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Root System Response of Soybean to Microplastics of Varying Types and Concentrations

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By

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Root System Response of Soybean to MP of Varying Types and Concentrations

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ABSTRACT

Biosolid fertilizer use causes the input of microplastics into the rhizosphere where they may interfere with root interactions with the soil and microbial community and thereby hinder plant acquisition of nutrients and water. However, it is unclear what microplastic concentration or types affect plant root system and when these impacts manifest. Using the rhizobox method, soybeans were grown in soil was dosed with microplastic mimics (PET sheets and PP beads at 2,000 and 15,000 particles / kg dry soil) and biosolids. A time-series analysis was conducted on plant root traits using non-destructive imaging with an Epson 12000xl scanner of the entire plant root system on a weekly basis until maturity. Microplastic concentration was positively correlated with total soybean biomass and the ratio of above to belowground biomass was statistically different relative to the control at the highest microplastic concentration (Tukey HSD $p<0.05$). Root systems of longer length occurred in the microplastic treatments, with concentration positively correlated with root length and PP treatments having higher root length than PET. Time-series analysis by root diameter (3 classes, 0-0.5, 0.5-1, >1mm) revealed higher portions of the total root length were thinner roots and that the root systems of the microplastic treatments grew at a faster rate until saturation whereas the control continually grew. Linear regression predicting root length based on week number suggested that 0-5mm roots grew at a rate of 811.8mm per week in the control versus 1,186.7 and 1,593mm for PET at 2,000 and 15,000 particles/kg dry soil respectively. Overall, microplastics increased the root biomass, root surface area, and duration of contact which overall increase the interface between roots and soil.

DEDICATION

I would like to dedicate this thesis to my mother, Rahma, and my siblings, Ahmed, Zaki, Khadija, and Zeinab. Thank you for always being there for me and somewhat willingly listening to my ranting and raving over the past two years. I especially dedicate this to Ahmed, though you are not here to see me graduate, I love you and I hope I am making you proud every day.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 Thesis Overview

Biosolids are an affordable source of nutrients that can be used as a soil amendment in agriculture (Crossman, Hurley, Futter, & Nizzetto, 2020), however, their application introduces microplastics whose ecotoxicity to the root-soil-microbial system is largely unknown (Guo, et al., 2020). There is a growing body of evidence that microplastics (MP) pose a risk to agroecosystems and can adversely affect soil physical, chemical, and microbiological properties, and consequently have a strong influence on the traits of plant root systems (Boots, William Russell, & Senga Green, 2019). Many studies have reported an increase in root biomass or decreases in root biomass when grown in soil amended with microplastics (Machado, et al., 2019) (Qi, et al., 2018). Polystyrene MP were also ecotoxic to the legume *Vicia faba*, and there are also concerns that MP could leach toxic additives into soil, or MP could facilitate the transfer of biosolid antibiotics and heavy metals to the root surface via water uptake (Jiang, et al., 2019). The wide range of studies suggesting direct toxicity or indirect alteration of soil physiochemical structure can be attributed to the diverse nature of MP properties and the multitude of mechanisms that affect plant growth. However, there are few studies that attempt to mimic real world MP properties (e.g., concentration, types, and size fraction) of the agroecosystems utilizing biosolids in their nutrient management plans. There also remain several gaps regarding MP effects on root system architecture (the spatial configuration of the root system and its features like root length, branching

frequency, number of lateral roots, among others). Although root biomass is an important metrics, other root traits such as root length, the portion of fine roots, root growth rates, and root development are better proxies for the interactions between roots, the soil, and the rhizosphere microbial community (Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes).

Root system architecture (RSA) encompasses the rooting strategies and behaviours of plants as well as their physical characteristics that allow them to respond dynamically to environmental change to better acquire nutrients and water (Mi, Chen, Yuan, & Zhang, 2016). RSA also responds to toxic soil contaminants, which induce considerable changes in root system architecture and root growth patterns, with plants avoiding contaminated areas and seek out non-contaminated areas for colonization (Remans, et al., 2012). Studies focused on plant root interactions with abiotic contaminants found that stress-induced morphogenic responses typically included reduction in primary root elongation compared to uncontaminated soils, and increased density of lateral root growth (Potters, Pasternak, Guisez, Palme, & Jansen, 2007) and the number of lateral roots (Betti, et al., 2021). In particular, heavy metals have been found to inhibit cellulose production, which reduces overall plant biomass (Khan & Frankland, 1984). Aside from their general toxicity, specific heavy metals affect plant root systems by different mechanisms, such as the production of reactive oxygen species or by interfering with nutrient uptake due to their similarity to essential ions. For instance, lead significantly affects nutrient uptake which can delay the plant

developmental process, while cadmium limits the activity of many enzymes which leads to increased number of root tips, increased root diameter, and cell walls thickened with suberin (Shahid, et al., 2015) (Sabella, et al., 2022).

Although changes in RSA have been linked to the mode of action of specific toxins, root toxin mitigation strategies vary in relation to toxin concentration. At low concentration root systems may combat toxins through exudation of detoxifying organic acids (Role of root exudates in metal acquisition and tolerance). At higher concentrations, roots may exhibit conservation strategies, alter root diameters, and change the ratio of absorption to transport roots. Only above certain concentration thresholds of heavy metals did aboveground and belowground biomass decreases, and the decrease was more severe with increasing concentration (van Dijik, Kranchev, Blust, Cuypers, & Vissenberg, 2020). At large enough concentrations, heavy metal effects on roots can be quite large. In an analysis of 19 studies, copper, cadmium, and zinc contamination was found to decrease primary root length between 0-100%, with the average reduction being 47.75%, with a standard deviation of 25.23% (van Dijik, Kranchev, Blust, Cuypers, & Vissenberg, 2020). Heavy metal effects can further be specific to certain root types. Stressors caused by copper, cadmium, and zinc contamination on the soil can also inhibit the growth of primary roots, as well as decrease the density of lateral roots. Lateral roots are often exploratory and nutrient-seeking in nature and their reduction may indicate that exploration is reduced as it poses risk to the plant system due to increased contamination (van Dijik, Kranchev, Blust, Cuypers, & Vissenberg, 2020). However, a contrasting study related to lateral root

growth in the presence of heavy metal contamination found that lateral root formation increased in soils contaminated with lead, chromium, zinc, and cadmium (Riyazuddin, et al., 2021). This increase in lateral root growth in turn decreases the specific root length for the entire root system as more of the belowground biomass is included in the thinner lateral roots rather than the thicker primary roots (Riyazuddin, et al., 2021). The complexity of toxic effects on roots suggests that biomass alone maybe an insufficient metric to gauge MP affects, especially as at low concentrations where MP affects maybe subtler or only afflict a portion of the root system.

RSA is dependent on soil properties, meaning MP alterations to soil properties affects both the total biomass and other traits such as root length and diameter (Machado, et al., 2019). In a pot experiment using common bean, RSA changed alongside increasing MP concentration with LPDE increasing root biomass and bioplastic reducing root biomass. A meta-analysis by Qie et al. (2022) found that MP in soil promoted root biomass with an effect size (lnRR) of 0.1235 (95% CI: 0.059 to 0.4, p = 0.003), suggesting that MP have a positive effect on root biomass in general. Plastic films also had a stronger effect size than fibres or fragments on root biomass, possibly due to improved water holding capacity (Soil microplastic characteristics and the effects on soil properties and biota: A systematic review and meta-analysis), Root biomass is not the only RSA trait affected by MP. In the Meng et al. (2021) study, specific root length (cm/g) was up to 1.36 times higher in MP treatments (i.e., 20,047 ± 989 cm/g in the 2.5% LDPE-MPs treatment versus 16,604 ± 1082 cm/g in the control treatment and 22,550±1816 cm/g in the 2.0% bioplastic treatment versus 16,604 ± 1082 cm/g in the

control treatment) suggesting the roots were getting narrower, or the portion of thin absorptive roots to thicker transport roots had shifted towards thinner roots. Shifts towards more thin roots are likely to increase the root surface area or interface area with soil, and thus amplify the influence of roots on soil physiochemical properties. As few studies examine root traits beyond biomass, there is limited evidence for effects of specific MP properties, albeit plastic chemical composition has been shown to influence root length and diameter (Machado, et al., 2019). Since MP influence multiple RSA components, methods that concurrently capture the entire root system are required to fill in data gaps and evaluate MP effects on root systems from a functional perspective.

Most studies investigating MP effects on plant roots have utilized destructive harvesting and root washing. Typically, plants are seeded in pots and allowed to grow before harvesting them. The harvesting process requires the plants to be removed from the soil, the roots to be carefully separated and washed to remove all remaining soil before drying. This process is known to be more damaging as the washing and separation stages allow for great fragmentation of root segments and large amounts of fine root loss (Oburger & Jones, 2018). Common alternatives such as hydroponics are rarely in studies involving microplastics as the suspensions of microplastics in hydroponic tanks requires many steps including ultrasonic ice baths, and intermittent 'dunking' in MP-laced water (Dong, Gao, Song, & Qiu, 2020). The arduous and damaging nature of both of these methods led to the creation of the rhizobox method, where plants are grown in thin, long planters with one side having a clear face to view roots during the entire growth period (Luster, Gottlein, Nowack, & Sarret, 2008) .The planters are often set at

an angle to encourage root growth against the imaging face. The first use of the rhizobox method was in 1983, with a study on root-induced pH changes with regards to different nitrogen sources (Marschner & Romheld, 1983). Rhizoboxes are a flexible method to investigate many belowground processes (e.g., Schmidt, Lowry, & Gaudin, 2018) and have proven to be a reliable means to image whole root systems and derive RSA traits. Since it is non-destructive, time-series imagery of root system development can be acquired. In this study, extra-large rhizoboxes were employed to monitor root growth from seeding to maturity on a weekly imaging schedule to quantify RSA components and maximize the accounting of fine roots which are the predominant means for plants to interact with the soil and microbial community.

Previous MP studies typically use concentrations which are not found in the agroecosystem, with up to 10% of soil dry weight being comprised of MP (Meng, Yang, Riksen, Xu, & Geissen, 2021) whereas soils amended with biosolids have concentrations three to four orders of magnitude lower. Previous studies have found that some of the most MP contaminated soils in Sydney, Australia from the city's biggest agricultural area had a contamination range of 0.03% MP to 6.7% in seventeen soil samples with an average of 0.3% (Fuller & Gautam, 2016). Few studies have investigated MP effects on plant root traits at concentration ranges that plants would be exposed to through biosolid application, and fewer studies have attempted to mimic biosolid MP properties such as the size fraction proportions. There has also been little effort to distinguish MP effects on specific roots such as fine absorptive and thick transport roots. Studies with targeted methods that explore these gaps in knowledge are extremely important as

more is learned on the amount of microplastics that find their way into soil, especially as applications of biosolids directly place these MPs into contact with plants with no knowledge of their effects on these roots and the long-term health of the soil ecosystem.

This project as a whole was intended to provide scientific evidence to help inform biosolid policy for government agencies. This investigation was centered on the impacts of MP concentration, types of plastic (form and chemical composition), and the influence of biosolid MPs on soybeans root systems in agricultural soils. This study grew soybeans in controlled growth conditions with 6 soil treatments comprised of a control, four MP lab-created mimics (PET and PPE at 2,000 particles and 15,000 particles per dry soil kg) homogenized throughout the soil, and a biosolid treatment where biosolids were mixed into the top 15cm of soil at typical application rates (i.e., 100 wet tons/acre). Plants were grown for 11 weeks until maturity at which point, they were harvested, and the biomass of different organelles measured. To investigate differences at the whole root system scale, root to shoot biomass and root length from snapshot imagery was investigated in relation to MP concentration, type, and the biosolid treatment relative to the control at plant maturity (Chapter 2, see below). To provide a more functional assessment of differences in RSA as a function of concentration, type, or biosolid application, a time-series analysis on the root-system imagery was performed. First, individual roots were classified into diameter classes that roughly align with absorption versus transport function. Second, growth rates were estimated from linear or linear piecewise regression depending on whether during the experiment root

system development experienced a switch from the foraging to senescence phase (Chapter 3, see below). This research has implications for policy makers as biosolid fertilizer use is likely to increase and recommendations on policy to inform how MP contamination may have harmful effects has become a mainstream issue. Knowledge of whether root system imaging suggest that biosolid MP have a toxic effect on root system or affect the soil properties that influence RSA are important for farmers to assess whether the use of biosolids as a fertilizer is warranted and whether any changes in RSA are liable to incur short or long-term repercussions.

1.1 Biosolid Usage and MP Concerns

One of the main pathways for MP to enter agricultural soils is through biosolids applied as a nutrient source. Biosolid usage is increasing as it is an inexpensive and environmentally friendly fertilizer, with previous study showing that repeated use can increase organic matter content within soil by up to 60% (Sullivan & Matthew, 2015). The MP load in soils of agricultural and horticultural sites exposed to sewage sludge and mulching film has been documented as averaging 13,000 pieces in 1 kilogram of dry soil (Büks & Kaupenjohann, 2020). Other studies have stated that farmed soils in North America could have 300,000 tonnes of MP added annually through biosolid applications (Nizzetto, Futter, & Langaas, 2016). The quantities of applied biosolids are sufficient to change the properties of soil, such as soil moisture retention in the case of plastic sheets, through the addition of MP as well as the nutrient-rich properties of the biosolids themselves (Gravuer, Gennet, & Throop, 2019). The addition of this contaminant at such high doses with little study of what is considered the average

amount of MP contamination in soil lends itself to uncertainty surrounding the sustainability of the practice of applying biosolids.

Textile production and daily use of plastic-based textiles, such as polyester clothing, can shed millions of tons of MP annually (Carr, 2017). Many clothing items are laundered, and the shed MP make their way into water treatment facilities. There they are partially filtered out as a part of the treatment process, with wastewater treatment facilities utilizing oxidation ditch filtration removing 97% of MP from influent and membrane bioreactor systems removing 99.5%, but this filtration and removal still concentrates the MP through their effluent into what is further processed to become biosolids (Lv, et al., 2019). The use of engineered systems like wastewater facilities to remove and concentrate waste during purification leads to the concentration of MP in their effluent by-products such as biosolids has been a common practice for many decades.

Many areas have combined sewer and stormwater collection systems that allow MP collected in stormwater to end up in biosolids (Beni, et al., 2023). Tire wear on roads is one of the most common forms of MP found in the terrestrial environment and can form the majority of what is known as ‘road dust’ in many areas (Jarlskog, et al., 2020). Previous studies have found that the ambient aerosol surrounding well-trafficked roads can contain up to 90% traffic-related abrasion particles (Sommer, et al., 2018). Of the most found MP in environments, some studies have found that tire wear particles are the second most abundant source of plastic contamination (Leads & Weinstien, 2019). Tire wear particles are also significantly linked to leachate due to the rubber additives,

and these leachates can cause teratogenic, mutagenic, and estrogenic effects (Wik & Dave, 2009). As such, it is extremely difficult to isolate MP from agricultural soil and to isolate the impacts of just one type of MP is unrealistic as there are likely man other types present in the soil from decades of tire wear, atmospheric deposition, and many different pathways which contribute many types of plastic.

The creation of biosolids, which contain a concentrated amount of MP as a by-product of water treatment, allows for a wide variety of sizes, shapes, and types of plastic to be present in soils amended with biosolids. MP quantified in cattle manure were found to be 44% fragments, 39% fibres, 10% films, 5% beads, and 2% foam with 34% comprised of polyethylene, 17% polyethylene terephthalate, 13% polypropylene and six other plastics each less than 8% (Meng, Yang, Riksen, Xu, & Geissen, 2021). However, biosolids from a wastewater treatment plant in Queensland, Australia was dominated by polyethylene terephthalate (43%), then polyethylene (19%), and polyamide (17%) and only six plastics were detected (Rashti, et al., 2023). The variation of MP in biosolids is tied to the varying ways these MP enter wastewater treatment plants, such as washing plastic textiles during the laundering of clothes, atmospheric deposition, and road dust entering storm drains.

The various morphologies of the plastics that can be found in soil vary and these different types can have a range of contamination effects. Soil properties can be changed with the inclusion of MP. The varying shapes of MP have an impact on what exactly changes in the soil properties, such as pore space, aggregation of soil, and soil moisture. In a study conducted on spring onion using MP size fractions between 200 to

800um created by freezing plastics reduced in size using cryomilling before grinding in a centrifugal mill, the variance in spring onion roots was related to the different types of plastics (Machado, et al., 2019). The polyester fibers used were added in at 0.2% fresh weight while the remaining five types were added in at 2% soil fresh weight. The results found that only polyamide plastic did not increase root biomass while all other plastic types increased it, and the strongest increase was found in polyethersulfone and high-density polyethylene. These two plastic types also increased the total biomass of the entire plant system.

Plastics in soil and their varying forms, such as fibres, fragments, and sheets, can change soil properties, and these known effects on soil properties are sometimes utilized by farmers in their purposeful addition of plastics like sheets. Plastic sheets are commonly used in agriculture as a vapor barrier, preventing soil moisture loss (Boots, William Russell, & Senga Green, 2019). MP size can also affect the likelihood of ingestion and can cause injury during digestion, inhibited growth rate, and reproductive stress (Xu, et al., 2020). As MP degrade and a turned into smaller plastics through photochemical or physical breakdown, the smaller size fractions are more readily up taken by soil-based invertebrates which are key to the nutrient cycle (Koutnik, et al., 2021). As an MP in the soil, sheets may act as similar barriers which could interrupt the flow of water in the soil column, clog macropores, and could even trap water. Unlike flexible sheets, plastic fibres are linear, hold their shape and are comprised of harder plastics. When blended into soil matrices, they reduced the surface area and prevented moisture loss in soils (Rillig, Ingraffia, & de Souza Machado, 2017). The opposite was

true of MP films which have been found to facilitate artificial pore formation associated with soil moisture loss and soil density reduction (Lehmann, Leifheit, Gerdawischke, & Rillig, 2021). However, some studies have shown that the increase of plastics in soil can eventually reach a threshold where further applications of fibres can negate positive soil aggregation caused by soil biota (Lehmann, Fitschen, & Rillig, 2019).

Determining whether MP are a rhizosphere contaminant is very challenging given the wide variety of MP properties and their combined positive and negative effects. The earliest studies on MP in soil tended to focus on the novelty of their presence and were used to explore the positive impacts of plastic in soil, such as through plastic mulching in later years, and few studies explored the many types of plastics and how their intentional addition to the terrestrial environment may effect soil fertility (Carpenter & Smith, 1972). Furthermore, many studies use short-term growth experiments which do not account for the ever-increasing plastic pollution and that plastics will further degrade in the environment. There is also a great variety of plants grown in these contaminated soils, which have varying needs and rooting behaviours that make a definitive study on these MP-rhizosphere interactions complex. In attempting to isolate what effects MP have on plant roots and the rhizosphere, there are many factors to take into consideration, such as root exudation, nutrient availability, soil fertility, and pollutant immobilization responses (Bouaicha, et al., 2022).

There is an inherent difficulty in studying RSA, especially as many plant species have varying RSA and vastly different root systems sizes (Reubens, Poesen, Danjon, Geudens, & Muys, 2007). Furthermore, the thinnest roots are the most fragile and

hardest to quantify. For example, in American tree species, it has been confirmed that most lateral roots (the root type primarily involved in uptake) are less than 0.5mm in diameter, yet this size fraction can account for up to 75% of total root system length, despite accounting for less than 20% of belowground biomass (Pregitzer, DeForest, Burton, Allen, & Ruess, 2002). Methods of quantifying RSA all have considerable drawback, although non-destructive methods are preferred as extraction of plants from soil has considerable risk for losing fine roots (Luster, Gottlein, Nowack, & Sarret, 2008). Most MP studies on root systems use root washing, which has been known to cause underestimation of root segments (Norvel & Cary, 1992). To accurately represent the growth of root systems and obtain information on changes in RSA during growth, rhizobox approaches allow for a non-destructive view of roots during growth. The rhizobox approach, while beneficial, is not without its drawbacks; its non-destructive window allows for imaging to be done without disturbing plants during growth, but its shallow depth still may allow roots to grow inside the soil substrates rather than against the clear face. This may be reduced through growing plants in rhizoboxes at an angle that, while not so steep as to effect growth of aboveground biomass, is enough to encourage roots to grow directly downwards, and come into contact with the clear acrylic imaging surface. While not all roots are guaranteed to be visible, during the analysis phase a normalization of results can be undertaken to ensure that the roots being include in the statistical analyses are reflective of the actual root biomass.

The rhizobox approach is non-invasive and provides a chance to explore roots over time rather than at a single point during harvesting. In imaging as part of a time-

series, root growth can be understood as it relates to the various growth phases of plant roots, with plants reaching maturity and increasing their exploratory root-growing capacity. There are few studies with time-series data on root traits in relation to MP exposure, despite some evidence the severity of MP impacts manifest at different life stages. In a pot experiment, root biomass of Glycine max grown Loess soil with biodegradable mulch film was statistically different from the control only at the flowering stage (~60 days), but not at the harvesting stage (~120 days)) (Li, Huang, Haoming, & Lie, 2021) . Furthermore, root systems undergo unique development phases, from early emergence and development of the root apical meristem, to foraging, to senescence and eventual cell death, with each phase characterized by unique processes and interactions with the soil. During foraging, root exudation and nutrient uptake are at their highest and other growth associated processes soil such as root border cell loss and mucilage secretion are higher. Differences in the quantity and quality of root exudation are not the only significant factor shaping the rhizosphere, the duration of root exudation increases the selective pressure the root exerts on the rhizosphere microbial community, with Dennis et al. (2010) arguing that a growing root has a lesser influence on rhizosphere microbial communities as exudation occurs primarily at root tips which can pass through soil quickly during foraging. Hence, a root that has ceased growth has greater capacity to shape the rhizosphere microbial community. However, as the majority of the MP studies have utilized destructive sampling there is little documentation of MP influence on root system foraging and senescence phases, especially for the fine root fraction which is the portion of the root

system primarily involved in interactions with soil through uptake of water and nutrients and carbon loss through exudation.

1.2 Thesis Objectives

This study provides a unique opportunity to determine if real world MP biosolid concentrations, plastic types, or top biosolid application affects root biomass and metrics of RSA such as root length and root diameters derived from time-series imagery. The experimental design using biosolid mimics is novel and could help resolve the conflicting findings in the literature which have suggested microplastics can act as a soil contaminant with toxic effects, or a soil amendment that changes soil properties with potentially beneficial effects. The objective of this thesis is to conduct dose-response study using biosolid mimics and direct biosolid application that will grow soybean plants from seed in rhizoboxes with imaging of the root system and biomass quantification at plant maturity. Treatment differences in root biomass and root length due to MP concentration, plastic type, and biosolids was assessed by classifying the imagery into root and non-root classes, and quantifying RSA traits from the binarized imagers over time.

The study was carried out in a controlled environment facility that grew soybeans for 11 weeks in 18 rhizoboxes with weekly imaging of the complete root systems. The treatment groups included a control with no MP addition, four biosolid mimic treatments; Polyethylene terephthalate (PET) in sheet form and polypropylene (PPE) in fragment form at two concentrations, 2,000 and 15,000 particles/kg dry soil. These MPs are then homogenized in the soil, with the final treatments having direct biosolid

application to the top 15cm at an application rate of 100 wet tonnes/acre. Rhizobox soil moisture content was monitored in one representative box per treatment and water was conducted weekly to keep the soil moisture at the percentage at the start of the experiment. Weekly imaging was conducted at 600dpi using a vertical mounted scanner. Scanned images were processed into a binarized version with all pixels belonging to a root given a unique class. Metrics of RSA were quantified using a standard processing protocol.

Chapter 2 focused on laboratory-based assessments of mature soybean root systems. The objective of this chapter was to assess whether biomass and total root system length differed due to MP of different types, concentrations, as well as biosolid MP relative to the control. The soybean plants were grown for 11 weeks with imaging being completed weekly for each plant. Maturity was determined to have been reached by week 8 for all plants, as all plants had grown to the complete vertical length of their rhizobox. This lack of further soil column to colonize encourages plants to focus on their next phases of growth beyond soil colonization. The images taken during plant growth were then analyzed and the final binarized images were placed into our root trait quantification software, Rhizovision Explorer. The output of this software included traits like total root length of the system, average root length, and average root diameter. Following the completion of plant growth, the boxes were also harvested with the aboveground and belowground biomass being extracted from the soil where they were separated, washed, dried, and weighed. As MP can illicit toxic effects or alter soil properties with positive repercussions, two hypotheses related to microplastic

concentration were proposed. First, increasing microplastic concentrations would be negatively correlated to both root biomass and root length if MP were toxic to soybeans. As with other contaminants, it was expected if MP were toxic, they would negatively affect the growth process and thus have overall smaller root systems relative to the control. Second, increasing microplastic concentration would be positively correlated to root biomass and length. If MP altered soil properties such as aiding in moisture retention the additional soil resources would permit larger growth and increased root branching, increasing both biomass and length. Although high MP concentrations could induce negative soil properties such as blocking oxygen, many studies at much higher MP concentrations did not show negative effects, and the range of MP concentrations in this study were expected to be insufficient to incur any negative effects. For plastic type, it was hypothesized that plastic type which encompasses chemical composition and shape would have slightly different scale of effects. Although the plastic types differed chemically, the duration of the experiment would be insufficient for weathering. Hence, the main difference of would be due to MP shape. It was expected that MP sheets would have a larger effect on root biomass and length than fragments as their larger surface area would increase their effect on soil properties. For biosolid application, it was also expected that the effect of root biomass and length to be insignificantly different from the control, largely as the biosolid MP are confined to the top 15cm of soil which represents a small percentage of the total root system and thus would have a minimal effect on root biomass and length.

Chapter 3 focussed on a time-series analysis of soybean rhizobox imagery between the control and MP amended treatments to determine if MP concentration, type, and biosolid MP had an effect on the growth rates of thin and thick roots, which served as proxies for absorptive and transportive roots. Root diameter classifications were chosen based on previous studies that explored root diameter classes as a definable way of grouping roots into orders. While there were no papers found which focussed on soybean plants specifically, the logistics for the papers on similar plants, such as the common bean, could be applied to this plant species quite effectively, with some modification (Meng, Yang, Riksen, Xu, & Geissen, 2021). The objective of this study was to divide the root system into a functional root classification and separately model the growth rates of each fraction over time to discern if and when the root development phases changed from foraging to senescence. Root systems were divided into three diameter classes (0-0.5mm, 0.5-1mm, >1mm) and the root length for each class was quantified for each image in the time-series using Rhizovision Explorer. For a continuously growing root system, linear regression was used to predict root length by imaging week number. A linear piecewise regression with a single breakpoint was utilized for time-series with phase changes between foraging and senescence. Choosing between linear and piecewise regression models was based on the degree of improvement of model fit on a 30% validation data set. The hypothesis for the root diameter classes is that an increase of microplastics will increase the number of fine roots and reduce the number of thick, transport roots. The researcher also hypothesize that the type of plastic will have an effect on the number of fine and thick roots.

Previous studies have not looked into the specific presence of contaminants and the impact they may have on root diameter during growth, but contaminant-based studies also have shown that certain contaminants can stunt growth as they limit nutrient access. This hypothesis was reached as the presence of contaminants often causes a reduced ability for a root system to access and absorb nutrients, which will encourage the plant system to grow more roots which have an increased ability to absorb nutrients, which in this case are the thinnest diameter class of roots. The hypothesis for growth rates of the entire root system is that the presence of microplastics, regardless of type or concentration, will delay the rate of growth and increase the time it takes for the plant root system to shift from growth to saturation. This hypothesis was reached as an increase in thinner, absorptive roots delays the growth of thicker transport roots which are indicative of a more established soil presence, and the fewer thick transport roots there are the less mature a root system is, as the proportion of thicker roots is related to total root system maturity.

CHAPTER 2

MICROPLASTIC CONCENTRATION AND TYPE INFLUENCES ON PLANT BIOMASS AND ROOT LENGTH

2.0 Introduction

Microplastics (MP) are an emerging contaminant rarely studied in agricultural soils which has shown increasing concentrations of plastic of various types. Previous studies have demonstrated the negative impacts of MP contamination on the natural environment, though a considerable focus has been on aquatic ecosystems (Koutnik, et al., 2021). In terrestrial ecosystems, previous research has focused on the pathways of MP into soil, especially through atmospheric deposition and biosolid application, yet there is little understanding of their impacts to the plant itself under realistic soil MP concentrations. In particular, MP effects on root systems have been studied at soil concentrations three of four order of magnitude higher than what has been reported in soils with biosolid application. Within soil, the most active region is the rhizosphere, a narrow region surrounding roots where many interactions occur between roots, the soil, and the microbial community. Thus, the rhizosphere may be particularly sensitive to MP contamination. For instance, MP affect soil bulk density, aggregation, and pore space size, which may negatively affect symbiotic fungi thereby hinder plant acquisition of nutrients and overall decrease in agricultural yields (Leifheit, Lehmann, & Rillig, 2021) The rhizosphere is a distinctive region of soil, approximately 5mm in diameter, surrounding any given root that is characterized by its interaction with plant root systems and the non-living components that make up soil (Hinsu, Panchal, Pandit, &

Kothari, 2021). It is specifically in this narrow zone of the ecosystem that the many constituents of this nutrient rich soil, such as bacteria, invertebrates, and fungi all interact, communicate, and provide exchanges with plant life (Venturi & Keel, 2016). Alongside this spatial aspect of the rhizosphere there is also a temporal dimension to these ecosystem interactions. Many temporal relationships within rhizosphere signalling are crucial to plant survival, such as the use of specific signalling chemicals exuded from plant root tips that are associated with altered rhizosphere microbial community composition. For example, cucumber secretes citric acid which attracts Bacillus amyloliquefaciens, which helps fight root pathogens through the production of antibiotic proteins and by outcompeting these pathogens (Jamil, Mukhtar, Foillaud, & Dufosse, 2022) (. Roots can further exude inverse signalling chemicals which create a reduction in bacteria cell density surrounding root tips (Watt, Silk, & Passioura, 2006). As MP can alter soil physiochemical properties, release toxins, and create physical barriers to soil, nutrients, and water they may hinder rhizosphere interactions, with the effects manifesting at larger scales on the entire plant root system in terms of root biomass and RSA. According to the review by Qiu et al. (2022) there were 48 studies that had investigated MP and root biomass, yet few have investigated RSA metrics such as root length. The majority of studies have used pot experiments which extract roots from the soil and therefore have limited ability to discern RSA.

2.0.1 Selection of Soybeans for Study

Due to the many different ways plant roots can interact with soil, MP affects are hypothesized to be species dependent. In order to learn more about MP effects on

plants with high degrees of symbiosis with soil partners, soybeans were selected for this experiment. Furthermore, soybeans are a vital plant used for human consumption, biofuel, animal feed, and myriad other purposes, soybean plants are of great importance economically (Valliyodan, et al., 2017). One of the world's most widely grown crop, research into characteristics that can influence their yield and resource utilization, such as distribution, shape, and branching patterns, has been taking place for decades (Lynch, 1995).

2.0.2 Study Objectives

MP from biosolids may have an ecotoxic effect that alters root traits. The specific objective of this research is to utilize a snapshot image of mature soybean root system to investigate root biomass and length to MP type, concentration, and direct biosolid application, which related to MP locality within soil.

2.1 Methods

Through a dose-response study, the treatment effects of biosolid MP on the root systems of soybeans was investigated in soil spiked with two plastics (Polyethylene terephthalate (PET) in sheet form and polypropylene (PPE) in fragment form) at two concentrations (2,000 and 15,000 particles/kg dry soil) which reflect the estimated soil concentration after 4 biosolid applications and the biosolid MP concentration levels. A control treatment of no MP addition and a treatment of direct biosolid application were also included for a total of six treatments. Each treatment had three replicates (n=18). To assess MP impacts, rhizobox images were taken weekly using an Epson 12000XL

(Epson, Markham, ON, Canada) scanner during plant growth and root traits were analyzed using root image analysis software to gain greater knowledge of how increased plastic presence at known concentrations with known types of plastic can affect plant root traits and growth. After growth and imaging were complete, the boxes were harvested for aboveground and belowground biomass.

2.1.1 Rhizobox Soil and Biosolid Mimic Treatments

MP mimics were constructed from sheet PET and bead PPE by manual grinding with a coffee grinder and subsequent size fractionation using 2mm - 5mm, 500um-2mm, 90-500um, and 20-90um metal sieves. The percentage of each size fraction was based on MP documented in soil in the region (Crossman, Hurley, Futter, & Nizzetto, 2020). Approximately two million microplastic particles were utilized, the majority the 90-500 µm size fraction (**Error! Reference source not found.**). Due to the large difference in p article size, the larger MP particles outweighed the smaller particles, albeit the number of particles was orders of magnitude higher in the smaller particle size fractions. Dry biosolids collected from an Ontario wastewater treatment plant were added to distilled water at a 2.5% biosolid to 97.5% water ratio. The amount of MP in the biosolid treatment is unknown. The biosolids were applied to the top 15cm of soil at a rate of 100 wet tons per acre.

Burford clay-loam soil was collected from a local farm in Essex County. This soil in other studies was known to be strongly calcareous, with a large proportion of the soil being calcium carbonate (Evans & Cameron, 1982). The soils in Essex County were also

found to have illitic mineralogy, or high amounts of non-expanding clay minerals (Evans & Cameron, 1982). This soil chemistry was extremely common in southwestern Ontario (Evans & Cameron, 1982). The combination of both high levels of chalkiness and clay lend itself to soil that had poor drainage and high organic matter content (Richards, Caldwell, & Morwick, 1949). This poor drainage was not seen to limit the high value of the soil for agricultural purposes as the profitable agriculture of the area provided opportunities to invest in tile drainage infrastructure to expand on farming in the region (Richards, Caldwell, & Morwick, 1949).

Table 2.1. MP size fractions and weight amended to each rhizobox at the two experimental concentrations.

Size Fraction	Percentage (%)	Particle Mass (g)	MP/box in Soil Concentration (g)	MP/box in Biosolid Concentration (g)
2mm-5mm	0.6	$1.27x10^{-3}$	0.73770	6.56553
500µm-2mm	9.9	$2.84x10^{-5}$	0.27878	2.47045
90-500µm	71.0	$1.59x10^{-8}$	0.00111	0.00991
20-90µm	18.5	$2.00x10^{-9}$	0.00004	0.00033
Total:	100%	N/A	1.01643	9.04620

2.1.2 Root Growth and Imaging Schedule

Seeds were water germinated until sufficient roots for implantation was grown. Water germinated seeds were buried at a depth of 1cm. The plants were grown for a minimum of 10 weeks, with some grown for an additional 2 weeks as the harvesting period took place. The lighting used during growth was Hedera Apex lighting (Linneaus Lighting, Oakville, Ontario, Canada), which used 2 channels (3000k and 5000k) at a 50/50 ratio to ensure that the plants were kept under 200µmol/m^2/s of

photosynthetically active radiation with a 16 hour on, 8 hours off 24-hour cycle. Light intensity was confirmed using a Photobio LGBQM2 Advanced Quantum Sensor PAR sensor (Indoor Farmer, Waterloo, Ontario, Canada).

To minimize MP movement due to watering, rhizobox irrigators (Rhizosphere Research Products, Wageningen, The Netherlands) were used to supply water at 30cm depth through a porous tube as well as top-watering by hand at rates based on average growing season (May-September) precipitation from 2000-2020 climate normal from the Windsor Airport monitoring station. Onet 10HS soil moisture probes (Hoskin Scientific, Oakville, Ontario, Canada) were installed at 4 inches depth, 12 inches depth, and 18 inches depth on one rhizobox per treatment to ensure moisture levels were similar between treatments. No supplemental watering was necessary. Rhizoboxes were imaged weekly using a custom vertical lift that permitted the scanner to image the entire length of the rhizobox with three scans with 15% overlap. Images were taken from the first week after germination until week 11.

2.1.3 Image Processing

The image processing of the root images was split into 5 major steps: 1) The images were buffered and split into smaller sizes to be binarized into root and non-root pixels, 2) Sub-images were then segmented by an image processing neural network that was trained on hand-classified images to train the software to differentiate root and non-root pixels, 3) Segmented sub-images were then recombined into the correct order and configuration of their original images, 4) The three scan lines per rhizobox were

stitched together for the complete rhizobox scan images, 5) These fully binarized whole-root system images were used for root analysis. A total of 422 images were captured.

2.1.4 Image Buffering and Splitting

The rhizobox window covered a 16” W x 36” L window which was scanned horizontally in 12” swaths by an EPSON 1200xl scanner, requiring 3 vertical scans for the entire rhizobox. However, depending on the stage of growth, the entire root system was captured with fewer images. To correctly capture the thinnest roots, image scans of the rhizobox were captured at 600dpi resulting in three individual 7,246 x 10,189 images stacked vertically. The dimensions of the original root scanning images were too large for deep learning approaches which were chosen for the large amounts of data in this analysis. To use these deep-learning methods, the images were first sub-divided into smaller subsections (Figure 2.1). A custom Python code was used to buffer the original images into an equal grid of 768 x 768 images (600 dpi). A total of 59,080 sub-images was created.

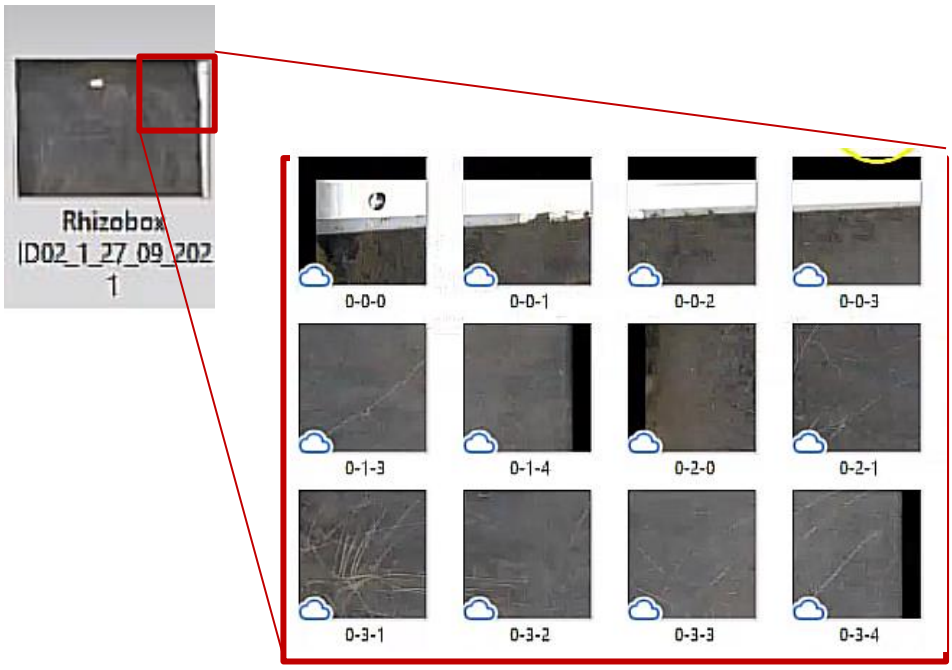


Figure 2.1 Example of the splitting of Rhizobox 2 prior to segmentation

2.1.5 Root Segmentation

Each sub-image was then individually segmented into a binary image where all background elements were coded 0, and root pixels were coded 1. Rootpainter, a convolutional neural network-based image segmentation tool, was used to segment all sub-images (Figure 2.2). The software was trained on approximately 50 sub-images that were hand annotated, delineating roots from the background in an interactive training process where initial segmentation predictions were refined with user input. Default model training parameters were used (e.g., a maximum of 60 epochs per model, 20% annotated training data used for validation). Initial models were re-trained to correct for confusion with water vapour and the rhizobox frame. Approximately 20 model iterations were performed until performance was visually determined as satisfactory (e.g., no confusion with scratches, water bubbles, condensation, and roots were correctly classified regardless of colour). Total training time exceeded 72 hours.

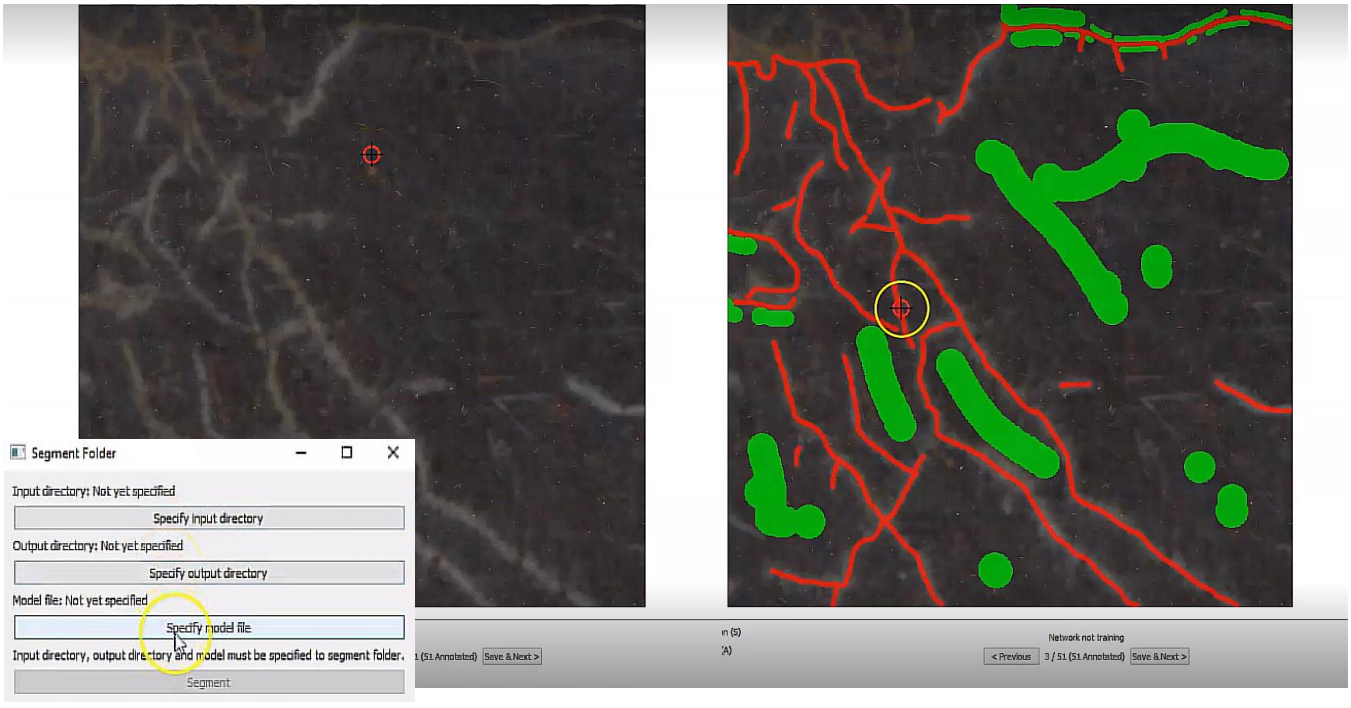


Figure 2.2: Training process to identify root and non-root pixels (red and green, respectively)

2.1.6 Image Recombination

The Rootpainter outputs were segmented sub-images that were recombined to reconstitute the original images (Figure 2.3). The image buffering and splitting process added the grid numbers to each filename which allowed the sub-images to be merged into a single image that perfectly aligns with the original. A custom Python code merged the images.

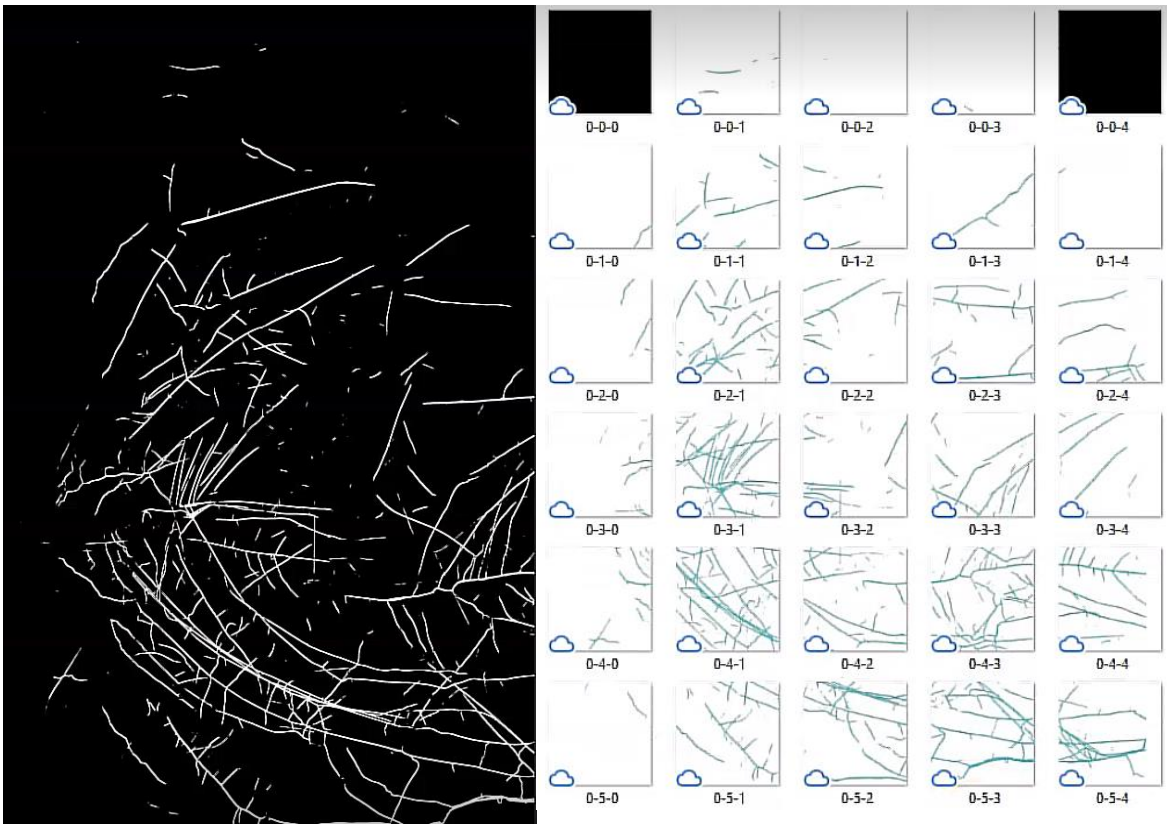


Figure 2.3: The segmented and binarized images before and after re-stitching

2.1.7 Image Stitching

A custom R-Studio code created a standard sized blank image of the rhizobox window and pasted the 1-3 images in vertical order to create the final combined image for analysis. Each image was denoted by the sampling date, which allowed for the

quantification of root traits over time. Note that the image acquisition was not aligned and therefore the time-series "jitters" as there as slight positional changes in the root system between weeks. The jittering was aesthetic only and did not affect the root analysis results as the root length quantification did not depend on the position or alignment of roots between weeks.

2.1.8 Biomass Measurements

At the end of the experiment, the entire plant system was harvested and the biomass for the belowground and aboveground plants was determined. Each rhizobox was opened and the entire root biomass was removed. Following the careful excavation of the root system, the roots were washed in type 1 water to remove any remaining soil. All plant parts were dried at 65 Celsius for 24 hours in a drying oven (Isotemp, Fisher Scientific), and weighed on a Entris II 0.001g accuracy analytical balance (Sartorius, Goettingen, Germany). The above to belowground biomass ratio was calculated as the ratio of dry weight of each fraction.

2.1.9 Root Trait Quantification Software

There were several software options for analysing root traits from segmented rhizobox images, mainly differing in their breadth of root traits, the scales of quantification (e.g., root segment to the entire root system), and the requirements of the imagery. Many software programs require the complete root system to be visible, a requirement that was not met with this image set as sections of individual roots were often blocked by soil. Rhizovision Explorer was used to quantify total root system length

(cm) per rhizobox. The setting for each type of analysis were standardized using batch processing, outputting all traits into a csv file for subsequent analysis.

2.1.10 Root Trait Quantification

Many different root metrics and traits were calculated using Rhizovision Explorer. After the images were bulk input into the software and the roots are normalized to fill in pixel size gaps in the roots, typically caused by small gaps between the imaging face and the roots, the processing was completed with little other input. Regions of interest, which are areas from each image that are highlighted in Rhizovision Explorer to be isolated from each image being processed, were selected from the images, such as specific depths in the soil column, or specific soil regions like the rhizosphere. The output of the program was a fully sorted set of images and a .csv file. The traits that were included in the csv file are the number of root tips, number of branching points, total root length, branching frequency, average and median diameter, surface area, and average root length. Some of these features extracted were more prone to error, such as branching points, due to the inability to connect root sections that at some points during growth weave in and out of the soil in the rhizobox. The most reliable measurements found were the total root length and diameter, and they were used as the primary metric for MP effects in Chapter 2 and 3, respectively. Root length of a system was an important indicator of plant health as more root typically means more access to water and nutrients.

The total root length for the entire system was explored to determine the impact of MP, both in the two types used as well as the two concentrations. To ensure there were differences in root system length between treatments during this univariate analysis, ANOVA was first performed on all 6 treatments to determine whether any treatment was more variable from the control. In the case of a statistically significant ANOVA (i.e., $p < 0.05$), a post hoc Tukey HSD test was used to compare individual treatments to the control. In particular, the effects of concentration between the low and high concentrations of the PET and PPE mimic-spiked treatments were analyzed individually. To evaluate the effect of plastic type, the PET and PPE treatments were individually compared at the same concentration level. The biosolid treatment was compared to the control.

The top 15cm soil layer that has direct biosolid application was investigated to determine if this highly concentrated area of MP application had any impacts on the earliest stage of root growth. The first 15cm of both treatment 1 and treatment 6 (the control and biosolids treatment) respectively, were compared. While this did assume no vertical MP migration, this depth was chosen as top watering and irrigation displacement of MP during growth could not reasonably be quantified during the course of the experiment. The data collected for the soil below this region of interest was also used to explore any changes in the ratio of total root surface area and root length above and below the biosolid enhancement zone between the two treatments. The traits used were total root surface area and total root length because they were the most reliable root traits.

2.2 Results

The data collected during the imaging process, once fully binarized and processed for root analysis created a total of 183 whole root images. The three rhizoboxes for each of the six treatments were all imaged for 10 weeks (**Error! R eference source not found.**). The images were processed with filters within the Rhizovision Explorer software to ensure that non-root objects in the images were filtered out, typically scratches on the acrylic rhizobox front window which were binarized alongside the roots. There was also the matter of roots thin enough that small pixel sized holes were present in the binarized images. This was filtered out using the 'fill holes in root objects' function, which allowed the user to select the maximum pixel size of root holes and ensures that small gaps in the images were filled to make sure that the total pixel count of roots accurately reflected the true amount of root mass in the root system.

The root segmentation process performed well, with only small misclassifications. Primary image objects that were confused with roots were scratches in the acrylic and condensation build-up during the growing process. However, Root Painter successfully segmented both newer white roots and aged roots (**Error! R eference source not found.**4 A and C). Although classification performance was good, individual roots would often disappear beneath soil only to reappear later or appear broken following segmentation. Based on a visual inspection, these errors appear random and were deemed not a significant factor as the majority of the root systems were segmented such that the overall root system was discernable.

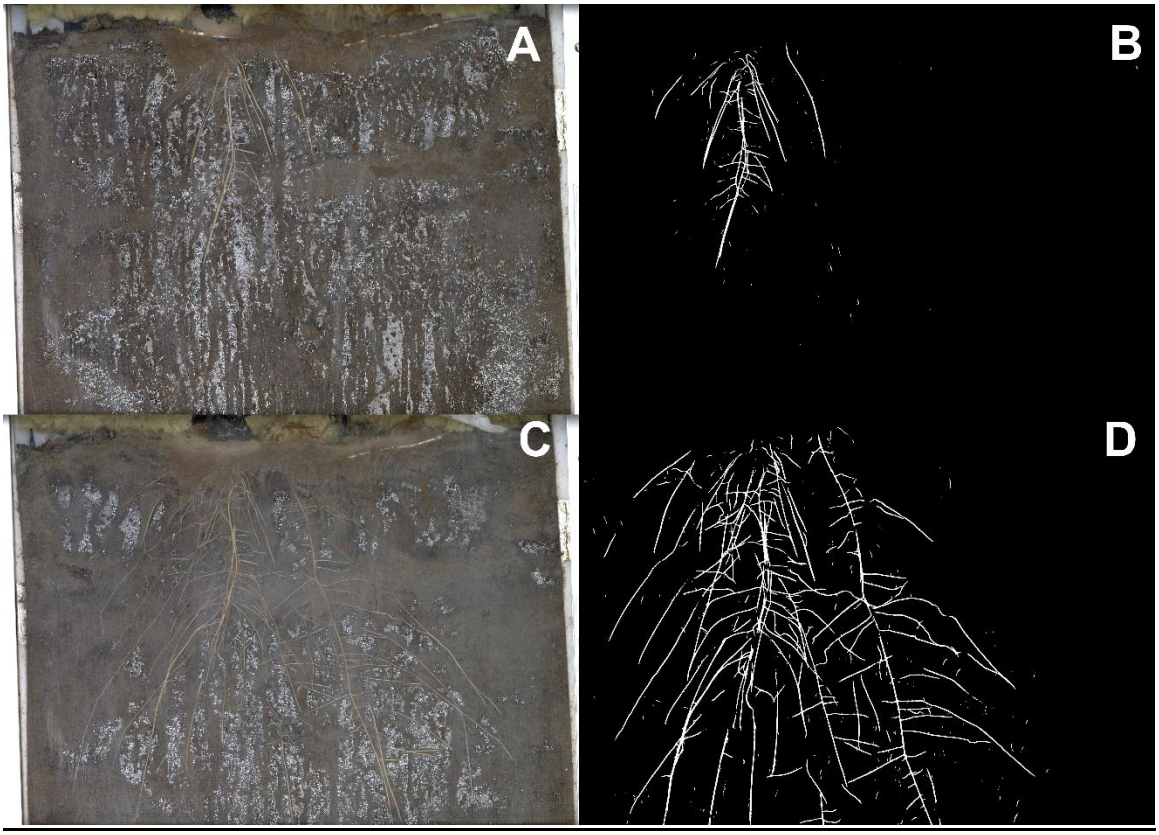


Figure 2.1. Images of rhizobox 46 (treatment 4) at week one (A and B) and week 10 (C and D). Original images (A and C) and segmented images (B and D).

2.2.1 Biomass by Treatment

The plants did produce seedpods at the end of the experiment and the seedpod biomass was only included in the total aboveground biomass. Compared to the control, treatments 2 and 6 had the lowest difference in their above- and belowground biomass (A/B) ratio (**Error! Reference source not found.**). Treatment 4 had a slightly lower A/B r atio, but still within the error bars. The lowest A/B ratio was found in treatments 3 & 5, which were the highest mimic treatments. In these treatments the total biomass (above

and below) was ~36% higher than other treatments, but the belowground biomass was considerably greater.

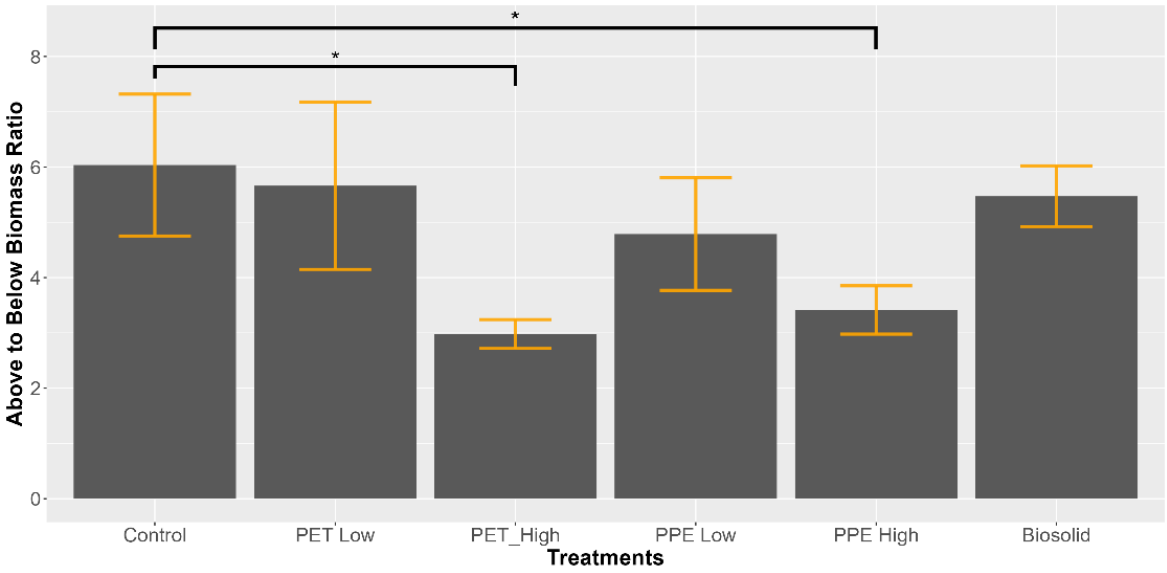


Figure 2.2. The average ratio of aboveground to belowground biomass (dry weight) per treatment. The orange bars are the standard deviation per treatment and the line indicates which treatments were statistically significant ($p<0.05$ Tukey HSD).

Comparing the A/B ratio between different treatments and the control did not have a statistically significant difference for all treatments (Tukey HSD $p<0.05$), however the sample size per treatment was low (n=3). The treatment with the lowest A/B ratio was Treatment 3. The effect of concentration on the A/B ratio showed that the concentration of the MP, regardless of type, had a consistent effect for the higher concentration treatments. The PET low treatment (treatment 2) had almost no difference from control. The effect of plastic type showed that PP had a lower A/B ratio than PET treatments. This lower A/B ratio indicates that there is a larger effect, which can mean that PP has a lower ecotoxicity threshold than PET. The biosolid treatment in

comparison to the control treatment had a non-statistically significant difference. This can be explained due to the smaller area in which the MP were dispersed; the biosolids were only applied to the top 15cm, making their time of contact to roots minor in comparison to the extended contact times found in other treatments where the dispersal of MP was throughout the whole rhizobox.

2.2.2 Root Length by Treatment

Average root length varied between treatments (**Error! Reference source not found.**), with the means of the MP mimic spiked treatments, 2, 3, 4, & 5, were 68,448, 76,826, 67,987, and 53,835mm respectively, approximately 4-48% greater than the control. ANOVA performed on these treatments found there was a statistically significant difference ($p<0.05$, F-value 22.82), suggesting the means of at least two groups were unique.

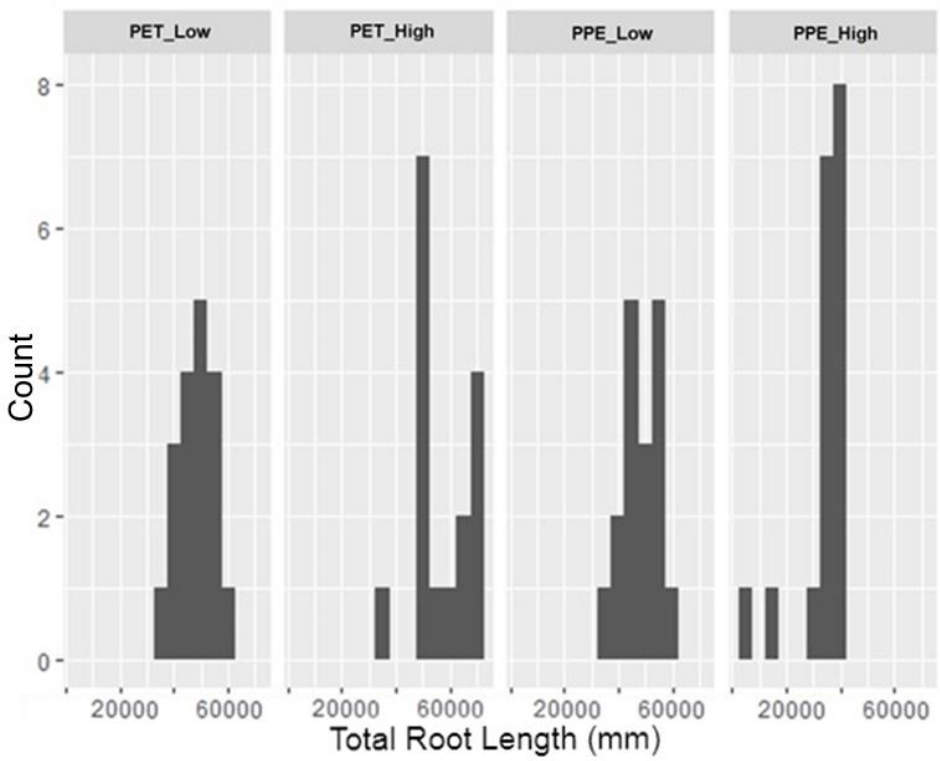


Figure 2.3. Histograms of root length for the biosolid mimic treatments.

The Tukey test results based on the concentration of the mimic treatments indicates that all biosolid plastic mimic treatments were statistically significant (**Error! R eference source not found.**). MP concentration was shown to be a strong influence on root length, with higher MP concentrations showing a larger root length.

Table 2.2. Tukey HSD for the root length between treatments

Comparison	Difference	Lower to Upper	Adjusted p-value
PET Low – PET High	8,378	873, 15,883	<0.01
PP Low – PP High	-14,152	-21,540, -6,765	<0.01
PET Low – PP Low	-461	-7,848, 6,927	<0.01
PET High – PP High	-22,991	-30,496, -15,486	<0.01

Differences between plastic types at the same concentration were also noted, with the polypropylene treatments featuring a larger root length. Root length was also

investigated between the control and biosolid treatments (**Error! Reference source not found.**). This was done to discern the difference in growth during the early phases of plant development with regards to the impact of the biosolid enhancement zone. The target trait for this analysis was the average root length for each box per week. The Tukey HSD results of the statistical difference between the two treatments, the control and biosolid, were not found to be statistically significant. The control treatment had a higher variation in its numbers as well as a higher average. The control treatment had a lower average and higher variance, while the biosolid treatment had a higher average root length and a smaller variance. The result was p-value = 0.077, which shows this trait was not statistically significant in its difference between the two treatments.

Table 2.3. Pairwise Tukey HSD Results with the statistically significant results ($p < 0.05$) between pair of treatments indicated by an asterix (*).

Treatments	DFn	DFd	p	p<0.05
Control				
1-2	1	34	6.7 e-07	*
1-3	1	32	5.23 e-08	*
1-4	1	33	1.65 e-06	*
1-5	1	34	0.018	*
1-6	1	34	0.077	
Mimic				
2-3	1	32	0.008	*
2-4	1	33	0.842	
2-5	1	34	2.99 e-06	*

2-6	1	34	1.99 e-05	*
3-4	1	31	0.007	*
3-5	1	32	6.81 e-08	*
3-6	1	32	7.36 e-07	*
4-5	1	33	7.82 e-06	*
4-6	1	33	6.62 e-05	*
5-6	1	34	0.594	

2.3 Discussion

The soil used in the experiment was chosen as it is naturally occurring soil in southwestern Ontario that is used for agricultural purposes. To mimic as close to real growing conditions as possible within the constraints of the experimental design, it was decided that the best soil substrate to use was one that is commonplace and sourced from a farm that has no known history of biosolid application. While it is not possible to determine the exact amount of MP in the soil prior to experimentation as the most feasible methods of quantification are destructive, it was assumed that the baseline amount of MP present in the soil would not have a strong effect on the experimental design. Throughout the growing period great care was taken to ensure that the

experimental design considered the likelihood of MP shifting and migration were reduced. The earliest phases of watering were done through subsurface irrigation, and later watering was completed through hand watering at the surface of each individual box multiple times a week to mimic natural precipitation cycles. The growth of the plants was at an angle to ensure that the roots grew towards the face of the rhizobox for ease of imaging, but this angle also potentially may have caused the roots to experience greater contact with MP during watering and any migration that occurred in the MP. While this may have impacted the study, such an angle was necessary to ensure that root imaging was as successful as possible.

The effects of MP in soil on the root system as a whole have been studied through testing different plastic types, sizes, shapes, and concentrations on a variety of crop types. In a pot experiment, a 2% concentration of PET fibers, and a 2% concentration of PP fragments in a separate treatment in soil where spring onions were grown caused no effect on their growth (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). One study found that concentrations of 10% to 20% of PVC powders in soil where wheat was grown caused a positive impact on the growth of the crop (Zang, et al., 2020). In a review article, of the nine different plastic types used in MP studies, which were in varying shapes and concentrations that ranged from 0.1% to as a high as 20%, approximately 20% of the data pointed towards a stimulation of increased plant growth, while 42% remained neutral compared to control treatments and 38% experienced negative impacts on growth (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). These studies all focused on common agricultural crops, wheat and corn,

as well as spring onions. In a meta-analysis and review completed by Qiu et al (2022), it was found that all shapes of MP increased root biomass, with the strongest influence on root biomass being caused by films, with the effect size having a confidence rate or p-value of 0.003 (Qiu, Zhou, Zhang, Zhou, & Qin, 2022). In a study done on wild carrots grown in pots which were amended with MP of four shapes, fibers, films, fragments, and foams, at four concentrations, 0.1%, 0.2%, 0.3%, and 0.4% w/w, the majority of treatments caused an increase of root biomass (Lozano, et al., 2021). The plastics were cut to size fractions below 4mm using a blender, which resulted in plastics that were likely larger than those used in this study. The most dramatic effect was found in the foam treatments, with the increase in root biomass being 160% of the root biomass found in the control. The only treatment that did not increase root biomass in comparison to the control was the fiber treatment, which may be related to the increased water holding time in soil that was observed in the presence of fibers during the study.

The research in this experiment in particular chose to focus on soybeans as they have a distinct relationship with the rhizospheric community, which includes close communication and symbiosis with fungi and the microbial community. An experiment conducted by Meng et al on the common bean plant, *Phaseolus vulgaris L.*, grown with low density polyethylene and bioplastics in sandy soil at doses ranging from 0.5% to 2.5% dry soil weight found that three of the five LDPE plastics showed an increase in root biomass, though no consistent trend could be identified (Meng, Yang, Riksen, Xu, & Geissen, 2021). The particle range of <53um to 1000um was consistent with the size

ranges of the plastic used in our study as well. The bioplastics had a slight increase in root biomass at their lower concentrations and showed a moderately statistically significant decrease in root biomass at their highest three concentration in comparison to the control, a decrease ranging from 9% to 18% root biomass (Meng, Yang, Riksen, Xu, & Geissen, 2021).

The effects of MP extend to the whole plant. It is important to note that the ratio of aboveground to belowground biomass can indicate an uneven energy allocation, which in the case of agriculture can be very important. If a plant is focusing much of its energy into growing roots rather than the aboveground portion, there may be an effect its ability to spend energy on its growth of fruiting bodies, especially if the crop in question has much of its value based on its growth above ground, such as cash crops like corn and wheat. However, some studies are finding that the effect of MP on the aboveground portion of plants are inconsistent, and largely dependent on soil properties (Qiu, Zhou, Zhang, Zhou, & Qin, 2022). The factors that are more often found to have direct impacts are things like soil nutrient availability, dissolved organic matter, and the size and distribution of the root system itself (Qiu, Zhou, Zhang, Zhou, & Qin, 2022). One study found that there are some cases in which the increase in root biomass may be related in some way to increases in shoot mass, but more study may need to be done to understand if or how these two are related to MP within soil (Lozano, et al., 2021). The results of our experiment did find that there was an increased root biomass in comparison to the aboveground biomass, which was also in line with 5 of the 6 tested MP in the study by de Souza Machado et al, which found that the ratio of roots to leaves

was increased in the majority of their treatments (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). Meng et al also found similar effects during their study on the common bean plant, and their results also pointed to a correlation between the increasing concentration of MP to the increase in root biomass to aboveground biomass in their plants. The strain on plant resources as well as a plants ability to access water, nutrients, and symbiotic soil partners may be related to the increased concentration of MP in the soil. Though the exact mechanism that causes the increase in root growth is unknown, it could also be attributed to some other form of root growth stimulation that is related to the presence of plastics in the soil. It is becoming clearer that studies are finding that MP in the soil has some form of relation to the ratio of aboveground to belowground biomass in many plant species, which may be a key factor for future study.

The length of roots was a key factor under examination during this experiment. Past studies have focused on biomass. As such, there is a distinct lack in specific information about all relevant root traits as well as the influence of MP of differing size shape, type, and concentration in other similar studies. One study that noted any particular change in root biomass found that in their growth of spring onions, the increase of root biomass in their high-density polyethylene, polyethersulfone, polyethylene, and polypropylene plastic spiked treatments also increased root length, and that the one outlier plastic type, PA, did not increase root biomass still was found to increase root length (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). While root biomass and root length are positively correlated, the root diameter and specific root length is still a mediating factor. When comparing two root systems with the same

overall root biomass, the thinner the roots the more length those roots must have compared to a smaller root system with thicker average roots. The spring onion study by de Souza Machado et al points to this, with all MP increasing total root length and decreasing total root diameter (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). While not measured in this study, this suggests that that there is an increase in the specific root length, and how long a root is per gram weight. This also points to there being an increase in root surface area in the presence of MP (de Souza Machado, Kloas, Zarfl, Hempel, & Rilling, 2017). The common bean plant study by Meng also found that specific root length was increased compared to the control for the majority of their treatments (Meng, Yang, Riksen, Xu, & Geissen, 2021). The strongest trend for increasing root length was associated with LDPE plastics and bioplastics, with the LDPE increasing specific root length 21% relative to the control and the bioplastic treatment increased the specific root length by 36% relative to the control (Meng, Yang, Riksen, Xu, & Geissen, 2021). Changes in specific root length have been suggested as a response to stress in plants, with studies that fertilized plants in acidic soils were found to have decreased specific root length (Ostonen, et al., 2007).The specific root length of trees were also linked to shading, irrigation, and soil disturbance. In a study comparing phosphorus efficiency in soybean, sunflower, and maize, the specific root length of the three crops were consistently linked to soil nutrient availability, with thicker roots being associated with a long-term investment by a plant in areas of soil with high available nutrients (Fernandez, Belinque, Gutierrez Boem, & Rubio, 2009). These thicker roots are more able to withstand disease and pests, while thinner roots are associated less with

nutrient availability as these roots are more able to explore a greater area of soil, and only are found to thicken in soil with high nutrient availability once a stronger root presence has been established. The increase of specific root length and the smaller diameter of roots grown in soil with high concentrations of MP could potentially reflect less long-term investment by the plants in their root growth; if the soil has a high nutrient availability but the roots remain thin and long, the plant may still be growing the highly explorative roots that will continue to seek less contaminated soils.

The effects of concentration of MP on root length may be related to the ecotoxicity threshold of these specific types of plastics. The effects of concentration of MP on root length has been observed in many studies, and it is also observed in our own. When comparing our concentration of MP to that of others to determine their relation, we find that our own concentrations were in some cases much lower than the number of plastics used in others. Some studies do outline that there may be an inconsistent effect caused by concentrations, and our own experiment saw only one of the mimic plastics having an increased ecotoxicity by concentration. Through only studying two different types of specific plastics as well as biosolid MP, there is not enough to be certain what influence concentration has on ecotoxicity from our results alone. The effects inconsistency may be related to the environment as well as situation dependency, with different plant species and their root systems behaving differently, causing them to show ecotoxicity or MP effects in different ways. Plastic shapes like sheets can impact plant roots by having more surface area and causing greater obstruction for roots to access soil and symbiotic partners. Sheets also can increase soil

moisture by effecting soil pore space, which may in turn have positive effects on plants as there is less struggle to find adequate water, and it may offset some more deleterious effects of plastic on root health by doing so. The long-term effects of concentration of MP in soil are also not known as this field is relatively new. Studies into the residence time and long-term presence of MP in soil are few, and in the case of biosolids, only approximately 1% of plastics remain following the application of biosolids and regular growth and harvesting of plants (Crossman, Hurley, Futter, & Nizzetto, 2020). With MP being applied at high amounts due to their concentration in biosolids, it is still unlikely that their concentration levels will reach 15% in soils without repeated applications for decades as the export of applied MP is reliably removing the vast majority of MP.

The ecotoxicity threshold of plastic types varies greatly in many studies. Different chemical compositions of plastics in many studies are seen to have varying effects, with some behaving similarly in their influence on plant roots, and others causing little change or even positive effects on root biomass and total plant growth. However, the varying shapes of plastics can have many different effects as not only do they impact the roots themselves, but they can affect the conditions of the soil. Plastic sheets are known to increase soil moisture, which may be beneficial in drought conditions, and fibers are known to impacts soil aggregation by reducing pore space and increasing the compacting of soil (Qiu, Zhou, Zhang, Zhou, & Qin, 2022). The weathering of the plastics found in the soil may not have as much of an impact as other factors such as shape, type, and size. Plastics like PET, which are used in plastic mulching, do not have as much negative effect as other plastics, though this may again be related to PET application

being in sheets to increase soil moisture, which is why their purposeful application is so often seen in agricultural practices (Qiu, Zhou, Zhang, Zhou, & Qin, 2022). While there is some evidence that supports that certain plastic types can have different impacts than others, there is no concrete evidence to suggest any specific type of plastic's chemical composition has any stronger or weaker effect on plant root traits, whether positive or negative. While studies have often been broad and related to simply examining the changes in concentration in different plastic types, more research must focus on understanding what, if any, plastics may have the strongest effects on plant roots.

The results of the comparison between the biosolid treatment and the control treatment seem to indicate that the MP concentration, while high, may not have as much influence as previously hypothesized. With regards to the experimental design, previous studies which found concentration may have an effect may have only found these effects as they used concentrations which are not agriculturally relevant and likely do not exist in any environment. The enhancement zone of the MP in the biosolid application is only 15cm deep, and the roots that are present grow beyond that quickly during the earliest stages of growth, limiting the amount of contact that layer has with the most vulnerable fresh roots. The was little detectable difference in root traits for this reason. The MP may have migrated further down during growth and watering, but still the impact was not enough to cause any statistically significant results. There were also no visual differences between the two treatments roots. Previous research does indicate that the MP presence in biosolid appears to have a very short residence time, which may explain the lack of impact in this case.

2.4 Conclusions

In conclusion when looking at the span of this whole project, the inclusion of MP in soil, whether through purposeful additions in the case of biosolids, or the varying pathways like road dust and atmospheric deposition, there is much to study with their impacts on plant growth. The many types of plastic and their varying chemical compositions indicate that the problem is extremely multifaceted and as such should involve much research to truly conclude what impacts can occur in plants grown in the presence of MP. While some may have limited effects, others may impact plants and soil in a way that is positive or negative. These variations in impacts lead me to conclude that while this research may point in some cases to there being little harmful effects to plants, the scope of this project simply does not encompass all required to conclude anything with finality.

Following conducting this experiment more needs to be done to examine how the positive impacts of some plastic shapes, like sheets, may influence the outcome of research. While it cannot be stated for certain whether any outcomes are positive or negative, quite yet the seemingly positive short-term impacts lead me to encourage future research examinations of the long-term effects. This study shows me little in the way of deleterious effects of MP in the soil for the plants grown, but this study was also done on soil with no previous history of known biosolid enhancement. In soils with a long history of such, perhaps the few remaining MP particles every year may eventually add up and cause a change in effects. They may also cause a change in the symbiotic relationships that the plants grown in later seasons may form with their soil partners.

The relationship that plants have with fungi and microbes in the soil is extremely complex and still not fully understood to this day. To say with certainty that a conclusion can be reached with this study is impossible, especially as more needs to be done to understand other, highly complex aspects of rhizobiology. The impacts of this study hopefully will lead to further research into the many types and shapes of plastics and their impacts on a wide variety of plants. The introduction of this strategy of imaging, it is also hoped will incite the creation of software that is better able to gain more information from root imaging, as this is a promising method of understanding roots in greater detail than previous methods, like root-washing.

CHAPTER 3

MICROPLASTIC CONCENTRATION AND TYPE INFLUENCES ON ROOT DIAMETER; A TIME-SERIES ANALYSIS

3.0 Introduction

Although total root length at maturity is a good indicator of the degree of root-soil-microbial interactions, it ignores the root system development process and assumes that all roots are equally important. However, fine roots, also known as functional roots, perform essential requirements for plant health in ways that coarser, older plant roots do not. As a function of their thinner diameter, higher surface area, and low cost of production, fine roots are better suited to absorb and transport essential soil resources like water and nutrients and have a larger role in the mediation of biogeochemical cycles (McCormack, et al., 2015). However, destructive methods for extracting roots often lead to incomplete accounting for total belowground biomass as the thinnest fine roots are often the most lost portions of the root system (Do Rosario, et al., 2000). As a result, important root system traits such as the amount of surface area are often underestimated.

Quantifying roots by size fractions are necessary for studying plant-soil interactions. Improper size fraction cut-offs can make it difficult to interpret the different types of roots are their specialized roles in plant physiology. Typically, thinner fine roots are involved in uptake of soil resources of nutrients. Absorptive roots are the most distal parts of plant root system as they are the most exploratory and perform the

valuable function of seeking and bringing in nutrients and water which are distributed to aboveground tissues. Transport roots typically are those tasked with transporting the resources absorbed by absorptive roots to other parts of the plant. Specialized internal cells develop within transport roots to aid resource transport, increasing their thickness. Transport roots thus increase in abundance towards the center and top of the root system, with the taproot often dedicated to transport.

The traditional method of defining absorption roots is a ≤2mm diameter. However, this cutoff was designed for trees and though more recent studies suggest that this cut-off limits understanding the diversity of functions of the many size fractions within the class of 'fine roots' (McCormack, et al., 2015). The diameter cutoff method is simple and faster than other methods like order-based classification. In order-based classification, root branching is used to define the role of the roots within the root system. Root-order is key when seeking out roots for their specific traits and services to the plant system; as root-order goes higher, the roots' ability to transport increases while its uptake decreases (McCormack, et al., 2015). The older a root is, the higher its root-order may become as oldest roots tend to be suberized first, meaning the oldest roots tend to be the thickest and only used in the root system for transportation. The youngest roots typically have the finest diameter (McCormack, et al., 2015). The younger a root is, the more it is responsible for seeking and up-taking valuable nutrients and water for the plant, which is why understanding the functional qualities of different plant root types also means having a robust system to define was exactly a fine root is.

Root systems change as they grow in a similar way to plant leaves. There is a hypothesis in the study of plant leaves that there comes a shift in prioritization from resource acquisition to conservation as they age. Plants in more nutrient dense areas grow their leaves quickly as the bigger a plant becomes, the more it succeeds in out competing others for resources like sunlight (Doussan, Pages, & Pierret, 2009). This is echoed in resource competition in plant roots. Plants in areas where resources are scarce have less competition over the smaller amount of resources. Thus, they tend to be growth adverse and prefer to allocate their resources to increasing root defences, such as developing a thick layer of suberin in cell walls that protects against both biotic and abiotic threats (Chen, Liu, Wang, & Chen, 2022)

Studies exploring what is sometimes called “root economics” have shown that in cases where resources are abundant, specific root length and specific root area increase as roots grow over a larger area and are thinner (de la Riva, et al., 2021). Conversely, areas with low resource availability cause roots to grow closer together, with lower specific root length (de la Riva, et al., 2021). This low resource availability creates roots that have a longer lifespan than in plants with high resource availability, which is in part due to the low return on investment associated with the expenditure of energy required to grow the root system. The costs associated with root growth decrease linearly with the increase of soil nutrient availability, and peak when water availability is moderate (de la Riva, et al., 2021). Furthermore, environmental factors can shift root economic behaviour. Studies exploring the impact of temperature on herb, shrub, and tree plants

found that the higher the temperature, the greater the decrease of root width, depth, and branching (Luo, Xu, Chu, He, & Fang, 2020).

Specific root length can be altered due to a number of factors as well as heavy metal contamination. Acidification of soil can also reduce specific root length by 20%, and aluminium stress from soil can reduce it by 13%. This study also found that shading of plants reduced specific root length by 12%, elevated CO2 reduced it by 712%, and heavy metal stressors reduced specific root length by 727% (Ostonen, et al., 2007). The addition of nutrients can increase specific root length by making longer roots a better long-term investment of plant energy, but the addition of contaminants or other stresses may reduce the specific root length. This is of interest to this research as the addition of microplastic contaminants at the same time as nutrients, such as through the application of biosolids may negate or lessen the impacts of the contaminant, if the MP have a negative impact on the specific root length. However, it is unclear how MP alter the root traits that control the surface area between the roots and the soil and the degree of root-soil-microbial interactions.

The root growth process further influences the degree that roots, and soil interact since a non-growing root exerts a stronger influence on the soil microbial community. A growing root is principally concerned with providing adequate anchoring as well as foraging the soil for water and nutrients. Plants tend to produce new roots until the benefits of the nutrient uptake in that area are outweighed by the cost of root growth itself. As such, roots will often forage for nutrients and water in soil regions where there is little to no competition. Without competition, roots grow until it senses a

physical barrier, and even then, it may either diverting its course or in some cases, such as tree roots specifically, they may attempt to pass through the barrier. Root growth can persist if the plant is not resource limited. However, plants also regulate their growth by exuding signalling chemicals into the soil, usually to signal to their symbiotic partners that they need specific nutrients or a stronger symbiotic connection, but as they exude more, root growth is suppressed as the root system encounters more of its own exudates. As the plant sends out these signalling chemicals, the more it encounters them the more the root system communicates with itself that there is already a strong root presence in an area. This may be used to encourage growth and colonization into other areas of the soil rather than concentrating root presence in an already saturated region. In contrast, a non-growing root exhibits more of a conservation strategy and attempts to recruit soil microorganisms to aid in nutrient acquisition and protection from pests. The longer timeframe of root-borne compound release into the soil increases the likelihood of symbiosis formation as well as the accumulation of plant defence compounds that inhibit the growth of fungal and bacterial pathogens (Dennis, Miller, & Hirsch, 2010).

Understanding the change in root traits over time can also provide cues about root vulnerability and physiology. As roots age, their diameter and length increase while specific root length decreases (Guo, Mitchell, & Hendricks, 2004). Often the speed of the aging process is related to nutrient availability and other deleterious soil properties. Root aging is considered beneficial for first order roots, which are the youngest and most active roots that are also the most vulnerable part of the plant. Fresh root tips

have a very thin endodermis which allows for them to transfer ions between soil and root (Miyashima & Nakajima, 2011). This ion-transfer allows for nutrient transfer to occur but also has the adverse effect of potentially allowing heavy metal to infiltrate and contaminate the root structure. Root development with age can prevent this, through the development of suberin lamellae, which makes roots more tolerant and reduces transport efficiency for both nutrients and heavy metals (Miyashima & Nakajima, 2011). This lack of transferability also causes plants to delay suberin development in root aging where soil nutrient availability is low; the less accessible nutrients are the more transferability is needed to ensure plant needs are met (de Silva, et al., 2021). However, most studies on MP effects on root traits have utilized destructive methods, and few have monitored the change in root traits over time.

3.1 Methods

Time-series imagery of binarized root systems of soybean plants (n=18) grown in rhizoboxes and imaged weekly was investigated to determine whether microplastic concentration, type, or locality influenced root length by root diameter class, with the principal focus on the thinnest root diameter class. For each treatment (n=3), the weekly root length per diameter class for all three replicates was predicted from week number to infer growth rates and assess whether they were consistent over time. Treatments where root growth was continuous throughout the experiment were modelled by linear regression. Treatments with a phase shift from foraging to senescence where root growth ceased were modelled by linear piecewise regression

with a single breakpoint. Whether a piecewise model was justified was based on the degree of fit to a 30% validation data set.

Root diameter classes for this analysis were chosen to be in line with previous research on orders of fine roots (Table 3.1). Prominent studies such as that of McCormack et al, suggest that cutoffs between root diameter, based on root age and suberization occurred at varying diameters depending on plant type (McCormack, et al., 2015). Previous study has given rise to a 2mm or less classification for fine roots, though many past studies were done on tree roots. As outlined by McCormack et al (2015), 1st order roots, which are the thinnest and freshest root tips, have diameters of 0.5mm or less. This definition led to the creation of the first diameter class in this analysis, and through further background, the second class of 0.5mm to 1mm was chosen as the gap between 2nd and 3rd order roots was the least distinct of the smallest of root orders. As the plants in this analysis were from a much smaller organism than trees, the 3rd and final diameter class of 1mm and above was chosen to ensure that all thicker, older, and suberized roots were considered. Variations in these diameter cutoffs were assessed multiple times using Rhizovision Explorer using a randomly chosen young, mature, and mid-age root system for each treatment.

Table 3.1. Classification based on root diameter range.

Root Diameter Class	Diameter Range
1	<0.5mm
2	0.5mm-1mm
3	>1mm

To predict root length as a function of week number, linear regression was used. The assumption of a linear growth rates of the plant roots, including the rate of roots suberization and thickening with age, was justified based on the controlled growth conditions and equal weekly supply of photosynthetically active radiation. The sole explanatory variable was the week number, with week 0 representing the date of seed planting and weeks 1-11 the imagery acquired using the weekly imaging schedule which was performed on the same day weekly. As root length was zero at week zero, the linear model had no intercept. The goal of the regression analysis was first to determine if the growth rates per diameter class were constant within and between treatments. Linear regression was performed using the *lm* function in in RStudio (R version 4.2.1, http://www.r-project.org) based upon the following equation:

$$y = mx \tag{3-1}$$

where y is root length at week x, x is the week number, and m is the derived linear model coefficient. Model performance was evaluated using the adjusted R-squared value.

In cases of multiple phases of growth (i.e., a transition from a foraging phase to a senescence phase with minimal change in root length), linear piecewise regression was used. The piecewise regression allowed for there to be multiple growth rates between the dependent and independent variable while still maintaining a linear model. To balance model complexity and minimize the potential for overfitting, only a single

breakpoint was permitted. Piecewise regression was performed using the *segmented* package in RStudio (R version 4.2.1, http://www.r-project.org).

$$y = m_1 x + m_1(x - \psi) \quad (3\text{-}2)$$

where m_1 is the left slope, m_2 is the difference in slopes, x is the week number, and ψ is the breakpoint. Regression coefficients and the breakpoint were extracted from the segmented model.

To compare different types of regression models, there was difficulty in determining which one best suited the dataset as a model with more flexible is likely to better fit to the data, allowing the regression to explain more of the variation between the dependent and the independent variables. Too much flexibility, however, and the model would too closely fit to the training data. To avoid an underfitted model that lacks specificity and does not perform well in its predictive goal, or an overfitted model that does not show the reality of the dataset's variation, model validation was used. In this analysis the data was randomly split into 70% training and 30% validation. The model was trained on the majority portion of the data randomly selected and a smaller subsection was used to test the predictive abilities of the fitted model. This ensured that the model was not being overfitted while gaining a good view of the regression's predictive abilities.

3.2 Results

Classification of roots into diameter classes resulted in reasonable delineation between recently formed roots and lateral roots (**Error! Reference source not found.**1). A n individual root segment was often divided into one or two diameter classes as sections

such as the tip and parts that deviated from the root window could be delineated as different diameter classes. However, the chosen root diameter classes were able to separate the thicker roots from the thinner roots.

The share of total root length by diameter class during week 8 (Figure 3.1) demonstrates that the control treatment has the least fresh roots as a share of the total length while the spiked treatments have slightly larger quantities of fresh root tips. As root diameter increases, so does specific root length (SRL), so the increase in the thickest roots in the spiked treatments leads to an increase in SRL for treatments 2 to 6. However, the increase in the amount of thin, fresh roots limits the increase SRL as the higher proportion of fine roots, the lower the SRL on average.

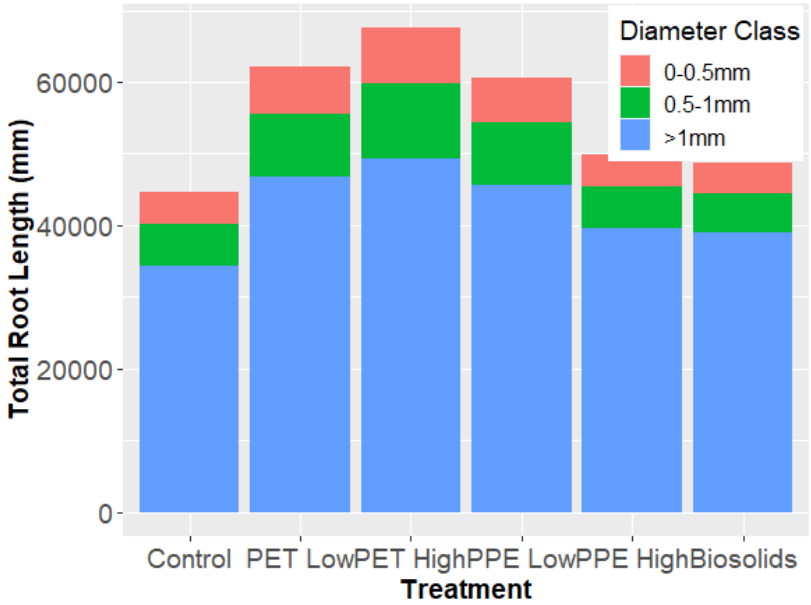


Figure 3.1. Total root length per treatment separated by diameter class.

The share of total root length by diameter class during week 8 demonstrates that the control treatment has the least fresh roots as a share of the total length while the

spiked treatments have slightly larger quantities of fresh root tips. As root diameter increases, so does SRL, so the increase in the thickest roots in the spiked treatments leads to an increase in SRL for treatments 2 to 6. However, the increase in the amount of thin, fresh roots limits the increase SRL as the higher proportion of fine roots, the lower the SRL on average.

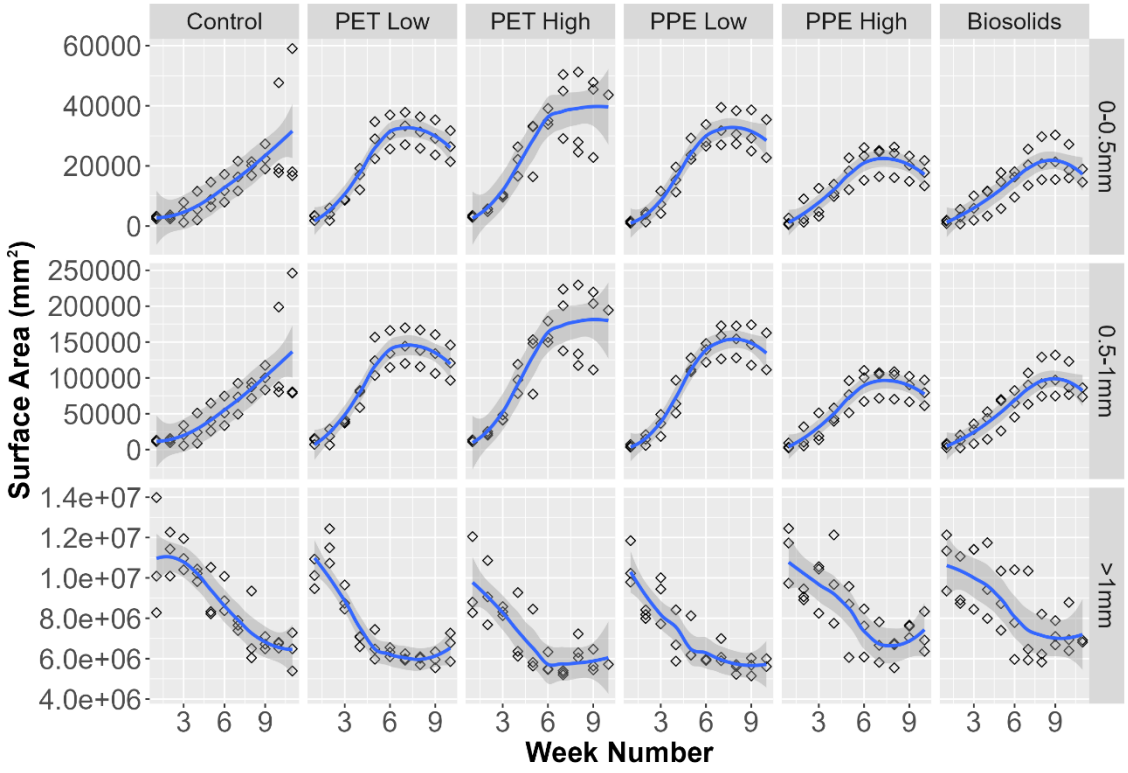


Figure 3.2. Loess spline of root surface area (mm^2) by week number per diameter range class by treatment.

The regression shows that the thickest root class, >1mm, starts high but declines similarly among all treatments (Figure 3.2). However, in the final week of growth treatments 2, 3, and 4 all have less of the thickest transport roots than the control treatment. This may indicate that an increase in plastic concentration could have an

increase in root system maturation speed. They also have the fastest rate of growth for the first two root thickness categories as well as the quickest plateau. This plateau indicates an early achievement of full root system maturity. Treatments 2 through 5 had the earliest cessation of growth but their breakpoints are uncertain, which could potentially mean that while they grew to the full length of the rhizobox, they did not display a distinct breakpoint of maturity (Table 3.2). Treatment 3 is the only treatment to continue to have positive growth after the breakpoint of maturity.

Table 3.2 Piecewise regression cross validation results

Treatment	Diameter Class	Piecewise Breakpoint	Piecewise 1st Coeff.	Piecewise 2nd Coeff.	Piecewise RMSE
1	<0.5	4.3	428.5	742.3	1,243.7
1	0.5-1.0	3.2	432.2	1,408.7	1,282.6
1	>1.0	1.1	12,640.0	4,307.5	6,223.5
2	<0.5	7.0	1,464.1	-2,169.5	1,790.8
2	0.5-1.0	7.3	1,980.0	-1,380.5	2,537.4
2	>1.0	4.9	11,406.1	968.16	4,531.7
3	<0.5	6.6	1,693.8	1022.2	3,291.1
3	0.5-1.0	6.7	2,300.8	202.69	2,354.5
3	>1.0	5.1	11,919.0	1,090.7	7,969.9
4	<0.5	6.4	1,407.4	-1554.4	2,273.0
4	0.5-1.0	7.0	1,898.0	-45.8	2,252.0
4	>1.0	5.5	10,666.0	1,391.5	5,566.0
5	<0.5	6.3	1,850.0	-321.5	1,480.6
5	0.5-1.0	7.4	1,163.9	-492.8	1,862.9
5	>1.0	6.0	8,630.8	840.2	3,965.7
6	<0.5	8.0	802.1	-1,149.4	1,924.5
6	0.5-1.0	9.5	1,024.7	-1,243.7	1,905.9
6	>1.0	2.7	10,897.0	4,031.8	9,350.5

Treatment 6 has a slower rate of growth compared to the other spiked treatments, but the largest exposure to the microplastic layer is limited to the earliest weeks of growth. Treatment 6 also had the latest breakpoints of the spiked treatments, which indicates that outside of control it took the longest to reach full root system

maturity. There is no indication that plastic type is a factor in root system maturity, and the coefficient breakpoints also depict a range that is inconclusive with regards to plastic type.

3.3 Discussion

Root diameter and specific root length are two metrics of rhizospheric and root system study that are very closely linked. The different ranges in diameter for roots of varying order are tied to SRL through the root benefit-root cost ratio that is observed during the study of plant root systems. Studies have shown that fine roots of 0-1mm can account from anywhere from 62% to 72% of the root system and are focused on nutrient and ion adsorption and transfer while the more stable 1-2mm orders of roots are responsible for transporting nutrients and water to the rest of the plant system (Ostonen, et al., 2007). Changes in SRL can be observed and caused by many factors, such as changes in the microbial community, soil moisture, soil contaminants, and nutrient content (Ostonen, et al., 2007).

The effects of concentration of microplastics on root length may be related to the ecotoxicity threshold of these specific types of plastics. The effects of concentration of microplastics on root length has been observed in many studies, and it is also observed in our own. When comparing our concentration of microplastics to that of others to determine their relation, we find that our own concentrations were in some cases much lower than the number of plastics used in others. Some studies do outline that there may be an inconsistent effect caused by concentrations, and our own experiment saw

only one of the mimic plastics having an increased ecotoxicity by concentration. Through only studying two different types of specific plastics as well as biosolid microplastics, there is not enough to be certain what influence concentration has on ecotoxicity from our results alone. The effects inconsistency may be related to the environment as well as situation dependency, with different plant species and their root systems behaving differently, causing them to show ecotoxicity or microplastic effects in different ways. Plastic shapes like sheets can impact plant roots by having more surface area and causing greater obstruction for roots to access soil and symbiotic partners. Sheets also can increase soil moisture by effecting soil pore space, which may in turn have positive effects on plants as there is less struggle to find adequate water, and it may offset some more deleterious effects of plastic on root health by doing so. The long-term effects of concentration of microplastics in soil are also not known as this field is relatively new. Studies into the residence time and long-term presence of microplastics in soil are few, and in the case of biosolids, only approximately 1% of plastics remain following the application of biosolids and regular growth and harvesting of plants (Crossman, Hurley, Futter, & Nizzetto, 2020). With microplastics being applied at high amounts due to their concentration in biosolids, it is still unlikely that their concentration levels will reach 15% in soils without repeated applications for decades as the export of applied microplastics is reliably removing the vast majority of MPs.

Plant root system architecture has a genetic basis with a high degree of plasticity that allows individual plants to tune root traits such as growth rates, diameter, growth direction, and lateral development to environmental conditions. Primary root growth

inhibition and the effects on the density of lateral root growth can be seen when soils are contaminated with heavy metals such as cadmium and zinc, with even non-contaminated areas seeing reduced rates of root colonization throughout the plants life (Remans, et al., 2012). Root system architecture, or RSA, can determine the range within the soil that the root system explores. This in turn effects the amount of nutrients and water the roots may be able to access during a plant's life. The more limited a root systems area of exploration and colonization becomes, the larger the root diameter is on average, which reduces SRL (Tracy, et al., 2012). There is also evidence that the soil conditions early on in root development can have lasting impacts on the root system beyond its exposure time to the contaminants; roots exposed to contaminants may become thicker on average with a shorter SRL even after they have cleared the area of soil contamination (Slovak, Ogura, Satbhai, Ristova, & Busch, 2016). This genetic memory during a root systems development may cause certain temporary exposures to contaminants, like biosolid topsoil which has high amounts of microplastics, to cause RSA changes in the entire root system even in the deeper layers of soil. Due to the more recent nature of microplastic addition to the soil it is not likely that they are causing any genetic triggers in root behaviour that change RSA; it is more likely that their more immediate indirect effects on soil moisture, soil density, and their ability to act as a physical barrier are the causes for these changes.

Root system architecture and any factors that influence it can cause changes in the number of fine roots in a plant root system. Fine root hairs are responsible for the majority of nutrient uptake in plant systems and can alter their traits to become more

efficient depending on the availability of nutrients within the soil (Yan, Jia, & Dai, 2019). Fine root hairs are also very sensitive to changes in soil conditions like soil moisture. Soil moisture changes can be attributed to the addition of microplastics, with microplastic spiked soils having anywhere from 7% to 35% higher soil moisture (Machado, et al., 2019). In studies such as that of Pardales et al, excessive soil moisture increases during root development of sweet potato plants caused an increase in root length, root biomass, and greater amounts of 1st and 2nd order lateral roots (Pardales, Banoc, Yamauchi, Iijima, & Esquibel, 2015). This increase in the fine roots was shown to also fluctuate if the soil moisture began low and increased later or if the soil moisture conditions began high and decreased; any changes to reduce soil moisture caused a lengthening of 1st order roots which is in line with studies that demonstrate fine root changes can be a response to reduced resources and increased resource competition (Pardales, Banoc, Yamauchi, Iijima, & Esquibel, 2015).

The implications of the changes in root structures when exposed to microplastics can be very influential for the agricultural industry. The larger a root system the more it interacts with soil and gains access to nutrients and soil moisture. However, this may cause increased resource competition in times of scarcity. If the field can maintain nutrient and moisture levels, this may be less of an issue. The biggest negative implication that a larger root system with more sensitive 1st order roots may have is that an increased energy expenditure on the belowground biomass may mean reduced output and energy into the aboveground biomass. In crops where the aboveground biomass contains the most economic value, this reduction can lead to reduced yield,

which reduces the economic viability of these crops for farmers. To increase microplastics in the soil may allow roots to grow more resilient to changes and reduction in moisture and nutrients, but it can also have the adverse effect of increasing the resources required by the root system to keep the plant alive, making the plants energy focused less on growing things like flowers, buds, and seedpods. Microplastics in soil can also cause, as seen in this study, changes in the duration in which the plant roots develop. Microplastics were also found to increase the amount of thin fresh roots in the root system. An increase in the time it takes for plant roots to reach the stage of maturity where the thickest and oldest roots become more prevalent can increase plant vulnerability. The thinnest freshest roots are best able to acquire nutrients due to their thin dermis, but this makes them vulnerable to disease as well. The longer a plant root system remains immature the more at-risk it is to diseases.

3.4 Conclusions

In conclusion, the rate at which roots grow, reach maturity, and thicken were affected by the inclusion of microplastics. Changes to a plants root system rate of growth may cause a plant to have more vulnerability to root-borne diseases. The longer a plants root system is immature the longer it remains susceptible. Plant root systems that are grown in the presence of microplastics also have altered root traits that potentially cause them to grow more thin roots than plants without increased microplastic presence. The increase of thin roots can indicate resource scarcity whether or not that is the actual case within the soil. This increase in fresh thin roots also requires a larger energy expenditure from the plant to continue colonizing the soil,

which may lead to reduced energy for the aboveground portion of the plant. Economically, this could mean that an increased presence of microplastic in the soil leads to less viability for a profitable yield.

Future studies should focus on how an increased level of microplastic on the soil effects both the root system and overall crop yield. A larger scale experiment that is designed to examine output of cash crops which are commonly grown with the addition of biosolid fertilizer would be very beneficial in exploring the economic impacts on this widely used practice. The practice of applying biosolid fertilizer reduces costs for the average farmer as well as closes the nutrient cycle, taking what would have previously been a wastewater effluent byproduct and putting it to use. As more is known about applying microplastics to soil and its potential impacts on plants, practices that involve the direct application of contaminants must be re-examined with our increased knowledge.

CHAPTER 4

SYNTHESIS

4.0 Experimental Design Novelty and Limitations

The field of study of microplastics is relatively new, and the study of terrestrial microplastics with regards to agriculture is quite novel. This study sought to underline and investigate the influences of microplastics on RSA with a focus on realistic size fractions and concentrations of microplastics. Unlike most past studies, this study utilized the rhizobox method which allowed for non-invasive and destructive imaging of the roots during growth, rather than a single snapshot of the root system during harvest. The production of an imagery time-series is a novel component that captured when and where roots are growing, and avoided the loss of fine roots which is what would have happened if the pot and root-washing method was used. This study was the first to explore root length and diameter in relation to MP contamination, with a focus on varying types and concentrations of MP.

Although the rhizobox method is novel, the method and experiment design had some limitations. First, the MPs were distributed homogenously into the soil in the biosolid mimic treatments prior to the experiment which ensured that there was MP exposure in all layers of the soil column. This even distribution likely varies from real life conditions where applications of biosolid are only on the top layer of the soil horizon with very limited amounts of MP making it downwards in the soil horizon. Second, the MP used for the biosolid treatment are extremely variable, with there being little way to predict the exact makeup of the plastic ‘cocktail’ that comprises biosolid MP at any

time. Hence, the biosolid “mimics” aren’t true mimics as they were made up of a single plastic type and many other MP properties could not be aligned. Third is that the number of replicates was low, and plants can be quite variable. In particular, treatment 5 displayed extremely variable root traits throughout the study period. Future studies would benefit from much larger replication to ensure a similar irregularity in one or more treatments has a limited detrimental effect on their results. Fourth, this study also investigated only a small subset of all possible RSA traits. Although root length is a serviceable metric for understanding root system growth, additional traits could add useful contextual information. For instance, branching frequency (the number of times a root created branches of exploratory roots off itself) is of great interest as how often a root grows laterally is related to contaminant avoidant behaviour. Lastly, things like the choice of the researcher in things like selecting classes for root diameter may impact the study results. It is important to ensure that while the research remains objective it is also grounded in past research, which is why certain choices like selection of root diameter classes were made.

4.1. Key Conclusions

Regarding the hypothesis of negative MP affects by MP concentration, there was no evidence to support this hypothesis. Rather than a reduction in overall root length and belowground biomass, there was an increase in total root length and biomass for the biosolid mimic treatments, and the increase scaled with concentration. The consistent pattern of increased root length and biomass relative to the control indicates that the increase in MP contamination of the soil can stimulate root growth as well as

increase the average length of roots. The lowering of the aboveground to belowground biomass with MP concentration suggests that the plants are not only growing larger, suggesting improved growth conditions, they are allocating more photosynthates belowground. Growth rates were also positively influenced by MP concentration, with the fine root fraction growing 229.7 cm/week faster during the foraging phase in the PET 15,000 vs the 2,000 particles per dry soil kg and 442.6 cm/week faster in the PPE 15,000 vs PPE 2,000 particles per dry soil kg treatments. The breakpoint between the foraging and senescent phase for the biosolid treatments was roughly 6-7 weeks, suggesting they all grew towards saturation much quicker than the control treatment which never achieved saturation and was still growing linearly at nearly half the rate as the biosolid mimics. Considering that root length, biomass, growth rates, time to saturation were all higher in the biosolid treatment and the effects were greater at 15,000 particles per dry soil kg supports the second hypothesis that MP are altering soil properties in a beneficial manner for this soil type.

Both plastic types were consistent in having a positive effect on the soybean root systems. However, the degree of effect differed by plastic type. PET at 2,000 particles per dry soil kg did not have as large an effect as PPE 2,000 particles per dry soil kg. In terms of the above to belowground ratio, PET Low was quite similar to the control. Although the PPE Low was not statistically significant from the control, the above to belowground ratio was considerably lower. Root length between PET and PPE at 15,000 particles per dry soil kg was statistically significant and the growth rate was 156.2 cm/week higher during the foraging phase. These results suggest that at low

concentrations PPE had a larger positive effect than PET, and at high concentrations PPE had a slightly stronger effect. The effect is attributed to the MP shape rather than chemical composition as both plastics were clear (did not contain dyes) and likely did not weather significantly during the experiment. Although the review by Qiu et al. (2022) suggests sheets have a larger effect size than fragments, this study found differing effects based on concentration. It should be noted that the PPE High treatment was the most variable of all treatments. Hence, the inconsistency may be due to the low number of replicates. Overall, there is support for the hypothesis that plastic type is an important factor to the alteration of soil properties. Practically, the difference is insignificant, especially as biosolids contain plastics of many types and removal of a single chemical type would be challenging. Efforts would need to be made to remove the input from a single MP source prior to entry into the wastewater system.

The minimal effect of the biosolid treatment relative to the control indicates the impact of biosolid microplastics may be stronger than hypothesized, albeit there is conflicting evidence. The above to belowground ratio, root biomass, and root length all suggest there is no difference from the control. This is unsurprising as the depth of biosolid application was only to the top 15cm, thus has minimal interaction between the root system and the biosolid MP. However, the biosolid treatment had a development phase different from the control and akin to the biosolid mimics. The root system grew at about the same rate as the control, but the system grew for only eight weeks whereas the control continued to grow. Hence, the biosolid treatment reached saturation approximately 1-2 weeks after the biosolid mimics. It is possible that growing

through the topsoil with microplastics was sufficient to alter the root development process.

4.2 Future Research

With regards to any future research that this work could inspire, it is important to impart as much of the knowledge learned from the successes and shortcomings of this study as possible. The method of using the rhizobox and imaging rather than growth in pots and performing a simple root washing and analysis at the end of growth was one of the most successful elements of this experiment's design. The ability to make multiple snapshots of the root system throughout the growth period enabled us to get further insights into the changes a root system undergoes throughout the entire time of growth. Much of the study of contamination requires that the most minute details, like the thinnest of roots in a root system, be accounted for. This method allows for the thinnest roots to be imaged and can help ensure that there is minimal damage to them during the study period, where other methods like root washing can increase fine root loss and fragmentation of root systems. The main drawback, however, of this method is that it does not accurately replicate the conditions of farmers' fields. This study design tried to remain as accurate as possible to growing conditions in an agricultural setting but are still limited to the researcher's ability to combine a study method that allows for intimate and detailed knowledge of roots while also attempting to replicate as much real-life growing conditions as possible. While the rhizobox method was very successful at ensuring that the whole root system was studied at all points of growth, perhaps

future studies can find a way to merge this component with actual agricultural fields, such as through in-soil cameras, to fully immerse the study in agricultural reality.

The use of the mimics in this study was in line with previous studies and their use of lab created mimics during their soil microplastic experiments. The size fractions in this study were chosen as they were representative of the range in size fractions found in biosolid microplastics. While any studies that focus on more long-term effects may focus on smaller size fractions, ranging into nanoplastics to represent further degradation and fragmentation of microplastics, the size fractions used in this study did not need to do so as the time it would take for these MPs to breakdown is outside the scope of this study. Nanoplastics may be an important focus of any future studies regarding rhizospheric microplastics as the smallest size fractions of plastics may be uptaken into plant roots during the drawing up of nutrients, ions, and water by the plant roots during growth. The plastics used in this study were also non-weathered, which may be something for other studies to consider. The weathering of MP surfaces can allow for an increased surface area as well as increased colonization of bacteria, which may be harmful, on the MP surface. One key aspect for a future study would be the addition of weathered plastics and the creation of a bacterial biofilm prior to the amendment of the MPs into the soil. The potential for microplastics bacterial biofilm to represent a potential disease vector in agriculture is one that provokes future study as it has yet to be explored. Our microplastics were also uncolored and not ones in which harmful chemical leaching may have occurred. This is not the case for all MP found in the natural environment so any future study may wish to replicate this experiment as

well as add in MPs which have been dyed or have components that leach potentially harmful chemicals into the soil if the MPs breakdown during their study period.

This study also used standard common plastic types, while some other studies also included bioplastics in their experiments. Similar experiments to this one have been completed by this research group and, while unpublished as yet, it points to the inclusion of bioplastics in soil leading mostly to a strong increase in mold growth within the soil. This is likely caused by the starch components of bioplastics, which when broken down, largely encourage the growth of mold at such a rate that any impacts they may have during growth are very much overshadowed. This study also focused on keeping the concentrations of microplastics toa a realistic level in comparison to biosolid inclusions. This low-dosage study allowed the research to focus on real-world implications of MP additives in soil and how these realistic concentrations can affect soil properties.

The imaging in this experiment was quite successful, with the only critiques to be improved on in future study would be to find other alternative ways to ensure that condensation within rhizoboxes is as limited as possible during growth. The condensation in the boxes created some difficulty during the binarization process while image processing. A different type of imaging, such as a time-lapse recording of the boxes throughout growth may also be very valuable as it can potentially be sued to determine which time periods had the most accelerated growth, which can assist in any pre- and post-root system maturity analyses being performed. This study would also

recommend that more attention is paid to soil moisture during the study as it became more obvious with further research that soil moisture has a greater impact in root growth, especially given the impacts MP can have on soil moisture. This study's recommendation would be soil moisture probes in at least one box per treatment at least 2 different soil depths, preferably one 6 inches into the soil column and another 6 inches from the bottom of the soil column. This can allow for more understanding as to how soil moisture levels change before and after watering throughout the box and may also allow for studies to determine the amount that MP contamination can affect soil moisture levels and distribution.

Finally, given that this study was focused on soil from the Essex region, it may be of value to recreate this experiment on soils from different areas. The soil used in this study is known for its increased soil moisture, which combined with the potential for soil MP to increase soil moisture may have limited the visibility of any moisture-related effects during growth. This experiment may be of great value if replicated on soil that has high amounts of drainage and low soil-moisture holding capacity, such as areas in which sand makes up a higher percentage of the soil chemistry.

4.3 Broader Impact

This experiment was conducted using funding from Environment and Climate Change Canada to determine the effects of biosolid microplastics on agricultural soils and the health of agricultural crops. The results of this study demonstrate that policy regarding biosolids may not need to be restrictive as there were few, if any, negative impacts associated with the biosolid treatment compared to the control. Based on the

results of this study, there is not sufficient evidence for any restrictive MP policy related to biosolids in agriculture. However, it is also the recommendation of this study that while there may be no apparent deleterious effects, this study is short-term, and there remains the question of whether biosolid MP can have long-term effects, either positive or negative, on plant root systems as a whole.

The diversity of plastics that constitute biosolid MPs due to the many pathway's plastics can have to enter wastewater treatment makes it unique to any other potential plastic sources in the environment. This uniqueness means that biosolid MPs should still be part of future study on terrestrial microplastics, especially within the agricultural field. The lack of long-term studies is understandable as terrestrial MP studies on biosolid MPs are still rare, but it is the opinion of this researcher that these long-term studies are key to ensuring that all aspects of increased MP in soil are understood and examined, especially as the addition of biosolid fertilizer is becoming more common.

APPENDICES

Appendix A

Copy of R code for stacking scanned images together

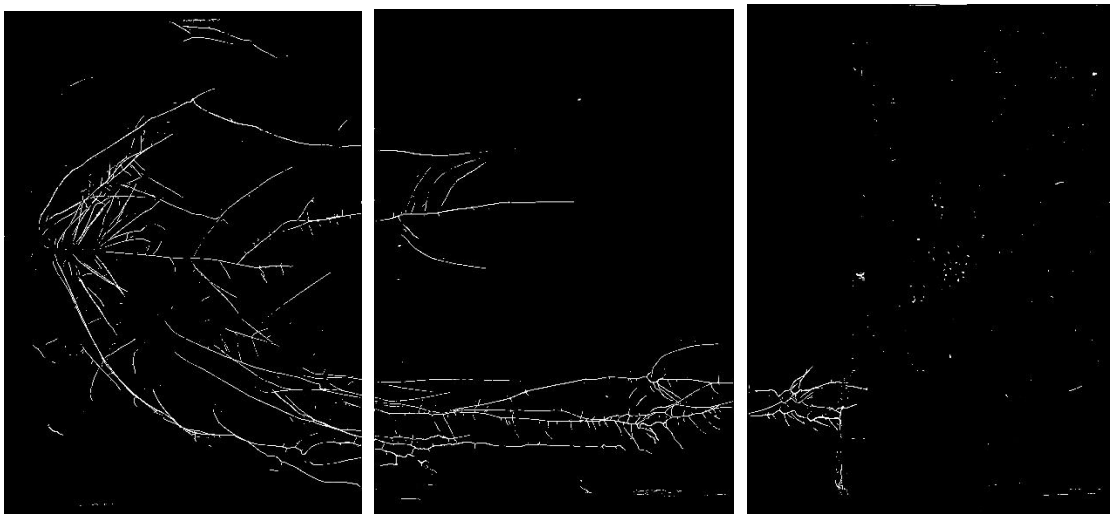
```
library("jpeg") # for reading in PNGs
library("rstudioapi")
library("magick")
library("data.table")
library("lubridate")
setwd(dirname(getActiveDocumentContext()$path))
#MagickNET.SetTempDirectory("f:/Projects")
img<-list.files(path=".", pattern="*.jpg", all.files=FALSE,full.names=FALSE)
# get info from filnames
boxID<-substr(img,12,13)
ScanLine<-substr(img,15,15)
Date<-dmy(gsub("_", '/',substr(img,17,27)))
#create a new dataframe and find unique box numbers
img<-data.frame(cbind(img,boxID,ScanLine))
img<-data.frame(img,Date)
uboxID<-unique(img$boxID)
#create blank images
blank<-image_blank(width = 4608, height=3072, color = "black")
blankcrop<-image_blank(width = 4608, height=130, color = "black")
```

```
order<-c("a","b","c","d","e","f","g","h","i","j","k","l","m","n")

# loop by box then by date

#concatenate all scanlines and write in order of date

for(box in 1:length(uboxID)) {

allBoxes <- subset(img, boxID == uboxID[box])

uDates <- setorder(data.frame(unique(allBoxes$Date)))

print(paste("box",box,sep=""))

for (ddate in 1:nrow(uDates)) {

allDates <- subset(allBoxes, Date == (uDates[ddate,1]))

print(paste("date",ddate,sep=""))

if (length(allDates$boxID) == 1) {

cmb <- c(image_rotate(image_read(path=file.path(allDates$img[1])),90),
blank, blank)

final = image_append(cmb, stack = TRUE)

image_write(final,
paste(uboxID[box],"_",order[ddate],"_RhizoboxCmb_","_",uDates[ddate,1],".j
pg",sep=""))

print(paste("scanlines1"))

} else if (length(allDates$boxID) == 2) {

cmb <- c(image_rotate(image_read(path=file.path(allDates$img[1])),90),
image_crop(image_rotate(image_read(path=file.path(allDates$img[2])),90),"46
08x2942+0+130"),blankcrop,blank)

final = image_append(cmb, stack = TRUE)
```

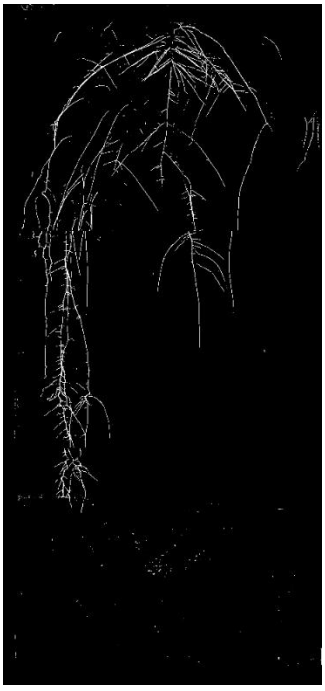
```
image_write(final,
paste(uboxID[box],"_",order[ddate],"_RhizoboxCmb_","_",uDates[ddate,1],".j
pg",sep=""))

print(paste("scanlines2"))

} else if ((length(allDates$boxID) ==3)){

cmb <- c(image_rotate(image_read(path=file.path(allDates$img[1])),90),
image_crop(image_rotate(image_read(path=file.path(allDates$img[2])),90),"46
08x2942+0+130"), blankcrop,
image_crop(image_rotate(image_read(path=file.path(allDates$img[3])),90),"46
08x2942+0+130"),blankcrop)

final = image_append(cmb, stack = TRUE)

image_write(final,
paste(uboxID[box],"_",order[ddate],"_RhizoboxCmb_","_",uDates[ddate,1],".j
pg",sep=""))

print(paste("scanlines3"))

}

gc()

}

}
```

APPENDIX B

Example Process for Imagery from Week 8, Box 3 (Control)



Appendix B 1Box 3, Week 8, Frame 1, 2, & 3



Appendix B 2Box 3, Week 8, Root Diameter Output

Appendix B 3 Box 3 Week 8 Whole Image

Appendix B 42Box 3, Week 8, Root Diameter Output Zoomed in; Blue: <1mm, Green: 0.5mm-1mm, Yellow: >0.5mm

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