The effect of sewage sludge containing microplastics on growth and fruit development of tomato plants

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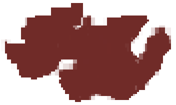
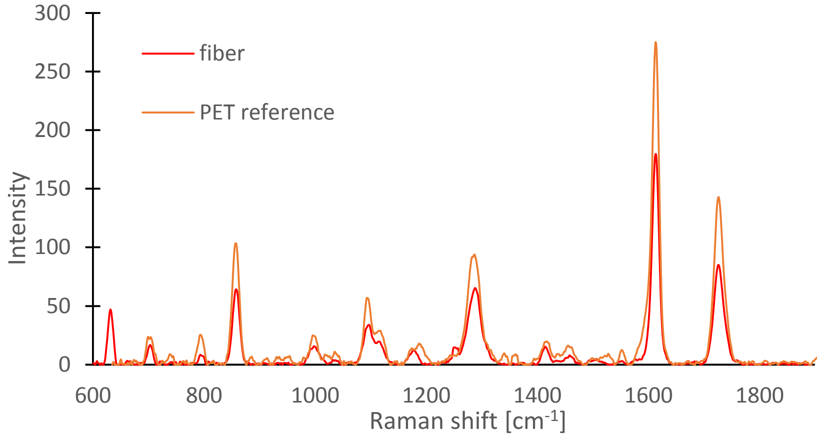


Extraction of the microplastics from sewage sludge

Characterization and quantification



Cultivation of tomato plants with sewage sludge

Measurement of morphological parameters, biomass and fruit production

The effect of sewage sludge containing microplastics on growth and fruit development of tomato plants

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ABSTRACT

Microplastics (MPs) are becoming an environmental growing concern, being the sewage sludge applied to agriculture fields one of the most important inputs to the environment. To date, there is no standardized protocol for their extraction and changes in vegetative growth and fruit maturation on cultivated plants induced by sludge containing MPs have not been studied yet. Sewage sludge from three different wastewater treatment plants located in Murcia, Spain, were studied. First, the microplastic concentration was estimated and, then, the effects of the sewage sludge in the development of tomato plants and fruit production was analyzed. The measured parameters in tomato plants were both, biomass and length, for shoot and root part, as well as, stem diameter and tomato production. The present work has developed and validated a protocol for the extraction and quantification of MPs comprising several shapes, materials and sizes from samples of sewage sludges, which offers a good compromise for the extraction of different types of microplastic. The protocol used for MPs extraction had a recovery efficiency of 80 ± 3 % (mean ± SE) and used bicarbonate, to maximize MPs extraction. The mean abundance of MPs in the studied sewage sludge samples was 30,940 ± 8,589 particles kg^{-1} dry weight. Soils with sludge containing MPs fostered the growth of tomato plants, while delaying and diminished fruit production. However, other factors or their interactions with MPs could have influenced the outcomes. Further studies are necessary to corroborate these findings and explain the mechanisms of possible effects of MPs on plants.

Capsule: In this work, the effects of sewage sludges containing microplastics on growth and fruit development in tomato plants have been evaluated.

KEYWORDS: Tomato plants, sewage sludge, microplastics, biomass, fruit crop

1. Introduction

Microplastics (MPs) are an environmental growing concern, with an annual input to the environment of 11 million tons (Boucher and Friot, 2017). Agriculture fields receiving sludge from sewage treatment plants as fertilizers constitute one of the largest inputs of MPs to the environment (63000-430000 t of MPs per year; (Nizzetto et al., 2016). Only in Europe, wastewater treatment plants (WWTPs) produce 13 million tons of dry weight sludge per year and approximately 99% of MPs can be retained in the sludge (Magnusson and Norén, 2014). 80% of this sludge is used in agriculture (Mahon et al., 2017). Despite the importance of quantifying the concentrations of MPs in sludge (de Souza et al., 2018; Corradini et al., 2019), to date, there is no standardized protocol for their extraction and quantification (Rocha-Santos and Duarte, 2015; Rochman et al., 2017).

Different methodologies have been proposed for the extraction of MPs from sludge samples, generally using oxidizing agents and acids to remove part of the organic matter and a density separation of the MPs by means of saline solutions (Lusher et al., 2017; Sujathan et al., 2017; Hurley et al., 2018; Lares et al., 2018; Li et al., 2018). However, one of the drawbacks of using high concentrations of these agents to remove organic matter is the possible degradation of some polymers, (Avio et al., 2015), difficulting their identification by using Raman and FT-IR spectroscopy (Lares et al., 2018).

Additionally, estimates of MPs concentrations must be accompanied by a recovery test of the most abundant types of MPs comprising different sizes. Previous studies quantifying MPs in sludge or in soil samples have generally performed partial recovery tests only using one (Mahon et al., 2017; Sujathan et al., 2017; Li et al., 2018) or two shapes of MPs (Hurley et al., 2018; Corradini et al., 2019) and in some cases a recovery test was not applied (Lusher et al., 2017; van den Berg et al., 2020). Nevertheless, recovery tests are key in assessing the effectiveness of the extraction methods, necessary to design standardized procedures that can precisely estimate MPs concentrations in sewage sludge.

MPs are expected to alter the biophysical properties of the soil, in terms of water retention capacity, bulk density, microbial activity and formation of aggregates, which could lead to an alteration in the water cycle in the soil (de Souza Machado et al., 2018; Wan et al., 2019). However, the effect that MPs accumulation in the soil will generate on the vegetation has been scarcely studied. MPs present in sludge from WWTPs used in agriculture can remain in soil for a long-time affecting the vegetative growth (van den Berg et al., 2020). In this sense, it has been reported that MPs can induce changes in both aerial and below-ground parts during vegetative growth in the wheat plant (Qi et al., 2018) while reducing the germination rate and root growth of *Lepidium sativum* as the concentration of MPs to which plants were exposed increased (Bosker et al., 2019). Although sewage sludge has been employed to improve soil fertility in agriculture (Llorens et al., 2012), to the best of our knowledge, changes induced by sludge containing MPs in vegetative growth and fruit maturation of cultivated plants have not been studied.

The aim of this work is to assess the efficiency of a protocol for MPs extraction and quantification from sewage sludge samples and evaluate the effect of sewage sludge containing MPs on tomato plants once applied in soil. To assess the efficiency of the extraction and quantification protocol, a recovery test was carried out using different types of particles, materials and sizes. At last, the effects of sewage sludge containing MPs on tomato plants were evaluated by means of response variables related to growth and fruit production.

2. Materials and Methods

2.1 WWTPs and sludge samples

Sludge samples were obtained from the municipal WWTPs located in Librilla (W1), Alhama de Murcia (W2) and Totana (W3) (Murcia, Spain) (Table S1).

2.2 Microplastics (MPs) extraction procedure from sludge samples

Three replicates of 20 mL sludge from each WWTP were introduced in 250 mL pre-washed glass beakers. Afterwards, 40 mL of solution H_2O_2 (30 wt %) was added to the beakers and left for 2 d to remove most of the organic matter present in the sample. Then, MPs were extracted through density separation by adding 150 mL of a NaCl saturated solution and stirring with a glass rod for 3 minutes. The solution was then allowed to settle overnight. Afterwards, the supernatant was decanted and filtered. By adding 150 mL of the saline solution, MPs decantation was facilitated by minimizing resuspension of the settled particles.

This process was repeated four times. Since the density of the used saline solution was 1.2 g cm^{-3}, some polymers, mainly polyethylene terephthalate (PET; (density 1.29 – 1.40 g cm^{-3}) and polyvinyl chloride **(**PVC; density 1.30 – 1.58 g cm^{-3}) (Duxbury, 1992) may not float in the solution but settle on the bottom of the beaker. To prevent this, in the last repetition of the process, 6 g of sodium bicarbonate were added to the saline solution. Then, the solution was heated for approximately 4 minutes until the temperature reached between 45ºC and 50ºC and stirred with a glass rod to facilitate the release of CO_2 bubbles that promote the flotation of MPs to the top of the saline solution (per. obs.). The supernatant was filtered through glass fiber filters with a pore size of 1.2 µm (Frisenette ApS, Knebel, Denmark).

Filters were left in petri dishes and the remaining organic matter was removed by submerging them with approximately 20 mL of 1:1 (v/v) H_2O_2 (30 wt. %) + H_2SO_4 (96 wt. %) (Merck, Darmstadt, Germany) solution and left overnight. Since the H_2O_2:H_2SO_4 solution produces a very exothermic reaction, it was left to cool down before being used to prevent possible damage to MPs. The used solution was chosen after several tests using the following proportions of H_2O_2 (30 wt. %) and H_2SO_4 (96 wt. %): 1:1, 1:2 and 1:3 aiming to find the best compromise between organic matter removal and avoidance of PET microfibers disintegration, which are the most sensitive component to be disintegrated out of the main plastic debris found in the sludge (per. obs.). The best results were obtained by using the 1:1 ratio in which fibers were not visually affected. Higher proportions of H_2SO_4 lead to total or partial disintegration of the fibers.

After the exposition of filters to the acid solution, the liquid content in the petri dishes was filtered. Both petri dishes and filters were rinsed off several times with distilled water to ensure that no particles were retained. Then, particles in the filters were identified by Raman spectroscopy and quantified. Since some particles were retained on the walls of the bottom part of the funnel of the filtering equipment, after each filtration, the funnel was separated from the main structure, washed with distilled water and the liquid collected was filtered again in the same filtration unit to avoid MPs losses.

2.3 Recovery test

A recovery test was performed to estimate the extraction efficiency and reproducibility of the protocol used in this study. The recovery test was carried out using commercially available plastic materials with different shapes and sizes (Table S2) representative of the MPs found in sludge. The MPs were spiked into 20 mL of sludge from W3. The particles used in the recovery test had specific colors and shapes to facilitate their differentiation from particles already present in the sludge. In addition, it was previously checked that the treatments applied to remove the organic matter did not alter the particles used in the recovery test.

Fragments were composed of high density polyethylene (HDPE) and polypropylene (PP) from a bleach bottle and a cap of a container, respectively. For films, HDPE and low density polyethylene (LDPE) were used, from different plastic bags. The sizes of fragments and films were: 0.4-0.6 mm, 0.8-1.0 mm, 1.9-2.1 mm and 2.4-2.6 mm. PET microfibers were obtained from two T-shirts with a length of *ca.* 2 mm. Microbeads were composed of butylene/ethylene copolymer (B/E) and they were obtained from a shower gel presenting a size between 0.6 and 0.8 mm. Fragments, films and microfibers were cut using a binocular magnifying glass (LFZ Dual, OPTECH, Ontario, Canada), a graduated scale and a scalpel to produce the desired sizes. To test the repeatability, a total of 10 particles of each size and type of material were added to 20 mL of sludge, summing a total of 80 fragments, 80 films, 20 PET microfibers and 10 B/E microbeads. The recovery test was performed six times to test its reproducibility, indicating the calculated variation in the extraction efficiency when the measurements are reproduced.

The recovery efficiency percentage was calculated as follows:

$$E\ (\%) = (P\ /\ Pr) \bullet 100$$

Where E stands for the Efficiency, P refers to the number of particles added and Pr is the number of particles recovered.

2.4 Characterization of MPs by Raman Spectroscopy

MPs were characterized by Raman spectroscopy following the procedure indicated in the Supplementary Data. Spectra of the suspected MPs were compared to the Raman spectra of reference materials (Figure S1), (Boerio et al., 1976; Andreassen, 1999; Long, 2004; Kida et al., 2016) (Table S3).

2.5. Microplastics count

The MPs present in each filter were counted using a binocular magnifying glass (LFZ Dual, OPTECH, Ontario, Canada) with the aid of a transparent circular template divided in eight sections that was placed at the top of the Petri dish.

2.6. Quality control

During the whole process of MPs extraction, blank filters were exposed to the air as quality controls to evaluate possible cross-contamination (Woodall et al., 2015). Particles found in these filters were analysed through Raman spectroscopy as explained previously. See Supplementary Data for a more detailed explanation.

2.7. Plant material and growth conditions

The tomato plant (*Lycopersicon esculentum Mill.*) was chosen since it is widely cultivated and shows low or no toxicity due to over fertilization with macronutrients (Sainju et al., 2003). One-month tomato plants were transplanted outdoors on the 14th of March of 2019 individually into a 3.3 L pot made of terracotta. The dimension of each pot was 19 cm high, diameter of 20.7 cm at the top and 12.7 cm at the bottom. Five soils, control (C), manure control (MC), and soils containing sludge of each WWTP (W1, W2 and W3), were created using 50:50:0:0, 45:45:10:0, and 45:45:0:10 proportions of peat moss (COMPO SANA ® UNIVERSAL, Münster, Germany), silica sand (grain size range: 0.4-0.8 mm), manure (FEMABÓN ®, Castellón, Spain) and sludge, respectively.

Control soil was used as the reference substrate, while manure control soil was used as a fertilizer-type amendment to soil aimed to simulate nutrient addition to the soil but with an insignificant MPs concentration. To ensure that there was no nutrient deficiency due to the different element composition of manure and sewage sludge in all soils, each plant was fertilized monthly with 10 g of a solid slow-release fertilizer (NPK 12-8-18) (Bluefficient Platinnum®, Salamanca, Spain), aiming to prevent changes in the development of tomatoes due to a specific element deficiency while avoiding fertilizer toxicity. Characteristics of the employed peat, manure, and fertilizer used are shown in Table S4. Each type of soil was replicated in seven pots, each one containing a tomato plant, so seven tomato plants were cultivated in each type of soil which made a total of 35 tomato plants.

The pots containing the plants were placed outdoors in an area of the campus of the University of Alicante that had direct radiation from the sun during the whole day. A wood stick was tied to each plant to prevent them from bending and a blue protective mesh was placed in each pot to avoid plants from animal attacks. The experiment lasted 109 d. Each pot was watered with 500 mL of tap water. Watering frequency was once every two days until June. Then, it was increased to twice per day until the experiment finished on the 1st of July. The position of the pots was changed every month to avoid possible effects due to placement.

2.8 Measurement of pH and soil salinity

Every month, pH and soil salinity were measured to monitor nutrient availability and water absorption capacity of the tomato plants as it is described in Supplementary Data.

2.9. Collection of tomatoes

The fruits of all tomato plants began to be harvested after three and a half months of cultivation and the fruit selection was carried out according to their maturation status with the purpose to collect tomatoes in a similar maturation stage, using as a criterion the color of tomato fruits (Figure S2).

2.10. Measurements of tomato plant growth parameters

Once the experiment was concluded, tomato plants were taken out of the pots and roots were cleaned in a cube with water to remove soil particles attached. Shoot length of tomato plants was measured as the maximum length of the main stem, whereas root length was determined as the maximum length of the longest root. Stem diameter was measured with a digital vernier caliper (Mitutoyo CD-6 C5, Neuss, Germany). Then, tomato plants were cut into the shoot and root parts and dried during 72 h at 70°C to obtain the dry weight of each part.

2.11. Statistical analysis

The experiment consisted of a one factor (type of soil) design with five types of soils: C, MC, W1, W2 and W3. Each treatment was replicated three times. Significant differences among the response variables measured in the different types of soils were analyzed through a one-way analysis of variance (ANOVA). Before ANOVA, the normality was tested through Kolmogorov-Smirnov's test and heterogeneity of variance of the data was tested using Cochran's test. If the data did not meet these assumptions, transformations were applied, and assumptions were checked again. When significant differences were found in the ANOVA main test, the *post-hoc* Tukey's test was used to find which treatments significantly differed (Table S5). Statistical analyses were performed with the R statistical software (v. 3.6.0; R Core Team, 2019). All statistical tests were conducted with a significance level of α = 0.05. Data were reported as mean ± standard error (SE).

3. Results and discussion

3.1 Method extraction efficiency

The mean average extraction efficiency for the total of particles spiked in the sludge samples was 87 ± 2% (Figure 1). The range of particle size with the highest extraction efficiency was 2.4-2.6 mm (91 ± 5%) whereas the lowest extraction efficiency was obtained in particles with size in the range 1.9-2.1 mm (87 ± 3 %). Fragments and films were extracted with an average of 91 ± 2 % and 86 ± 3 %, respectively. Microfibers had a mean extraction efficiency value of 83 ± 3%. Spiked microbeads showed 90 ± 6 % of extraction efficiency. As regarding the type of material PP, HDPE, LDPE and PET were extracted with an efficiency of 91 ± 2%, 89 ± 2%, 88 ± 3% and 83 ± 3% respectively.

Figure 1

The obtained percentage values of extraction efficiency are, in general, comparable with previous studies (Mahon et al., 2017; Sujathan et al., 2017; Hurley et al., 2018; Li et al., 2018; Corradini et al., 2019). Only in some cases are slightly lower, which could be due to the wide range of sizes of particles used in this study, generally reaching lower sizes than other studies (Huerley et al., 2018). In addition, the recovery test carried out in this study is comprehensively complete compared to previous ones where only a few types of particles, materials and sizes were used (Mahon et al., 2017; Sujathan et al., 2017; Hurley et al., 2018; Li et al., 2018;). Specifically, in this work the recovery test uses microfibers, which is generally the main component of sludge despite not being a particle type commonly used in recovery tests (Mahon et al., 2017; Sujathan et al., 2017; Li et al., 2018). Our results indicate that this extraction method is capable of efficiently recover PP, LDPE, HDPE and PET microfibers, which are the most commonly used plastics (PlasticsEurope, 2019) and the most common types of MPs found in sludge (Mahon et al., 2017; Li et al., 2018; Corradini et al., 2019). Thus, this study demonstrates that the present extraction method is suitable for the extraction of MPs in sewage sludge, while being inexpensive and safer than others that use other salts, such as ZnCl and NaI.

3.2 Characterization of microplastics in sludge samples

The concentration of MPs was 17,870 ± 2,174; 27,821 ± 1,357 and 47,130 ± 3,002 particles kg^{-1} dry weight of sludge in W1, W2 and W3, respectively (Figure 2). The sizes of the MPs were in the range of 0.31-2.11 mm (Figure S2).

Figure 2

W3 showed a significantly higher concentration of MPs in comparison with W1 and W2. Concerning MPs shape, microfibers were the most abundant particle followed by fragments, films and microbeads.

Figure 3

Microfibers represented the 93 ± 2 %, 89 ± 5 % and 97 ± 1% of the total MPs in W1, W2 and W3, respectively. All microfibers analyzed were made of PET, so it is suspected that they were synthetic fibers used for acrylic garment, since microfibers in sludge mainly come from cloth washing (Browne et al., 2011; Folkö, 2015; Åström, 2016). Additionally, microfibers, since they are made mostly of PET, their density, higher than water and along with their high surface:volume ratio, they are prone to precipitate in the sludge during wastewater processing in the WWTP. This explains that PET microfibers were the most abundant MPs in sludge as previously reported (Mahon et al., 2017; Li et al., 2018; Corradini et al., 2019).

Fragments accounted for the 6 ± 2%, 9 ± 4% and 2 ± 1% of the total MPs in the sludge from W1, W2 and W3, respectively. Films were uniquely found in W2 (2 ± 1%). Fragments and films found were generally irregular. PE was the predominant polymer in these types of particles, while PP showed a limited presence.

The lesser abundance of fragments and films in sludge in comparison to fibers sides with previous studies (Mahon et al., 2017; Li et al., 2018; Corradini et al., 2019). This may be due to PE, has lower density than water and it tends to float, and it is less likely that their fate is the sludge. Additionally, because of its shape, fragments and films, for a specific size, are more likely to be retained than microfibers in the pretreatment filters of WWTPs before entering in the degreaser-desander channel. In fact, W2, which was the WWTP with the largest mesh size pretreatment filter (70 mm) of all the WWTPs, had the greatest percentage of fragments (9 ± 4%) and films (2 ± 1%) compared to the percentage of fragments found in W1 (6 ± 2 %) and W3 (2 ± 1 %), which had a mesh size of 2 and 3 mm, respectively. These findings seem a plausible explanation of the low presence of fragments and films in sludge. Microbeads only constituted 0.4 ± 0.4 %, 0.4 ± 0.4 % and 0.2 ± 0.2 % of the total MPs in W1, W2 and W3, respectively as previously reported(Mahon et al., 2017; Li et al., 2018).

As regards the extraction process in the sludge samples, out of the total particles extracted, 61 ± 4 %, 19 ± 4 %, 13 ± 2 % and 7 ± 2 % of the particles were recovered in the first, second, third and fourth extraction step, respectively. In the last extraction step, bubbles produced by the bicarbonate kept microfibers on the surface of the solution used for the separation of MPs through difference of density. Thus, the use of bicarbonate for MPs extraction could be advisable to maximize the process, especially in matrices with high microfibers content.

The average concentration of MPs in the sludge amongst the three WWTPs was 30,940 ± 8,589 particles kg^{-1} dry weight, being higher than previous studies(Corradini et al., 2019); (van den Berg et al., 2020); (Mahon et al., 2017).

3.3. Measurements of tomato plant parameters

After 109 d growth, significant differences among soil treatments were found in biomass production, but not in the morphological variables studied (Figure 4).

Figure 4

Shoot biomass was significantly higher in W2 (79.2 ± 7.3 g) than in C (35.8 ± 4 g), MC soil (41.4 ± 3.4 g) and W3 (53.4 ± 5.1 g). Significant differences were also found between W1 (60.9 ± 7.2 g) and C samples. For root biomass, W2 (67.8 ± 7.3 g) was significantly higher than C (23.4 ± 5.1 g), MC (36 ± 10.4 g) and W3 (32.4 ± 5.1 g). Total biomass was significantly higher in W2 (146.9 ± 11.3 g) than in C (59.1 ± 8.7 g), MC (77.4 ± 11.8 g) and W3 (85.8 ± 11.2 g) soils. Significant differences were found between W1 (60.9 ± 7.2g) and C samples. The values of stem diameter, height and root length ranged between 11.7 ± 1.5 and 10.3 ± 1.1 mm, 74.0 ± 5.4 and 61.9 ± 15.4 cm and 34.2 ± 6.0 and 27.4 ± 6.8 cm, respectively.

The number of tomatoes harvested on each tomato plant did not significantly vary as regards the type of soil. MC soil had the highest tomatoes production with 5 ± 1 tomatoes while W3 produced less than 2 fruits. During the experiment, the number of mature tomatoes produced in plants grown in C and MC soil was significantly higher (1.4 ± 0.3 and 1 ± 0.4 tomatoes, respectively) than the ones grown in soils containing sludge with MPs (W1; 0,13 ± 0,13; W2: 0,14 ± 0.14; W3: 0) (Figure 5).

Figure 5

At the end of the experiment, MC had the highest percentage of plants (75%) that produced tomatoes, whereas in C or soils containing sludge from W1, W2 and W3 this percentage was lower being 50, 37.5, 25 and 25%, respectively.

The mean values of pH obtained for W1 (6.4±0.1) and W3 (6.5±0.1) were considered slightly acidic, whereas W2 (6.6 ± 0.4), MC (7.2±0.1) and C (6.8±0.1) were considered neutral (Juárez et al., 2004), which limits nutrient deficiency problems of nutrient availability related to highly acidic or basic soils (Sainju et al. 2003). The mean values of electrical conductivity obtained from C (3.3 ± 0.2 dS/m), W1 (2.9 ± 0.2 dS/m), W2 (2.8 ± 0.1 dS/m) and W3 (2.7 ± 0.1 dS/m) were considered very slightly saline (2-4 dS/m) whereas MC soil was considered slightly saline (5.3 ± 0.2 dS/m) (Schoeneberger et al., 2002). The sludge addition to soils generally results in an increase of their salinity, but in case of saline soils, it can reduce their salinity (Pomares et al. 1998). Peat moss, that was used as the base of the soil component in the present experiment, can retain a large amount of cations, which could be responsible of increasing the levels of salinity. The variety of tomato plants used, Raf, have a threshold of tolerance to salinity of 6 dS/m (Nelson, 2011). Thus, the levels of salinity in the different types of soil is not expected to negatively affect the growth and yield of tomato plants.

Nutrient levels in the different sludge used were in some cases notably different. For example, N was 7.6±0.9% in W2, while in W1 and W3 was 1.9±0.2 and 5.4±0.6, respectively (Table S1). High contents of N concentration can promote biomass at the expense of fruit yield (Sainju et al. 2003). Our results indicate a notable shoot and root growth of tomato plants in soil W2, which was significantly different to the plants grown in soil W3, but not in W1, which had the lowest N content (Fig. 4). Nevertheless, fruit production was not affected in plants grown in any of the soils treated with sludge (Fig. 5).

Phosphorous and K values were also different among the used sludges. Therefore, in our experiment a solid slow-release fertilizer containing N, P and K was applied to all the treatments aimed to prevent nutrient limitation. Other macronutrients, such as Mg and Ca, despite the content being different among sludges, the levels were high enough that no deficiency was expected. Nutrient toxicity due to the above-mentioned nutrients is not expected in tomato plants, apart from the commented issue with N (Sainju et al. 2003).

Our results suggest contrasting effects of sludge addition in soils since the biomass production was higher in plants cultured with sludge than the ones cultured in the C or MC soils. However, tomato plants cultured in soils treated with sludge from W3, which had the highest concentration of MPs, had the lowest biomass and tomato production, while it did not produce any mature tomato during the experiment. Plant biomass can be influenced by many factors, such as soil humidity, soil and air temperature, photoperiod, solar radiation, precipitations, genotype, etc. One of the most important factors influencing biomass is soil nutrient availability since both nutrient deficiency and toxicity negatively affect total biomass and fruit production (Msilini et al., 2009; Karim et al., 2012). The solid slow-release fertilizer aimed to prevent nutrient limitation. The shoot:root biomass ratio of cultured plants did not show significant differences among plants cultured in the different soils, suggesting non-relevant differences in nutrient availability among soils (Ericsson, 1995). Water was homogeneously provided, while it was ensured that all the plants were similarly exposed to light.

The effects of MPs in soil have been scarcely studied, but are expected to be highly influenced by the shape (Rilling et al., 2019) and size (Bosker et al., 2019), being microfibers among the type of MPs that is expected to have the strongest effects (de Souza Machado et al., 2019). In the present experiment, differences in plant biomass among soils with sludge were noticeable. Root mass is expected to increase in the presence of microfibers since they can lower soil bulk density (de Souza Machado et al., 2018) promoting increased root growth due to reduced penetration resistance for plant roots and improved soil aeration (de Souza Machado et al., 2019). These differences could also directly and indirectly affect soil structure through the modification of soil aggregation (de Souza Machado et al., 2018). Our results side with previous findings showing the largest biomass in the root, as well as, in shoot in soils with sludge, especially in W2 (de Souza Machado et al., 2018; de Souza Machado et al., 2019). Very high concentrations of MPs, as in W3, can have the opposite effect, reducing root biomass production (Jiang et al., 2019).

Our results indicate a lower crop in the tomato plants grown under soil treated with sludge at the time of the end of the experiment. This could be because of microfibers in increasing C:N ratio as a consequence of the modification of nutrient availability derived from alterations in water dynamics reported in onion plants (de Souza Machado et al., 2019). Nutrient alterations can lead to plant stress, lowering crop production (Li et al., 2009). Since the experiment did not last until the end of the crop season, we could not know if the tomato plants suffered a reduction in crop or only a delay in crop production. Nevertheless, a delay in the crop production is expected to lower the overall crop production that is possible to be harvested (Thomison et al., 2011).

When interpreting the results of this study, possible variables that were not controlled, such as nutrient level, could influence them and need to be taken into account. Despite the solid slow-release fertilizer provided to all the treatments aimed to prevent nutrient limitation, nutrient concentration was not equal in the different treatments. Further experiments, aiming to have a more similar nutrient concentration among treatments would be desirable.

Additionally, sewage sludge is a complex matrix that does not only contain MPs, but also metals, pathogens and organic toxicants. The levels of metal concentration found in sludge samples were below the ones established in the European Council Directive 86/278/EEC (Table S1). Sludge samples also contained bacteria such as *Escherichia coli* and *Salmonella sp.*, that inhabit human intestinal tracts (Table S1) and so are not necessarily pathogens, but certain strains can cause pathogenicity. Thus, nutrients, metals, bacteria and other variables, as well as the interactions of these variables with MPs could have influenced the results of this study. Because the above commented variables are not controlled, the experimental design of our study using different sludges does not allow us to demonstrate a direct cause-effect of MPs. However, this work allows us to test the real effects of sewage sludge containing MPs. Complementary studies would be needed to increase the insight on the possible effects of MPs from sewage on agriculture.

This study suggests, that despite the large number of MPs reported in sludge (Lusher et al., 2017; Mahon et al., 2017; Li et al., 2018; Corradini et al., 2019; van den Berg et al. 2020), these estimates could underestimate real concentrations or indicate that the number of MPs is growing. Under this scenario of high amounts of MPs exported through sewage sludge to the environment (De Souza Machado et al. 2018), it is urgent that policy makers define maximum levels of MPs in sludge used for agriculture as it has already been done with other toxic compounds, such as metals (86/278/EEC).

To the best of our knowledge, no study has tested the effects of sludge with MPs in the yield of agriculture crops. Our results show that sewage sludge with large concentrations of MPs can notably affect tomato plants, which could be due to a greater or lower extent to MPs. However, other possible factors may have been responsible or can produce additive or interactive effects with MPs. Thus, further work is necessary to corroborate these findings and to explain the mechanisms of possible effects of MPs on plants, such as tomato.

Author Contributions:

Conceptualization, C.S., A.B.,; methodology R.H., C.S., A.B., P.N.; formal analysis, R.H., A.B., C.S., ; investigation, R.H., P.N., A.B., C.S., ; resources, A.B., C.S.; data curation, A.B., C.S., R.H.; writing—original draft preparation, R.H., A.B., C.S.,; writing—review and editing, C.S., A.B., ; supervision, A.B., C.S. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare that there are no conflicts of interest

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FIGURES

Fig. 1. Percentage of particles extracted in the recovery test (mean ± SE; n=2) concerning size, type of polymer and particle.

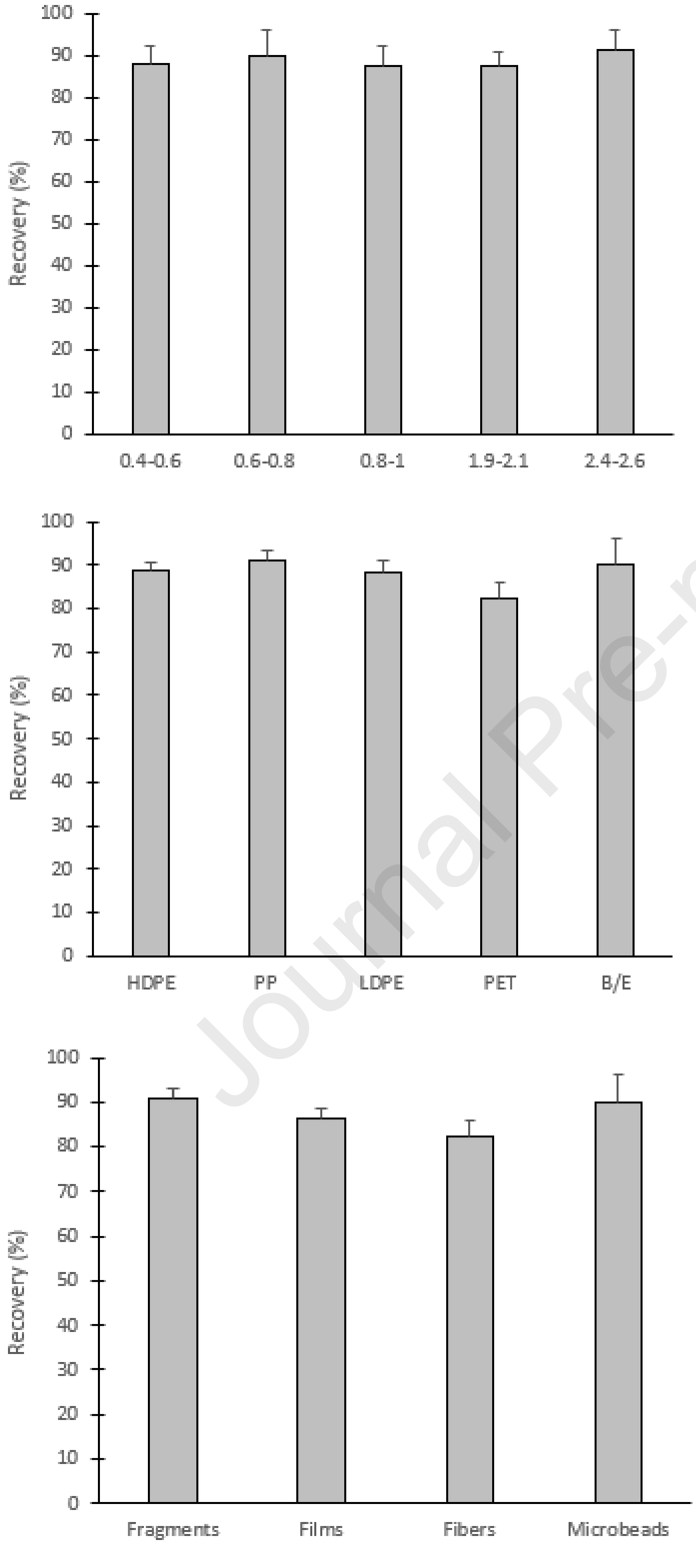
Fig. 2. Number of microplastics (mean ± SE; n=3) found in sludge samples from the wastewater treatment plants in the cities of Librilla (W1), Alhama de Murcia (W2) and Totana (W3) in Spain. Different letters on top of the bars represent statistically significant differences (Tukey-HSD test, $p < 0.05$).

Fig. 3. Percentage of types of polymers (A) and particles (B) (mean ± SE; n=3) found in the cities of Librilla (W1), Alhama de Murcia (W2) and Totana (W3) in Spain.

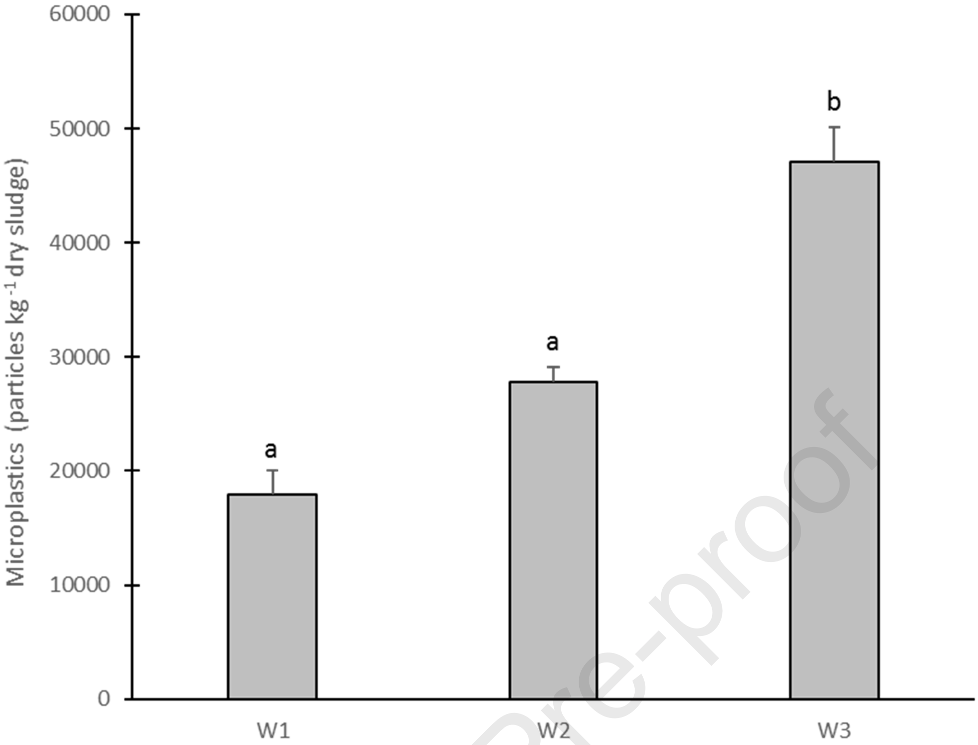
Fig. 4. Morphological and biomass parameters (mean ± SE; n=7) after 109 d of growth.. Different letters on top of the bars represent statistically significant differences (Tukey-HSD test, $p < 0.05$). Control (C), Manure Control (MC), Librilla (W1), Alhama de Murcia (W2), Totana (W3).

Fig. 5. Number of tomatoes (A) and number of mature tomatoes produced (B) (mean ± SE).. Different letters on top of the bars represent statistically significant differences (Tukey-HSD test, $p < 0.05$). Control (C), Manure Control (MC), Librilla (W1), Alhama de Murcia (W2), Totana (W3).

Figure 1


100
90
80
70
60
50
40
30
20
10
0
Recovery (%)
0.4-0.6
0.6-0.8
0.8-1
1.9-2.1
2.4-2.6
100
90
80
70
60
50
40
30
20
10
0
Recovery (%)
HDPE
PP
LDPE
PET
B/E
100
90
80
70
60
50
40
30
20
10
0
Recovery (%)
Fragments
Films
Fibers
Microbeads

641 Figure 2



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644 Figure 3

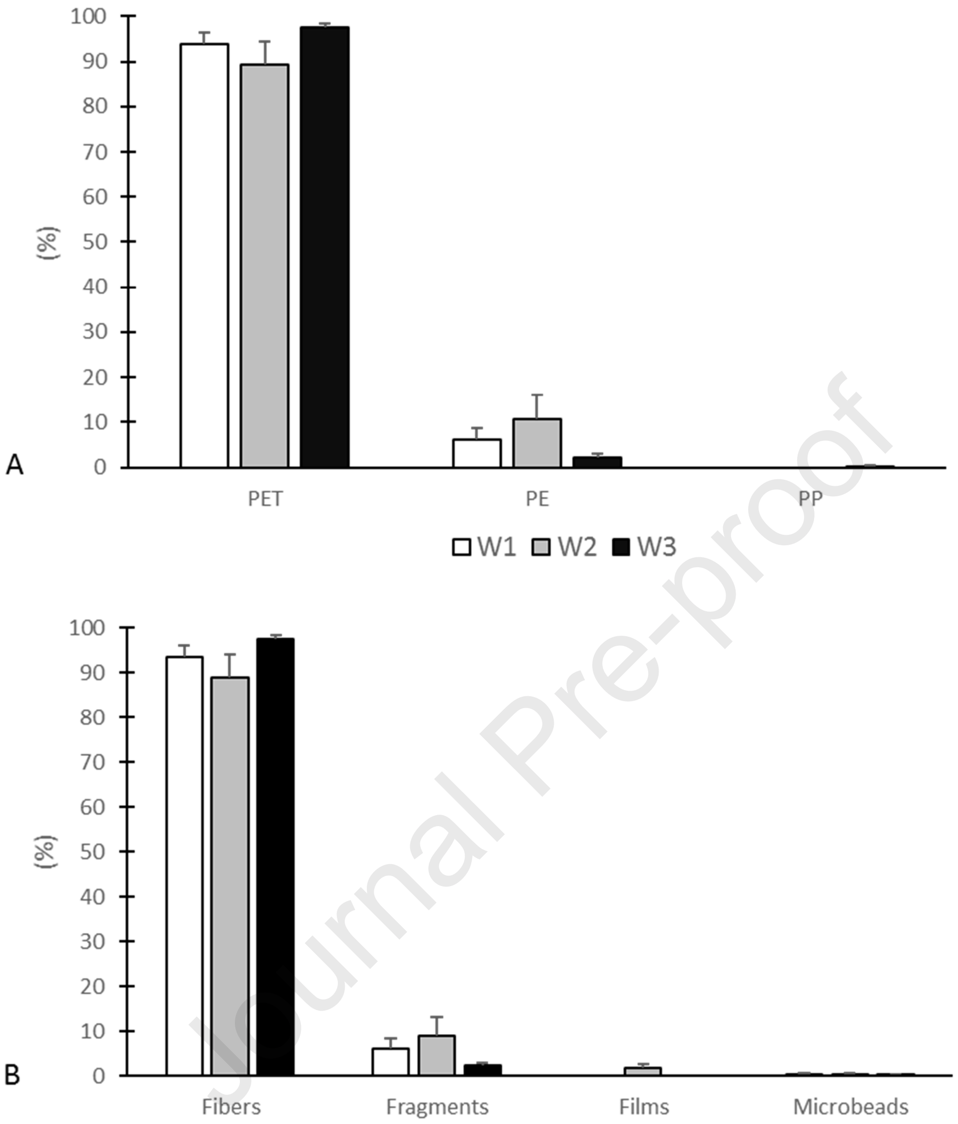

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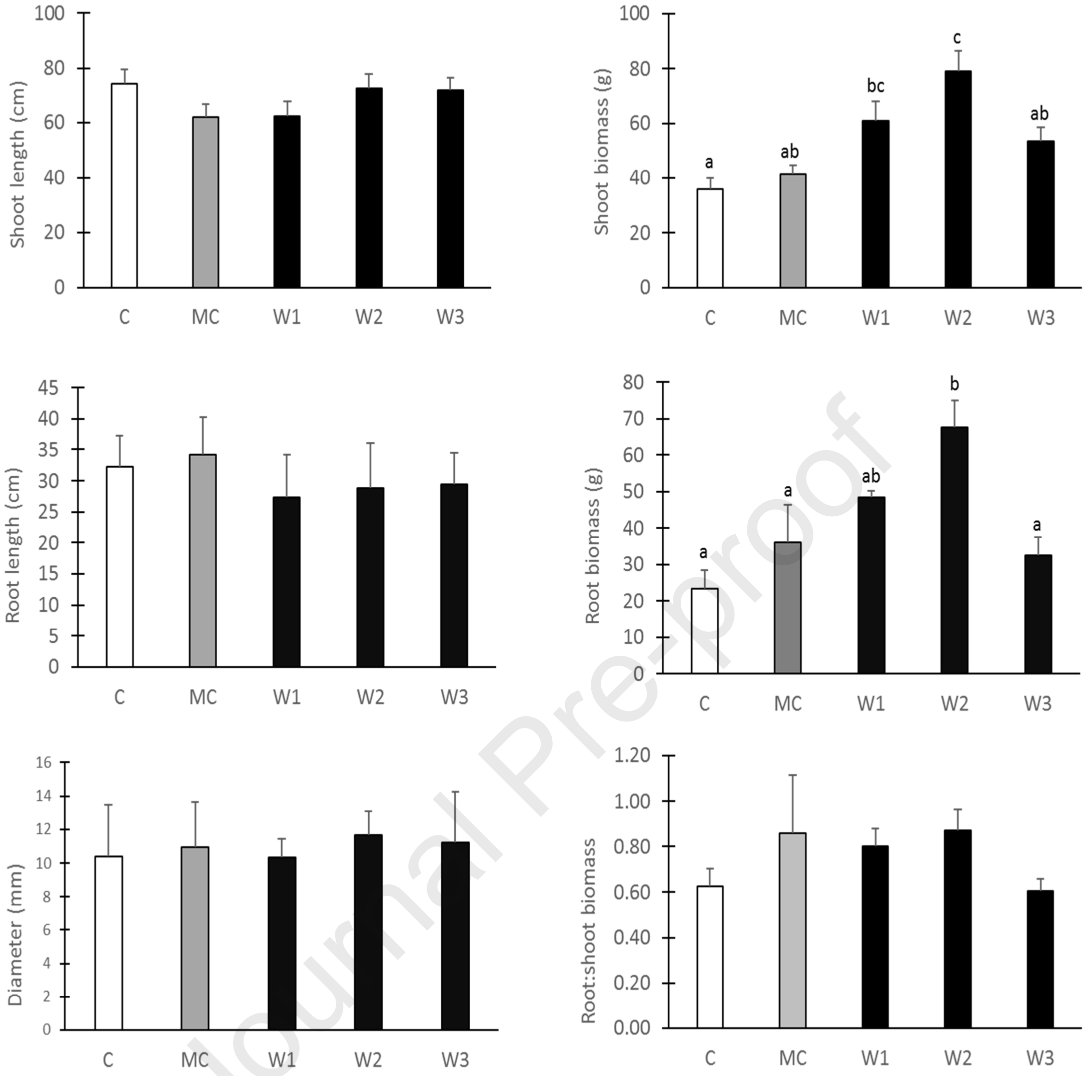
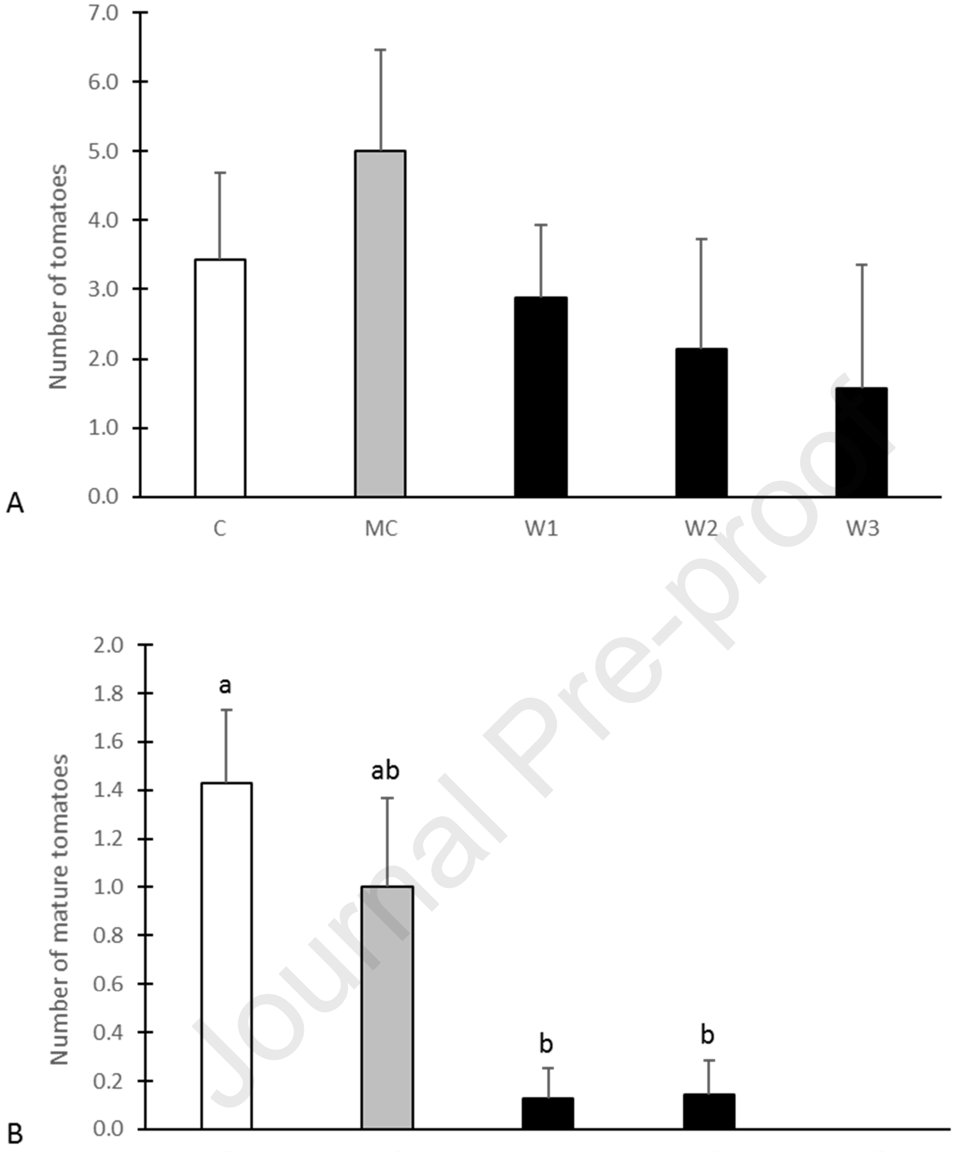
Figure 4


Shoot length (cm)
0
20
40
60
80
100
C
MC
W1
W2
W3
Shoot biomass (g)
a
ab
bc
c
ab
Root length (cm)
0
5
10
15
20
25
30
35
40
45
Root biomass (g)
0
10
20
30
40
50
60
70
80
a
a
ab
b
a
Diameter (mm)
0
2
4
6
8
10
12
14
16
Root:shoot biomass
0.00
0.20
0.40
0.60
0.80
1.00
1.20

650
651 **Figure 5**



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SUPPLEMENTARY DATA

2.4. Characterization of MPs by Raman Spectroscopy

MPs were characterized by using a Raman spectrometer NRS-5100 (Jasco, Madrid, Spain) equipped with LMU-20X-UVB lens. The laser excitation frequency and intensity used was 784.79 nm and 11.8 mW, respectively. Raman spectra were recorded with a charge-coupled device camera (UV-NIR range, 1024 × 255 pixels) electrically cooled to -70°C. Raman spectra were obtained between 162 and 1886 cm^{-1} with a spectral resolution of 2.47 cm^{-1}.

2.6. Quality Control

A total of 29 fibers were found in 3 blank filters. The mean concentration of MPs (9.7 ± 0.3 particles) in blank filters were subtracted to the mean value of the extracted MPs.

To test for modifications in the Raman spectra of the samples due to the chemical agents employed to remove the organic matter, a preliminary trial was performed with all the types of polymers used in the recovery test. The Raman spectra of all the types of polymers were obtained before and after performing the extraction protocol performed to the sewage sludge samples. No relevant changes in the intensity of the characteristic bands (Figure S3) were found in comparison with the spectra of the same materials before applying the extraction protocol.

To minimize contamination during the lab procedure all the containers were covered with aluminum foil, lab instrumentals were washed before use and were made of glass, the windows of the laboratory were closed, and 100% cotton coats were worn.

The Raman analysis of the fiberglass, the material the filter was made of, did not emit fluorescence that could interfere with the analysis of the samples, before and after applying the treatment to remove organic matter.

2.8. Measurement of pH and soil salinity

To determine pH values, air-dried soil samples from the pots were sieved through a 2 mm mesh to keep all primary soil particles and remove roots and bigger soil aggregates. Then, 40 mL of sieved soil was added to a glass beaker and slowly wetted with distilled water (*sensu* Juárez et al., 2004). After that, soil was stirred for 20 seconds with a glass rod and left 60 minutes before the pH was measured (pH Basic 20, Crison Instruments S.A., Barcelona, Spain). To monitor soil salinity, a container was placed under each tomato plant to collect 50 mL of the water lixiviated after watering the plants. Then, the electrical conductivity of the collected water samples was measured by using an electrical conductivity meter (Crison Basic 30, Barcelona, Spain).

Table S1. WWTPs and sludge samples characteristics.

Table S2. Characteristics of MPs used in the recovery test. Number of particles shown are referenced to one recovery test.

Table S3. PE, PET and PP Raman bands utilized for the identification of the microplastics.

Table S4. Label information of the products utilized in the composition of the soil treatments.

Table S5. Results of the ANOVA for the SL (shoot length), RL (root length), D (diameter), SB (shoot biomass), RB (root biomass), RB:SB (root:shoot biomass ratio), NT (number of tomatoes), NMT (number of mature tomatoes) and MPA (microplastics abundance). The factors were *WWTPs* (wastewater treatment plants) and *TOS* (type of soil).

Figure S1. Raman spectrum of a fiber and its similarity to a reference material.

Figure S2. Photographs of some microplastics found in the sewage sludge samples.

Figure S3. Tomato appearance and color at harvest time.

Figure S4. Raman spectra of a PP fragment (A), LDPE film (B) and PET fiber (C) before (grey line) and after (black line) the reagents used for removing the organic matter.

Table S1. Characteristics of the wastewater treatment plants (WWTP) and sludge. W1, W2 and W3, correspond to the three WWTPs located in Librilla, Alhama de Murcia and Totana, respectively in Murcia,Spain.

	W1	W2	W3
WWTP			
Population equivalent	4,312	17,293	27,628
Type of treatment	Secondary and tertiary	Secondary and tertiary	Secondary and tertiary
Water sources	Domestic	Domestic and industrial	Domestic and industrial
Treatment capacity (m3/year)	227,815	994,596	1,684,947
Sludge production (kg dry sludge /year)	55,632	317,280	695,592
Sludge			
Dry matter (%)	18±2	13±1	18±2
Total organic matter (%)	77±6	84±7	66±5
N (%)	1.9±0.2	7.6±0.9	5.4±0.6
P (P_2O_5) (mg kg^{-1})	54,204±10,841	36,846±7,369	37,716±7,543
K (K_2O) (mg kg^{-1})	9,220±1,660	3,484±627	6,570±1,183
Mg (MgO) (mg kg^{-1})	15,036±2,707	10,384±1,863	15,692±2,825

Ca (CaO) (mg kg^{-1})	31,906±6,381	31,668±6,334	70,714±14,143

Continuation Table S1

	W1	W2	W3
Sludge			
Cu (mg kg^{-1})	189±28	186±28	449±67
Cd (mg kg^{-1})	<2.0	<2.0	<2.0
Cr (mg kg^{-1})	27±5	21±4	32±6
Hg (mg kg^{-1})	0.23±0.08	0.24±0.08	0.27±0.09
Ni (mg kg^{-1})	20±3	16±2	19±3
Pb (mg kg^{-1})	18±3	25±5	40±7
Fe (mg kg^{-1})	4689±750	3850±616	8223±1316
Zn (mg kg^{-1})	511±87	608±103	1599±272
Escherichia coli (CFU/g; confidence interval)	140000±88000-220000	140000±88000-220000	17000±11000-27000
Salmonella sp.(presence/25g)	absence	presence	presence

Table S2. Characteristics of MPs used in the recovery test. Number of particles shown are referenced to one recovery test.

Polymer type	Number of particles	Particle shape	Color	Source	Size (mm)
HDPE	40	Fragment	Blue	Bleach bottle	0.4-0.6, 0.8-1, 1.9 2.1, 2.4-2.6
HDPE	40	Film	Black	Plastic bag	0.4-0.6, 0.8-1, 1.9-2.1, 2.4-2.6
LDPE	40	Film	Blue	Plastic package	0.4-0.6, 0.8-1, 1.9-2.1, 2.4-2.6
PP	40	Fragment	Red	Bottle tap	0.4-0.6, 0.8-1, 1.9-2.1, 2.4-2.6
PET	10	Fiber	Blue	100% polyester T-shirt	2
PET	10	Fiber	Yellow	100% polyester T-shirt	2

B/E	10	Microbead	Blue	Palmolive Thermal Spa Mineral Massage ®	0.6 – 0.8

Table S3. Examples of the Raman bands utilized for the identification of PE, PET and PP.

Polymer	Wavenumber (cm^{-1})	Assignment	Reference
PE	1063	Anti-symmetric stretching (C-C)	Kida et al., (2016)
	1080	Stretching (C-C)	
	1130	Symmetric stretching (C-C)	
	1298	Twisting (C-C)	
	1440	Bending (CH_2)	
	1460	Bending (CH_2)	
PET	1290	Stretching C(O)-C	Boerio et al., (1976)
	1414	CCH bending and OCH bending	
	1615	Ring mode 8a	
	1726	Stretching C=O	

Continuation Table S3

Polymer	Wavenumber (cm^{-1})	Assignment	Reference
PP	809	Rocking CH_2, Stretching CC_b, Stretching C-CH_3	Andreassen (1999)
	841	Rocking CH_2, Stretching CC_b, Stretching C-CH_3, rocking CH_3	
	900	Rocking CH_3, rocking CH2, bending CH	
	941	Rocking CH_3, stretching CC_b,	
	973	Rocking CH_3, stretching CC_b,	
	998	Rocking CH_3, bending CH, wagging CH_2	
	1040	Stretching C-CH_3, Stretching CC_b, bending CH	
	1152	Stretching CC_b, stretching C-CH_3, bending CH, Rocking CH_3	
	1219	Twisting CH_2, bending CH, stretching CC_b	

1330	Bending CH, twisting CH_2
1360	Symmetric bending CH_3, bending CH
1458	Asymmetric bending CH_3, bending CH_2

b: backbone

Table S4. Label information of the products utilized for the composition of the soil treatments.

Product	Label information
COMPO SANA ® UNIVERSAL	Composition: black peat, Perlita, Agrosil ® and lime; pH ($CaCl_2$): 5,0 – 6,5; Salt content (KCl) g/l: <3; Content in subscription (soluble nutrients): 200 – 400 mg/l Nitrogen (N), 200 – 500 mg/l Phosphate (P_2O_5), 300 – 500 mg/l Potassium oxide (K2O)
Manure FEMABÓN ®	Composition: herbaceous peat and compost manure; organic material (dry weight): 48%; bulk density: 610 g/l; electrical conductivity: 3 dS/m pH: 7.9
BLUEFFICIENT PLATINNUM®	12% Nitrogen; 7,5% (N) ammoniacal; 4.5% (N) ureic; 8% P_2O_5; 18% K_2O; 2% MgO 17% SO_3; 0.1% Mn; 0.1% Zn 3%; pH 6.0 inhibitor

Table S5. Results of the ANOVA for the SL (shoot length), RL (root length), D (diameter), SB (shoot biomass), RB (root biomass), RB:SB (root:shoot biomass ratio), NT (number of tomatoes), NMT (number of mature tomatoes) and MPA (microplastics abundance). The factors were WWTPs (wastewater treatment plants) and TOS (type of soil).

		SL			RL			D			SB		
Source of variation	d.f.	MS	F	P	MS	F	P	MS	F	P	MS	F	P
TOS	4	245.4	1.574	>0.2	51.85	1.507	>0.2	2.261	0.843	>0.5	2020	8.351	**<0.001**
Residual	30	155.9			34.40			2.681			242		
Total	34												
Cochran's C test			C = 0.30043, *P > 0.05*			C = 0.39329, *P > 0.05*			C = 0.49131, *P < 0.05*			C = 0.36118, *P > 0.05*	
Transformation			None			None			None			None	
Tukey's test												W2 > C	>0.0001
												W2 > MC	>0.001
												W2 > W3	>0.03
												W1 > C	>0.03

		RB			RB:SB			NT			NMT		
Source of variation	d.f.	MS	F	P	MS	F	P	MS	F	P	MS	F	P
TOS	4	2065.5	6.041	**<0.01**	0.1124	1.105	>0.3	8.896	0.513	>0.7	0.7198	4.755	**<0.01**
Residual	30	341.9			0.1017			17.343			0.1514		
Total	34												
Cochran's C test			C = 0.37135, *P > 0.05*			C = 0.69274, *P < 0.05*			C = 0.30208, *P > 0.05*			C = 0.43627, *P > 0.05*	
Transformation			None			None			None			logarithmic	
Tukey's test			W2 > C	>0.0008								C > W1	>0.01
			W2 > MC	>0.03								C > W2	>0.02
			W2 > W3	>0.009								C > W3	>0.005

		MPA		
Source of variation	d.f.	MS	F	P
WWTPs	2	663975790	34.97	**<0.001**
Residual	6	18987671		
Total	8			
Cochran's C test			C = 0.47455, *P > 0.05*	
Transformation			None	
Tukey's test			W3 > W1	> 0.03
			W3 > W2	> 0.0004

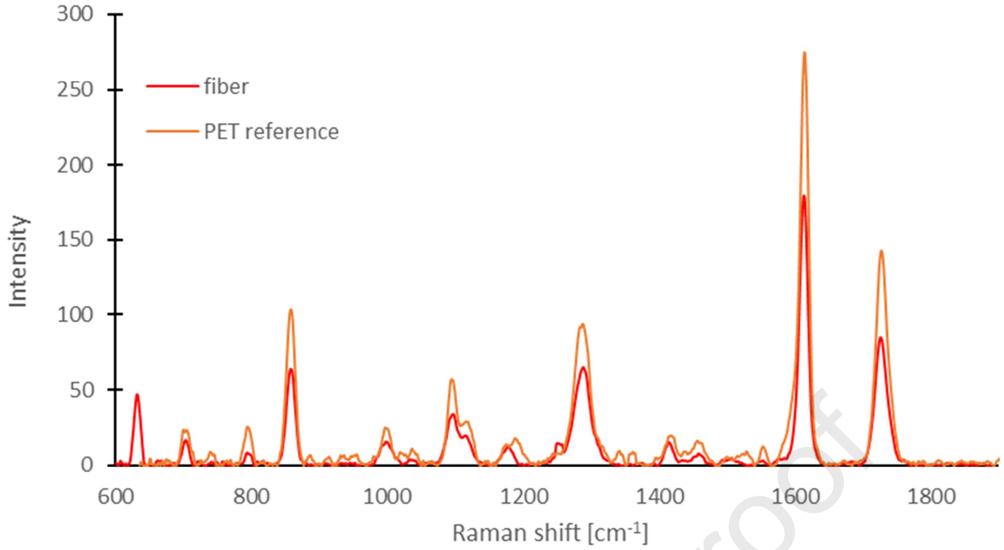

Fig. S1. Raman spectrum of a fiber and its similarity to a reference material.

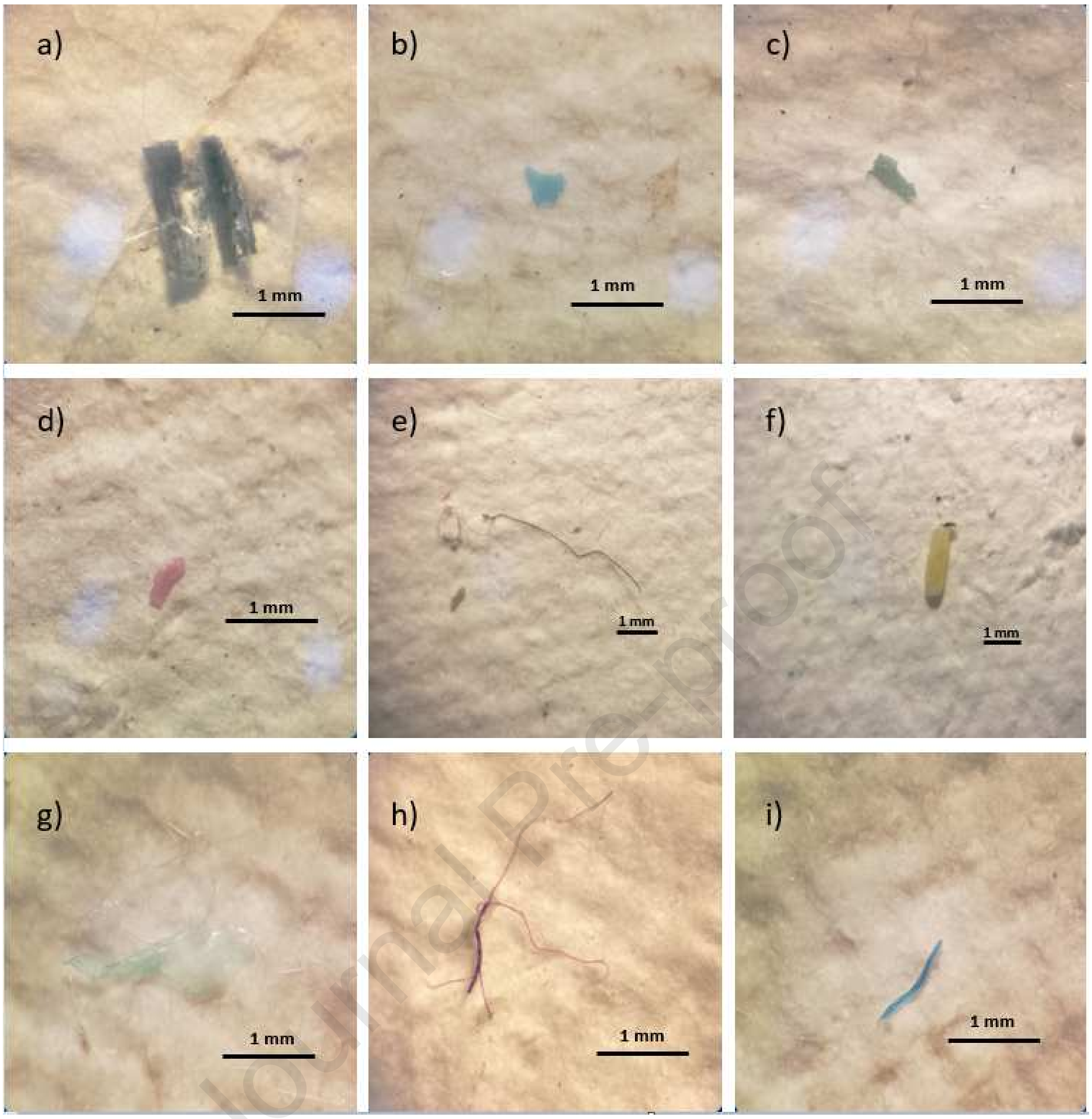

Fig. S2. Photographs of some microplastics found in the sewage sludge samples.

Fig. S3. Tomato appearance and color at harvest time.

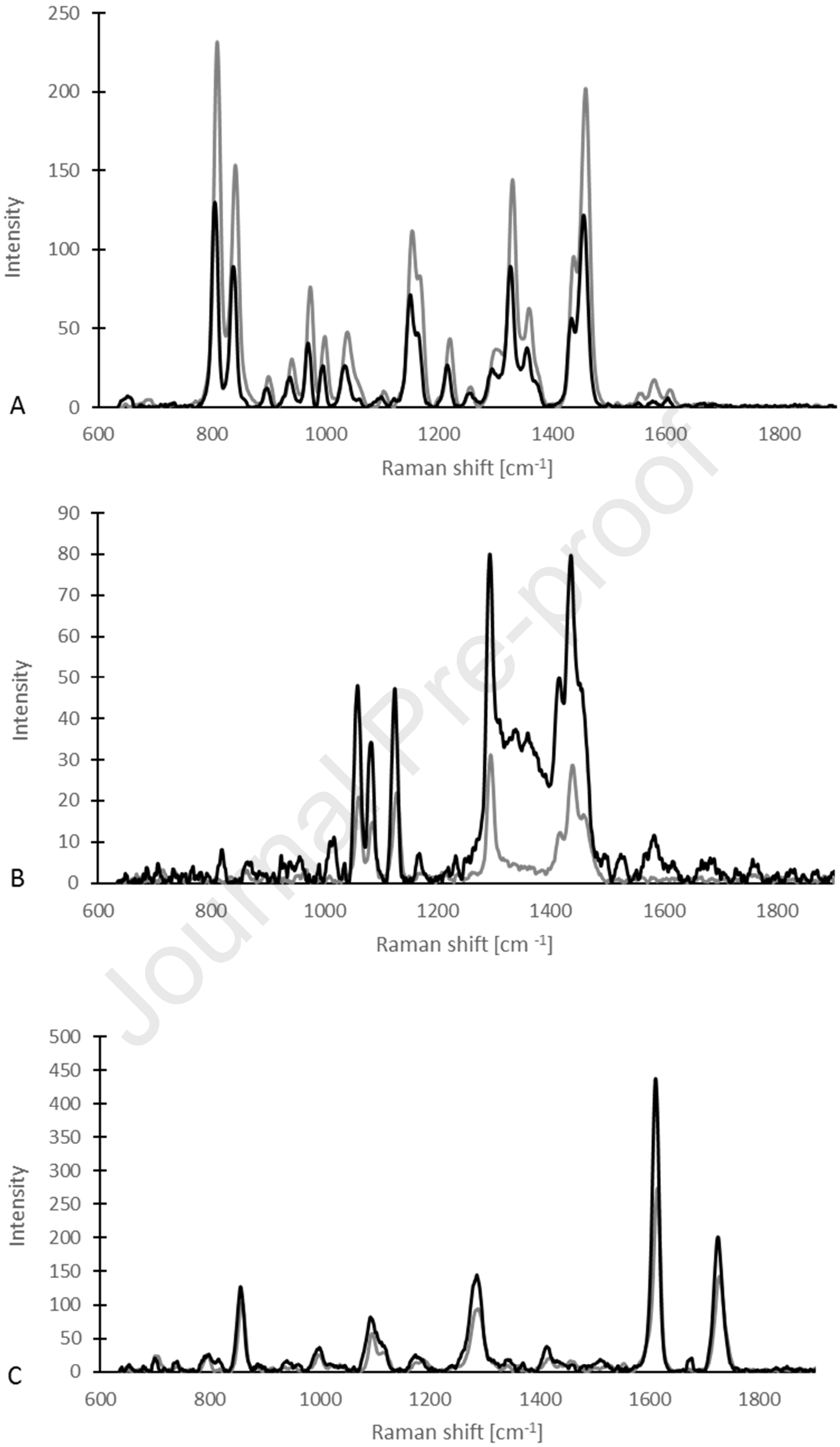

Fig. S4. Raman spectra of a PP fragment (A), LDPE film (B) and PET fiber (C) before (grey line) and after (black line) the exposition to the reagents used for removing the organic matter.

HIGHLIGHTS

An extraction protocol for MPs from sewage sludge has been validated.

Up to 31,000 ± 8,600 particles kg^{-1} dry weight in sewage sludge were estimated.

Sewage sludge containing MPs fostered the growth of tomato plants.

Sewage sludge containing MPs delayed and diminished fruit production.

Author Contributions:

Conceptualization, C.S., A.B.,; methodology R.H., C.S., A.B., P.N.; formal analysis, R.H., A.B., C.S., ; investigation, R.H., P.N., A.B., C.S., ; resources, A.B., C.S.; data curation, A.B., C.S., R.H.; writing—original draft preparation, R.H., A.B., C.S.,; writing—review and editing, C.S., A.B., ; supervision, A.B., C.S. All authors have read and agreed to the published version of the manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: