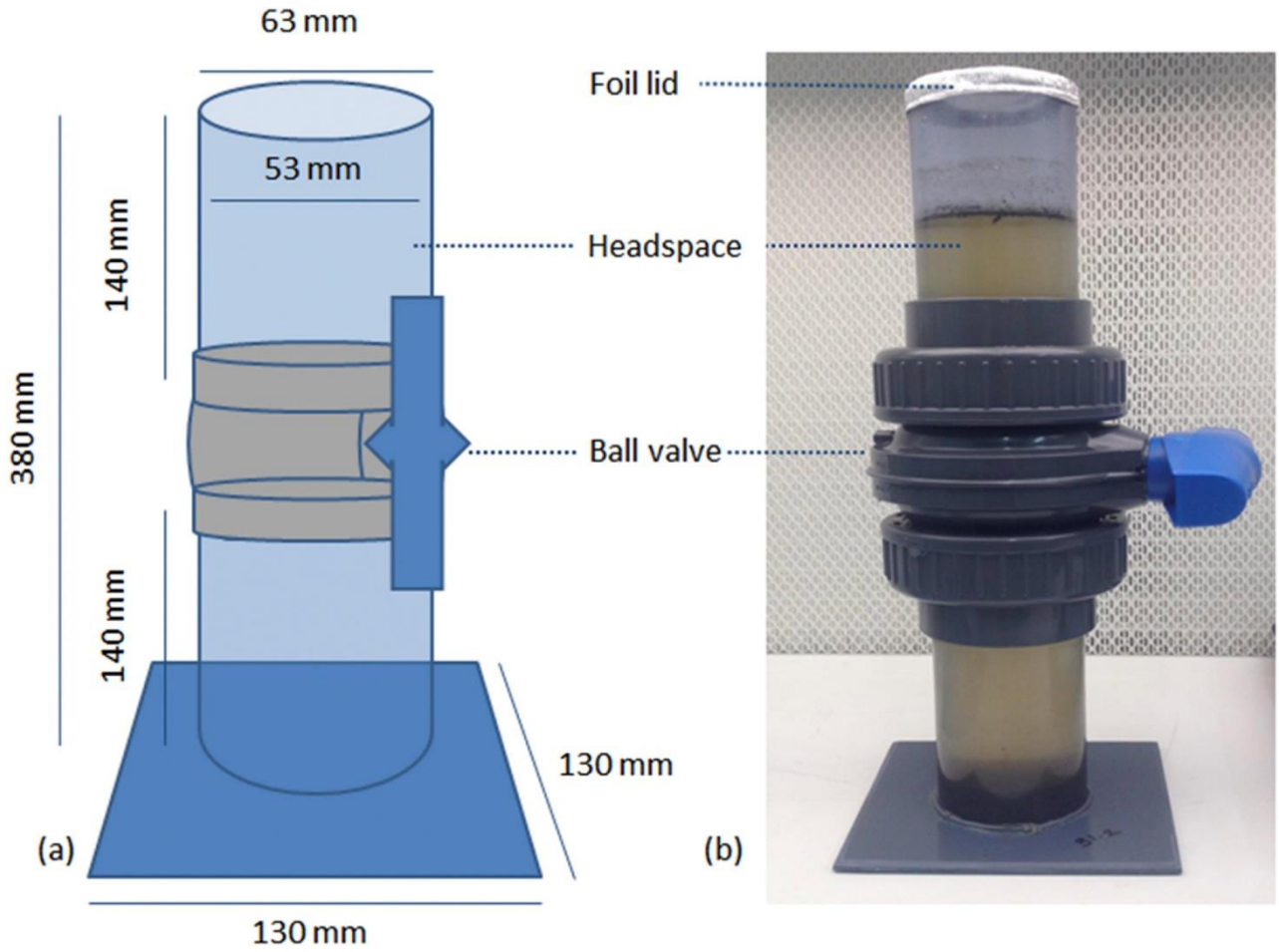

Figure 9 Soil Isolation Unit schematic and unit made from Poly-Vinyl Chloride tubing, and tin foil. Use granted courtesy of Dr. Coppock, Plymouth Marine Laboratory.

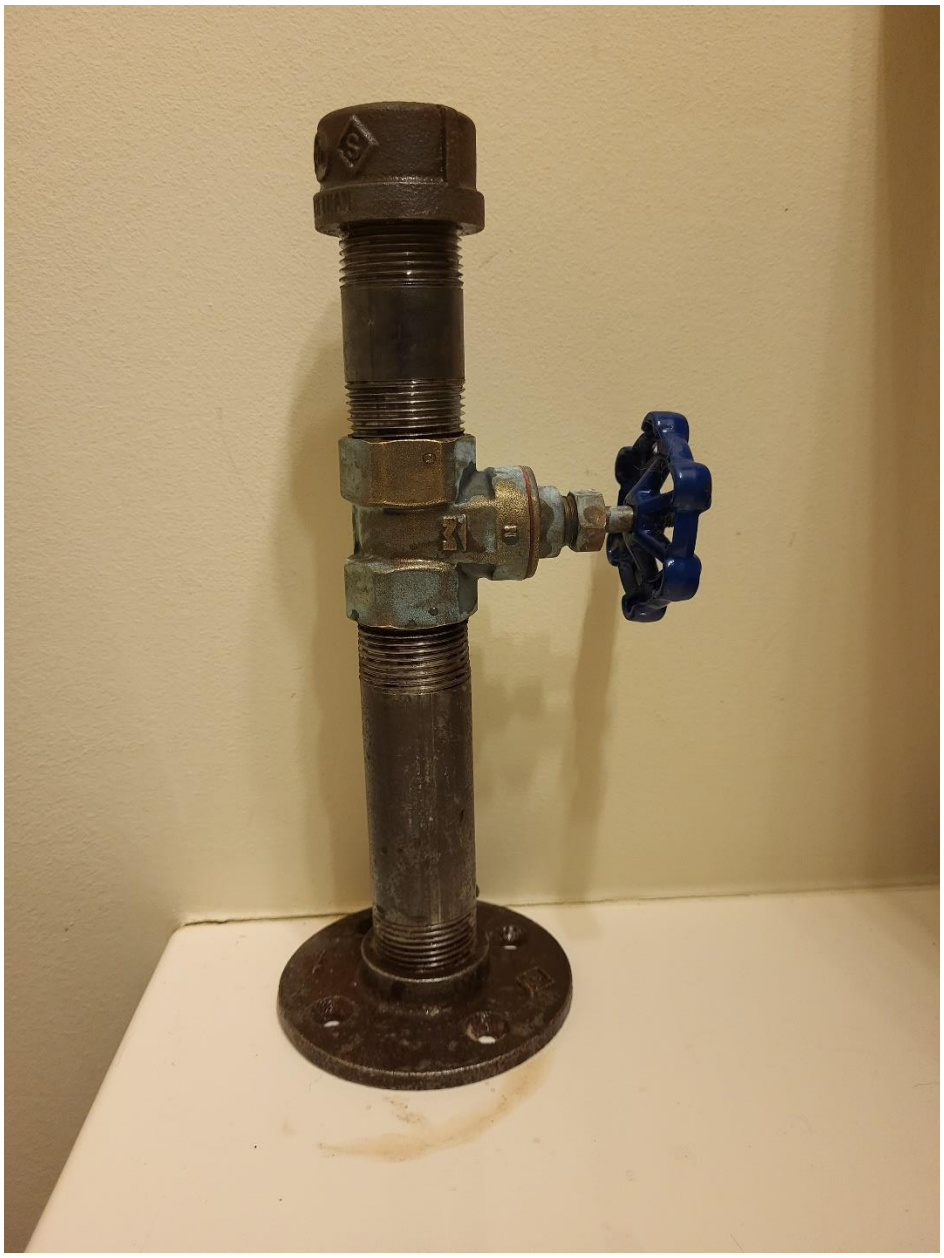

Figure 10 Improved SIU designed to prevent plastic contamination for use in small-scale density soil experiments

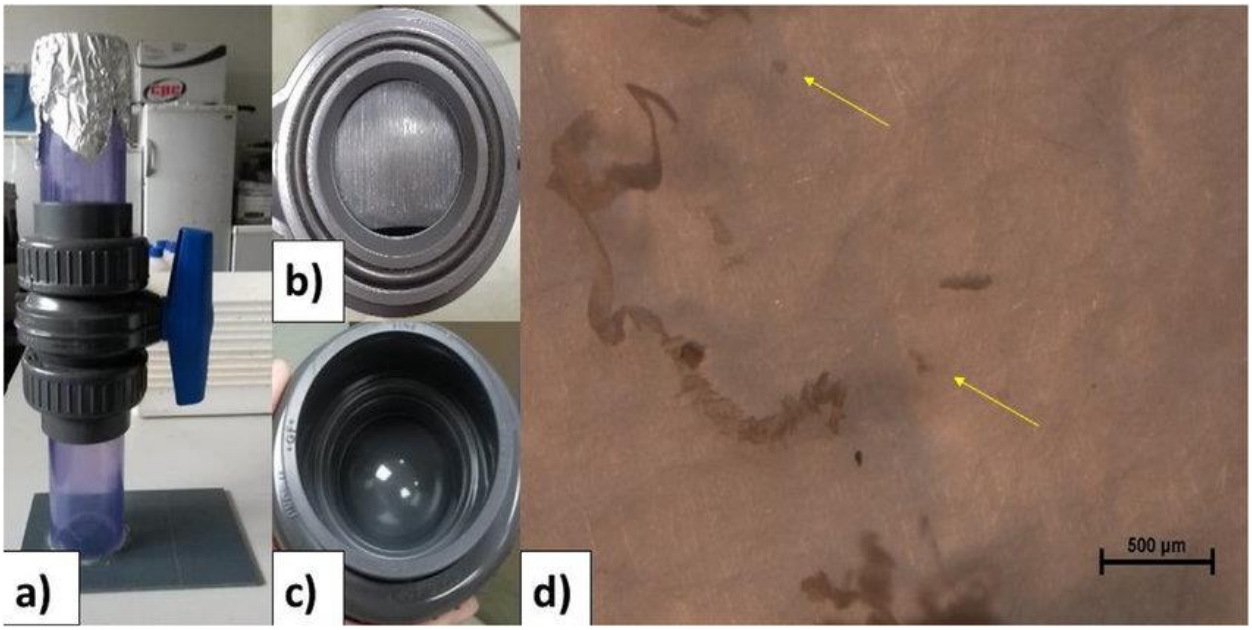

Figure 11 (a) Soil isolation unit as previously designed by Dr. Coppock; (b) degradation from wear and tear of unit; (c) Lubrication of unit to prevent degradation; (d) contaminated particles from device, Nel et al. 2019.

was repeated for a total of 100g of processed soil per site. Each volume of soil was placed into the SIU and shaken vigorously for 2-3 minutes. The resulting mixture allowed to separate and the low-density particles to float to the surface. Periods of 30 minutes or more resulted in no significant changes in settling in any samples. The ball valve was then closed to separate the supernatant from the dense soil particles. Each sample had additional Ludox poured after the initial separation to ensure any left-over particles were collected. The supernatant then followed the same procedure as previously outlined in the snail digestion. It was poured through a vacuum pump, 0.47 µm cellulose acetate filter and rinsed completely with deionized water. Samples were then placed in a covered glass petri dish to air dry for 7 days to await microscope examination. While running samples through the SIU unit, blank vials containing only Ludox were also analyzed. Outlined by Nel et al., testing for contamination via blanks is necessary despite our SIU containing metal components. Striations from repeated use are common and could potentially contaminate samples. Despite having no plastic material in our SIU, all fragments were tested to ensure that contamination was not occurring via the hot needle test.

Validation

Validation of the efficacy of the SIU was needed to determine the rate of recovery of plastic particles. Distinct plastic particles of PVC, PET, and PS were created in sizes from 800 µm to 3000 µm from commercially available plastics. Fifteen pieces per blank were randomly chosen, recorded, and placed within sediment. 5 total spiked sediment blanks were created and subjected to the same procedure as described above. Each blank was examined independently by two observers and recovery rate was recorded. Commonly, multiple observers are used to achieve an average observable mean of recoverable plastics. However, previous SIU experiments have shown that individual bias for controlled experiments on what is perceived as plastic can vary widely. To control for this, every blank was examined within the same session by two observers, counts were recorded, colors, size and every piece that was perceived as plastic was tested by the hot needle test. Further, a procedure for microscopic and filter examination (described below) was developed to allow observers to examine the location of perceived plastics for testing. This greatly enhanced the recovery rate for plastics and eliminated the need for ANOVA testing and caluclation of range differences between observers. It is suggested that SIUs are used with the ball valve open to reduce contamination, but the design and material, although costlier, in the this experiment's SIU eliminates these potential pitfalls as well.

Filter Reading Procedure

A filter reading procedure was used to help observers correlate previously seen plastic pieces and to also ensure that all observable particles were recorded (Figure 12). Pieces were only recorded if they were within the filtration perimeter defined by the chemical solution used. Often, filters that did not dry completely, or were exposed to slightly higher chemical concentrations for longer periods of time became curled or suffered a slight bend that made analysis difficult. This study elected not to glue down filters in order to minimize contact with the air. Filters that curled were examined thoroughly from different angles with adjustable lighting. Each filter was examined grid square-by-grid square, moving from right to left beginning at the same location by each observer. Although rare, some digestions resulted in scorching and dissolving of filters rendering it extremely difficult to detect MP particles. In these cases, filters were discarded and unable to be counted to prevent speculation of suspected particles. Digestion solutions were also diluted with 10ml of water and retested on blank filters.

Microscope Identification

Following digestion, the petri dishes and filters (both snails and soils followed the same process) were examined under a stereomicroscope with a camera attachment at 40-50x magnification. Samples were examined without removing the lids to the petri dishes to prevent any contamination unless physical testing was needed. Commonly, materials other than MPs are mistakenly categorized together, such as organic matter, glass, and sand (Shim et al., 2017; Chen et al., 2020). Microplastic identification was conducted visually and will follow previous guidelines outlined below by the Marine and Environmental Research Institute below. Shape, color, erosion, type, length, and number of particles were examined, and common particles were photographed and analyzed in Image-J (after Schneider, 2012). Due to the number of particles and samples, and time required to fully analyze each sample, not every MP particle was photographed. However, a variety of rules and tests were followed to ensure the accuracy of plastic particle identification (see following section).

Microplastics have typical characteristics that were considered each time a questionable piece was discovered. Particles were only counted if they were less than 5mm in longest dimension, but particles sizes are often exceedingly difficult to estimate due to curling or fragmentation. Therefore, sizes were estimated based on grid square sizes of 3mm with many difficult to size particles measured via Moticam measurement software.

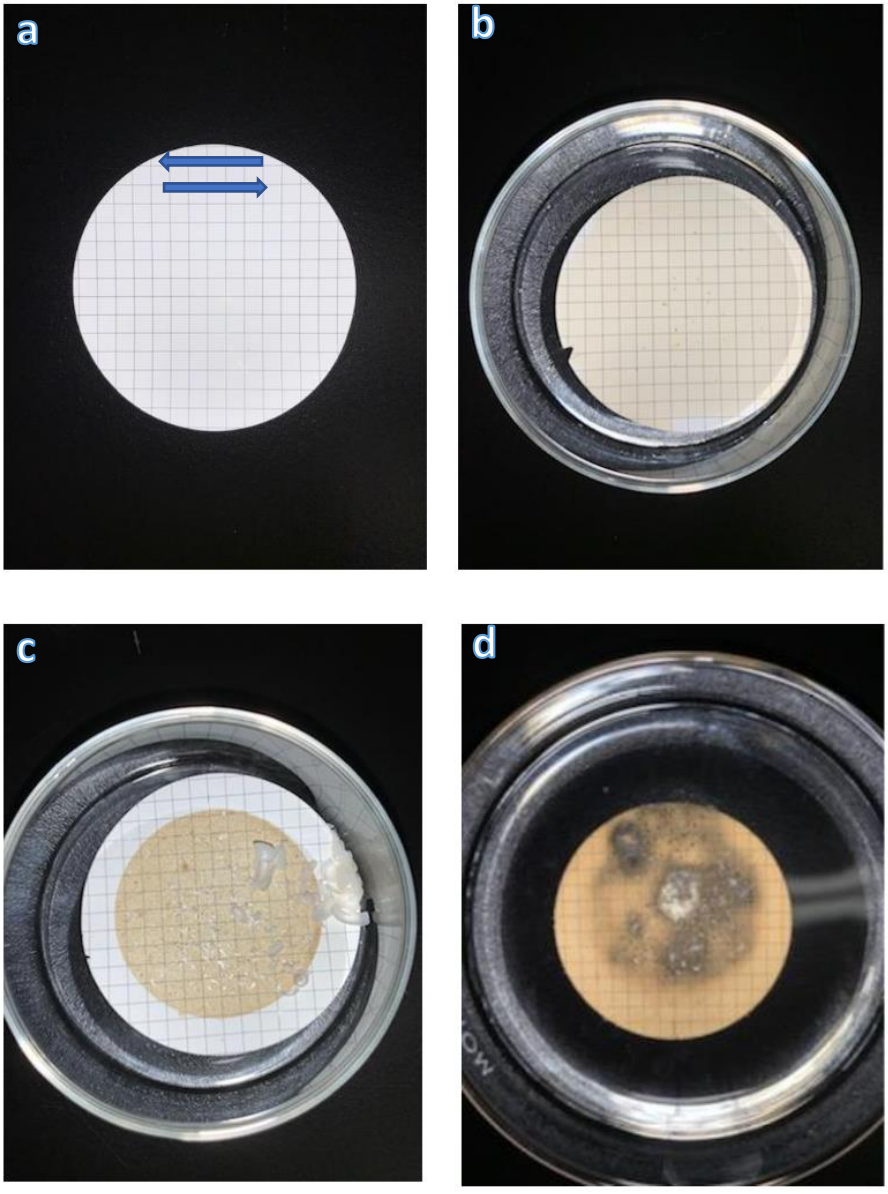

Figure 12 (a) filter reading procedure on a clean filter moving from top right corner left (b) good digestion low organic matter (c) bad digestion, shifting of shells and large organic matter (d) excessive concentration and digestion.

The length of all particles was examined throughout to find homogeneous color, shape, and thickness as most plastics show these qualities. There were rare instances in which severe damage was caused by bleaching or mechanical means. These pieces were examined more thoroughly on a case-by-case basis. However, all particles identified in this way lacked organic structures that are commonly seen in plant or animal matter.

The hot needle test (Figure 13) developed in 2014 is an easy and low-cost method to distinguish organic matter from plastic (De watt et al, 2014). Plastic has unique characteristics that differ from organic material and experience morphological change when exposed to high heat. The hot needle test is generally performed on pieces that are questionable, but this study laboriously conducted this test on every piece of plastic to compensate for inaccuracy in human perception while counting pieces. When plastic is exposed to high heat it will curl, melt, spread apart, or bubble. Non-plastic pieces will either persist with no changes or burn quickly. The only exception to this test is if the heat from the needle is not hot enough to melt plastic. Therefore, some unknown particles that did not burn were tested multiple times to ensure accuracy. Secondly, the spring test was performed in instances where neither visual inspection nor the hot needle test indicated plastic. Plastic will generally spring back when moved or forcefully squeezed by tweezers but will not break unless significantly damaged. This test was combined with earlier knowledge of the particles and the hot needle test to exclude many non-plastic pieces.

Identification Rules

Note: The rules used were referenced from the MERI guide for identification of microplastics. However, rules were adapted and changed to this specific project as outlined below. Modified rules are denoted by an asterisk with the original rule in italics below.*

Rule 1: Lack of cellular or organic structures

All examined pieces were viewed at the maximum allowable magnification to ensure any organic structures were observed prior to heat testing. Biofouling can result in the combination of organic material and MPs which can give the overall appearance of a non-plastic material. Biofouling in this instance is defined as any accumulation of organisms, organismal related shells, plants, algae, or any other unwanted organic material that obscures the view of MPs. Biofouling can occur by encapsulating an entire MP or be present in sections and therefore it was essential to examine the entire length of the MP.

Rule 2: MP structures should generally exhibit homogeneity

**(Original Rule 2: Fibers Should be Equally Thick Throughout Their Entire Length)*

Generally, the structure throughout plastic fibers is homogenous, but fraying is common due to strands splitting apart from physical stress. The original rule was not just applied to fibers but to all MPs. Although there were many MPs that were viewed as fragmented, sliced, or otherwise degraded, there were many others, besides fibers, that were homogenous throughout. Plastics in general, regardless of degradation, often have a defined structure that allows for homogeneity to be easily seen. These were key indicators for identification and this rule was used as a basis for further examination of a potential MP.

Rule 3: Particles should exhibit a similar color pattern

**(Original Rule 3: Particles should exhibit homogeneous color throughout)*

Particles regardless of type can appear in a wide range of colors. These colors can be changed drastically by the mechanisms that create secondary MPs, mainly chemical bleaching. A fiber for example, may appear to be translucent on one end and blue on the other and therefore may be mistaken as a different material or incorrectly measured. Further, it is essential that not only the colors be examined but the pattern as well. Although uncommon, many MPs are primarily made with a variety of colors that have not undergone environmental alteration. It is still highly likely that any MP found will be of a solid color, but all colors should be examined throughout the full length and on ends for internal examination if able.

Non-plastic materials

Calcium Carbonate Shells

The digestion process outlined above varied in Efficacy dependent on the size and amount of organic material. Since snail shells are notoriously difficult to digest, it was not uncommon for roughly 1 in every 10 samples to have substantial amounts of shells left (Figure 14). These samples were examined thoroughly and were more difficult to detect MPs within. A small metal prodding tool was used in conjunction with tweezers to test the structural integrity of smaller pieces of shell. Generally, the shells had become weak and brittle and were able to be moved quickly to examine contents underneath. White fibrous material that was suspected to have come from strands within the shells was present as well. These were clearly abundant in under-dissolved samples and were regarded as non-plastics.

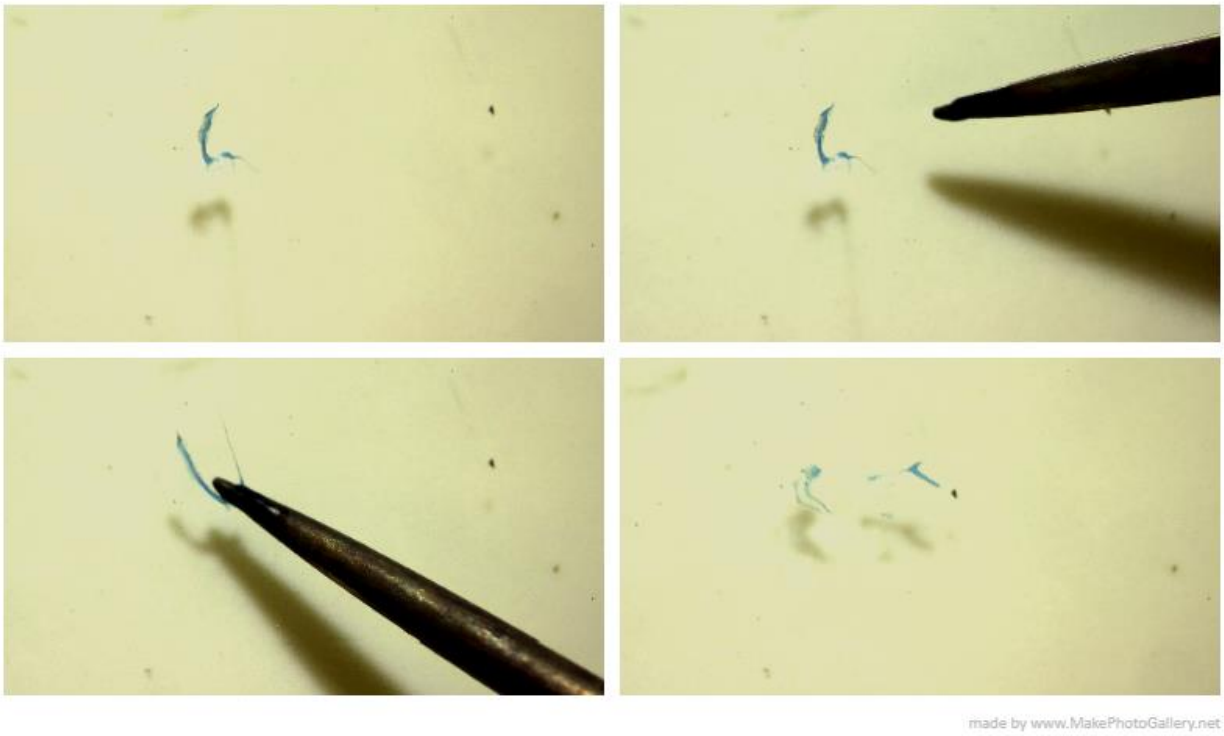

Figure 13 Hot needle test on a suspected MP particle. Confirmation from melting and separation.

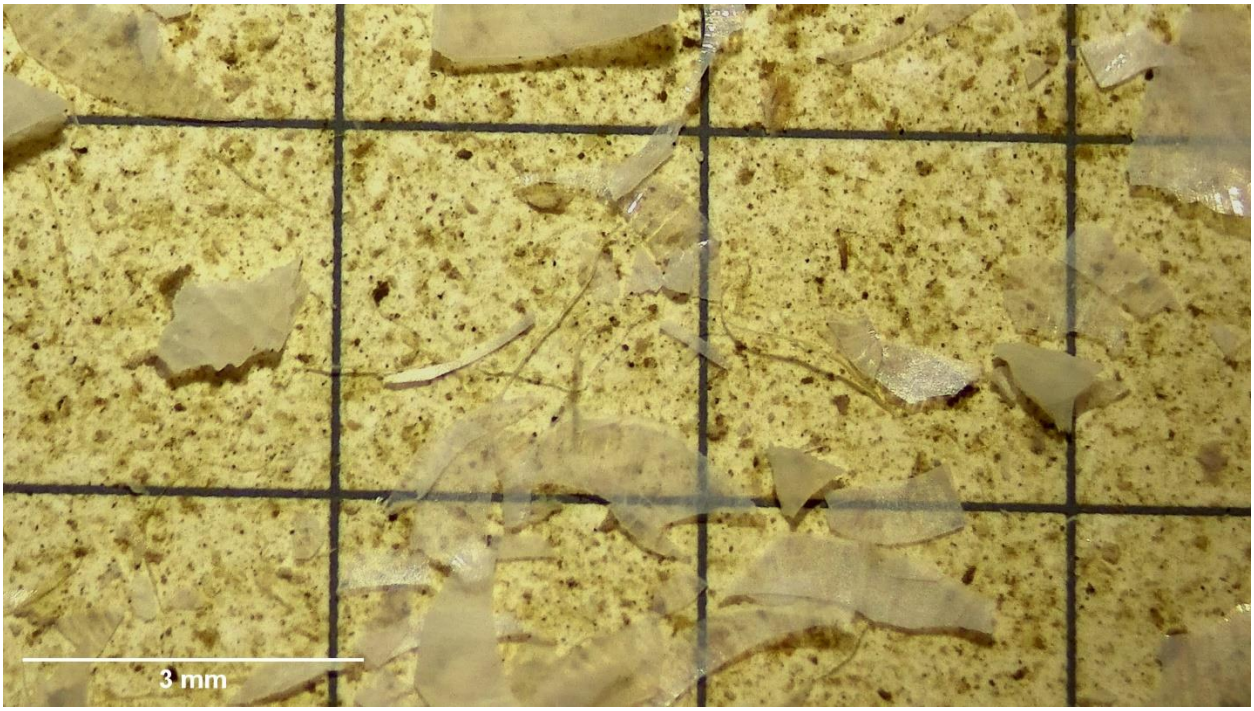

Figure 14 Undigested calcium carbonate snail shell

Plant material

Snails' diets consist largely of detritus or plant stems and roots. Like the shells, there is often (albeit in much smaller volumes) undigested material that can make identification difficult. As one of the rules outlined above, particles were examined for cellular structures and disregarded if any indication of such was present.

Contamination

Contamination from airborne sources has been rarely documented but was the main concern due to lab space conditions (Figures 15-17). Contamination reduction measures (see methods section above) were implemented upon discovery of contamination pathways. White and partially translucent fibers were found to be in abundance of 50+ particles in some snail samples with bundles of fibers making it nearly impossible to examine. It was assumed that the particles were a combination of cotton lab coat fibers and/or filter debris coming from the lab ventilation system. Similar observations of translucent and white fibers were made in background blanks and confirmed by examining separate blanks that had been placed inside drawers with no air flow. No such fibers were found on these blanks in any instance. Moreover, contamination from clothing underneath lab coats, although rare, was seen on some samples. Instances of filter grid lines that had excessive digestion were seen in some samples to peel away and visually look like MPs.

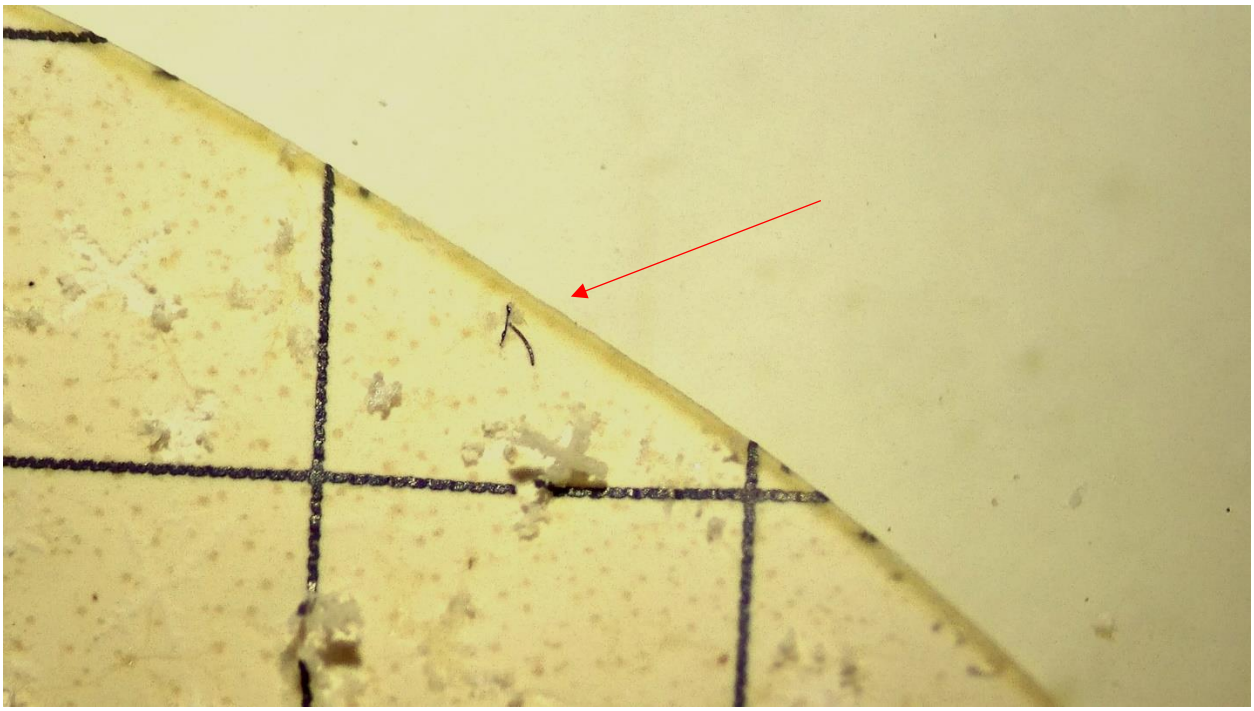

Figure 15 Filter grid line that has peeled away from chemical digestion

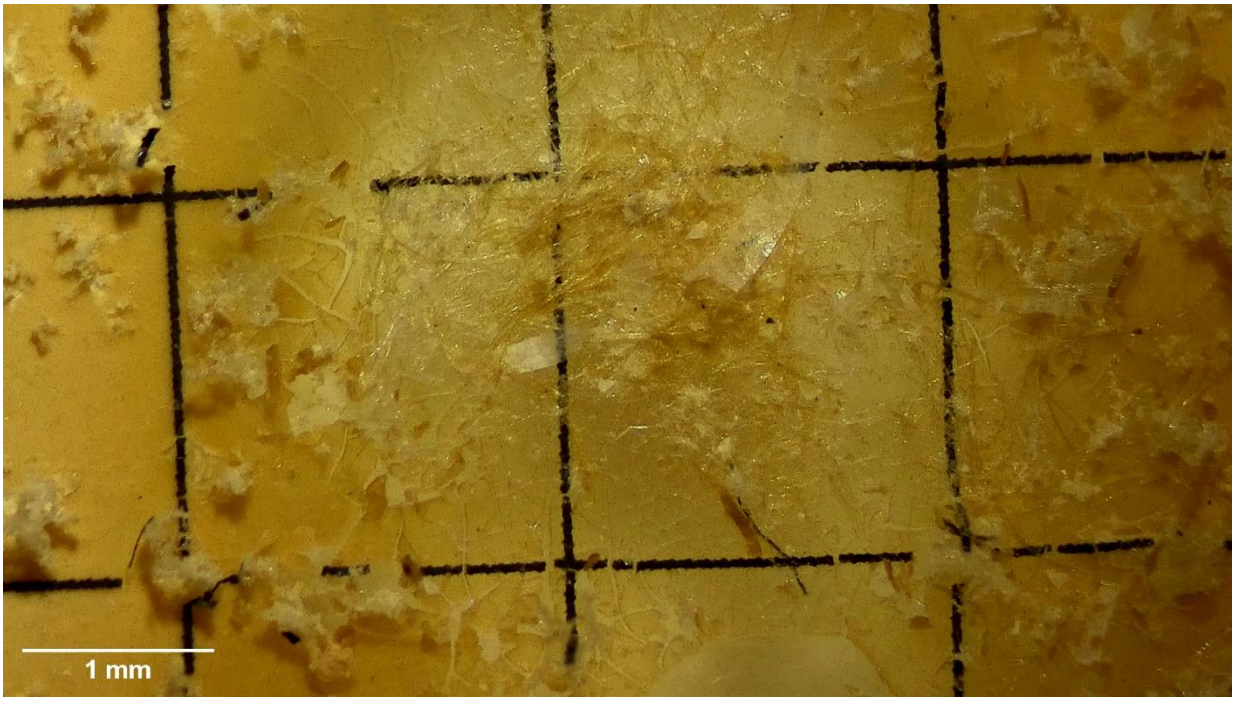

Figure 16 White translucent fibers and undigested snail shells

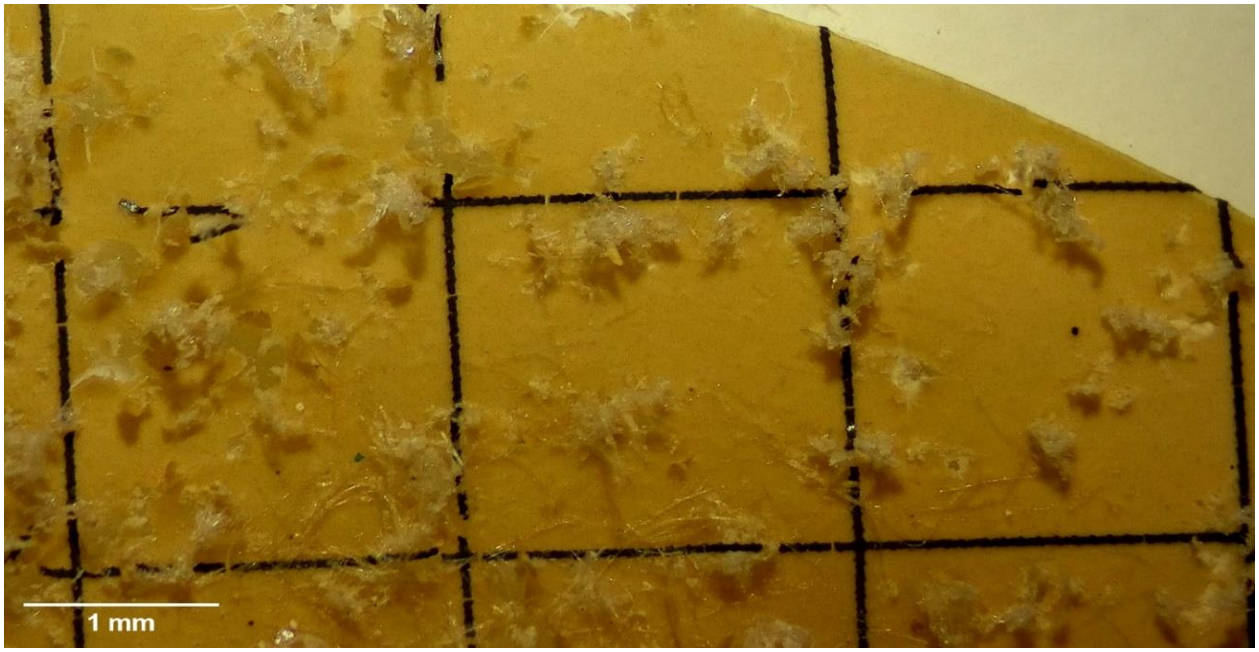

Figure 17 Bundles of white and translucent fibers

SECTION 4. RESULTS

Snail MP abundance

Within the Wildlife Management Area (WMA) sites a total of 214 MPs were recovered in 129 snail individuals (Figure 19) representing 8 genera. Total samples include all collections while positive samples examined only those that had positive MP presence. Summary characteristics for all samples are outlined in Table 2. Of the 129 snails examined, 87 (67.4%) contained MPs, ranging from 0 to 6 per snail sample, and a mean of 1.6 ± 0.14/total-samples and 2.5 ± 0.26 MPs/positive-samples. Total snail biomass analyzed was 14.7g with an average value of 0.06 ±0.006 MPs/g. Distribution of MP types was predominantly fibers (73.1%; n. 160), film (13.2%; n. 29), and fragments (11.7%; n. 25), with no recorded beads, pellets, or foams. Total length of MPs was 95mm (95644 µm) with a mean of 0.74 mm (741µm ±85.4 total-samples) and a mean of 1.1 mm (1138µm ±108.3 positive-samples).

UT campus sites had a total of 270 MPs from 130 snail samples (Figure 21) representing 10 genera. 103 (79.2%) were positive for MPs ranging from 0 to 8 per snail sample and a mean value of 2.07 ± 0.14/total-samples and 2.56 ± 0.14 MPs/positive-samples. Total snail weight was 23.0 g with an average value of 0.08 ± 0.01 MPs/g. MP types were fibers (75.5%; n. 204), films (13.7%; n. 37), fragments (10.3%; n. 28), pellets (0.3%; n. 1). Total length of MPs was measured at 147 mm (147701 µm) with a mean of 1.1 mm (1136 µm ± 92.6 total-samples) and a mean of 1.4 mm (1448.049 µm ± 97.4 positive-samples).

Color distribution was analyzed based on complete colors, i.e., no shading assessment. Thus, no distinction was made between light or dark colors due to observer bias and inability to distinguish some bleaching from manufactured colors. Color tracking was majorly used for contamination prevention by comparing found colors to clothing, equipment, and any materials used. The most common colors in both sites were found to be black, blue, and red (Figures 18 and 20). Clear pieces were generally discounted from results from contamination, but special cases were recorded for unique pieces that were not believed to be contamination from other colors present but not dominant.

Sub-Samples

Additional collections at previous sites were performed via hand sampling by metal tweezers and glass vials. These samples were separated and examined against board sampled sites to check for any artificial contamination. A total of 35 MPs were hand sampled from the WMA sites in 35 snail individuals representing 4 genera. 22(62.9%) resulted in positive indication for MPs with a range from 1 to 3 per sample and a mean value of 1.0 ± 0.16/total-samples and 1.59 ± 0.19 MPs/positive-sa

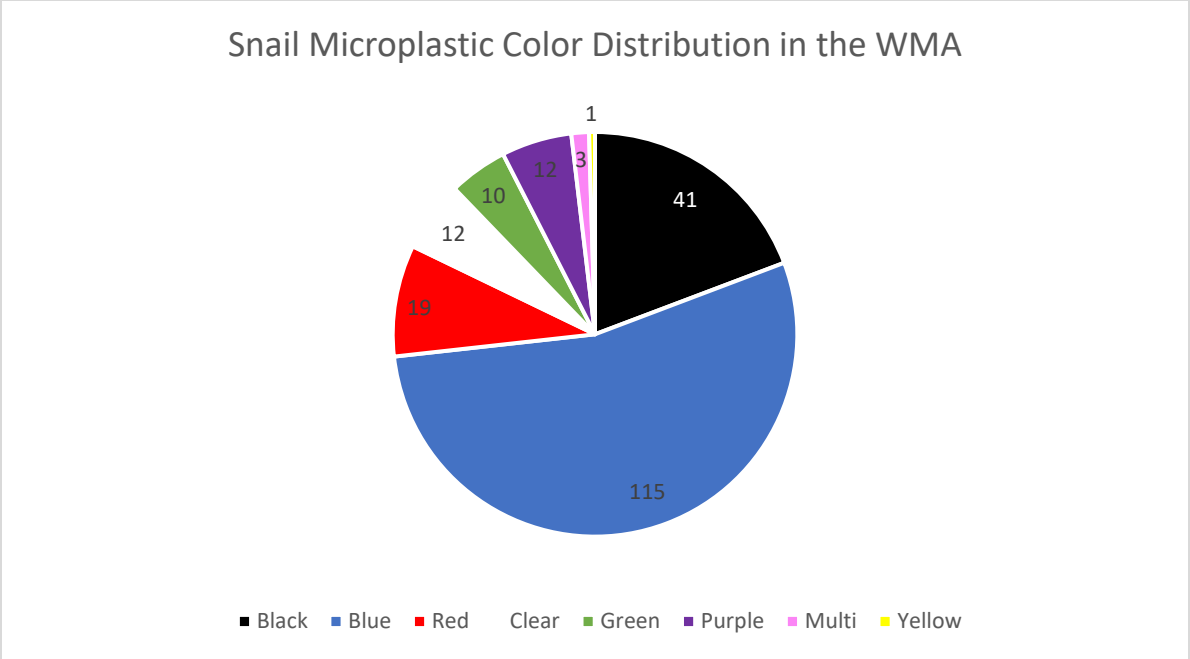

Figure 18 Color distribution of WMA Snails

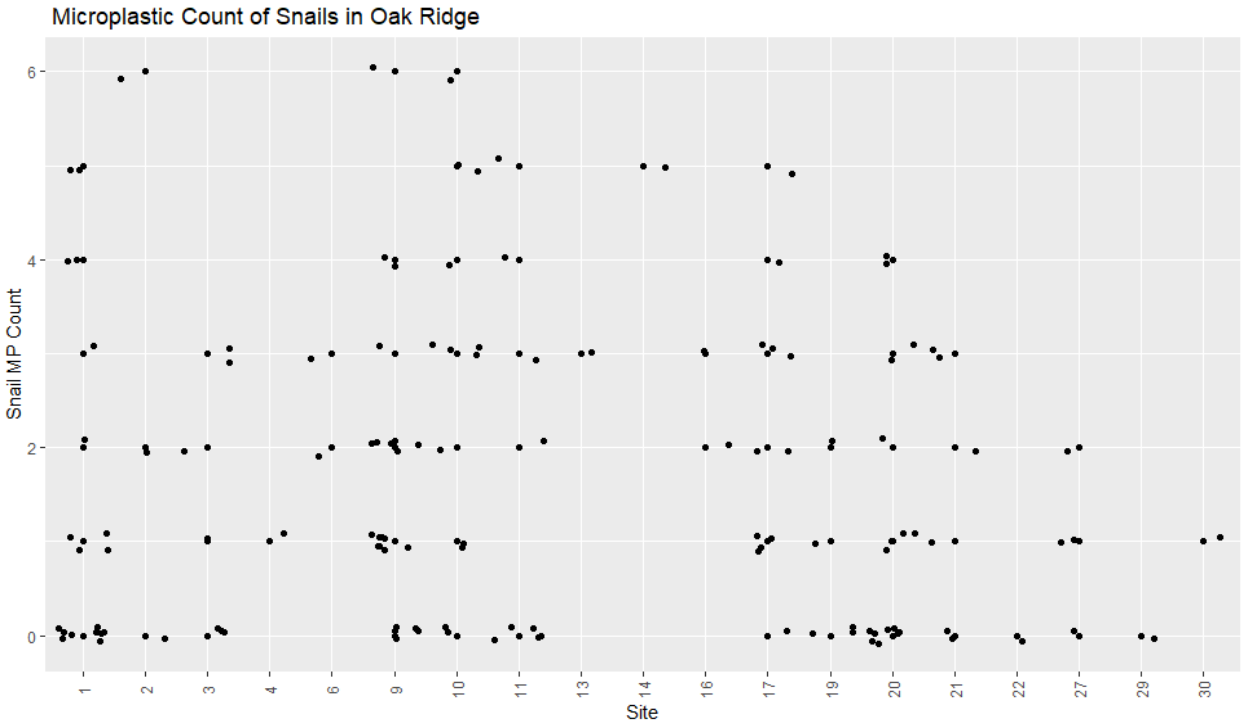

Figure 19 Jittered graph of MP concentration by site in the WMA

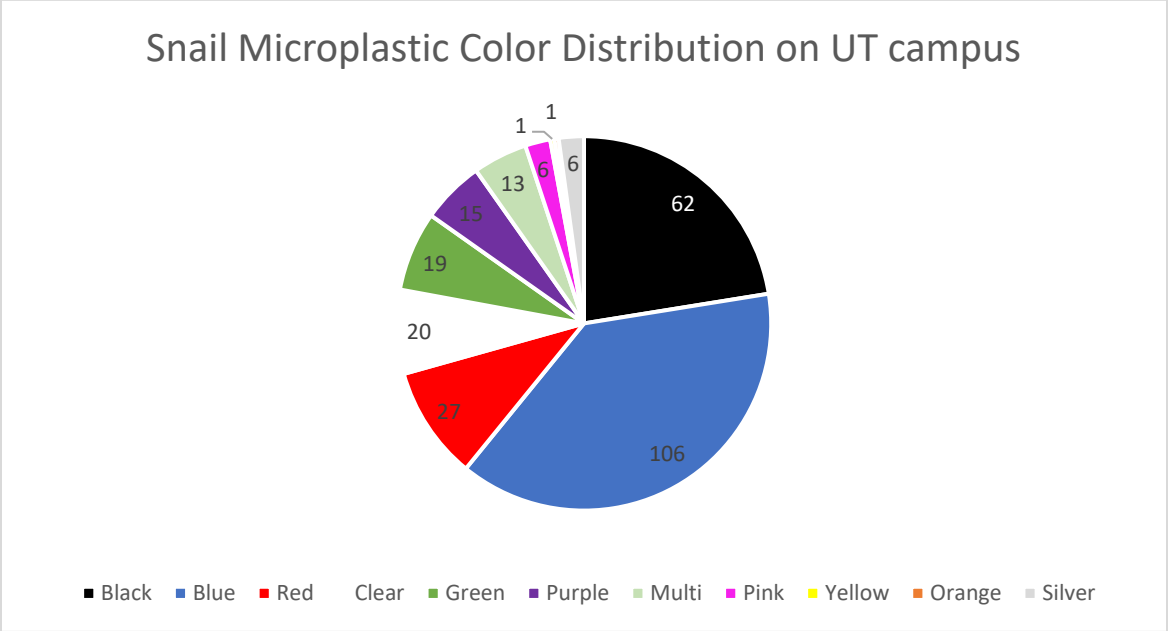

Figure 20 Color distribution of UT campus snails

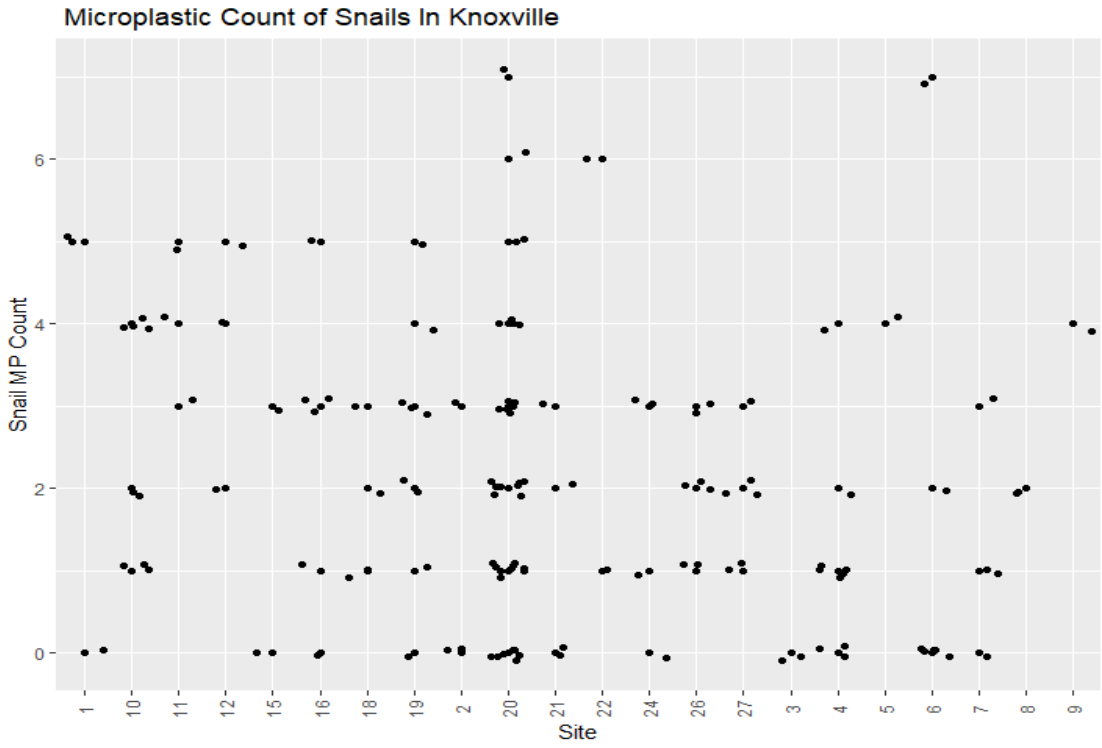

Figure 21 Jittered graph of MP concentration in UT campus snails

Table 2 Summary characteristics comparing WMA vs UT campus snails and soils

	Microplastic Sample Characteristics										
Snails	Samples	Mps/g	MP Count	Mean	MP Length (µm)	Fibers	Films	Fragments	Beads	Pellets	Foams
WMA	129	0.06 ±0.006	214	1.6 ± 0.14	741 ± 85.4	160 (73.1%)	29 (13.2%)	25 (11.7%)	0	0	0
UT campus	130	0.08 ± 0.01	270	2.07 ± 0.14	1136 ± 92.6	204 (75.7%)	37 (13.7%)	28 (10.3%)	0	1 (0.37%)	0
Soils											
WMA	30	0.2 ±0.034	478	15.9 ± 1.2	1465.8 ± 183.7	189(39.5%)	107 (22.3%)	158 (33.0%)	24 (5.2%)	0	0
UT campus	30	0.37 ± 0.056	540	18 ± 0.14	1595 ± 216.8	283 (52.4%)	59 (10.9%)	114 (21.1%)	36 (6.0%)	25 (4.6%)	23 (4.2%)

mples. Total snail weight analyzed was 2.7 g with an average value of 0.04 ±0.008 MPs/g. Distribution of total types of MPs was predominantly fibers (54.2%; n. 19), films (31.5%; n. 11), and fragments (8.0%; n. 3), with 2 samples containing 1 foam and 1 pellet (Insert figure). Total length of MPs was measured at 28.1 mm (28150 µm) with a mean of 0.84 mm (804 µm ±148.7 total- samples) and a mean of 1.27 mm (1279 µm ±167.4 positive-samples).

Separately, 46 more MPs were hand sampled from UT campus sites in 30 individuals representing 4 genera. 23(76.6%) resulted in positive indication for MPs with a range from 1 to 4 per sample and a mean value of 1.4 ± 0.21/total-samples and 2 ± 0.18 MPs/positive-samples. Total snail weight analyzed was 1.9g with an average value of 0.05 ±0.013 MPs/g. Distribution of total types of MPs was predominantly fibers (80.1%; n. 37), films (6.0%; n. 3), and fragments (12.0%; n. 6). Total length of MPs was measured at 30.8 mm (30885 µm) with a mean of 1.2 (1235 µm ±215.6 total samples) and a mean of 0.9 (996.2 µm± 194.8 positive-samples).

Soil Samples

For the WMA sites, 1000 grams of soil per site were collected and sample summaries are reflected in Table 2. WMA sites contained a total of 478 MPs (Figure 22), ranging from 5 to 33 per site with a mean of 15.9 ± 1.2. For this study, 50 g subsamples (100 g total per site) were analyzed in increments of 50 g per sample in order to reduce the time needed for separation and to decrease likelihood of contamination entering samples. Identification was easier with smaller samples as the amount of organic matter diminished greatly with weight. A total weight of 3000 g was analyzed with an average MP size of 1465.8 ± 183.7 and total length of 43.975 mm (43975 µm). UT campus soils contained a total of 540 MPs (Figure 23), ranging from 7 to 35 per site with a mean value of 18 ± 0.14. Average MP size was found to be 1595 ± 216.8 and total length of 47.850mm (47850 µm).

Size of MPs in the soil from both sites was generally larger and more intact when compared to the snail samples. This may indicate that smaller particles are more easily transported into snail digestive systems. MP type did not follow the same pattern as in the snail samples. At the WMA, proportionately far fewer fibers were in the soil (39.5%; n. 189 vs 73.1% in snails), and fragments (33.0%; n. 158), and films (22.3%; n. 107) were much more common in the soil than in the snails. At UTK all MP types were recovered from the soil, but not in snails, including Fibers (52.4%; n. 283), Fragments (21.1%; n. 114), Films (10.9%; n. 59), Beads (6.0%; n. 36), Pellets (4.6%; n. 25), and Foams (4.2%; n. 23). Examples of MP particles found throughout snail and soil samples are found in Figures 24- 28.

Statistical Analyses

Statistical analyses were carried out using open-source R Studio. A Shapiro-Wilks test was used to check for normality ($p < .05$) among sites. Normality was not achieved likely due to the wide fluctuation in the number of snail individuals per site. Power testing was performed using the 'pwr' package to determine reliability of T-tests and was found to be 0.85 and therefore was within an acceptable range. A nonparametric Wilcoxon signed rank test showed no significant differences ($p > .05$) between the hand sampled sites and board sampled sites. Therefore, we infer no evidence for contamination from the use of coverboards to gather snail samples.

There were significant differences ($p < 0.05$) found between UT campus and WMA in number of MPs in snails. Likewise, there were significant differences ($p < 0.05$) found in MPs between UT campus and WMA sites in soil and MP type. No statistical prevalence was found for colors in any instance and no correlation was found between MP abundance in soil and MP abundance in snails at either location. However, there were significant differences ($p < 0.05$) in both UT campus and WMA when examining snail's vs soils for MP type.

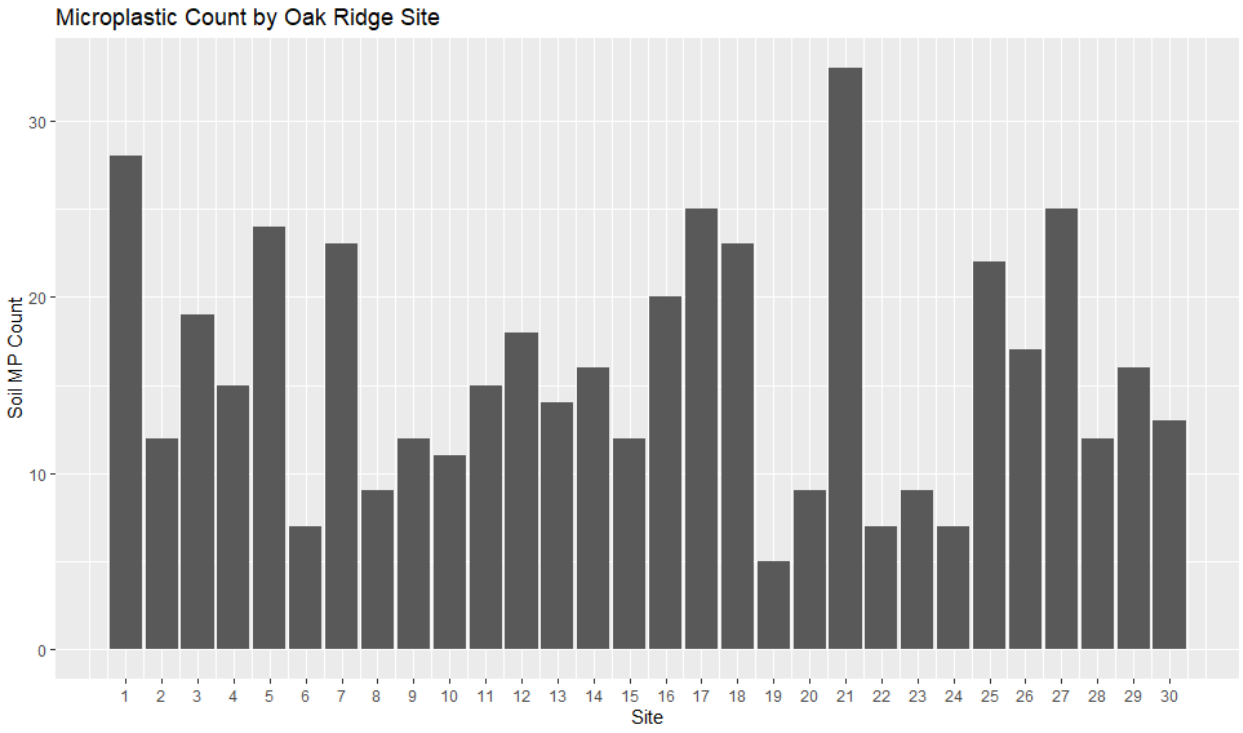

Figure 22 Total MP count of soils by site in the WMA

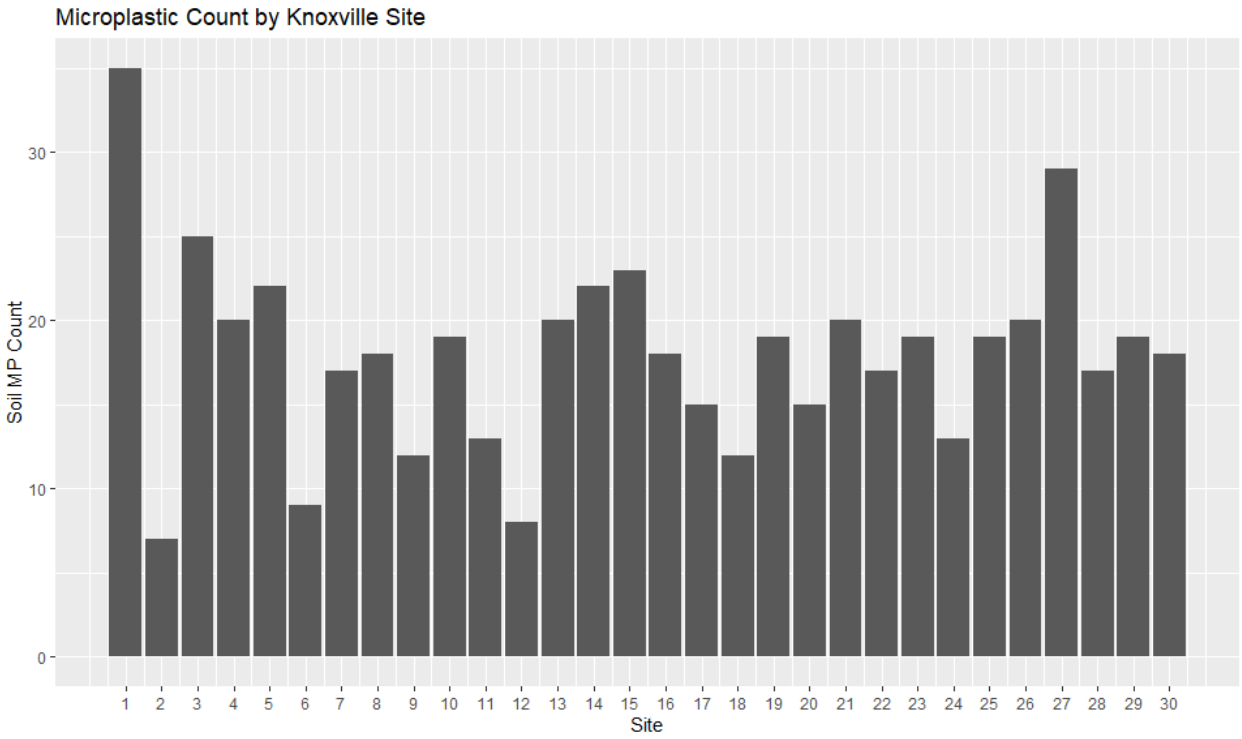

Figure 23 Total MP count in soils on UT campus

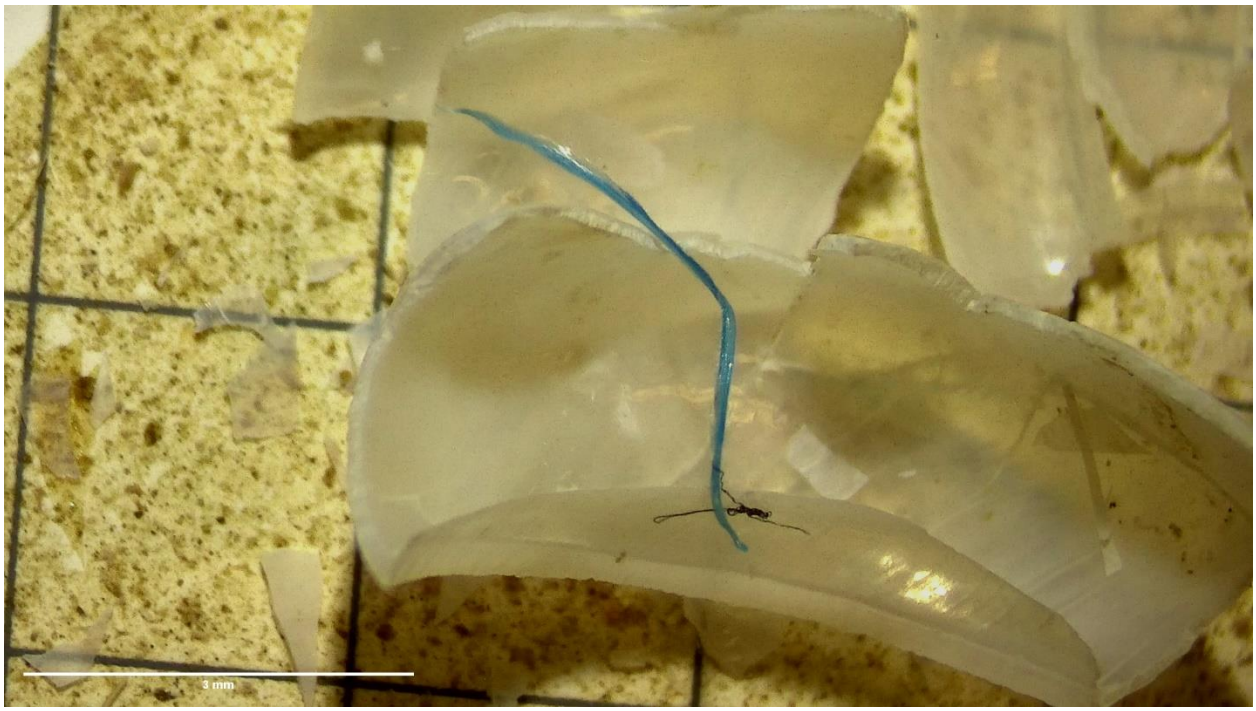

Figure 24 Large Blue MP thread found in a UT campus Mesodon snail sample

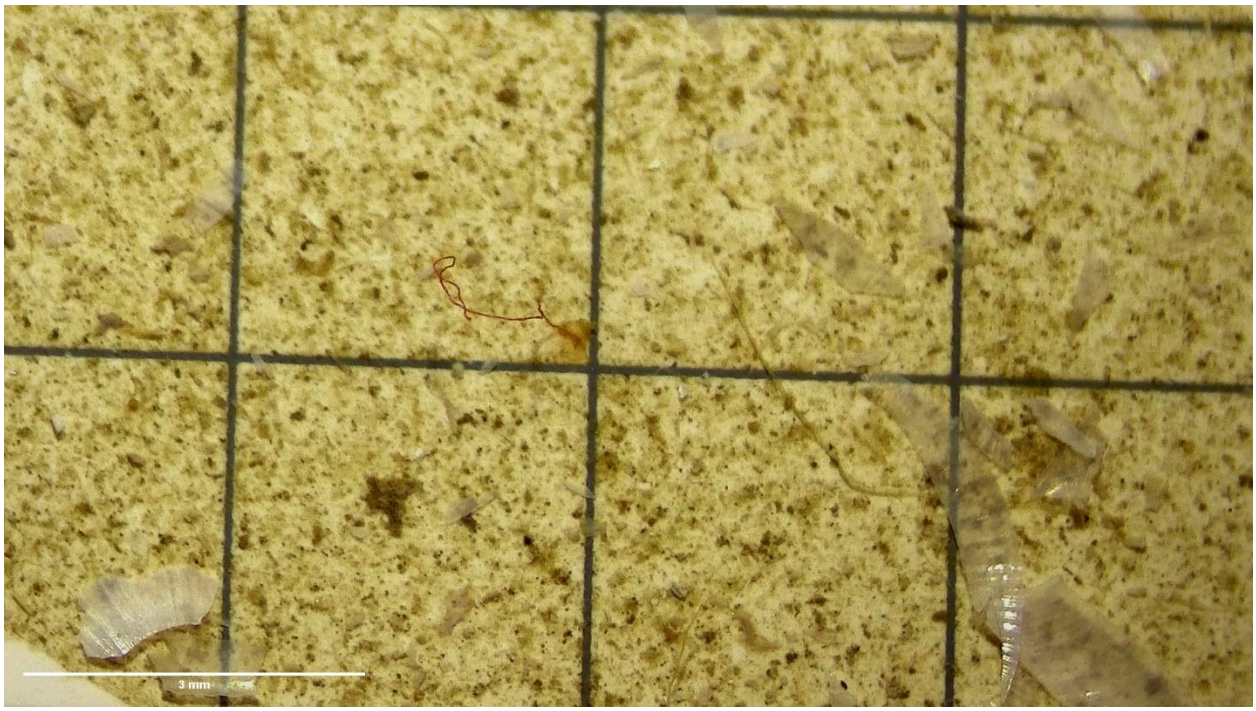

Figure 25 Red microplastic thread found from a WMA snail sample

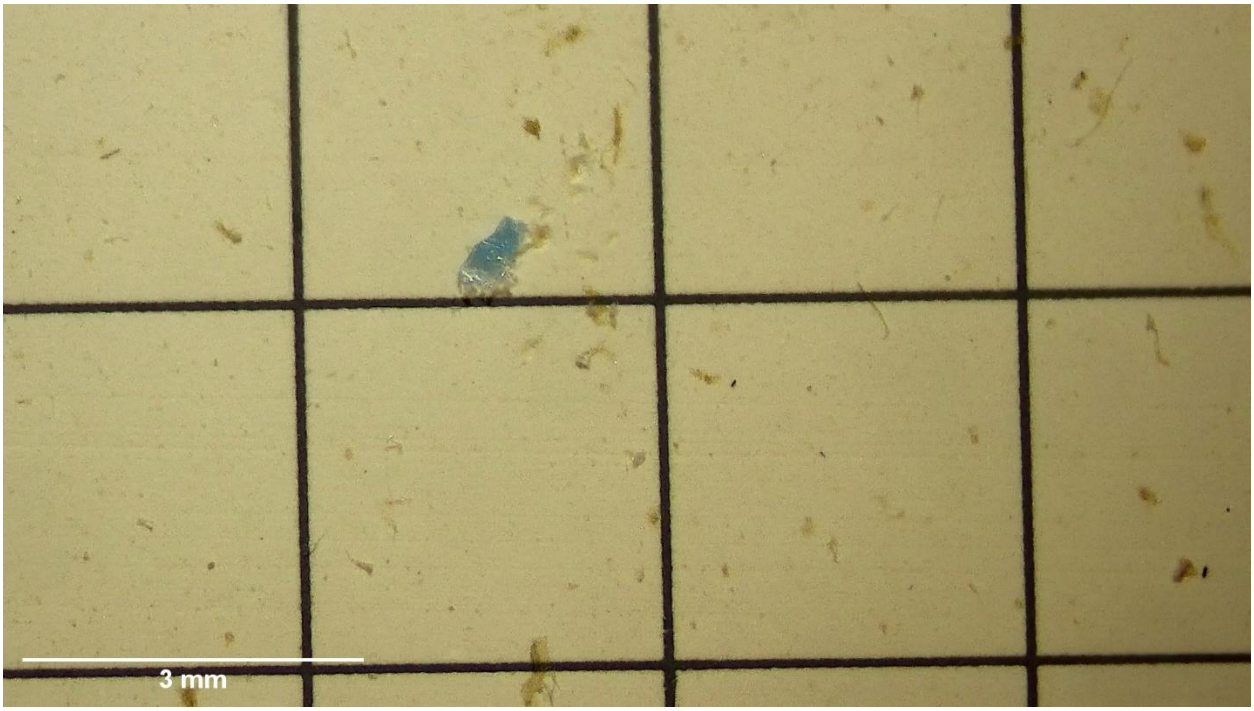

Figure 26 Blue MP film found in WMA soil

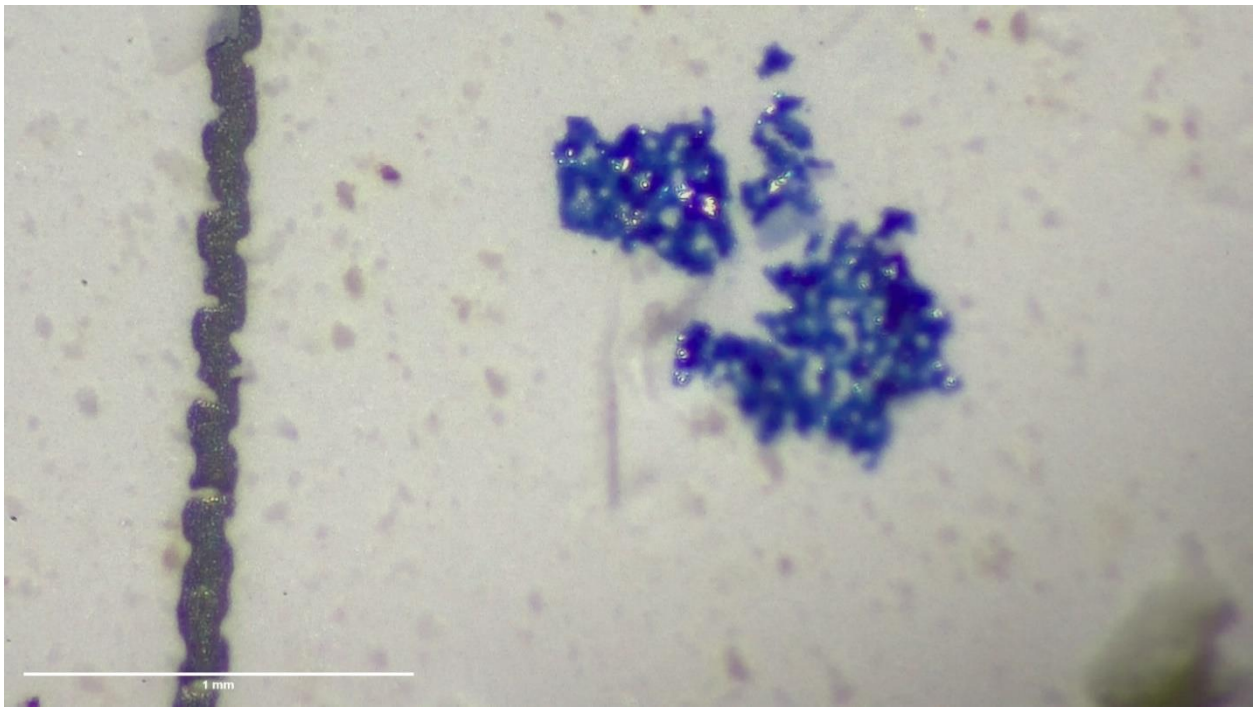

Figure 27 Blue MP flakes found in UT campus soil

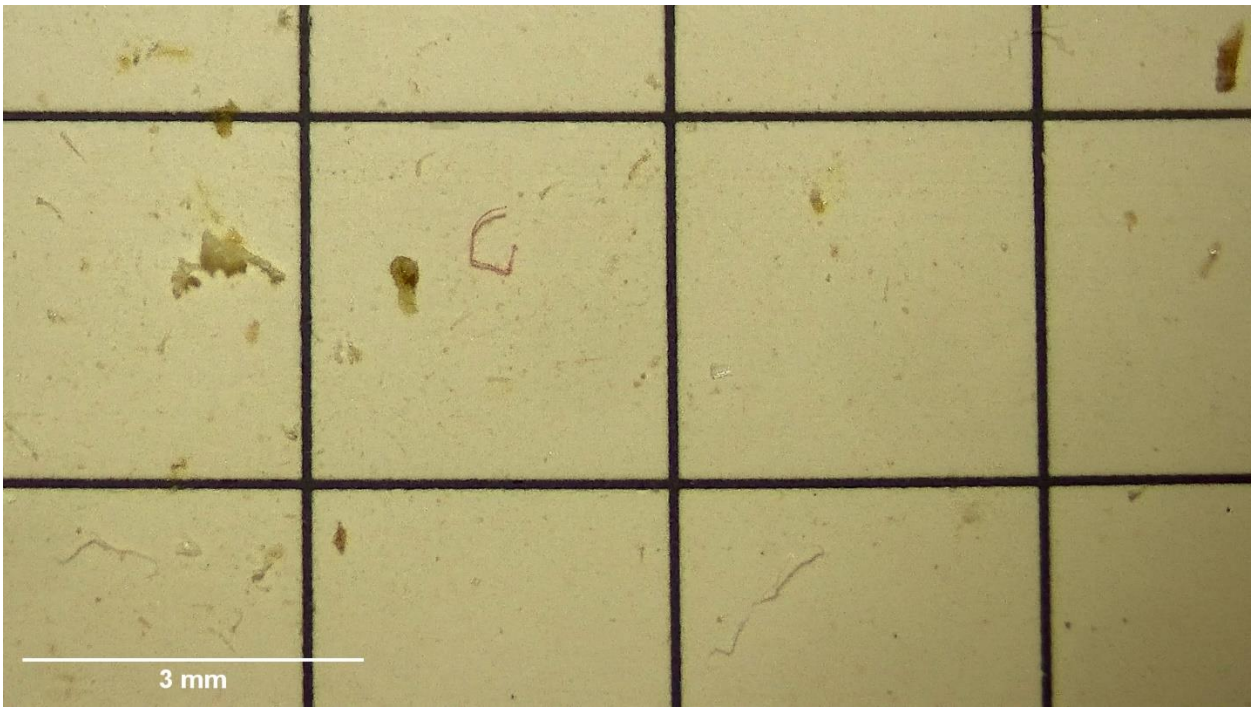

Figure 28 Red MP thread amongst undigested material found in a WMA snail

SECTION 5. DISCUSSION

Although MP accumulation in marine snails (Exposito et al., 2022; Li et al., 2018) and, to a lesser extent, freshwater snails (Weber et al., 2021) is relatively well studied, data on microplastics in wild terrestrial snails is limited to a single study (Panebianco et al., 2019). Thus, information regarding abundance and potential pathways of MP accumulation in snails is nearly non-existent. This study is therefore one of the first examinations to give insight into the concentration and potential pathways of MP accumulation in terrestrial snails.

Microplastic Abundance and Type In Soils and Snails Varied According to Disturbance Level

This study showed that soils on the UT campus, with higher disturbance characteristics (areas of high foot traffic and intensive landscape maintenance) had a higher average abundance of MPs than those within a less disturbed habitat (a wildlife management area). This is not surprising given that MP sources are entirely anthropogenic, and in this case the higher population density and foot traffic on a campus will likely produce more litter and all sizes of plastic debris. In addition, the regular landscaping activities of weeding, mulching, and soil amendments often include the introduction of plastics into the soil (Zhang et al., 2018; Sobhani et al., 2022).

The higher MP levels in the disturbed campus soils are reflected in the higher MP levels in the snails from these sites. However, at the site scale no statistically significant correlation was found between the number of MPs in snails and the number in soils in either the campus or WMA sites. This has been recorded in several similar studies. A study on crabs in an India lagoon failed to establish a link between sites and MP abundance alone and referenced a large matrix of factors (Haite, 2017). Likewise, Doyle (2019) could not find a correlation between site and MP abundance in periwinkles. A confounding factor could be that organisms seemingly consume MPs at random (Li et al., 2019). This indicates that a linear relationship between abundances in media and organism is difficult to establish and may include a matrix of factors that have not been fully recognized.

There are many potential explanations for the lack of correlation. This study did not consider the previous use for each site such as agricultural, industrial, or commercial. Previous MP accumulation over time could affect the type and abundance of MP particles in the soil. It is possible that although the sites we chose were within proximity, the uses of those areas in the past differed greatly. For example, in sites that were closer to buildings on the UT campus, plastic weed barriers were often used under bushes and were not completely pulled up after use. This could result in major

differences in microplastic types in those areas. Although these sites reported higher than average abundances the type of MP that was present could be a confounding factor. This is reflected in studies in the Beibu Gulf of Guangxi (CITE THIS) where no correlation was found as well, except in places where land use changed from industrial to agricultural. Other factors such as elevation, floodplain, season, and rainfall amount could affect the flow of MPs into each site.

This study also found that soils tend to have a different suite of MP types than snails, with proportionately far more fragments, films, pellets and foam in the soils of both sites (compared to the snails at those sites). It is likely that the pathways that enable uptake in snails favor inherently smaller and lighter MPs such as fibers. Fragments, films, foams and beads could require significantly longer degradation which can explain the difference in MP types.

It is difficult to speculate how each type of MP will travel and break down in order to explain prevalence in soils vs. snails. Data shows that common secondary microplastics such as fibers are widespread from clothing production and are more easily tracked (Suaria et al., 2020). However, fragments, films, foams, and beads can take on many shapes, sizes, and forms and it is nearly impossible to explain the heterogeneity seen. It is possible that in contrast to the pathways that fibers travel (easily through waterways and airways) other MP types may have limited mobility. Color may follow this pattern and could be used as an indication of MP travel. Blue and black were the majority of MP colors in snails from both sites, but little data regarding MP origin can be obtained by color alone. This is supported by studies that have examined MP color in fish and have found that black and blue were also the top colors. It is inferred that these types and colors of plastics are simply in greater use in the two study areas as there is no data that that supports color affecting fate or transport of the MP particles. However, in certain industrial applications colors such as blue and black are often used in conjunction with specific MP types such as polyvinyl chloride. This can give insight to the origin of the MPs in certain applications.

Theorized Methods Of Accumulation

Our finding that snails have a greater concentration of fiber MP’s than the surrounding soil indicates a selective pathway that enhances the likelihood of snail MP fiber uptake. The processes by which microplastics accumulate in organisms are varied and complex. Feeding behaviors, locomotion, digestion, and metabolism could all potentially influence accumulation. Land snails are mainly herbivores and detritivores with diets that can include plant stems, leaves, fungus, decaying animals, decaying vegetation, and many other types of organic matter. Snails discover food by utilizing

chemoreceptors on the head to survey chemical gradients. Of 4 tentacles total, two on the head are used to discover food by scent, and two "feelers" physically examine or taste potential food sources. Snails utilize both of these appendages to distinguish food from non-food sources (Carnegie, 2022). It has been shown that dousing foods with chemicals like acetic acid, which is found commonly in plastics, can cause snails to avoid consuming the food (Montana, 2022). However, the small size of MPs combined with the composition of soil aggregates could reduce and mask scents and prevent snails from detecting MPs by chemical means.

The main pathway of MP intake could, therefore, be coincidental uptake during ingestion of the surrounding detrital organic matter. This hypothesis has been supported by experiments in juvenile snails (Colpaert, 2021) in which land snails were intentionally fed food spiked with polyethylene pieces. No avoidance behavior toward food spiked with plastic was discovered, indicating an inability for snails to detect and avoid MPs. This study supports the incidental ingestion hypothesis as MPs were found in high abundance in soils that contained common snail food sources.

Many other studies of controlled experiments of land snails have examined the fate of MPs after ingestion and give insight on how accumulation, or lack thereof, occurs (Colpaert et al 2021.; Song et al., 2019). Snails utilize a cartilage jaw and numerous, small radulae to break down food. The process of consuming food should promote the fragmentation of MP particles as well, but chemical breakdown in gut biomes may also play a significant role (Song et al., 2019). It has been suggested that other organisms such as earthworms possess the capability to fragment particles but driving mechanisms may include the presence of mineral particles in the gut that can physically break down the plastic pieces (Kwak et al.).

Chemical Sorption onto Plastic Surfaces

Chemical and physical characteristics of MPs have profound effects on the sorption capability of pollutants onto their surfaces. Kinetic and isothermic models (Figure 29) are commonly used to help understand the environmental and localized factors that can affect diffusion, transport, and interaction between molecules (Guo and Wang., 2019; Koelmans et al., 2019). These factors are numerous and complicate the determination of baseline levels of MPs harmful to organisms. For example, MP properties, pollutant properties, and environmental factors must all be considered simultaneously and each of these contain a multitude of variables that are continuously changing.

The main mechanisms for sorption are dependent largely on the chemical composition of the MP and pollutant. As shown in figure 28, these can include hydrological interactions, covalent

bonding, electroactivity, size, pH, and temperature among many others (Fue et al., 2021). Some plastics, such as HDPE, can take up to 44 months before it ceases to absorb toxins (Rochman et al., 2012. HDPE, LDPE, and PP comprise over 50% of all plastic produced globally, implying that certain plastic types may be a greater threat than others. Fibers, which are common and easily transported, may be an even greater threat when they are made of these types of plastics.

Potential Harmful Effects

Larger plastic sizes >5mm have been found in many organisms blocking gastrointestinal tracts and pose a direct physical threat. Examples include birds, fish, sea turtles and whales (Wang et al., 2021; Ready, 2018). The chemical effects of MPs have been shown to vary widely across both media and organisms, and can potentially induce harmful effects (Campanale et al., 2020; Bhuyan, 2022). Generally, plastic has been considered inert in biological processes and it has not yet been shown to pose a direct threat to the environment by chemical structure alone (Teuten et al., 2009). However, as plastics transform from macro to micro sizes they can undergo physical transformations that enhance the ability of pollutants to adhere to surfaces (Munier and Bendell, 2017). As plastic particle size decreases to the MP scale (<5mm) the surface area of the plastic particles increases proportionally, resulting in an enhanced ability to react with and retain contaminants. At this size, pieces are small enough to travel within physiological systems and even to penetrate cellular systems, affecting cell metabolism (Jewett et al., 2022). Indeed, there is an extensive and growing literature on nanoplastics (<1 μm) that document impacts of very small plastic particles on subcellular and metabolic systems (Lai et al., 2022).

The physiological effects of MPs in snails have been examined (Song et al., 2019; li et al., 2020). Weights of PET fibers in snails as low as 0.01-0.71 g have been shown to increase oxidative stress, lower food intake, and damage gastrointestinal walls of terrestrial snails (Blanco et al., 2021; Seuront, 2018). In addition, enzymatic activity in the intestinal tract can change due to lipid peroxidation from MPs and this may be an indicator of toxicity (Qu et al., 2020). Changes in growth trajectories by body weight and shell size have been recorded in some species of giant snails from the effects of MP ingestion (Felice et al., 2021). These impacts are often delayed and tend to increase with exposure time and concentration of MPs until an end morphometric point. Behavioral studies of land snails have looked at ranges to better understand where endpoints lie and at what concentrations the effects taper off (Seuront et al., 2018; Felice et al., 2021). One study concluded that mortality and

reproduction were not altered, but reaction time to outside stressors hindered movement in land snails and was drastically increased until a maximum concentration of 4000 mg/g was reached (Blanco et al., 2021)

Such changes in reaction time may not cause mortality directly but can have sublethal impacts on survival rate. Sea snails such as the common periwinkle have been observed to act differently in predator-prey interactions when impacted by high levels of MPs (Seuront et al., 2018). When hunted by a natural predator such as a crab, the periwinkle will normally exhibit avoidance and rapidly retreat. However, periwinkles fed MPs will retreat slowly, or not at all. This is likely caused by the ingestion of toxins sorbed onto the surfaces of the MPs (Gallo et al., 2018). No such behavioral changes have been recorded in terrestrial snails, but similar observations have been recorded in other land animals such as mice and hermit crabs (Araujo and Malafaia et al., 2021; Cunningham et al., 2021).

Other kinds of behavioral changes from MP ingestion are documented in a variety of organisms, ranging from failure to reproduce, loss of social interaction, and loss of locomotion, among others. Examples include hermit crabs' inability to choose shells, a decrease in discus fish hunting behavior, a decrease in zebra fish defensive mechanisms, and slower locomotion in mice. Reviews of MP-affected behavioral changes can be found in Cunningham et al. (2021); Salinas et al. (2019); and Araujo and Malafaia (2021).

Baseline levels for health impacts on many organisms, including urban snails studied herein, have not yet been determined due to the lack of studies documenting behavioral changes in most habitats. Of course, MP impact studies require more than simply measuring weights and numbers of particles per volume of media. A variety of factors, such as species-specific traits, contaminant and MP type, and abundance if MPs undoubtedly interact in complex ways (Binda et al., 2021; Zhang et al., 2022).

In our study, the abundance of MPs in both soils and snails fall within abundance ranges that have been found to cause harmful effects. Fish were found to have lipid oxidative damage with as little as 0.054 ± 0.099 MP items/g; and snails had lower reproductive rates with 5-10 MPs per individual. Any exposure to MPs in rice fish was found to alter brain activity (Zolotova et al., 2022; Weber et al., 2021; Barboza et al., 2020). Due to the tendency for snails to accumulate contaminants in their tissues, should contaminants sorb onto MPs in snails at rates recorded in prior studies, it is likely that with further testing a similar biophysical response will be seen.

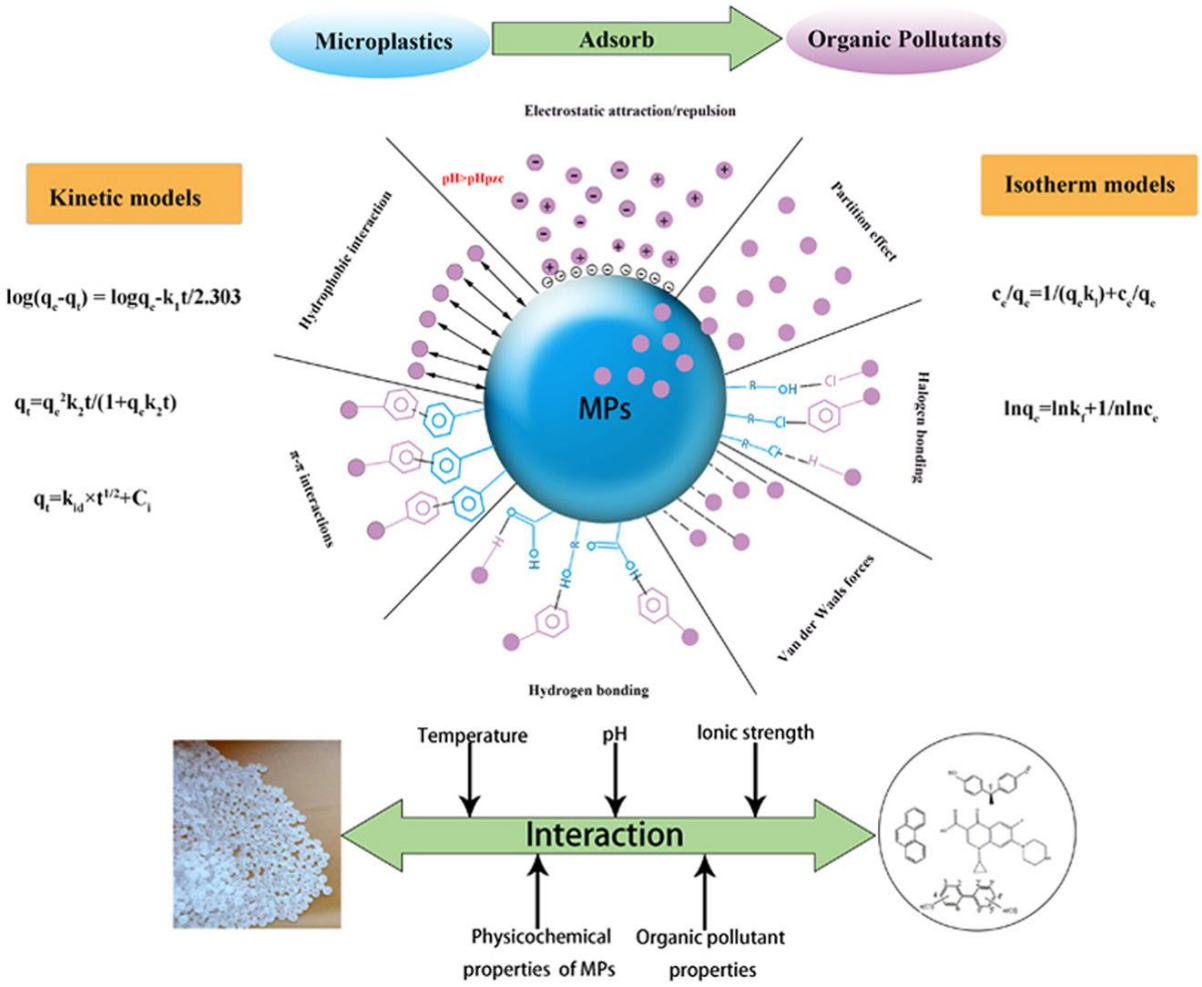

Figure 29 Kinetic and Isothermic modeling for mechanisms of pollutants on MPs.

SECTION 6. SUMMARY

This work represents one of the first studies to examine microplastics in terrestrial snails. It confirms the presence of multiple types of MPs in both soil and land snails, and it established cost-effective methods to examine both land snails and the soil on which they live for MPs. Two different areas were examined in East Tennessee: a wildlife management area (WMA) in Oak Ridge, TN that had significantly less disturbance and potential for plastic contamination, and a highly disturbed area on the University of Tennessee campus (UT). At the WMA, MPs were confirmed in 67% of snails for a mean value of 1.6 MP and with 100% of sites testing positive for MPs in the soil with a mean value of 15.9. The campus had confirmed MPs in 79.2% of snails for a mean value of 2.07 MP with 100% of sites testing positive for MPs in the soils with a mean value of 18.0. These values are much higher than the single known previous study of MPs in terrestrial snails and indicates that major knowledge gaps exist in this area of research.

This study utilized several previously published research methods but also sought to expand and improve upon them. Snails were digested using sodium hypochlorite and potassium hydroxide at a 1:1 ratio on an orbital shaker for 12-24 hours. They were vacuum filtered and transferred to a 0.47-micron filter. Stereo microscopes were then used for the examination process at 50x magnification in a double-blind observation. A combination of microplastic identification guides were all considered and previous rules were adapted to this project. Also, filter reading procedures were adopted to ensure that observers always followed the same procedure. The hot needle and spring test were used in order to distinguish plastic and non-plastic particles. Soil identification methods utilized a combination of experimental procedures by density separation with Ludox TM-50. A soil isolation unit was prototyped and modified to create a durable, non-plastic separation unit. This allowed the flotation of lower-density plastic from higher-density non-plastic particles. Methods used for identification and digestion were both cost efficient and provided low contamination levels.

Although there was no found correlation between abundance of MPs in soil and snails, there were significant differences in the MP types between soils and snails. This is likely due to the size differences and breakdown mechanics that exist from radula teeth and gut digestion aiding in the breakdown of plastics. This could allow smaller types such as fibers and smaller fragments a pathway into snails, explaining why there are proportionately more fibers in snails than soils. Predictably, there were also significant differences between sites for total MP count in both soils and snails, with the UT campus having more plastic pollution than the WMA. There are many more opportunities for plastics

to fragment and break down into MPs in more disturbed areas. Colors were similar across all locations with black and blue being the primary colors. This is not unusual and was useful in cross examination of contamination. Though colors alone do not provide useful insight on origins, examining degradation and color fading could provide insight on the degradation time.

Fates of microplastics in snails are still largely unknown but this research provides more data toward the development of a model of how contamination may occur. Size, location, environmental, and chemical characteristics influence how and where MPs are transported. Snails utilize tentacles to detect chemical gradients of potential food sources but may be unable to detect MPs in media. Accidental ingestion is likely the main source of uptake, and this can lead to a variety of harmful effects from oxidative stress, reproductive harm, and predator-prey behavioral changes. Snail toxicity studies are limited but inferences can be drawn from work in other organisms. Ultimately, the variety of contaminants and plastic types make it difficult to determine how different species and organisms will react.

SECTION 7. FUTURE WORK

Building upon previous microplastic work is essential to understand the complexities that influence both the effects and distribution of MPs. This study utilized a variety of methods that were combined and altered to test Efficacy and accuracy. Despite thorough planning, this study is not without flaws. A very common occurrence and perhaps the biggest threat to MP research is contamination. The use of glass instruments, blanks and air filters should continue to be used in all cases but there may other suitable options that could perform better. Clean boxes could be used to help filter air from the inside of the MP containment area and keep unwanted particles out. This is in stark contrast to the common use of examination under fume hoods which work by cycling air from outside-in. The use of Honeywell filters and creating a closed space compensated for this but could be improved on. The emergence of atmospheric particles creates an environment where the air flow could contaminate samples.

The large variety of digestion methods needs to be addressed as well. Seemingly, it is not suitable to have a method that is a one-size fits all. The variety of organismal skeletons, shells, tissue, and general makeups make it difficult to find chemicals that can digest efficiently without damaging plastics. Exploration of new, or lesser used, combinations such as those in this study should be performed and documented so they can be referenced for comparison.

Understanding the toxic effects of MPs lies in accurate identification. All current methods used have some drawbacks in the form of interference, cost, or limited examination. FTIR machines that have high accuracy can cost thousands of dollars. Low-cost stereomicroscope work is very laborious. Inflorescent particles are limited to certain types in limited conditions. MP research needs to be performed across many environments and disturbance levels to get good baseline levels. Attempts like this have been made by organizations such as citizens science, but the methods being implored can result in nearly 100% over estimation. However, the research is still meaningful and helps develop other low cost-efficient methods.

REFERENCES

Akindele, E. O., Ehlers, S. M., & Koop, J. H. E. (2019). First empirical study of freshwater MPs in West Africa using gastropods from Nigeria as bioindicators. *Limnologica*, *78*(August), 125708. https://doi.org/10.1016/j.limno.2019.125708

Akhbarizadeh, R., Dobaradaran, S., Nabipour, I., Tajbakhsh, S., Darabi, A. H., & Spitz, J. (2020). Abundance, composition, and potential intake of microplastics in canned fish. *Marine Pollution Bulletin*, *160*. https://doi.org/10.1016/j.marpolbul.2020.111633

Al Hamra, A. J., & Patria, M. P. (2019). Microplastic in Gonggong snails (Laevistrombus turturella) and sediment of Bintan Island, Kepulauan Riau Province, Indonesia. *AIP Conference Proceedings*, *2202*. https://doi.org/10.1063/1.5141692

Andrady, A. L. (2011). MPs in the marine environment. *Marine Pollution Bulletin*, *62*(8), 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Barboza, L. G. A., Lopes, C., Oliveira, P., Bessa, F., Otero, V., Henriques, B., Raimundo, J., Caetano, M., Vale, C., & Guilhermino, L. (2020). Microplastics in wild fish from North East Atlantic Ocean and its potential for causing neurotoxic effects, lipid oxidative damage, and human health risks associated with ingestion exposure. *Science of the Total Environment*, *717*. https://doi.org/10.1016/j.scitotenv.2019.134625

Bhuyan, M. S., Rashed-Un-Nabi, M., Alam, M. W., Islam, M. N., Cáceres-Farias, L., Bat, L., Saiyad Musthafa, M., Senapathi, V., Chung, S. Y., & Alfaro Núñez, A. (2022). *Environmental and Morphological Detrimental Effects of Microplastics on Marine Organisms to Human Health*. https://doi.org/10.21203/rs.3.rs-1290795/v1

Campanale, C., Massarelli, C., Savino, I., Locaputo, V., & Uricchio, V. F. (2020). A detailed review study on potential effects of microplastics and additives of concern on human health. In *International Journal of Environmental Research and Public Health* (Vol. 17, Issue 4). MDPI AG. https://doi.org/10.3390/ijerph17041212

Carlin, J., Craig, C., Little, S., Donnelly, M., Fox, D., Zhai, L., & Walters, L. (2020). Microplastic accumulation in the gastrointestinal tracts in birds of prey in central Florida, USA. Environmental Pollution, 264, 114633. https://doi.org/10.1016/j.envpol.2020.114633

Carretero, O., Gago, J., & Viñas, L. (2021). From the coast to the shelf: MPs in Rías Baixas and Miño River shelf sediments (NW Spain). *Marine Pollution Bulletin*, *162*(October 2020). https://doi.org/10.1016/j.marpolbul.2020.111814

Chen, B., Fan, Y., Huang, W., Rayhan, A. B. M. S., Chen, K., & Cai, M. (2020). Observation of MPs in mariculture water of Longjiao Bay, southeast China: Influence by human activities. *Marine Pollution Bulletin*, *160*(May), 111655. https://doi.org/10.1016/j.marpolbul.2020.111655

Chen, M. C., Chen, T. H., Wang, Y., Zou, X., Peng, C., Qiao, S., Wang, T., Yu, W., Khokiattiwong, S., Kornkanitnan, N., Khalik, W. M. A. W. M., Ibrahim, Y. S., Tuan Anuar, S., Govindasamy, S., Baharuddin, N. F., Syakti, A. D., Hidayati, N. V., Jaya, Y. V., Siregar, S. H., … Zhou, Y. (2020). Guide to MP Identification. *Marine Pollution Bulletin*, *154*(November), 110729. https://doi.org/10.1016/j.marpolbul.2020.111027%0Ahttps://doi.org/10.1016/j.marpolbul.2019.110729%0Ahttps://doi.org/10.1016/j.marpolbul.2019.110687%0Ahttps://doi.org/10.1016/j.ecoenv.2019.109913%0Ahttps://doi.org/10.1016/j.marpolbul.2019.07.072%0Ahttps://

Cole, M., Lindeque, P., Halsband, C., & Galloway, T. S. (2011). MPs as contaminants in the marine environment: A review. *Marine Pollution Bulletin*, *62*(12), 2588–2597. https://doi.org/10.1016/j.marpolbul.2011.09.025

Coppock, R. L., Cole, M., Lindeque, P. K., Queirós, A. M., & Galloway, T. S. (2017). A small-scale, portable method for extracting MPs from marine sediments. *Environmental Pollution*, *230*, 829–837. https://doi.org/10.1016/j.envpol.2017.07.017

Cunningham, E. M., Mundye, A., Kregting, L., Dick, J. T. A., Crump, A., Riddell, G., & Arnott, G. (2021). Animal contests and microplastics: Evidence of disrupted behaviour in hermit crabs Pagurus bernhardus. *Royal Society Open Science*, *8*(10). https://doi.org/10.1098/rsos.211089

da Costa Araújo, A. P., & Malafaia, G. (2021). Microplastic ingestion induces behavioral disorders in mice: A preliminary study on the trophic transfer effects via tadpoles and fish. *Journal of Hazardous Materials*, *401*. https://doi.org/10.1016/j.jhazmat.2020.123263

Davison, P., & Asch, R. G. (2011). Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. *Marine Ecology Progress Series*, *432*, 173–180. https://doi.org/10.3354/meps09142

de Felice, B., Ambrosini, R., Bacchetta, R., Ortenzi, M. A., & Parolini, M. (2021). Dietary exposure to polyethylene terephthalate microplastics (PET-MPs) induces faster growth but not oxidative stress in the giant snail Achatina reticulata. *Chemosphere*, *270*. https://doi.org/10.1016/j.chemosphere.2020.129430

Dioses-Salinas, D., Pérez-Baca, B., & De-la-Torre, G. (2019). Ecotoxicological effects of microplastics and adsorbed contaminants on aquatic organisms. *Manglar*, *16*(2), 173–182. https://doi.org/10.17268/manglar.2019.024

Doyle, D., Gammell, M., Frias, J., Griffin, G., & Nash, R. (2019). Low levels of microplastics recorded from the common periwinkle, Littorina littorea on the west coast of Ireland. *Marine Pollution Bulletin*, *149*. https://doi.org/10.1016/j.marpolbul.2019.110645

Dris, R., Gasperi, J., Saad, M., Mirande, C., & Tassin, B. (2016). Synthetic fibers in atmospheric fallout: A source of microplastics in the environment? *Marine Pollution Bulletin*, *104*(1–2), 290–293. https://doi.org/10.1016/j.marpolbul.2016.01.006

E J Carpenter, S. J. A. G. R. H. H. P. M. B. B. P. (1972). Polystyrene spherules in coastal waters. *Science*.

Enders, K., Lenz, R., Beer, S., & Stedmon, C. A. (2017). Extraction of MP from biota: Recommended acidic digestion destroys common plastic polymers. *ICES Journal of Marine Science*, *74*(1), 326–331. https://doi.org/10.1093/icesjms/fsw173

Eriksen, M., Mason, S., Wilson, S., Box, C., Zellers, A., Edwards, W., Farley, H., & Amato, S. (2013). Microplastic pollution in the surface waters of the Laurentian Great Lakes. *Marine Pollution Bulletin*, *77*(1–2), 177–182. https://doi.org/10.1016/j.marpolbul.2013.10.007

Expósito, N., Rovira, J., Sierra, J., Gimenez, G., Domingo, J. L., & Schuhmacher, M. (2022). Levels of microplastics and their characteristics in molluscs from North-West Mediterranean Sea: Human intake. *Marine Pollution Bulletin*, *181*. https://doi.org/10.1016/j.marpolbul.2022.113843

Foekema, E. M., de Gruijter, C., Mergia, M. T., van Franeker, J. A., Murk, A. J., & Koelmans, A. A. (2013). Plastic in north sea fish. *Environmental Science and Technology*, *47*(15), 8818–8824. https://doi.org/10.1021/es400931b

Filgueiras, A. V., Gago, J., García, I., León, V. M., & Viñas, L. (2021). Plackett Burman design for MPs quantification in marine sediments. *Marine Pollution Bulletin*, *162*(October 2020). https://doi.org/10.1016/j.marpolbul.2020.111841

Frias, J. P. G. L., & Nash, R. (2019). MPs: Finding a consensus on the definition. *Marine Pollution Bulletin*, *138*(September 2018), 145–147. https://doi.org/10.1016/j.marpolbul.2018.11.022

Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., Nadal, A., & Romano, D. (2018). Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. In *Environmental Sciences Europe* (Vol. 30, Issue 1). Springer Verlag. https://doi.org/10.1186/s12302-018-0139-z

Gola, D., Kumar Tyagi, P., Arya, A., Chauhan, N., Agarwal, M., Singh, S. K., & Gola, S. (2021). The impact of microplastics on marine environment: A review. In *Environmental Nanotechnology, Monitoring and Management* (Vol. 16). Elsevier B.V. https://doi.org/10.1016/j.enmm.2021.100552

Guo Xuan, J. W. (2019). *The chemical behaviors of microplastics in marine environment: A review*.

Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., & Thiel, M. (2012). MPs in the marine environment: A review of the methods used for identification and quantification. *Environmental Science and Technology*, *46*(6), 3060–3075. https://doi.org/10.1021/es2031505

Hodges, M. N., & McKinney, M. L. (2018). Urbanization impacts on land snail community composition. *Urban Ecosystems*, *21*(4), 721–735. https://doi.org/10.1007/s11252-018-0746-x

Ivar Do Sul, J. A., & Costa, M. F. (2014). The present and future of MP pollution in the marine environment. *Environmental Pollution*, *185*, 352–364. https://doi.org/10.1016/j.envpol.2013.10.036

Jewett, E., Arnott, G., Connolly, L., Vasudevan, N., & Kevei, E. (2022). Microplastics and Their Impact on Reproduction—Can we Learn From the C. elegans Model? *Frontiers in Toxicology*, *4*. https://doi.org/10.3389/ftox.2022.748912

Karakolis, E. G., Nguyen, B., You, J. B., Rochman, C. M., & Sinton, D. (2019). Fluorescent Dyes for Visualizing Microplastic Particles and Fibers in Laboratory-Based Studies. *Environmental Science and Technology Letters*, *6*(6), 334–340. https://doi.org/10.1021/acs.estlett.9b00241

Kleinschmidt, J. M., & Janosik, A. M. (2021). Microplastics in Florida, United States: A Case Study of Quantification and Characterization With Intertidal Snails. *Frontiers in Ecology and Evolution*, *9*. https://doi.org/10.3389/fevo.2021.645727

Koelmans, A. A., Mohamed Nor, N. H., Hermsen, E., Kooi, M., Mintenig, S. M., & de France, J. (2019). Microplastics in freshwaters and drinking water: Critical review and assessment of data quality. In *Water Research* (Vol. 155, pp. 410–422). Elsevier Ltd. https://doi.org/10.1016/j.watres.2019.02.054

Li, J., Liu, H., & Paul Chen, J. (2018). MPs in freshwater systems: A review on occurrence, environmental effects, and methods for MPs detection. *Water Research*, *137*, 362–374. https://doi.org/10.1016/j.watres.2017.12.056

Li, R., Zhang, S., Zhang, L., Yu, K., Wang, S., & Wang, Y. (2020). Field study of the MP pollution in sea snails (Ellobium chinense) from mangrove forest and their relationships with MPs in water/sediment located on the north of Beibu Gulf. *Environmental Pollution*, *263*, 114368. https://doi.org/10.1016/j.envpol.2020.114368

Liu, F., Vianello, A., & Vollertsen, J. (2019). Retention of MPs in sediments of urban and highway stormwater retention ponds. *Environmental Pollution*, *255*, 113335. https://doi.org/10.1016/j.envpol.2019.113335

Mariano, S., Tacconi, S., Fidaleo, M., Rossi, M., & Dini, L. (2021). Micro and Nanoplastics Identification: Classic Methods and Innovative Detection Techniques. *Frontiers in Toxicology*, *3*. https://doi.org/10.3389/ftox.2021.636640

Maricela, R., & Espinoza, B. (2019). *Microplastics in Wastewater Treatment Systems and Receiving Waters*.

Marine Debris Program, N. (2015). *Laboratory Methods for the Analysis of MPs in the Marine Environment: Recommendations for quantifying synthetic particles in waters and sediments*. *July*.

Marsden, I. D., & Baharuddin, N. (2015). Gastropod growth and survival as bioindicators of stress associated with high nutrients in the intertidal of a shallow temperate estuary. *Estuarine, Coastal and Shelf Science*, *156*(1), 175–185. https://doi.org/10.1016/j.ecss.2014.05.032

Munier, B., & Bendell, L. I. (2018). Macro and micro plastics sorb and desorb metals and act as a point source of trace metals to coastal ecosystems. *PLoS ONE*, *13*(2). https://doi.org/10.1371/journal.pone.0191759

Naji, A., Nuri, M., & Vethaak, A. D. (2018). MPs contamination in molluscs from the northern part of the Persian Gulf. *Environmental Pollution*, *235*, 113–120. https://doi.org/10.1016/j.envpol.2017.12.046

Panebianco, A., Nalbone, L., Giarratana, F., & Ziino, G. (2019). First discoveries of MPs in terrestrial snails. *Food Control*, *106*(April), 106722. https://doi.org/10.1016/j.foodcont.2019.106722

Qu, H., Ma, R., Barrett, H., Wang, B., Han, J., Wang, F., Chen, P., Wang, W., Peng, G., & Yu, G. (2020). How microplastics affect chiral illicit drug methamphetamine in aquatic food chain? From green alga (Chlorella pyrenoidosa) to freshwater snail (Cipangopaludian cathayensis). *Environment International*, *136*. https://doi.org/10.1016/j.envint.2020.105480

Reichert, J., Tirpitz, V., Anand, R., Bach, K., Knopp, J., Schubert, P., Wilke, T., & Ziegler, M. (2021). Interactive effects of microplastic pollution and heat stress on reef-building corals. *Environmental Pollution*, *290*. https://doi.org/10.1016/j.envpol.2021.118010

Rochman, C. M., Hoh, E., Hentschel, B. T., & Kaye, S. (2013). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: Implications for plastic marine debris. *Environmental Science and Technology*, *47*(3), 1646–1654. https://doi.org/10.1021/es303700s

Rocha-Santos, T., & Duarte, A. C. (2015). A critical overview of the analytical approaches to the occurrence, the fate and the behavior of MPs in the environment. *TrAC - Trends in Analytical Chemistry*, *65*, 47–53. https://doi.org/10.1016/j.trac.2014.10.011

Romero-Blanco, A., Remón-Elola, A., & Alonso, Á. (2021). Assessment of the Effects of Environmental Concentrations of Microplastics on the Aquatic Snail Potamopyrgus antipodarum. *Water, Air, and Soil Pollution*, *232*(10). https://doi.org/10.1007/s11270-021-05379-7

Samsi, N., Asaf, R., Sahabuddin, S., Santi, A., & Wamnebo, M. I. (2017). Gastropods As A Bioindicator and Biomonitoring Water Pollution. *Aquacultura Indonesiana*, *18*(1), 54. https://doi.org/10.21534/ai.v18i1.42

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. doi:10.1038/nmeth.2089

Seuront, L. (2018). MP leachates impair behavioural vigilance and predator avoidance in a temperate intertidal gastropod. *Biology Letters*, *14*(11). https://doi.org/10.1098/rsbl.2018.0453

Shim, W. J., Hong, S. H., & Eo, S. E. (2017). Identification methods in MP analysis: A review. *Analytical Methods*, *9*(9), 1384–1391. https://doi.org/10.1039/c6ay02558g

Simmerman, C. B., & Coleman Wasik, J. K. (2020). The effect of urban point source contamination on microplastic levels in water and organisms in a cold-water stream. In *Limnology And Oceanography Letters* (Vol. 5, Issue 1, pp. 137–146). John Wiley and Sons Inc. https://doi.org/10.1002/lol2.10138

Singh, N., Mondal, A., Bagri, A., Tiwari, E., Khandelwal, N., Monikh, F. A., & Darbha, G. K. (2021). Characteristics and spatial distribution of MPs in the lower Ganga River water and sediment. *Marine Pollution Bulletin*, *163*(December 2020), 111960. https://doi.org/10.1016/j.marpolbul.2020.111960

Song, Y., Cao, C., Qiu, R., Hu, J., Liu, M., Lu, S., Shi, H., Raley-Susman, K. M., & He, D. (2019). Uptake and adverse effects of polyethylene terephthalate MPs fibers on terrestrial snails (Achatina fulica) after soil exposure. *Environmental Pollution*, *250*, 447–455. https://doi.org/10.1016/j.envpol.2019.04.066

Suaria, G., Achtypi, A., Perold, V., Lee, J. R., Pierucci, A., Bornman, T. G., Aliani, S., & Ryan, P. G. (2020). O C E A N O G R A P H Y Microfibers in oceanic surface waters: A global characterization. In *Sci. Adv* (Vol. 6).

Teuten, E. L., Saquing, J. M., Knappe, D. R. U., Barlaz, M. A., Jonsson, S., Björn, A., Rowland, S. J., Thompson, R. C., Galloway, T. S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P. H., Tana, T. S., Prudente, M., Boonyatumanond, R., Zakaria, M. P., Akkhavong, K., … Takada, H. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2027–2045. https://doi.org/10.1098/rstb.2008.0284

Turner, S., Horton, A. A., Rose, N. L., & Hall, C. (2019). A temporal sediment record of microplastics in an urban lake, London, UK. *Journal of Paleolimnology*, *61*(4), 449–462. https://doi.org/10.1007/s10933-019-00071-7

Townsend, K. R., Lu, H. C., Sharley, D. J., & Pettigrove, V. (2019). Associations between MP pollution and land use in urban wetland sediments. *Environmental Science and Pollution Research*, *26*(22), 22551–22561. https://doi.org/10.1007/s11356-019-04885-w

Turner, S., Horton, A. A., Rose, N. L., & Hall, C. (2019). A temporal sediment record of MPs in an urban lake, London, UK. *Journal of Paleolimnology*, *61*(4), 449–462. https://doi.org/10.1007/s10933-019-00071-7

USGS. Removal of organic matter with hydrogen peroxide. OFR01-041: Procedures - Acetic Acid Treatment to Remove Carbonates, https://pubs.usgs.gov/of/2001/of01-041/htmldocs/methods/h2o2.htm.

Vaughan, R., Turner, S. D., & Rose, N. L. (2017). MPs in the sediments of a UK urban lake. *Environmental Pollution*, *229*, 10–18. https://doi.org/10.1016/j.envpol.2017.05.057

Waite, H. (n.d.). *Investigating the Quantity and Types of Microplastics in the Investigating the Quantity and Types of Microplastics in the Organic Tissue of Oysters and Crabs in the Indian River Lagoon Organic Tissue of Oysters and Crabs in the Indian River Lagoon*. http://library.ucf.edu

Wang, L., Nabi, G., Yin, L., Wang, Y., Li, S., Hao, Z., & Li, D. (2021). Birds and plastic pollution: recent advances. In *Avian Research* (Vol. 12, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40657-021-00293-2

Wang, L., Nabi, G., Yin, L., Wang, Y., Li, S., Hao, Z., & Li, D. (2021). Birds and plastic pollution: recent advances. In *Avian Research* (Vol. 12, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40657-021-00293-2

Weber, A., von Randow, M., Voigt, A. L., von der Au, M., Fischer, E., Meermann, B., & Wagner, M. (2021). Ingestion and toxicity of MPs in the freshwater gastropod Lymnaea stagnalis: No MP-induced effects alone or in combination with copper. *Chemosphere*, *263*. https://doi.org/10.1016/j.chemosphere.2020.128040

Willis, K. A., Eriksen, R., Wilcox, C., & Hardesty, B. D. (2017). MP distribution at different sediment depths in an urban estuary. *Frontiers in Marine Science*, *4*(DEC), 1–8. https://doi.org/10.3389/fmars.2017.00419

Wright, S. L., Thompson, R. C., & Galloway, T. S. (2013). The physical impacts of MPs on marine organisms: a review. *Environmental Pollution (Barking, Essex : 1987)*, *178*, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031

XiaoZhi Lim, B. (n.d.). *MICROPLASTICS ARE EVERYWHERE-BUT ARE THEY HARMFUL? Microplastics collected from the Magothy River in*.

Xu, X., Wong, C. Y., Tam, N. F. Y., Lo, H. S., & Cheung, S. G. (2020). MPs in invertebrates on soft shores in Hong Kong: Influence of habitat, taxa and feeding mode. *Science of the Total Environment*, *715*, 136999. https://doi.org/10.1016/j.scitotenv.2020.136999

Zaki, M. R. M., Zaid, S. H. M., Zainuddin, A. H., & Aris, A. Z. (2021). Microplastic pollution in tropical estuary gastropods: Abundance, distribution and potential sources of Klang River estuary, Malaysia. *Marine Pollution Bulletin*, *162*(November 2020), 111866. https://doi.org/10.1016/j.marpolbul.2020.111866

Zhang, Y., Kang, S., Allen, S., Allen, D., Gao, T., & Sillanpää, M. (2020). Atmospheric microplastics: A review on current status and perspectives. In *Earth-Science Reviews* (Vol. 203). Elsevier B.V. https://doi.org/10.1016/j.earscirev.2020.103118

Zhang, G. S., & Liu, Y. F. (2018). The distribution of microplastics in soil aggregate fractions in southwestern China. *Science of the Total Environment*, *642*, 12–20. https://doi.org/10.1016/j.scitotenv.2018.06.004

Zolotova, N., Kosyreva, A., Dzhalilova, D., Fokichev, N., & Makarova, O. (2022). Harmful effects of the microplastic pollution on animal health: a literature review. In *PeerJ* (Vol. 10). PeerJ Inc. https://doi.org/10.7717/peerj.13503

VITA

Gregory Bonilla was born in Maryville, Tennessee in 1995. After high school graduation and spending half a year pursuing a humanity degree at the Tennessee Technological Institute in Cookeville TN, he went on to join the United States Army to become an infantry man. Following honorable discharge after service, he returned to school at Roane State Community College in 2015 to study geology and environmental sciences. He received an associate of science with honors and proceeded to complete his bachelor's degree in geology at the University of Tennessee, also with honors. While attending the University of Tennessee he had many opportunities to work with a variety of projects and professors. These projects included examining bird strike abundances on campus, environmental restoration projects on over run areas in many parks in East Tennessee and mapping out various potential threats to ecologically fragile areas in Tennessee. He also had the privilege of working in the engineering department where he was co-author on 2 papers examining socioeconomic factors on solar adoption and cities that could be vulnerable during the covid-19 pandemic. On acceptance into the master's program, he sought to fill in knowledge gaps concerning he fate and transport of microplastics in the environment. This led to an extensive project covering many departments and colleagues over 2 years. He presented his findings at the 2022 Southeastern Geological Society of America meeting and was invited afterwards to speak at Roane State Community College. He hopes to work in non-profit and continue his work in environmental consulting.