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Exposure to nanoplastics disturbs the gut microbiome in the soil oligochaete *Enchytraeus crypticus*☆

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ABSTRACT

Microplastics are emerging pollutants that have recently aroused considerable concern but most toxicological studies have focused on marine biota, with little investigation of the influence of microplastics on terrestrial ecosystems. Here, we fed the soil oligochaete *Enchytraeus crypticus* with oatmeal containing 0, 0.025, 0.5, and 10% (dry weight basis) nano-polystyrene (0.05–0.1 μm particle size) to elucidate the impact of microplastics on the growth and gut microbiome of *Enchytraeus crypticus*. We observed a significant reduction of weight in the animals fed 10% polystyrene and an increase in the reproduction of those fed 0.025%. More importantly, using 16S rRNA amplification and high-throughput sequencing we found a significant shift in the microbiome of those fed 10% microplastics with significant decreases in the relative abundance of the families Rhizobiaceae, Xanthobacteraceae and Isosphaeraceae. These families contain key microbes that contribute to nitrogen cycling and organic matter decomposition.

1. Introduction

Many polymers are used for a relatively short time because they are used to manufacture single-use disposable products. Hence many plastics are discarded into the environment relatively quickly and comprise 54% (by mass) of the human waste released to the environment (Hoellein et al., 2014). Although biodegradation of plastics does occur, these artificial polymers are extremely resistant to degradation in the environment and the majority of plastic wastes persist in the environment and become pervasive and persistent pollutants (Thompson et al., 2005). Plastic debris is classified into multiple types according to particle size with particles <5 mm defined as microplastics (Duis and Coors, 2016; Horton et al., 2017; Thompson et al., 2004). Under various forces, such as mechanical abrasion, microplastics will eventually be weathered into nanoplastics with diameter <1 μm. (Mattsson et al., 2015). UV radiation can also further modify the plastic particles and release the embedded materials. Representative microplastics include chemicals such as polystyrene that are often ubiquitous in marine samples (Hidalgo-Ruz et al., 2012) and are widely used in personal care products (Fendall and Sewell, 2009).

Much of the research on microplastics to date has focused on the marine environment and studies involving planktonic, benthic, and pelagic food webs have demonstrated effects of microplastics on marine ecosystems (Huerta Lwanga et al., 2016). However, knowledge of how microplastics influence the terrestrial ecosystem is limited and very few studies have quantified the risk of microplastics in soil ecosystems. This is despite the fact that a very large proportion of plastics are used and disposed of on land the terrestrial ecosystem is therefore greatly exposed to microplastics (Horton et al., 2017). Land application of sewage sludge or municipal wastewater can introduce considerable amounts of microplastics into soils. For example, approximately 4000 microplastic particles kg^{-1} of dry sludge (~0.04%) were found at

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agricultural and landfill sites in Europe (Magnusson and Norén, 2014; Mahon et al., 2016; Mintenig et al., 2017; Zubris and Richards, 2005), likely derived from synthetic fibers and small polymer particles from cosmetic products, household products and personal care products (Habib et al., 1998; Zubris and Richards, 2005). The fragmentation of agricultural plastics including plastic films covering polytunnels and plastic films used as mulches to control agricultural soil moisture and temperature, and fragmentation of plastic seed coatings used to regulate germination is another source of microplastics in terrestrial systems (Clayton et al., 2004; Kasirajan and Ngouajio, 2012; Kyrikou and Briassoulis, 2007). Plastic debris remaining on the soil surface is more exposed to UV light than polymers in the marine environment, leading to an elevated release of microplastic particles to terrestrial ecosystems.

Due to the size of microplastics/nanoplastics, soil fauna may ingest them when feeding. Decreased fecundity and growth and many other adverse physiological effects have been observed in a range of organisms exposed to microplastics (Browne et al., 2008; Graham and Thompson, 2009; Huerta Lwanga et al., 2016; Sussarellu et al., 2016; Wright et al., 2013). Studies indicate that the detrimental effects are mostly due to physical harm such as internal abrasion and blockage (do Sul and Costa, 2014). Other investigations also point out that nanoplastics can enter the circulatory system (Browne et al., 2008) or cross the gut wall and be translocated to other tissues in mussels (Farrell and Nelson, 2013), leading to other potential pathways that can induce additional detrimental effects. However, the hypothesis that ingestion of microplastics can alter the gut microbiota of animals remains to be investigated. Some studies have found that small particles can impose a strong influence on gut microbial community structure. For example, nanosilver particles can shift the murine gut microbiota structure (Wilding et al., 2016), the gut microbiota of zebrafish was disrupted by metal nanoparticles (Merrifield et al., 2013) and dietary ZnO nanoparticles altered the intestinal microbiota in weaned piglets (Xia et al., 2017). Therefore, given that microplastics are emerging pollutants in terrestrial ecosystems and that the gut microbiota of soil fauna plays a key role in soil decomposition processes (Toutain, 1987; Van Vliet et al., 1995; Zhu et al., 2017a,b), it is important to investigate whether or not microplastics can affect the gut microbiota.

Soil invertebrates provide valuable services that sustain soil quality (Zhu et al., 2016a, 2016b). They are key players in the mineralization, decomposition and distribution of soil organic matter, release of mineral-N and storage of nitrogen (Höfer et al., 2001; Lepage et al., 2006). However, until recently few studies have recognized the important role played by the gut microbiome. Some studies have indicated that many bacteria in the gut of soil fauna have obtained new metabolic functions such as nitrogen fixation to adapt and reshape terrestrial ecosystems (Zhu et al., 2018). We therefore selected *Enchytraeus crypticus*, a soil oligochaete that plays an important role in nutrient turnover, organic matter decomposition and soil structure in reshaping the terrestrial ecosystem (Van Vliet et al., 1995) and can be regarded as a representative animal in soil ecosystems, to investigate effects of different levels of nanoplastics exposure. Since long-term exposure is time-consuming and the microbiota may respond really quickly to pollutants, we adopted short-term exposure (7 days) in this study. Physiology changes of *Enchytraeus crypticus* were determined. In addition, 16S rRNA gene amplification and high-throughput sequencing were used to investigate how the gut microbial community was affected by exposure to nanoplastics.

2. Materials and methods

2.1. Experimental work

Individuals of *Enchytraeus crypticus* were kindly donated by staff of Aarhus University in Denmark. The worms were raised in *E. crypticus* medium (2 mmol $CaCl_2$, 1 mmol $MgSO_4$, 1 mmol $NaHCO_3$, 0.1 mmol KCl, and 17 g agarose L^{-1}, pH 7.8) and fed with oatmeal (Pepsi Food Co., Beijing, China). The stock culture was maintained in the laboratory for more than two years following the OECD guidelines (OECD, 2004). The experiment was conducted using Petri dishes (diameter 3.5 cm) containing *E. crypticus* medium. Ten sexually mature *E. crypticus* of similar length were randomly selected from the stock culture and transferred to each Petri dish. Latex bead polystyrene (Klamar Co., Shanghai, China) was chosen as a representative nanoplastic in the environment. The polystyrene beads ranged in diameter from 0.05 to 0.1 μm and were prepared as an aqueous suspension (2.5% w/v). Oatmeal was mixed with the polystyrene suspension and in accordance with similar toxicological studies on nano-polystyrene (Huerta Lwanga et al., 2016), four different weight percentages of the nanoplastic particles in the oatmeal-polystyrene mixture were used, 0, 0.025, 0.5 and 10%. The mixtures were prefrozen at −80 °C and then dried using the vacuum freeze dryer. After drying, the mixtures were ground to a powder and kept at −20 °C until use. There were six replicates of each concentration, giving a total of 24 Petri dishes, each with 20 mg of one of the powders and ten *E. crypticus* individuals which were stored in a thermostatic box (Safe Co., Ningbo, China) at 18.5 ± 2 °C and 75% relative humidity (RH) with a 16:8 h dark:light, 800 lux diurnal regime for seven days.

2.2. Collection of reproduction and growth data and detection of nanoplastic particles

The reproductive ability of the animal was evaluated by the number of cocoon produced during the seven days. Growth was assessed by recording the total weight of worms in each petri dish at the beginning and the end of the experiment. Each individual was gently washed with sterile water and then carefully dried with paper tissue to remove the medium and food remains on the surface of body before weighing. Considering the death of worm at the harvest time, we finally compared the average weight increase per individual worm. The average weight of individual worm in each petri dish was obtained by dividing number of worms by the total weight.

After the collection of reproduction and growth data, worm samples of each replicate were divided into duplicate. A part was used to detect nano-plastic particles, and the other part was used to analyze microbial community of worm. Before the detection of nanoplastic particles, the worm samples were carefully washed by ultrasonic 30 s and rinsing three times with ultrapure water to remove all particles on the surface of the worm. The fluid which has washed worms was detected by the field emission scanning electron microscope (SEM, S-4800, Hitachi, Japan), and we have not found nanoplastic particles in the last wash. The cleaned worms including four treatments (0, 0.025, 0.5 and 10%) were respectively digested using sodium hydroxide and nitric acid according to the method of Roch and Brinker (2017). The 0% treatment (*E. crypticus* without exposure) was selected as control to exclude the outside contamination of tissues. The detected liquid was driped on the aluminum foil and dried at 50 °C. The obtained samples were mounted onto an epoxy resin and sputter-coated with a thin layer