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Horizontal subsurface flow constructed wetlands as tertiary treatment: can they be an efficient barrier for microplastics pollution?

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ABSTRACT

The presence and fate of microplastics (MPs) in wastewater represent a subject of major concern, as wastewater is one of the main inputs of MPs to the environment. This study deals with the ability of horizontal subsurface-flow constructed wetlands (CWs), as tertiary treatment, to reduce the MPs concentration of secondary effluents. Different locations of a wastewater treatment plant (WWTP) including raw wastewater, CW influent and final effluent, were sampled. Macroinvertebrates were collected from the CW to evaluate their potential role in the MPs distribution along the wetland. The global WWTP efficiency for MPs removal was 98%. MPs removal efficiency by CW was on average 88%, causing a significant reduction of the MPs concentration from 6.45 to 0.77 MP/L ($p<0.05$), thus preventing them from entering vulnerable aquatic systems. The areal removal rate and the first order areal rate coefficient (k_A) were estimated to be 3120 MPs/m^2/d and 1.70 m/d, respectively. The most abundant size fraction was the one comprising MPs between 75 and 425 µm (51%), while the other size ranges analysed (40-75 and 425-5600 µm) accounted for 25 and 24%, respectively. Fiber was the most abundant shape in the WWTP influent (75%), the CW influent (54%) and effluent (71%). Non-significant differences were found between sites regarding size and shape distributions ($p>0.05$). Macroinvertebrates can ingest a non-negligible quantity of MPs, with an average content of 166.2 MPs/g or 0.13 MPs/individual. Therefore, they could play a certain role in the MPs distribution inside CWs. Fiber was the most abundant shape for macroinvertebrates as well (89%), so attention should be paid to reduce their contamination at source. This study provides the first results on MPs removal in CWs as tertiary treatment and assesses the potential role of macroinvertebrates in their distribution along the CW, thus filling this gap of knowledge.

1. Introduction

Microplastics (MPs) are defined as plastic particles smaller than 5 mm and include primary, i.e. plastic particles originally produced in the size of microns, and secondary MPs, resultant from the degradation or fragmentation of larger plastics. MPs are currently recognized as a global environmental problem of major significance, proof of that is the exponential increase of the number of publications in recent years (Figure S1).

The effects of MPs on aquatic organisms have been the object of numerous studies, which have detected a wide variety of ecotoxicological effects, including reduction of feeding activity and increase of energy consumption, oxidative stress, neurotoxicity, growth delay, reduction of reproductive fitness or even death, among others, according to a recent critical review (de Sá et al. 2018), which also concludes that more research using actual concentrations in the environment and multispecies exposures is necessary. Once the MPs reach the aquatic environment, they start aging and additives are leached which, depending on their composition, can give rise to toxic effects. For instance, Luo et al. (2020) have found toxic effects on the freshwater algae *Microcystis aeruginosa*, because of the leaching of lead chromate pigmented MPs. Taking into account all these potential effects, it could be said that MPs can endanger the biodiversity of aquatic ecosystems and represent also a concern for human health in the medium and long term.

The sources of MPs production are diverse, including both marine and land-based activities. The latter are mainly caused by mismanagement of the waste system (low recycling, leakage from uncontrolled landfills), leakage from industrial plants, urban runoff with tire wear as one of the main sources of MPs (Hüffer et al. 2019), and urban wastewater (Talvitie et al. 2015) (i.e. wear of synthetic clothing and utensils or primary MPs from cosmetics, personal care and pharmaceutical products, among others). Therefore, wastewater treatment plants (WWTPs) act as a potential barrier to MPs before they enter the aquatic environment, even though they are not able to completely remove them (Ngo et al. 2019).

Several studies have targeted MPs in wastewater and their removal in each of the depuration steps (Correia Prata 2018; Sun et al. 2019). A wide range of concentration values and removal efficiencies of pre-treatment, primary, secondary and tertiary treatments have been reported. In general, pre-treatment and primary treatment removes the majority of MPs in wastewater, with removal efficiencies ranging from 35 to 59% for the pre-treatment and from 50 to 98% after the primary treatment, while secondary and tertiary treatments manage to get a further decrease, with secondary effluent containing between 0.2 and 14% of the WWTP influent concentration and tertiary effluent containing 0.2-2% relative to the influent, according to the review of Sun et al. (2019). Therefore, it has been shown that conventional WWTPs perform efficiently at removing MPs. Nevertheless, given the large volumes constantly discharged to the aquatic environment they actually represent a significant source of MPs in terms of load (Mintenig et al. 2017; Wolff et al. 2019; Conley et al. 2019).

So far, the research carried out on WWTPs has focused on plants that provide service to large populations, whereas treatment systems for small communities have received less attention (Gatidou et al. 2019). Constructed wetlands (CWs), also known as treatment wetlands, represent a nature based solution commonly used for wastewater treatment in small communities, but also widely used as tertiary treatment in large plants (Rousseau et al. 2008). Used as tertiary treatments, CW are able to further remove suspended solids, pathogens or nutrients, among other variables, allowing both water and nutrient reclamation (Rousseau et al. 2008).

As the MPs are filtered out of the wastewater, one may expect accumulation inside the system. For instance, in vertical flow CW, there is a higher accumulation of total suspended solids (TSS) on the top layer of media, while in the horizontal flow CW solid particles distribute along a horizontal gradient, high near the inlet area and gradually decreasing toward the outlet zone (Dotro et al. 2017). This raises the question if those MPs somehow interfere with treatment processes? It is well-known for instance that macro-invertebrates like bristle worms, snails, beetle larvae etc. can occur in high numbers in CWs, and play a crucial role in controlling sludge accumulation (Ouattara et al. 2009). That has even lead to successful experiments in which such organisms have been purposely introduced into CWs to remediate clogging problems (Li et al. 2011). However, it has been shown that MPs, even at relatively low concentrations, can have a profound effect on the survival and fitness of macro-invertebrates (Huerta Lwanga et al. 2016). Other studies have reported reduced feeding activity, reduction of lysosomal membrane stability or increase of energy consumption (Besseling et al. 2013; Van Cauwenberghe et al. 2015). Hurley et al. (2017) have shown that *Tubifex* worms retain microplastics for longer than other particles of the ingested sediment matrix. It can thus be hypothesized that MP accumulation in CW affect macro-invertebrate communities and thus may have an indirect effect on sludge accumulation and clogging problems. Alternatively, macro-invertebrates could have an effect on the distribution of MPs within the CW because of the bioturbation process.

In summary, as CWs are efficient filters for total suspended solids, it can be expected that they are efficient filters for MPs as well. For instance, horizontal subsurface flow CWs present TSS removal efficiencies higher than 80% and vertical flow and French CWs offer efficiencies higher than 90% (Dotro et al. 2017). Some researches about MPs removal in stormwater wetlands or ponds have been performed (Liu et al. 2019; Ziajahromi et al. 2020). However, to the best of our knowledge, there is no mention in the literature of studies on the contribution of CWs to control MPs pollution in wastewater. It is fundamental to fill this knowledge gap in order to correctly manage the existing CWs as a first step to prevent MPs from entering aquatic environments. Therefore, the general objective of this study is to gain insight about how to decrease the contribution from WWTP to aquatic ecosystems. This general objective can be broken down into the following specific objectives: to evaluate the removal efficiency of MPs in CW, looking at the removal efficiencies for different MPs sizes and shapes, and to evaluate the content of MPs in aquatic macroinvertebrate and the potential role of these organisms in MP spreading within the filter material.

This study raises the following hypotheses: 1) constructed wetlands can further reduce the MPs concentration still present in the effluent from secondary treatment, giving rise to a final effluent with a significantly lower concentration; 2) the size of MPs in CW effluent is smaller than those in the influent; 3) macroinvertebrates can play an important role in MPs fate and transport in CWs.

2. Materials and methods

2.1 Aalbeke WWTP

Aalbeke WWTP (Kortrijk, Belgium) was built in 1996 for serving a population of 450 population equivalents (P.E.). The main units of this plant are a primary settler, two rotating biological contactor (RBC) in series followed by a secondary settler, as secondary treatment, and a 500 m^2 horizontal subsurface CW, as tertiary treatment. The CW also functions as a stormwater treatment unit, taking any flow higher than 3 Q_{14}. It is filled with gravel (2.4 mm < 50% < 5.6 mm, 5.6 mm < 50% < 8.0 mm) and planted with common reed. Available data from 2019 (until 11/13/2019) indicate an average daily inflow of 261 m^3/day though with considerable variation (min. 106 – max. 927 m^3/day), a plant-wide TSS removal of >98% (with all effluent concentrations consistently below 5 mg/L) and a plant-wide COD removal of >92% (Flemish Environment Agency, geoloket.vmm.be).

2.2 Wastewater and macroinvertebrate sampling

Wastewater was collected from three different sites along the WWTP: raw wastewater in the influent to the primary settler, effluent from the secondary settler and effluent from the CW. Six sampling campaigns were carried out in the following dates: Dec 17th (2018) and Jan 22nd, Feb 14th, Oct 22nd, Nov 12th and Dec 4th (2019). Grab samples of different volumes of water were taken for analysis: 2.5 L for the raw wastewater, 7.5 L for the CW influent and, 100 L for the CW effluent. All the samples were taken in duplicate with a metal bucket and filtered by a stack of sieves (5600 µm, 425 µm, 75 µm, 40 µm) in situ to avoid contamination. After that, the residue remaining on the sieves (425 µm, 75 µm, 40 µm) was transferred into glass beakers covered with aluminium foil and transported to the laboratory for further processing.

On the same dates, two substrate samples were also taken from the CW for collecting the macroinvertebrates living in it. A volume of 5 L of substrate per sample, consisting of the gravel and the solids trapped in the wetland, was taken with a metal shovel. Different sites in the wetland were sampled, depending on the accessibility. The CW presented symptoms of clogging, with the initial and central parts usually flooded. The substrate sample was collected from the top 30 cm, which is the normal depth where macroinvertebrates live. These samples were stored in large glass containers covered by aluminium foil and transported to the laboratory for further processing.

2.3 Extracting MPs from the water samples

The beakers containing the residue collected from the sieves were put in the oven at 70 °C to dry up, higher temperature was avoided since the shape of plastics could be affected by elevated temperature. After that, 20 mL of a diluted H_2O_2 30% solution (1:1 for raw wastewater and 1:2 for

CW influent and effluent) was added into the beakers for the digestion of the organic matter (Hurley et al. 2017). In order to speed up the digestion, the beakers were put back in the oven at 60 °C. Once the digestion was completed and the solution dried out, a density separation was conducted. A fixed volume (20 ml) of a high density solution ($CaCl_2$ 1.34 g/cm^3, prepared by dissolving 558 g of CaCl2 in 1 l of demineralized water) (Stolte et al. 2015) was added to the beakers and mixed with the particles inside it, then the mixture was transferred from beakers to glass vials, the beakers were washed three times to ensure the recovery of all the particles and the solution introduced to the vials. Then, the vials were centrifuged for 5 minutes at 3500 rpm, and the supernatants were vacuum filtrated with glass membrane filters (1.5µm, 47mm diameter) to collect the MPs from the samples. The vials were filled in with the high density solution and centrifuged one more time to ensure the MPs recovery. Finally, a Rose Bengal solution (0.2 mg/ml) was added to stain organic natural (non-plastic) particles and fibers on the filters (Ziajahromi et al. 2017). After 5 minutes of reaction, the dye solution was washed off with distilled water, and the no-plastics particles would be stained into pink in order to distinguished from MPs. The filters were finally placed in aluminium containers and put in the oven for 5 minutes to dry off before the MPs counting.

2.4 Extracting MPs from the Macroinvertebrates

The substrate samples were sieved with 2 sieves (2000 µm and 300 µm) in order to separate the gravel and other larger or smaller particles. The material retained on the 300 µm sieve was transferred with tap water to a white tray to facilitate the detection of macroinvertebrates. Each macroinvertebrate was picked up with help of tweezers, rinsed with distilled water to remove possible particles present on their skin and put in a beaker containing 70% EtOH conservation solution to prevent gut content excretion (Windsor et al. 2019). This part of the procedure was done as soon as possible after sampling, as it is easier to identify the macroinvertebrates if they are alive. After that, they were introduced in the oven at 60 °C until the solution dried off, to obtain their dry weight. Next, a digestion with 10% KOH was performed (Kühn et al. 2017), this step was carried out in the oven at a temperature of 60 °C for 24 hours. Once the organic matter was degraded, the samples were sieved by a 40µm sieve to get rid of the KOH solution, which interferes with the density separation by creating precipitates with the $CaCl_2$, and the sieve rinsed with distilled water. The following steps were the same as previously described for the water samples. The MPs concentration inside the macroinvertebrates was expressed in MPs per gram of macroinvertebrate (dry weight).

2.5 Microplastics counting and characterization

MPs retained on the filters were examined under stereomicroscopes with augmentations between x14 and x70 (SZM and SDZ-PL, Kyowa, Japan). The microplastics were identified following the rules indicated by Hidalgo-Ruz et al. (2012). During the filters examination, the hot needle test was also used to distinguish between microplastics and organic matter (Witte et al. 2014). Under the pressure of a hot needle, MPs should melt or curve, and biological or other non-plastics material not, organic matter usually breaks under the pressure with the hot needle. The filters were read from left to right, then move down one row from right to left. During the counting, MPs were classified into fibers, particles and films.

Some of the suspected MPs were isolated and analysed using Fourier Transform Infrared Spectroscopy (FTIR) with the Attenuated Total Reflectance (ATR) accessory (Thermo Nicolet FTIR, Nexus). Five particles were checked and the results of the FTIR confirmed that they were indeed MPs (Figure S2).

2.6. Quality assurance and contamination prevention

Before each analysis, all the equipment and working space were cleaned thoroughly to avoid contamination, using distilled water for the material and ethanol for the laboratory bench. During all the steps, including sampling, laboratory cotton coat and latex gloves were worn. Material made of plastic was avoided as much as possible. The beakers containing the solution and the filters were covered with aluminium foil while they were not under the microscope, to avoid contamination. The solutions applied in each step were filtered before being used. Four samples were counted in duplicate by different researchers and deviation was negligible.

Blanks were run for the water and macroinvertebrate procedures. For water two types of blanks were performed. On one hand, 20 l of distilled water were sieved through the 40 µm and no MPs were found; on the other hand, 100 l of tap water were sieved through the complete sieves stack and the full extraction procedure was performed, in this case an average of 11 MPs was found. For macroinvertebrate, the whole procedure was performed and an average of 5 MPs were found. These MP quantities were subtracted from the results of the samples. Similar background contamination was found by previous researches (Conley et al. 2019).

Total and volatile suspended solids were analysed according to Standard Methods (APHA, 2005).

2.7. Statistical analysis.

Statistical analyses were performed using SPSS 16.0 software (SPSS® software). The concentration and size and shape distributions were compared using parametric tests (ANOVA) if normality was satisfied test and nonparametric otherwise (Kruskal Wallis). Normality and homogeneity of variance were tested through the Saphiro-Wilk and Levene tests respectively. If the differences were significant, a posthoc test was performed (Tukey if homogeneity of variance was met and T2-Tahmane otherwise). Statistical significance was indicated by a probability of type I error of 5% or less ($p \leq 0.05$).

3. Results and discussion

3.1. Wastewater MPs concentrations and removal efficiencies

MPs concentration was measured in different sites of the Aalbeke WWTP. The results indicate a high variability in the influent to the WWTP, varying between 21.9 and 102.3 MPs/L (Fig. 1A and B). The concentrations are reduced along the treatment system and the range of variation is narrowed, varying between 4.0 and 10.3 MPs/L after the rotating biological contactor and secondary settler, and between 0.10 and 1.22 MPs/L in the final effluent, after the CW, highlighting its buffering capacity (Fig 1B). The decrease from one site to the next one was statistically significant ($p<0.05$). The removal efficiency was, on average, 87% in the combined primary and secondary treatment. The CW reduced the MPs concentration even more, with an average efficiency of 88%, providing a great environmental benefit that adds to the already known benefits of constructed wetlands. The removal efficiency of the whole WWTP, from the influent to the final effluent, was 98%. This is a value very similar to the TSS removal efficiency reported by the Flemish Environmental Agency (see section 2.1). Based on these results the first hypothesis can be accepted: CW can efficiently reduce the MPs coming from the secondary effluent, giving rise to a final effluent with a significantly lower concentration.

The efficiency of the CW monitored in this study is within the range of other tertiary treatments. For instance, biological filtration (84%) (Talvitie et al. 2015), discfilters (40.0-98.5%), rapid sand filters (97.1%), dissolved air flotation (95.0%) or membrane bioreactor (99.9%) (Talvitie et al. 2017) (see Table 1).

The MP concentrations reported in other studies present a wide range of variation, both in the influent and in the effluent (see Table 1). This wide range of variation could be related with the kind or size of the served population (Mason et al. 2016), as well as with the type of sewerage system (combined or separate), or even depend on some steps of the analytical procedure, such as the solution used in the density separation step or the pretreatment applied (Table 1). The concentration measured in this study falls within the first half of the variation range, a fact that could be related with the population served by the Aalbeke WWTP. Indeed, the Aalbeke WWTP serves a rural area where the use of plastics and original microplastics could be lower than in large populations, e.g. lower use of plastic packaged food or personal care products with MPs. The system is also fed by a combined sewer system which results in dilution of the wastewater (e.g. average data for 2019 indicate an influent with a BOD_5 of only 118 mg/L and COD of only 311 mg/L, data from geoloket.vmm.be).

Regarding the effluent concentrations reached in this study, they are comparable to other tertiary or advanced treatments, like membrane bioreactor treatment. The minimum concentration reached is in the level of the blanks performed, so the effluent produced by CW can be almost without MPs. In this sense, a significant linear relationship between the MPs and the total suspended solids has been found (Fig. 2) ($p<0.01$), so the TSS could be used as an indicator of the probable level of MPs in the wastewater. Therefore, when the CW properly filters out the suspended solids, it is also able to produce an effluent with a very small MPs concentration. Other authors have also recently observed a co-transport behaviour for MP particles and TSS, and also suggested considering organic solids as a good indicator of the fate of nanoplastics (Keller et al. 2019). However, in their study fibers did not correlate with TSS, whereas this relationship does exist in the present study ($r_{Pearson} = 0.96$, $p<0.01$).

Likewise, the MPs concentrations in the influent and the effluent were positively and linearly related ($r_{Pearson} = 0.73$, $p<0.05$), the CW showing a high buffer capacity for reducing peak concentrations but a certain dependence on the influent concentration.

Another interesting aspect to know are the characteristics of the MPs present in the wastewater, such as the size and shape distributions, which are shown in Figure 3 and pictures in Figure S3.. MPs in the medium size range (75-425 µm) were the most abundant, representing about 50% of the total MPs found in the samples. The larger and smaller MPs had a similar abundance, around 25%. The distribution did not vary significantly from one site to another ($p>0.05$). Therefore, the second hypothesis of this study cannot be accepted: the particles in the effluent were not significantly smaller than those in the influent. According to the literature, the influent usually contain a higher proportion of larger particles (MPs over 500 µm can reach over 70%) than the effluent, where up to 60% can be smaller than 100 µm (Sun et al. 2019; Talvitie et al. 2017), so treatments able to properly reduce this size fraction are of high interest. The most frequent fraction in this study comprises particles smaller than 100 µm, it could be necessary to add an extra size sieve to detect a significant difference between influent and effluent regarding the main size fraction. The average removal efficiency for the medium size fraction (75-425 µm) in the Aalbeke CW was 88%. Larger MPs were removed with a slightly lower efficiency (83%), whereas the efficiency for smaller MPs was somewhat higher (94%). As a rule of thumb in granular filtration, particles smaller than 15% of the granule size are retained (Crittenden et al. 2012). In Aalbeke CW, smaller particles than 15% of the granule size are efficiently retained, so biofilm, plant roots and other alive organisms might be contributing to enhance the removal efficacy of the granular media.

The shape distribution obtained in this study coincides with previous research as well, with fibers being the most predominant shape in wastewater (Sun et al. 2019). On average, they represented 67% of the MPs encountered in the samples, followed by particles (30%) and films were less abundant (3%). There were not significant differences between the sampling sites with regard to the shape distribution ($p>0.05$) (Fig. 3). The CW reduced the fiber concentration with a mean efficiency of 81%, while the other MPs were reduced with higher efficiency, of around 95%. Other authors have also noticed lower fiber removal efficiency, remarking that thinnest and longest fibers can pass longitudinally even through membranes under high pressure such as applied in membrane reactors (Raju et al. 2018). This fact, fibers representing the main shape, also can help to explain why non-significant differences were found between the size distribution in the influent and effluent, since fibers could pass through the sieves in spite of being more or less long.

Given the results presented above, the CW represents an efficient treatment system to further remove MPs from the wastewater, thus improving the global efficiency of WWTPs. Other kinds of CWs, such as vertical flow, could be equally efficient because of the existence of the filtration process through the filter media. Free water surface flow CWs could be somewhat less efficient as the main removal process would be sedimentation and some of the MPs could float and not be trapped in the wetland. In this sense, researches conducted on stormwater treatment wetlands, including retention ponds (Liu et al. 2019) and floating wetland (Ziajahromi et al. 2020), have shown their ability to trap MPs. Both studies concluded that the monitored wetlands were capable to retain a significant quantity of MPs, based on the MPs concentrations measured in the sediments. Ziajahromi et al. (2019) monitored the water influent and effluent as well. In the water monitoring, they found that effluent presented higher concentration than influent, what could be a result of a first flush effect. The study concludes that further research is necessary, including the characterization of the pollutograph in the influent.

Overall, even though WWTPs provide high removal efficiencies, efforts should be focused on reducing the contamination at the source, or even on methods that do not wait until plastic has already become pollution (Leslie 2019). The control measures for reducing the MPs inputs to the wastewater can range from simple gestures at home, like installing filters at the washing machine drain, to government regulations, such as the prohibition of using MPs in the manufacturing process (Correia Prata 2018).

3.2. Design and management indicators: MP emission per capita and removal rates.

The data obtained in this study provides information very useful for a first approach to key CW design parameters, such as the MP emission per capita, the first-order constant rate and the loading and removal rates. The emission per capita may vary depending on the kind of population served by each WWTP, but the values provided in this study could be considered valid for the design of CW as tertiary treatment, assuming that previous treatments can dampen large fluctuations associated with the population characteristics.

To calculate the MP daily emission per capita, the average of the last five years for the treated flow and the population equivalents (P.E.) actually served have been considered. The average flow was 275±14 m^3/d. The actual population served was calculated based on the P.E. definition in the Council Directive 271/91/EEC, i.e. 60 g BOD5/P.E./day, and the organic load received, resulting in an average value of 494 P.E, so the system is slightly overloaded. Taking into account the average MP concentrations in the WWTP influent and effluent, the following emissions can be estimated: 27381 and 428 MPs/capita/d for the influent and effluent respectively. In order to compare these emission rates with other studies, a transformation into mass of MPs can be realized. For this, it is necessary to make some approximations for the geometric form of the particles and the mean density. An approach based on Redondo-Hasselerharm et al. (2018), was adopted: a prism geometry was assumed for MPs, with a square basis equal to the average dimension of the most abundant size (i.e. 250 µm, as the average between 75 and 425 µm) and a half height (i.e. 125 µm), so the volume becomes $7.8 \cdot 10^{-6}$ cm^3/MP. For the mean density, an average value for the different polymer densities reported in Prata et al. (2019) was considered (i.e. 1.27 g/cm^3). These assumptions can lead to a non-negligible error in the estimation, but allow us to compare with previous reported values. This calculation gives to a MP loading rate of 98.9 g/PE/year (WWTP influent) and an effluent emission rate of 1.5 g/PE/year. The effluent emission rate is higher than previous reported values although in the same order of magnitude (0.68 g/PE/year, Conley et al. 2019). The estimated MP loading rate, in terms of mass, is significantly smaller than the TSS loading rate (around 105 kg/PE/year), representing barely 0.1% of the total solids entering the WWTP. By focusing on the CW, the MP loading rate results in 12.9 g/PE/year, representing 0.2% of the TSS entering the CW. Therefore, it could be concluded that, in terms of mass, MPs are not an important trigger of clogging CW.

The CW areal removal rate obtained was 3120 MPs/m^2/d or, on a volumetric basis, 3573 MPs/m^3/d. Besides areal or volumetric removal rates, other several design approaches for horizontal subsurface flow CW exist, among which the P-k-C* approach is the most recommended (Dotro et al. 2017). It is based on the tanks-in-series model and its equation is:

$$A = \frac{P \cdot Q_i}{k_A} \cdot \left(\left(\frac{C_i - C^*}{C_o - C^*} \right)^{\frac{1}{P}} - 1 \right) \qquad \text{(Eq. 1)}$$

where A is the area (m^2), P is the apparent number of tanks-in-series adopted (dimensionless), Q_i is the influent flow rate (m^3/d), k_A is the first order areal rate coefficient (m/d), C_i is the inlet concentration, C_o is the outlet concentration and C^* is the background concentration.

Considering the values indicated in Table 2 for the parameters included in Eq.1, a first approach for the MPs first order areal rate (k_A) in horizontal flow CW, as tertiary treatment, can be obtained. This gives a value of 1.70 m/d, or expressed in a volumetric basis, 5.22 d^{-1} or 0.22 h^{-1}. The last value (0.22 h^{-1}) can be interpreted as the reduction percentage per time unit, i.e., for each hour of retention the concentration is reduced by 22%.

3.3. Macroinvertebrates

In a first attempt to evaluate the potential role of macroinvertebrate on the MPs distribution inside the wetland, the MPs content inside the organisms was analysed. A total of twelve samples have been analysed. During the sampling campaigns the CW presented symptoms of clogging, with a water depth of about 15 cm in the initial part of the wetland. The macroinvertebrates found in the wetland substrate were mainly worms (annelids, with characteristics of *Tubificidae*) and few small beetles; some empty snail shells were also found. The abundance of worms in the substrate was highly variable, varying between 10.2 and 154.6 individuals/L. This range of variation falls within the ranges found in previous studies, for instance Ouattara et al. (2009) reported values between 24 and 303 individual/L in planted beds, decreasing from the upper surface layer (0-10 cm) to the bottom sampled layer (20-30 cm). Most of the worms found in the present study were very small, with a length between 1 and 3 cm and a weight ranging between 0.17 and 1.80 mg/individual. These size and weight characteristics are within ranges reported in the literature, e.g. 3−56 mm in length and 0.1-23.9 mg in weight were obtained by Hurley et al. (2017).

The concentration of MPs inside the macroinvertebrates also presented a wide range of variation: 10.2 – 700.8 MPs/g d.w. (mean = 166.2 MPs/g) (Fig. 4) or, expressed per individuals, 0.01 – 0.33

MPs/individual. The majority of the MPs found inside the macroinvertebrates were fibers (89%) and the rest were particles (11%), no films were found. Fibers might be more easily ingested than particles or fibers because of their small diameter. These results highlight again the importance of reducing the inputs of fibers to wastewater.

Figure 4 also indicates the zone of the CW from which the samples were taken. Looking at the figure, one could conclude that macroinvertebrates collected from the final zone of the wetland have a higher concentration of MPs and this could be due to a higher concentration in the substrate. This finding could be explained because some MPs can float on wastewater and, if the initial part is flooded, they settle further in the wetland. Nevertheless, it should be highlighted that depending on the flooding conditions, macroinvertebrates can be easily mobilized from one site to another. Another noteworthy point is that the samples were not taken on the same day, thus they may be non-comparable.

Overall, the results obtained indicate that macroinvertebrates can ingest MPs thus playing a certain role in the potential distribution of MPs along the wetland. Nevertheless, to certainly accept the third hypothesis of this study, the quantity of MPs that macroinvertebrates are able to mobilize, through their ingestion rate, should be related to the MPs total quantity present in the substrate. Future research should aim towards measuring the MP concentration in the substrate.

Regarding the toxicological effects that could be expected, the MPs concentrations measured in macroinvertebrates of the Aalbeke CW are in the same order of magnitude than those ingested by *Tubifex tubifex* living in the sediments of an urban river in United Kingdom (Hurley et al. 2017), where 129±65.4 particles/g were reported, the majority being fibers (87%). Although the results are not directly comparable because of different extraction procedure, these authors left the worms for 24 h in deionized water to depurate, then digested with KOH (10%) and the resulting slurry filtered, without a density separation step. Other authors have investigated the toxic effects of MPs on organisms like *Tubifex* spp. and no effects on their survival or growth were found when exposed to concentrations ranging from 0 to 40% in sediment dw (Redondo-Hasselerharm et al. 2018). These authors also demonstrated a positive linear relationship between the uptake of MPs by organisms (*Gammarus pulex*) and the MPs concentration in the sediment, varying between around 0.1 and 6 MP/organism for sediment MPs concentration between 0 and $1.2 \cdot 10^{11}$ MPs/kg sediment dw respectively. The content of MPs in *Tubifex* spp. was not reported. Conversely, Huerta Lwanga et al. (2016) found a reduced growth and higher mortality rates at concentrations significantly lower (0.4%) for *Lumbricus terrestris*. The different effect found could be related with the larger size of the earthworms, compared to freshwater annelids, and their ability to ingest larger MP particles.

The MP concentration in this study for organisms locate in the lower limit of the range reported by Redondo-Hasselerharm et al. (2018). Therefore, according to their findings, no toxic effects could be expected for the concentrations estimated for the CW substrate. Nevertheless, given the differences found among studies and the importance of the subject, we consider that further research is needed to validate this hypothesis about the potential effects of MPs on the macroinvertebrate activity inside wetlands.

4. Conclusions

The main conclusion drawn from this study is that horizontal subsurface flow CWs efficiently reduce MPs concentration, preventing them from entering vulnerable aquatic systems. As tertiary treatment, CW are able to significantly reduce the MPs concentration coming from the secondary treatment.

The size and shape composition of the MPs does not differ significantly from the influent to the effluent, so there is no size or shape removed significantly more efficiently than others.

Fibers represent the most abundant shape both in water and macroinvertebrates, so attention should be paid on reducing their contamination at source.

Macroinvertebrates ingest a non-negligible quantity of MPs, so they could play a role in the MPs distribution inside constructed wetlands. The authors consider it very interesting to further investigate this mobilization potential.

The MPs concentrations detected in the monitored CW do not reach concentrations able to induce toxic effects for *Tubificidae* annelids, according to previous literature. Therefore, the MPs retention inside the CW could be expected to non-negatively influence the important role that macroinvertebrates play regarding the control of sludge accumulation.

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TABLES AND FIGURES

Table 1. Review of MP concentration ranges in the influent and effluent of different WWTP around the world.

Separation method	Influent	Kind of treatment	Effluent	Volume (L)	Analysis method	Reference
Centrifugation	- -	Secondary Tertiary (gravity filter)	1 MP/ $5.68\cdot10^4$ L 0 MP/ $1.93\cdot10^5$ L	$4.23\cdot10^5$ $1.93\cdot10^5$ - $2.32\cdot10^5$	Microscope FTIR	Carr, Liu, and Tesoro 2016
Sonication, filtration	80 – 240 MP/L[1]	Tertiary (disinfection)	2.5 - 27 MP/L[1]	3.6 - 30	Stereomicroscope FTIR-ATR	Conley et al. 2019
	15 – 430 MP/L	Secondary Tertiary	0.25 - 50 MP/L $0.88\cdot10^{-3}$ - 9 MP/L			Correia Prata 2018[2]
Vacuum filtration	57.6 (±12.4) MP/L	Secondary (CAS) MBR	1.0 (±0.4) MP/L 0.4 (±0.1) MP/L	4.0 - 30	Optical microscope FTIR and Raman	Lares et al. 2018
		Primary Secondary Tertiary	1.54 MP/L 0.25 – 0.48 MP/L 0 – 10.05 MP/L			Lares et al. 2018 (review table)
Vacuum filtration	15.70 (±5.23) MP/L	Grit and grease Primary Secondary	8.70 MP/L 3.40 MP/L 0.25 MP/L	30 - 50	Dissection microscope FTIR	Murphy et al. 2016
Filtration	610 MPP/L	Primary Secondary Tertiary (biological filtration)	304.9 MP/L 82.4 MP/L 13.5 MP/L	10 - 20 35 - 50 30 - 285	Stereomicroscope	Talvitie et al. 2015
Filtration	0.5 (±0.2) MP/L$_{(S)}$[3] 2.0 (±1.3) MP/L$_{(S)}$ 0.7 (±0.1) MP/L$_{(S)}$ 2.0 (±0.07) MP/L$_{(S)}$ 6.9 (±1.0) MP/L$_{(P)}$	Discfilter1 Discfilter2 $_{(S)}$ Rapid sand filters $_{(S)}$ Dissolved air flotation $_{(S)}$ MBR	0.3 (±0.1) MP/L 0.03 (±0.01) MP/L 0.02 (±0.007) MP/L 0.1 (±0.04) MP/L 0.005 (±0.004) MP/L	2 - 50 2 - 50 70 - 1000 2 - 1000 140	Stereomicroscope FTIR	Talvitie et al. 2017
Density separation ($ZnCl_2$, 1.90 g/cm^3) and Centrifugation		Secondary	3.0-5.9 MP/L	84 - 214	Raman microspectroscopy	Wolff et al. 2019
Density separation (NaI, 1.49 g/cm^3) and Centrifugation		Primary Secondary Tertiary (desinf-ultrafiltration*, reverse osmosis**)	1.5 MP/L 0.48 MP/L 0.28* MP/L, 0.21** MP/L	3 - 100 27 - 150 200	FTIR spectroscopy	Ziajahromi et al. 2017

Notes: CAS (conventional activated sludge); MBR (membrane bioreactor); [1]read on a graph; [2]review article; [3]Influent comes from (S): secondary type effluent or (P): primary effluent.

Table 2. CW parameters for the calculation of the first order areal rate coefficient.

Parameter	Value
Area (m^2)	500
Average flow, Q (m^3/d)	275
Average influent concentration, Ci (MPs/L)	6.45
Average effluent concentration, Co (MPs/L)	0.77
Background concentration, C* (MPs/L)	0
Number of tanks (Dotro et al. 2017)	3

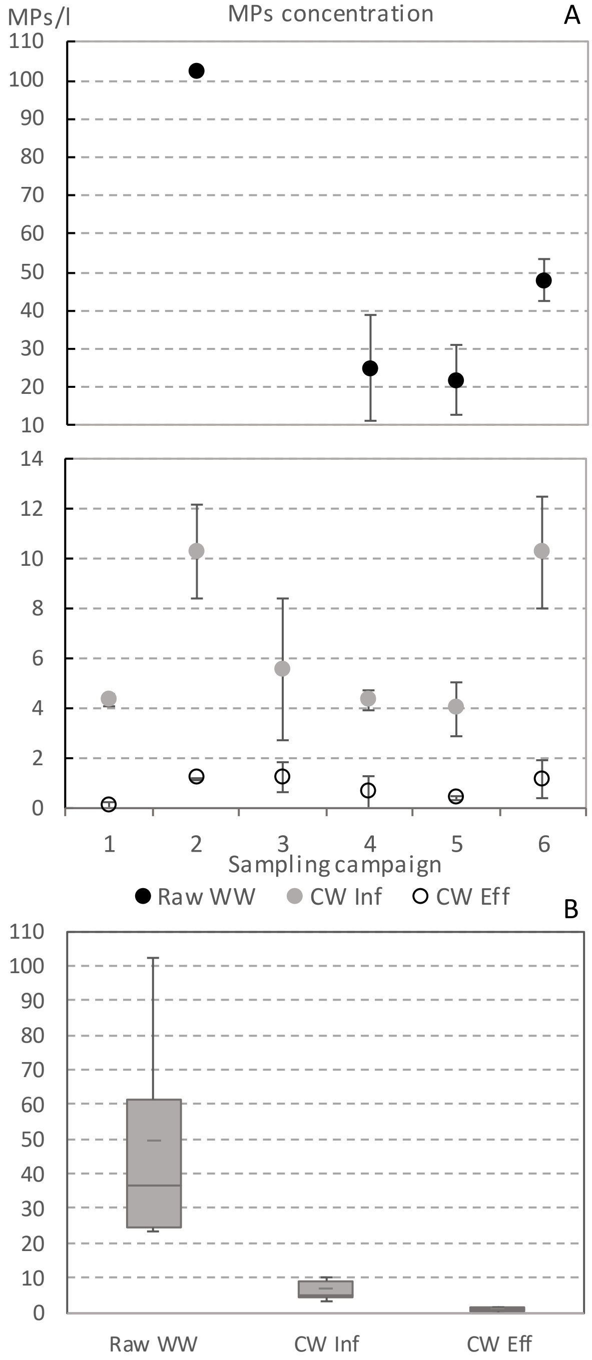

Figure 1. Results of MPs concentration measured in different sites of the WWTP. A) Average values and standard deviation (error bars) of the replicates analysed in each site and sampling campaign. B) Box-plot graph showing the variability of concentrations in each sampling site:

hyphens represent the average value. Raw WW is the influent to the WWTP; CW Inf is the influent to the CW; CW Eff: is the effluent from the CW and the final effluent from the WWTP.

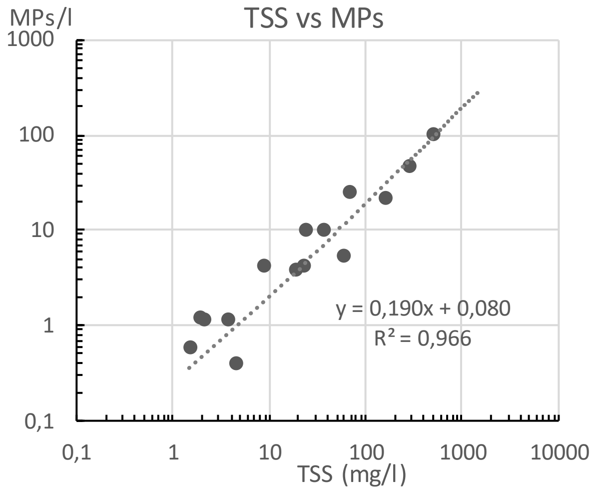

Figure 2. Relationship between TSS and MPs. Data from the three sampling sites (WWTP Influent, CW Influent and CW effluent) are represented.

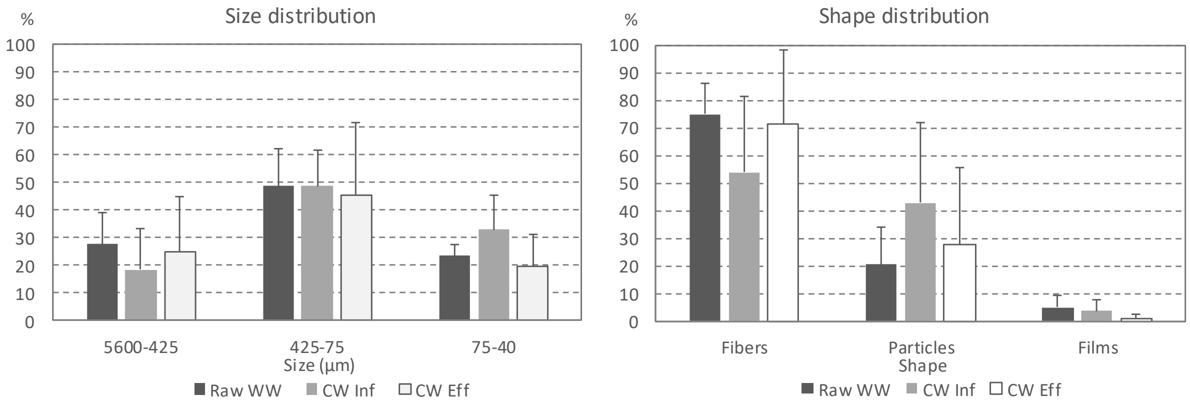

Figure 3. Distribution of MPs among the different size ranges and shapes. Error bars represent the standard deviation from all the sampling campaigns.

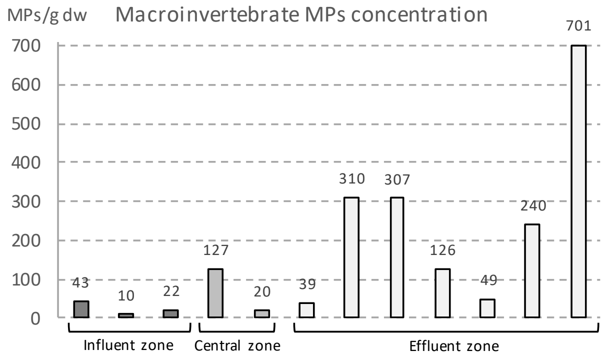

Figure 4. Macroinvertebrate MPs concentration in different zones of the CW: near the influent, central zone and near the effluent; dw: dry weight.

SUPPLEMENTARY INFORMATION

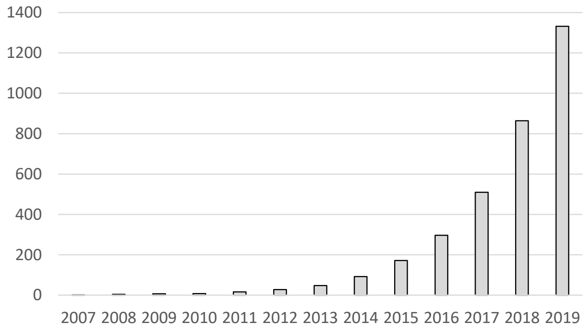

Figure S1. Number of peer reviewed publications in the category of environmental sciences and engineering and others related to them for the search term “microplastics”. Publications not related to environmental concerns about MPs have been discarded. Survey conducted using the ISI Web of Science database (Thompson Reuters).

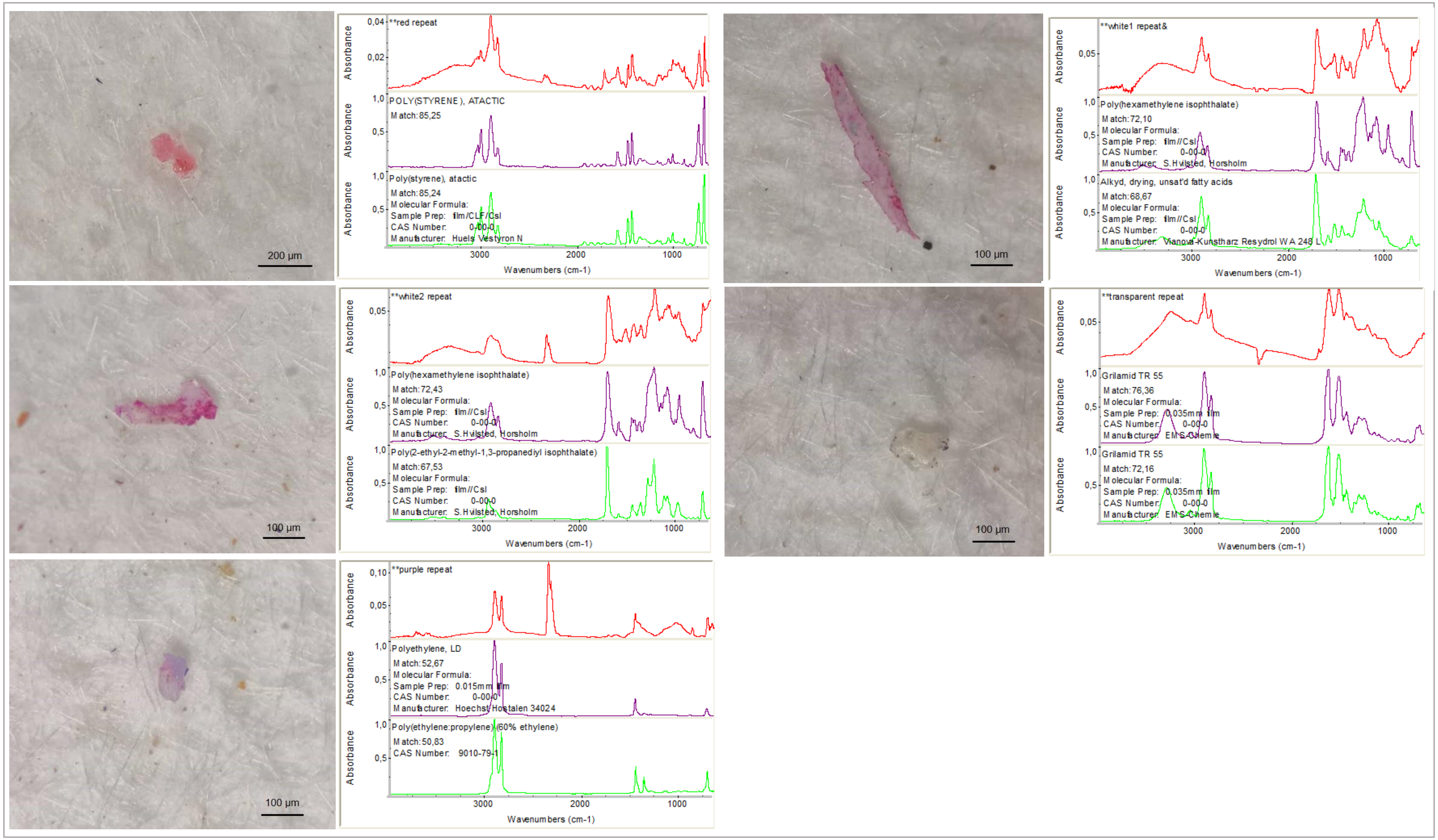

Figure S2. Images of MPs analysed using FTIR-ATR and spectrums obtained.

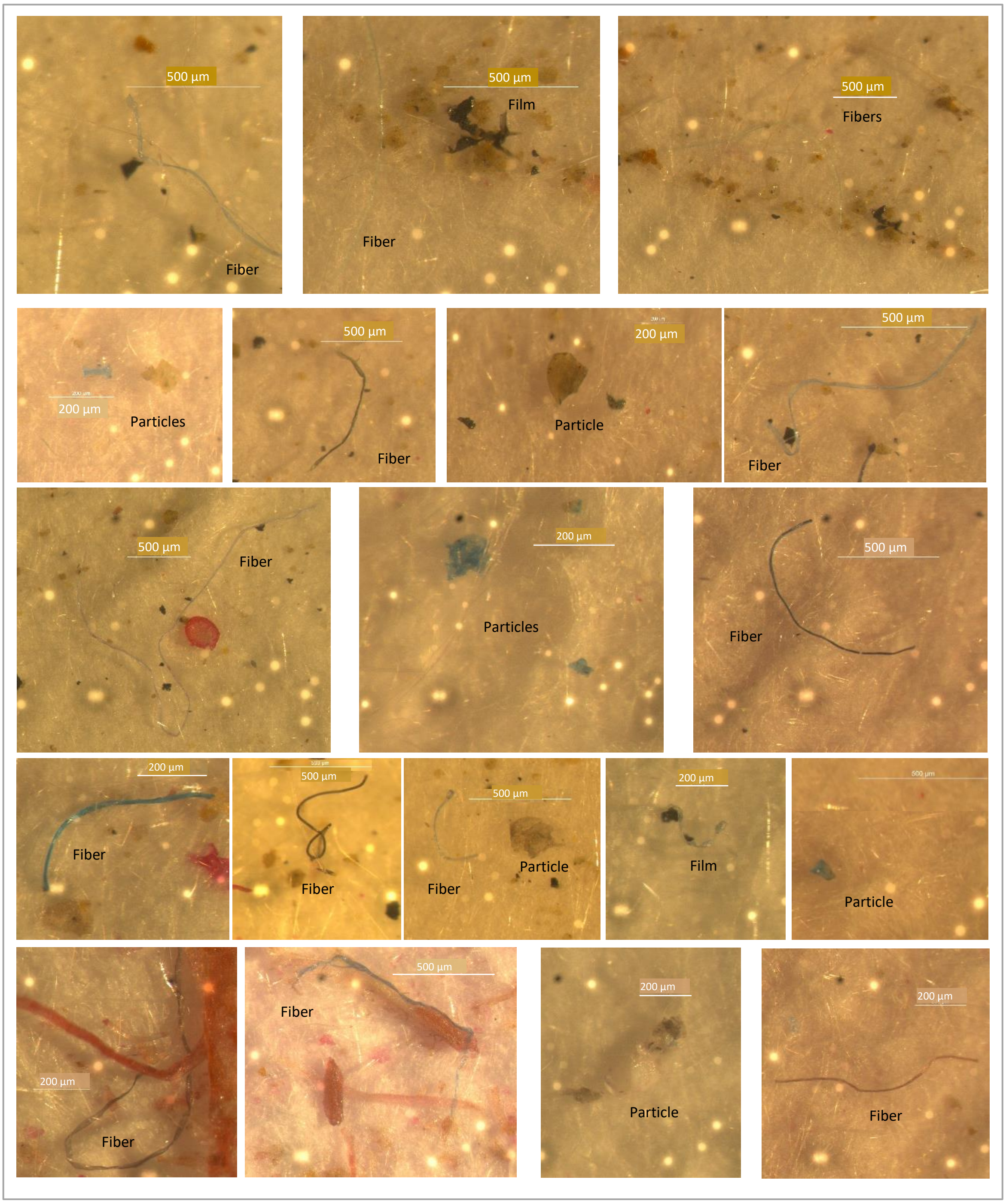

Figure S3. Pictures of different MPs found in the study, including fibers, particles and films.