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Abstract

Microplastics (MPs) can now be found in all the Earth's biomes, thereby representing a global change phenomenon with largely unknown consequences for biodiversity and ecosystem functioning. Soil protists are eukaryotic, primarily single celled organisms that play important roles in the soil food web. Microplastics have been shown to affect protist populations in freshwater and marine environments, yet the interactions between soil protists and MPs remains largely unknown. Here we examined whether phagotrophic soil protists can ingest MPs and experience declines in abundance. We exposed protists to soil treatments with different concentrations of MPs using commercial polymer fluorescent microspheres and used fluorescence microscopy to find evidence of MP ingestion. In addition, we quantified the total number of active phagotrophic protists over time. We show that most soil protists (>75% individuals) can readily ingest and keep MP within their food vacuoles, even at relatively small MP concentrations (0.1% w/w). There was a trend for higher prevalence of ingestion and for declines in protist abundance at the highest concentration of MPs (1% w/w). However, more data are necessary to further ascertain cause-effect relationships. This is the first report indicating that soil protists can play an important role in the transport and uptake of MPs in the soil food web.

Introduction

Plastic is one of the most abundant synthetic substances on the planet (Lambert et al., 2014). An estimated 381 million tons of plastics were produced in 2015 and this number keeps rising (Ritchie & Roser, 2018). Widely recognized since the 1950s as cheaper, lighter and more durable than other materials such as metal and glass, plastics (most commonly polystyrene (PS), poly(ethylene terephthalate) (PET), polyurethane (PUR), poly(vinyl chloride) PVC, polyethylene (PE) and polypropylene (PP); (Strungaru et al., 2019)) have become the primary material in disposable packaging, and are pivotal in the construction and automotive industries; applications are practically endless (Lambert et al., 2014). However, plastics accumulate in the environment (Chamas et al., 2020) and are emerging as a matter of concern in both aquatic and terrestrial systems (Andrady, 2011; de Souza Machado, Kloas, et al., 2018; Rillig, 2012).

Microplastics (MPs) are defined as any plastic particle <5 mm in diameter (Hidalgo-Ruz et al., 2012). They are produced either intentionally (e.g., by the cosmetic industry for use as skin exfoliants) or form through the breakdown of larger pieces (Wang et al., 2019). Weathering of plastic particles in the environment can occur via microbial action (Yuan et al., 2020), photodegradation by UV rays (McKeen, 2013), chemical action (Liu et al., 2020) and heat (Andrady, 2011; Pischedda et al., 2019; Wang et al., 2019). Microplastics can cause both physical and biotic effects in soil. Physical effects include changes in soil bulk density and structure, which can alter water holding capacity and nutrient availability (de Souza Machado, Lau, et al., 2018). In freshwater systems, MP particles can undergo changes in hydrophobicity and buoyancy, which can make them more bioavailable to certain organisms (Helcoski et al., 2020). Concentrations of MPs in

soil have been observed to vary widely, ranging from < 0.01 items/kg to > 10,000 items/kg (Jacques & Prosser, 2021) and they can be as high as 7% by volume in some of the most polluted top-soils where plastic mulches and biosolids are used (Fuller & Gautam, 2016). However, pinpointing environmentally relevant contamination levels remains difficult at this time, as publicly available large-scale terrestrial monitoring data and standardized quantification campaigns are limited or lacking altogether. In addition, a focus should be on potentially future, higher levels of this contaminant, at least within a global change biology framework (Rillig et al., 2021).

Although much of the study of environmental impacts of MPs in terrestrial systems has been focused on macrofauna (Prokić et al., 2021), such as birds (Azzarello & Van Vleet, 1987) and mammals (Zantis et al., 2021), new evidence shows that smaller soil organisms are also affected (Ren et al., 2021; Wang et al., 2019). For example, as much as 73% of springtails can ingest MPs < 2 µm, which contributes to slowing down their movement (Kim & An, 2020). In another study, three nematode species (*Caenorhabditis elegans* ,*Acrobeloides nanus* and *Plectus acuminatus*) could be seen ingesting MPs < 1 µm (Mueller et al., 2020), and the structure of soil microbial communities can also be affected by MPs (Huang et al., 2019; Zhang et al., 2019).

Protists are a diverse group of primarily microscopic, unicellular organisms that are abundant in both aquatic and terrestrial ecosystems (Adl et al., 2005; Geisen et al., 2018). A single gram of dry soil can contain 10^4 - 10^7 active individuals spanning from photosynthetic (algae), heterotrophic (phagotrophic), and mixotrophic (i.e., those capable of both photosynthesis and phagotrophy) protists. As such, protists are a pivotal, often neglected, component of the soil microbiota playing important roles in carbon and nutrient cycling (Adl & Gupta, 2006, Geisen et al., 2020). Phagotrophic protists ingest a variety of food sources including bacteria, fungi, plants and a range of mesofauna and other protists (Geisen et al., 2018). Many are free living and actively utilize locomotion by way of cilia or flagella to locate and capture prey and other resources, which they ingest through their oral groove into food vacuoles (Verni & Gualtieri, 1997).

Protists can serve as bioindicators of soil contamination. They have been used in toxicological studies as model species, for example to assess metal toxicity of cadmium and zinc (Johansen et al., 2018). Since all phagotrophic soil protist species are transparent, they are excellent candidates for use as indicator species as putative toxic particles can easily be seen internally. Despite this potential and their abundance in soils, the ecology of protists and their role in soil microbial communities is still poorly understood (Rillig & Bonkowski, 2018).

Whether phagotrophic protists can ingest MP is unknown. No papers exist that highlight both MP and soil protists specifically. A search for "microplastic*" on 'Web of Science' on December 3rd, 2020 resulted in 5531 papers. Searching for "Protist*" within "microplastic*" returned only three papers, two of which were focusing on aquatic systems and the other one was a call for research on soil protists and MPs (Rillig & Bonkowski, 2018). The fact that they are an important energy channel in the soil food web means that protists can serve as vectors of MPs to higher trophic levels (Setälä et al., 2014). This hypothesis is plausible considering that other soil microorganisms with equivalent mechanisms of food acquisition such as nematodes have been shown to readily ingest MPs (Kim, Kim, et al., 2020; Lei et al., 2018; Shang et al., 2020). In this study we test the following hypotheses: 1) phagotrophic soil protists ingest MP particles; 2) the number of protists ingesting MPs increases at larger MP concentrations and; 3) MPs reduce the abundance of active phagotrophic soil protists.

Methods

Soil collection and preparation

We collected soil from an undisturbed, forested area located within the Hiawatha Highlands conservation area in the vicinity of the city of Sault Ste. Marie, ON, Canada (46.5841277, -84.2850883) to reduce the incidence of any prior MPs contamination. The area is dominated by Balsam fir (Abies balsamea), White birch (Betula papyrifera) and Sugar maple (Acer Saccharum). We intentionally included leaf litter and the top 5 cm of the O horizon because this is where the abundance of protists is greatest (Adl & Gupta, 2006). We collected 1 kg of field soil and let it air dry at room temperature over the course of five days, allowing time for the protists to encyst. Once dry, the soil was stored in airtight zip-lock bags at 4.

Microcosm preparation and experimental design

Six weeks after the soil collection, we placed 10 g of soil in a 10 cm Petri dish (henceforth microcosm), ensuring that each had similar amounts of leaf and root material present. We repeated this process for a total of 30 identical microcosms and randomly divided them into three treatment groups (n=10). These treatments were prepared as follows: First, we prepared stock solutions of green fluorescent polymer microspheres 1-5 μm in diameter (Item # FMG-1.3, density 1.3 g cm^{-3}, Cospheric LLC, CA, United States) in deionized water (DI) at two concentrations (1 mg mL^{-1} (0.1g MP in 100ml of DI total) and 10 mg mL^{-1} (1g MP in 100ml of DI total)). According to the manufacturer, these "highly solvent resistant" microspheres are made of a thermoset amino formaldehyde polymer that is inert and fluorescent and is "excellent for PVC and other plasticizer applications". Second, we pipetted 10 ml of each appropriate MP solution (or DI water for the control) into each respective microcosm treatment for final concentrations of MPs to soil of 1 mg g-1 (0.1% w/w) and 10 mg g-1 (1% w/w). Despite the scarce availability of data, these concentrations of MPs may be orders of magnitude greater than those estimated to occur in the environment (Jacques & Prosser, 2021). However, this was deliberate to ensure that we could detect MP ingestion. In addition, the selected size of microspheres was consistent with the feeding preferences of soil protozoa (Adl & Gupta, 2006). We considered this the first day of the experimental trial. We shook the MP stock solutions vigorously while pipetting to ensure an even distribution of MP beads with each addition. After adding the MPs, we stirred the soil in each microcosm with a spatula, to ensure their homogeneous distribution into the soil matrix. We rinsed the spatula with deionized water in-between microcosms to avoid introducing MPs. We randomly placed the microcosms in an incubation chamber set to 22 °C and pre-punctured the lids with a needle in three spots to ensure proper gas exchange. Microcosms were randomly placed within the incubation chamber after each time-point assessment of protists. To confirm the reproducibility of the results we ran a second trial using the same procedure as in trial one but including the following treatment groups: no microplastic addition control, and 3 mg g-1 using the same MPs.

Protist abundance

We quantified protist abundance by direct counts of individual free-living ciliated and flagellated protists >30 μm in diameter using the non-flooded Petri dish method (Foissner, 1992). This involved first adding 10 mL of deionized water to each microcosm to bring the soil protists out of encystment and counting protists the following day for a total of 14 days or seven time points (trial 1) and 21 days or nine time points (trial 2). The method recommends eight sampling points at days 2, 4, 6, 10, 14, 20, 25 and 30. Our sampling timeline ensured that temporal declines in abundance were captured. More specifically, the extraction method consists of tilting the microcosm 45° and collecting a small amount of water escaping from the soil. Each time, we collected six individual 4 μl aliquots (i.e., a total of 24 μl per microcosm per time point) directly from each microcosm using a micropipette and placed each aliquot on a microscope slide without a cover slip. We then observed each aliquot for approximately one minute using a Leica DM5500B microscope at 50x magnification under phase contrast microscopy and recorded the total number of protists.

We switched pipettes between samples to avoid cross contamination.

Imaging of MP ingestion

To investigate whether protists can ingest MPs, each time a protist >30 μm was detected using phase contrast we switched to fluorescence microscopy (550 nm) and observed it for an additional minute to look for evidence of MPs (i.e., fluorescent light) within the food vacuoles. More specifically, if we could see the fluorescent MPs traveling through the field of view within the living motile protist's food vacuoles for at least one minute, we counted that as evidence of MP ingestion. We also investigated presence/absence of ingestion in the control group in trial 1 to confirm that other soil particles or the protist's organelles would not fluoresce in any measurable amount. We used the Microscope Software Platform Leica Application Suite (LAS X) (Leica Microsystems Inc., ON, Canada) to record video as evidence of MP ingestion while being careful to exclude any similarly sized organisms other than protists, such as rotifers, tardigrades and nematodes. Still images were captured from video using iMovie 10.2.3 (Apple Inc., CA, USA) and processed in Photoshop CC 20.0.10 (Adobe, CA, USA). We conducted this work on days 10, 12, 14 for all treatments (trial 1) and on days 2 and 6 for the single MP addition treatment (trial 2). We chose these days to capture a variety of time periods and to determine if MPs could be readily ingested.

Statistical analysis

The proportion between protists whose vacuoles showed fluorescence (i.e., evidence of MP ingestion) versus those that did not was compared between MP addition treatments for each separate trial using repeated measures ANOVA for days 10, 12 and 14 (trial 1) and days 2 and 6 (trial 2). The negative control treatments were not included in the analysis because there was no fluorescence detected for any protists in trial 1. Protist abundance was compared across all three (trial 1) and two (trial 2) treatment groups using repeated measures ANOVA with time and MP addition as main factors. The data were arcsin (ingestion proportions) and log (protist abundance) transformed to stabilize the residual variance. All analyses were conducted using JMP 15.2.1. (SAS Institute Inc., NC, USA) and data were plotted using DataGraph 4.6.1. (Visual Data Tools Inc., NC, USA).

Results

Evidence of microplastic ingestion

Evidence of MP ingestion was based on the observation of fluorescence present within the protists' food vacuoles (Figure 1 and Figure 2). None of the protists in the control treatments in trial 1 (a total of 80 individuals across the three sampling times) showed any evidence of MP ingestion (Table 1). In contrast, most protists showed signs of MP ingestion in all the treatments supplied with MPs and this was consistent in both trials (Table 1). In addition, overall, there was a marginally significant effect indicating that soil protists tended to ingest more MPs in the treatment with the highest concentration in trial 1 ($F_{1,9}$=22.29, P<0.0931) (Table 1). The number of protists ingesting MPs did not significantly change over time in trial 1 ($F_{2,8}$=0.05, P<0.813) and declined from day 2 to day 6 in trial 2 ($F_{1,9}$=0.87, P<0.020).

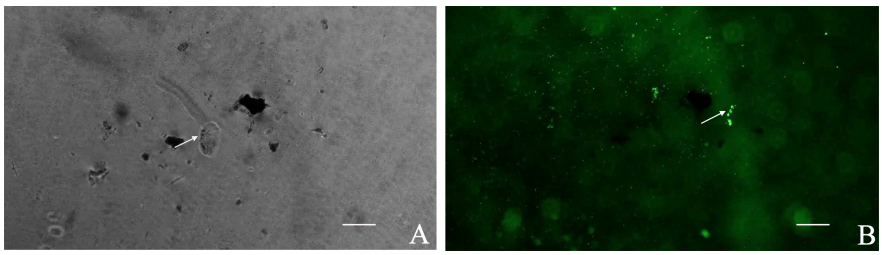

Figure 1: Ciliated protist observed under phase contrast (A) and fluorescence (B) microscopy using 50x amplification on a Leica DM5500B microscope. These images are still frames captured from a video supplied as supplementary material. The arrows indicate the food vacuoles containing fluorescent microplastic spheres 1-5 μm in diameter, and the scale bar is an estimated 50 μm.

Rich media available at https://drive.google.com/file/d/1laR27SuEzUTo5v3sRFhbzrC5prQ3kY_f/view?usp=sharing

	Trial 1*		
Microplastic treatment (mg g^{-1})	Day 10	Day 12	Day 14
0	0	0	0
1	85.2 +/- 11.26	72.9 +/- 10.42	69.7 +/- 12.6
10	100	93.5 +/- 6.25	94.4 +/- 5.55
	Trial 2*		
	Day 2	Day 6	
0	N/A	N/A	
3	100	86.3 +/- 5.5	

Table 1: Percentage of protists that ingested microplastics in the different microplastic addition treatments at different days after the onset of trials 1 and 2. *Values represent the mean +/- standard error of the mean (n=10). N/A – not applicable

Effect of microplastic addition on protist abundance

Overall, across trial 1, MP addition had an overall marginally significant effect on protist abundance (repeated measures ANOVA $F_{2,27}$=0.24, P<0.0540). In addition, protist abundance varied significantly over the course of the trial ($F_{6,22}$=1.50, P<0.0013) (Figure 3A). The treatment with the highest concentration of MPs tended to have the smallest number of protists after day 6 since the onset of the trial. In trial 2, there was no overall difference between the two MP addition treatments throughout the course of the trial ($F_{1,18}$=0.081, P<0.2433) (Figure 3B). However, time had a significant effect on abundance as demonstrated by the significant decline in abundance after day 10 ($F_{8,11}$=59.36, P<0.0001) (Figure 3B).

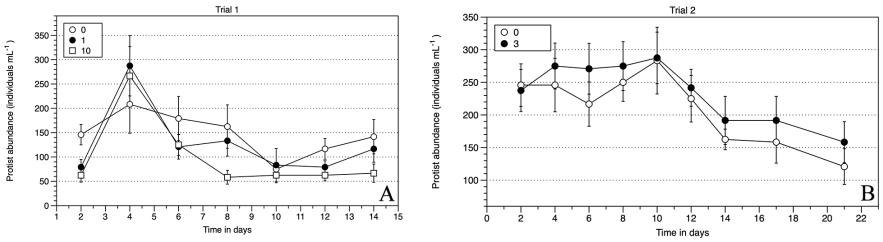

Figure 2: Figure 2. Abundance of protists observed in: (A) Trial 1 with three microplastic addition treatments (i.e., 0, 1 and 10 mg g^{-1}) up to 14 days after the onset of the trial, and; (B) Trial 2 with two microplastic addition treatments (i.e., 0, and 3 mg g^{-1}) up to 21 days after the onset of the trial. Each time point per treatment represents the mean of protists (n=10). Error bars represent the standard error of the mean.

Discussion

This is the first report showing that soil protists can ingest MPs and store them within their food vacuoles. In addition, our observations using fluorescence microscopy clearly showed that the majority of large (>30 μm) phagotrophic protists could readily ingest MPs in any MP addition treatment. This finding was robust because green fluorescence (550 nm) was restricted to MP addition treatments, showing that any background fluorescent MPs were not detectable and that the protists' organelles are not fluorescent at that wavelength. Approximately 75% of protists ingested MPs in the treatment with the lowest concentration MPs and a 10-fold increase in MP concentration resulted in nearly all protists ingesting MPs. These findings can have profound ecological and environmental implications given the important role that protists play in the soil food web (Adl & Gupta, 2006). Motile phagotrophic protists can serve both as vectors in moving MPs in the soil matrix and in transferring them to higher trophic levels, thereby potentially amplifying MP pollution (Geisen et al., 2018, 2020). Furthermore, we could observe several ciliate and flagellate morphotypes, which suggests that the feeding behavior for MPs may not be species specific. Conversely, we anecdotally observed that some protist morphotypes seemed to show a greater propensity for ingesting MPs than others as indicated by the intensity of the fluorescence within their food vacuoles. Certainly, more experimentation is required to understand if protists show morphotype or taxa specific feeding behaviors regarding MP ingestion.

The results of trial 2 show that soil protists can readily ingest MPs in as little as 24 hours after these are introduced. In addition, in the treatment with 1% MPs (w/w) nearly all protists ingested MPs. This is consistent with data for other soil biota such as bacterial-feeding nematode species using similar methods with fluorescent microspheres (Mueller et al., 2020). This technique could potentially be utilized in future experiments to quantify MP ingestion and to source track MPs.

We detected a significant overall temporal difference in protist abundance in both trials across treatments, which is explained by declines after approximately a week. This was expected since microcosms are closed systems. However, the hypothesis that the overall abundance of phagotrophic soil protists would be reduced by the addition of MPs particles to soil was not strongly supported by the data. Nevertheless, MP addition had a marginally significant effect on protist abundance in trial one. These results are interesting given that most protists were observed carrying MPs within their food vacuoles which would indicate, at least within short-time scales, that they may not be detrimental to the organisms' overall health. This is consistent with studies showing that MPs do not seem to cause considerable mortality in earthworms at environmentally relevant concentrations. However, mortality has been reported at relatively high concentrations (Jacques & Prosser, 2021). Likewise, the nematode *Caenorhabditis elegans* appears to be highly susceptible to high concentrations of MPs. In contrast, other species of nematodes may be more tolerant to MPs of similar

polymer composition to those used in this study (Mueller et al., 2020). It is possible that the commercial microspheres used in this study are indeed inert and their ingestion may not substantially reduce the protist's food intake. More research using different soils and a wider diversity of MPs types (e.g., varying the parameters shape (Lozano et al., 2021), polymer type (Waldman & Rillig, 2020), weathering status and additives (Kim et al., 2020)) will be required to clarify this.

If MPs particles were in fact safe to soil protists, given their high abundance in soils and important role as nutrient cyclers (Wood & Bradford, 2018), perhaps they may be able to further physically breakdown MPs. However, while they could probably use some of the additives, debris, or biofilms in the plastic particles it is unlikely that protists would be able to utilize the long carbon-backbone chains that makeup most plastics. The fact is, the MPs used in this experiment, although utilized in other MP-biota interactions research (Bringer et al., 2020), do not represent the average MP particle found in soil (Wang et al., 2019). Future studies could utilize other MPs commonly found in soil environments, such as polyacrylic fibers or polyethylene fragments. However, appropriate MP detection methods for those polymers (Shan et al., 2018) would be required in combination with methods to extract protists from soil and sediments (Alongi, 2018). Overall, our results show that large phagotrophic protists appear to have the ability to ingest MPs. More research is needed to verify to what degree MPs can in fact affect the abundance and community composition of soil protists and understand the effect of MPs on soil food webs.

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