Fate and removal of microplastics in unplanted lab-scale vertical flow constructed wetlands

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Influent: 50 particles/L per day of HDPE, PS, PP and Microbeads

10 cm

80 cm Sand

80 cm Sand with biofilm

80 cm gravel

15 cm

Efluent

100% MP removal

100% MP removal

96% MP removal

99.8% MP removal

Earthworms can ingest and transport microplastics

HDPE
PS
PP
Microbeads
1. Porous media of vertical-flow constructed wetlands (VFCWs) can remove microplastics.

2. Small size and spherical microplastics showed higher mobility in lab-scale VFCWs.

3. Microplastics were distributed throughout the full height of gravel-filled VFCWs.

4. Sand-filled VFCWs retained more than 98.8% of microplastics in the top 10 cm layer.

5. Earthworms can transport microplastics to the bottom of VFCWs and egest them.
Abstract

Many studies have reported conventional wastewater treatment plants as one of the main sources of microplastics (MPs). However, constructed wetlands (CWs) as a nature-based wastewater treatment system have received little attention. This study investigated the influence of biofilm, media type and earthworms on the fate and removal of MPs in a short-term (45d) experiment with unplanted lab-scale vertical flow CWs (VFCWs). In sand-filled VFCWs, MPs were retained in the first 10 cm, and the removal efficiency was 100%, regardless of the presence of a biofilm. When gravel was used as filling material, the removal efficiency of MPs was stable at 96%, but the MPs were distributed throughout the 80 cm high VFCWs. In the presence of earthworms, the maximum depth that MPs reached within sand-filled VFCWs increased from 10 to 15 cm. Furthermore, the MPs concentration at a depth of 3-6 cm and 6-10 cm increased 2 and 10 fold respectively compared to the same VFCWs without earthworms. Although no MPs were detected in the sand from deep layers (15-80 cm), transport of MPs from top to the bottom by earthworms was found, and a few MPs were detected in the effluent, leading to a removal efficiency of 99.8%. This study indicated that both a higher media grain size and the presence of earthworms have a small effect on the removal efficiency of MPs in VFCWs, but the effect on the
distribution of MPs was considerably. Longer-term studies in full-scale CWs are advised to perform under the influence of more practical factors.

**Keywords:** Microplastics; Constructed wetlands; Biofilm; Media; Earthworms

### 1. Introduction

Microplastics (MPs) pollution was first proposed by Thompson et al. (2004) and has gradually become a focus of global research. MPs are commonly defined as plastic particles smaller than 5 mm (Arthur et al., 2009). It was reported that MPs could be accumulated by fish, birds and mammals which are feeding on aquatic organisms or living in aquatic environments. The ingestion of MPs could be caused by the organisms’ inability to distinguish MPs from food, or through predation of lower trophic creatures containing these particles (Fendall and Sewell, 2009; Ivar do Sul and Costa, 2014). Several impacts of MPs on organisms have been reported, such as reduction of the predatory performance and efficiency (de Sá et al., 2015), oxidative stress (Della Torre et al., 2014), neurotoxicity (Ribeiro et al., 2017), genotoxicity (Della Torre et al., 2014), growth delay (Redondo-Hasselerharm et al., 2018) and causing death (Li et al., 2016). Human beings are also exposed to the ubiquitous MPs (Li et al., 2020), and its potential negative effects (Smith et al., 2018).
There are two main sources of MPs: primary MPs which are plastics that appear directly in the environment at micron scale, including microbeads from personal care products and microplastic powder used in paint and sand blasting (Cole et al., 2011), and secondary MPs which are formed from the large size plastic waste after aging in the natural environment (Rillig, 2012).

Human activities introduce large quantities of plastic waste into the environment, which is mainly a result of mismanagement of the waste system such as industrial pollution, urban surface runoffs and domestic wastewater. Previous studies have reported that domestic washing and personal care products were the major sources of MPs (De Falco et al., 2018; Fendall and Sewell, 2009). These MPs from households could be directly discharged into the sewer system with wastewater and end up at wastewater treatment plants (WWTPs) where the technologies used were not designed for MPs removal. Several studies have reported the MPs removal in each stage of WWTPs (Ben-David et al., 2021; Sun et al., 2019). The pre-treatment and primary treatment removed the most of MPs, with removal efficiencies of 35% - 59% in pre-treatment and 50% - 98% in primary treatment. After secondary treatment and tertiary treatment, MPs in wastewater were further decreased to 0.2% - 14% and 0.2% - 2% relative to the influent respectively. The total removal efficiencies of MPs in WWTPs
were reported to above 88% without tertiary treatment and to be up to over 97% with tertiary treatment (Murphy et al., 2016; Sun et al., 2019). Despite this large reduction, WWTPs are still releasing millions of MPs into receiving water every day (Mason et al., 2016; Murphy et al., 2016). It was reported that MPs concentration significantly increased in the downstream of WWTPs, which means that effluent from WWTPs is a major source of the MPs in the environment (Estahbanati and Fahrenfeld, 2016; McCormick et al., 2014).

Constructed Wetlands (CWs), as a robust nature-based solution for wastewater treatment (Rousseau et al., 2008), have been widely applied for small communities in Europe. As these CWs often discharge in small water courses with low dilution rates, the environmental impact can be quite high. CWs have a relatively good removal efficiency on total suspended solids (TSS) (over 80%) (Dotro et al., 2017) when compared with other stages in WWTPs (i.e. 50% - 70% in primary treatment and 80% - 90% in bio-treatment) (Silva et al., 2014). MPs as solid and micron particles can be considered to have the same properties as TSS in wastewater. Therefore, it can be expected that CWs may also have a good performance on MPs removal. Up to now, few studies have reported MPs removal in CWs. Wang et al. (2020) have studied MPs removal in a horizontal subsurface flow CW used as tertiary treatment in a small
community. The MP concentrations in wastewater were significantly reduced from 6.45 to 0.77 particles/L after tertiary treatment and the removal efficiency of the global WWTP was further increased to 98%. Wei et al. (2020) also have investigated four horizontal subsurface flow CWs which also served as tertiary treatments in Hangzhou, China. The results suggested that CWs could strengthen MPs removal in WWTPs. Nevertheless, it can be expected that the impact of different factors in CWs on MPs removal may vary largely, such as the biofilm present in vertical flow constructed wetlands (VFCWs), the choice of media type (sand or gravel) and the presence of macroinvertebrates. The biofilm in VFCWs has been reported to reduce the effective porosity (Zhao et al., 2009), which is expected to influence the removal and distribution of MPs. Also, the different possible media (e.g. sand and gravel) have different size, which would result in varied filtration capacities (Crittenden et al., 2012). As for macroinvertebrates and particularly earthworms, it has been reported that they were successfully introduced into CWs to remediate clogging problems (Li et al., 2012). However, the life and health of earthworms can be influenced, even with low concentrations of MPs (Huerta Lwanga et al., 2016). It can thus be hypothesized that MPs in CWs could have an effect on the clogging remediation of CWs by
affecting the earthworms. On the other hand, the earthworms may have an influence on the removal and distribution of MPs in CWs due to bioturbation. It has been reported that earthworms could be able to transport particles typically of millimeter size (0.5-1 mm), such as plant seeds and sand particles (Shumway and Koide, 1994). A recent study also showed that earthworms could transport MPs in soil from the surface to a depth of 10 cm (Rillig et al., 2017b).

The hypotheses of this study are (i) small size media and presence of biofilms can enhance the removal and retention capacity of VFCWs for MPs, (ii) earthworms in VFCWs can play an important role in MPs transport. The result of this study is expected to fill part of the knowledge gap and to give directions for further research on full-scale CW where additional factors are anticipated to play a role.

2. Materials and methods

2.1 Materials

The MPs used in this study were polystyrene (PS) particles, high-density polyethylene (HDPE) particles, polypropylene (PP) particles and PE microbeads (Table 1 and Fig. 1). Apart from the microbeads (45-53 µm) which were bought from Cospheric (California, US), the other three kinds of MPs were made from daily utensils which
are the main sources of plastic waste: plastic cups (PS), caps (HDPE) of water bottle and food containers (PP). To facilitate later identification, a different bright color was selected for every MP type. The selected utensils were first shredded into small pieces by a scissor. Then the pieces were further cut in a kitchen blender into microparticles. After that, the particles were collected and screened by a stack of sieves (5600 μm, 425 μm, 125 μm, 75 μm) (Wang et al., 2020). The particles remaining on the 125 μm and 75 μm sieves were collected. Before use, these MPs were separated by a sonication equipment in case they stuck together. The sand and gravel used in this study were bought from a DIY shop (Kortrijk, Belgium), and characterized in the lab. The average diameter of sand was 356 μm, and the size fractions were 10% < 231 μm, 50% < 400 μm, 90% < 668 μm. The diameter of the gravel (pebble) used was 2000 μm < 30% < 5600 μm and 5600 μm < 70% < 8000 μm. Earthworms were purchased from the company Eco-cult Worms (Roeselare, Belgium), including a mix of two species: *Eisenia fetida* and *Lumbricus castaneus*. Before the earthworms were added to CWs, they were washed with distilled water and kept on moistened filter paper at 22 ± 1 °C for 24 hours to void their gut contents (Wang et al., 2019).

2.2 Experimental design
To investigate the effect of media type, biofilm and earthworms on MPs fate and removal, 10 unplanted lab-scale VFCWs (Fig. S1) with a height of 100 cm and a diameter of 12.5 cm were constructed in a thermostatic room (22 ± 1 °C). Each VFCW was packed with 80 cm of the respective media for the main biologically active layer, and 5 cm gravel for a lower drainage layer. In order to separate particles from liquids of the effluent, funnels containing 40 μm sieves were attached to the bottom of each column. The solids on the funnels were collected for MPs analysis every 5 days. To avoid difficulties with the analyses, the top of the columns was covered with perforated aluminum foil to prevent MPs contamination from the air (e.g. operation accident with MPs used, fibers from clothes) and to prevent the earthworms from escaping. The specific conditions of each VFCW are shown in Table 2. VFCW1 and VFCW2 were run as blank control group (fed with tap water without MPs). VFCW3 and VFCW4 were filled with sand and run with tap water. VFCW5 and VFCW6 were filled with sand and run with synthetic wastewater to grow biofilms. VFCW7 and VFCW8 were filled with gravel and run with tap water. VFCW9 and VFCW10 were filled with sand and run with synthetic wastewater and earthworms were added after 20 days operation, allowing thus sufficient time for organic matter to accumulate inside the wetlands for earthworms to survive on. It
should be noted that vegetation was not selected as a variable in this initial study, to have maintain a clear focus on the processes of interest, i.e. physical filtration influenced by pore size, effect of biofilm development, and effect of earthworm bioturbation. In addition, there are two processes were vegetation is expected to have an impact, i.e. (1) physical disturbance of the top layer by wind-induced movement, and (2) formation of new channels by root growth decay. The former could not be mimicked at lab-scale, and the latter was estimated to be insignificant in a short-term study.

Synthetic wastewater was prepared with a simple formula, because its main purpose was to stimulate biofilm growth, and not to represent real wastewater (Hoagland and Arnon, 1950). Briefly, synthetic wastewater was prepared adding sugar (160 mg/L), milk (0.16 mL/L) and a nutrient solution (0.4 mL/L) to tap water, the latter based on Hoagland and Arnon (1950). The proportions of sugar and milk were estimated to have a concentration of biological oxygen demand (BOD) of approximately 150 mg/l, typical for the effluent of primary treatment. The fertilizer solution contained 21.85 mg/mL NO₃-N, 17.25 mg/mL NH₄-N, 29.9 mg/mL carbamide, 57.5 mg/mL P₂O₅, 69 mg/mL K₂O, 0.046 mg/mL Cu-EDTA, 0.4255 mg/mL Fe-EDTA, 0.0345 mg/mL Mn-EDTA, 0.01725 mg/mL Mo-EDTA and 0.1725 mg/mL Zn-EDTA. The tap water was
dechlorinated by settling down for at least 1 hour before being introduced into the VFCWs. The density of synthetic wastewater was $1.001 \pm 0.001\ g/cm^3$ (20 °C). As the density is very similar to tap water, the transport of MPs in the VFCW would not be affected. The hydraulic loading rate was set at a typical 0.08 m/d for VFCWs (Dotro et al., 2017), with the VFCWs being fed twice per day manually (0.5 L per feeding), and the feeding events were practical paused during weekends. The MPs were added with the synthetic wastewater or the tap water, 50 particles/L for each kind of MP. Each VFCW was run for 45 days.

2.3 MPs extraction methods

2.3.1 Extracting MPs from the effluent

The effluents of VFCWs were collected every 5 days for MPs analysis. The separation method was adopted from a previous study (Wang et al., 2020). For the effluent, the particles on the meshes in the funnels were flushed by distilled water into beakers which were put in the oven at 70 °C to dry up. After that, 20 ml high-density solution (CaCl$_2$ 1.34 g/cm$^3$) was added to the beakers and they were mixed with the help of a glass bar by hand (Stolte et al., 2015). Then the liquids were transferred from beakers to vials. To make sure all particles were recovered, the beakers were washed 3 times with the CaCl$_2$ solution, and the washing liquids were also added to
the vials. Next, the vials were centrifuged for 5 min at 3500 rpm, and the supernatants were vacuum filtrated with glass microfibers filters (Particle retention 1.5 μm, 47 mm diameter, VWR) to collect the MPs from the samples. The vials were then filled again with high-density solution and centrifuged one more time to ensure complete MP recovery. Finally, the filters were placed in aluminum containers and put in the oven at 70 °C for 5 min to dry before MP counting.

2.3.2 Extracting MPs from the solids

At the end of the experiment, the MPs inside the CWs were also analyzed with a separation method adopted from O'Connor et al. (2019). The solids were analyzed by layer. According to the previous study, the division of layers in the upper region was relatively dense. The division of layers in deeper regions was based on the analytical result of MPs in the above layer. So the CWs filled with sand were divided into 7 layers, which were 0-3, 3-6, 6-10, 10-15, 15-30, 30-50 and 50-80 cm, and CWs with gravel were divided into 9 layers which were 0-3, 3-6, 6-10, 10-15, 15-20, 20-30, 30-40, 40-50 and 50-80 cm. Each layer was first mixed to homogenize it and weighed before drying (W0), then two subsamples (30 g) were taken to assess humidity and loss on ignition (LOI), and two more subsamples (30 g) were taken to extract MPs. The solids samples were put in a flask and the same high-density CaCl2 solution was
added. Then the flasks containing the sand samples were put on a magnetic stirrer and
thoroughly stirred for 20 minutes in order to separate the MPs from the sand and have
them floating on the surface. The flasks containing the gravel samples were sealed
and shaken by hand and then inverted in order to let the MPs afloat. This shaking and
inverting step was repeated 3 times. After that, the solution was left to settle for 2
hours and the supernatant was collected to be further analyzed as before. The
concentration of MPs was calculated by Eq. (1):

\[ c = \frac{N_0 \times W_0}{W_1 \times V} \]  

(1)

where \( c \) is the MPs concentration (particles/cm\(^3\)), \( N_0 \) is the number of particles
detected in each subsample, \( W_0 \) is the total weight of each layer, \( W_1 \) is the fixed
subsample weight 30 g, \( V \) is the total volume of each layer (cm\(^3\)) and calculated by
height and diameter.

2.3.3 Extracting MPs from the earthworms

At the end of the experiment, earthworms were collected from the VFCWs by layer
and analyzed for MPs. The separation method was adopted from Wang et al. (2019).
Earthworms were first picked out with the help of tweezers and then washed with
distilled water thoroughly to remove the particles stuck to their surface. After that, the
Earthworms were put on wet filter paper in a clean beaker. The beaker was then put in the thermostatic room (22 ± 1 °C) for 48 h to let the earthworms egest. Then the beakers and earthworms were washed again with distilled water and the resulting liquid fractions were analyzed by the same method as used on the effluent. The earthworms were transferred to another clean beaker and put in an oven at 70 °C to dry off to obtain the dry weight. Then 10% KOH solution was added to the beakers for digestion in a 70 °C oven for 24 h (Kühn et al., 2017). After digestion, the samples were screened by a 40 μm sieve to remove the KOH solution. The residue on the sieve was then transferred into glass beakers with distilled water and the following MPs extraction procedure from water sample was as described in section 2.3.1. The MPs concentration inside earthworms and in excreta of earthworms were expressed in MPs per gram of earthworms (dry weight).

2.4 MPs counting

The filters which contained MPs were counted under stereomicroscopes with augmentations between x14 and x70 (SZM and SDZ-PL, Kyowa, Japan). The filters were read from left to right, then down one row from right to left and so on (Hidalgo-Ruz et al., 2012). During the counting, MPs were classified into PS, HDPE, PP and microbeads by color.
2.5 Quality control and contamination prevention

During all steps, including the setting up of CWs, sampling and analysis, materials made of plastic were avoided as much as possible. The filters and beakers containing the sample were always covered with aluminum foil when not being tested, to avoid contamination. Blanks, CW1 (filled with sand) and CW2 (filled with gravel), were run for the same period as the experimental group and fed with tap water. The analyses of MPs in effluent and its distribution were also performed in blanks, and no typical MPs used in this study were found. At the end of the experiment, the recovery rate of MPs in VFCWs was calculated for quality control. Each kind of MPs inside VFCWs, effluent and earthworms were compared with the total MPs in the influent to obtain the recovery rate.

2.6 Analytical methods

The size of sand was measured by Mastersizer S (Malvern, Netherland) and the size of gravel was measured by mechanical sieving. Chemical oxygen demand (COD) in wastewater and effluent was determined every 5 days and measured by Hach kits (Closed tube digestion, Hach) according to standard methods (APHA, 2005).
humidity and loss on ignition (LOI) were measured by oven and muffle furnace
(Touch et al., 2017). Each test was run in duplicate.

2.7 Statistical analysis

Statistical analysis was performed using SPSS 24 software (SPSS Inc., USA). The significant differences of MPs distribution by layers between the VFCWs were tested by paired t-test and one-way analysis of variance followed by post-hoc (Tukey's) test. The significance level was 0.05 (p < 0.05).

3. Results and discussion

3.1 General performance of different VFCWs

COD removal by VFCWs presumably indicates the existence of a biofilm inside VFCWs. In this study, the biofilm was designed to develop in sand-filled VFCW5/6 and 9/10 by feeding synthetic wastewater. The COD concentration in the influent of VFCW9/10 was a bit higher than VFCW5/6 in order to provide enough organic matter for earthworms. The results are shown in Fig. 2a and 2b; the average removal efficiency of COD in VFCW5/6 and 9/10 was 90.8% and 93.7%, respectively. This indicated the growth and activity of biofilm. The biofilm could also enhance the water retention, lead to higher humidity, and increase volatile content of solids. As shown in
Fig. 3, both VFCW5/6 and 9/10 had higher humidity and LOI than VFCW3/4 and 7/8, this also suggested the presence of biofilm.

The MPs removal was different between the VFCWs. In sand-filled VFCW3/4 and 5/6, no MPs were detected in the effluent and the removal efficiency thus reached 100%. In gravel-filled VFCW7/8, MPs were detected in the effluent, suggesting that MPs could penetrate the CWs filled with gravel. MPs were already detected in the first sample of effluent of VFCW7/8 and the average total concentration was 0.2 particles/L (Fig. 2c). With the continued operation of the VFCWs, the PS particle concentration in the effluent noticeably increased and reached 4.9 particles/L on day 41. Due to the lower relative density, HDPE particle (0.95 g/cm³) concentrations were much smaller than PS (1.05 g/cm³) in the effluent. The highest concentration of HDPE in the effluent was 0.6 particles/L. Because of their lowest density of only 0.90 g/cm³, the PP particles were hardly found in the effluent, and the average concentration of PP in effluent was only 0.07 particles/L. These three kinds of MPs had similar shape and size, thus the relative density could be the major effector on the removal of MPs. Low density MPs have more hindered penetration under infiltration because of the high buoyancy effect.
In sand-filled VFCW9/10, earthworms were added, and they influenced the removal of MPs. In the effluent of both VFCW9 and VFCW10, MPs were detected from day 25 onwards which was 5 days after adding the earthworms. During the operation of VFCW9 and 10, there was a 50 days suspension of feeding due to the COVID-19 pandemic from day 25. But the earthworms inside were still alive until day 75. At the end of the experiment, the MP concentrations in the effluent remained at a very low level. The average concentration of MPs from day 25 to the end was 0.4 particles/L, and the removal efficiency still reached 99.8% (Fig. 2d). Furthermore, only PS particles, HDPE particles and microbeads were detected in the effluent. Similar as gravel-filled VFCWs, PP particles were not found in the effluent due to the low relative density (0.90 g/cm³). The proportion of microbeads reached 65%, while the proportions of PS and HDPE particles were 30% and 5%.

3.2 Influence of biofilm on the fate of MPs

VFCW3, 4, 5 and 6 were filled with sand and used to investigate the influence of biofilm on the fate of MPs. VFCW3 and VFCW4 were fed with tap water and the others were fed with synthetic wastewater to develop the biofilm. The movement of MPs in the VFCWs could be influenced by external conditions such as flow and porosity, and MP characteristics such as density, shape and size (Yao et al., 1971). PP,
HDPE and PS particles were granular morphotypes, and the microbeads were spherical morphotypes (Table 1). Granular and spherical morphotypes are expected to have better mobility than fragments or filaments (O'Connor et al., 2019). However, in this group, none of the four kinds of MPs fully flowed through the sand filled CWs as in none of the effluents were any MPs detected during the 45 days. According to the distribution analysis (Fig. 4a and 4b), there was no obviously difference in the distribution of PP, HDPE and PS particles between VFCWs with and without biofilm (p > 0.05). Most of the MPs were detected in the top 6 cm layer. This agrees quite well with a previous study by O'Connor et al. (2019) who investigated MPs’ vertical migration in a sandy soil with wet-dry cycles, and found that the maximum depth that MPs could reach was 7.5 cm after twelve 83 mm infiltration events. In layer 1 (depth between 0 and 3 cm) and 2 (depth between 3 and 6 cm), the average total MPs concentrations in VFCW3/4 without biofilm were 17.2 and 1.9 particles/cm³, respectively. And the average concentrations of the same MPs in VFCW5/6 with biofilm were 17.8 and 1.7 particles/cm³, respectively. The slight difference between VFCW3/4 and VFCW5/6 could be caused by the biofilm (Vymazal, 2018), which slightly enhanced the filtering capability in the first layer. However, in the deeper layer 3 (depth between 6 and 10 cm) in VFCW5/6 with biofilm, the microbeads
concentration was 0.096 particles/cm³ and higher than PP, HDPE and PS (p < 0.05).

The major differences between microbeads and others were the particle size and morphotype. The size of microbeads was 45-53 µm which was much smaller than the other MPs used (75-425 µm). Recent studies also showed that smaller sized MPs had higher permeability (Rillig et al., 2017a).

The result showed that CWs filled with sand had a great removal capacity for MPs and that the removal efficiency for these four kinds of MPs reached 100%. The biofilm slightly enhanced the filtering capacity of CWs, leading to a tiny difference of MPs distribution. MPs were mostly retained in the first 6 cm. Nevertheless, due to the smaller size and spherical morphotype, microbeads had the highest concentration in the layer of 6-10 cm. O'Connor et al. (2019) also reported that the PE particles with an average size of 21 µm they used for the vertical migration experiments were the most mobile of the MPs tested, travelling to a maximum depth of 7.5 cm.

3.3 Influence of media type on the fate of MPs

Gravel-filled VFCW7 and 8 were used to investigate the influence of media type on the fate of MPs. The VFCWs were fed with tap water. The distribution of MPs in gravel-filled VFCWs was highly different from sand-filled VFCWs (p < 0.05), with
PS, HDPE and PP particles being distributed throughout the VFCWs. Layer 3 (depth between 6 and 10 cm) retained the most MPs, with an average total concentration of 3.98 particles/cm³ (Fig. 4c). In the top 30 cm layer, the concentrations of PP, HDPE and PS particles were similar. On the contrary, in the lower layer (50-80 cm), the concentration of PS particles was higher than PP and HDPE (p < 0.05). In the deepest layer, the concentration of PS was 0.081 particles/cm³, while the concentrations of HDPE and PP were 0.032 and 0.024 particles/cm³, respectively. These results are consistent with the results of concentrations in the effluent. Therefore, relative density is still the major factor influencing the distribution of MPs, and the particles with low relative density (lower than water) could be retained to a higher degree in VFCWs.

Compared with the VFCWs filled with sand, the gravel-filled VFCWs have a low capacity to retain the MPs, and high-density MPs more easily passed the VFCWs. In real-life applications, gravel-filled VFCWs usually have a dense sludge layer on top, known as French-style CWs which was reported to have a high capacity for TSS removal (Millot et al., 2016). Therefore, it is reasonable to assume that the sludge layer can enhance the MPs retention capacity of gravel-filled VFCWs.

The MPs were distributed throughout the VFCWs by water percolation, but the lowest removal efficiency of MPs for gravel-filled VFCWs still surmounted 96%. The result
indicated that the diameter of wetland media also played an important role in distribution and removal of MPs. A previous study also reported the effect of porous media diameter on filtration (Tarbuck et al., 2005). The filtering effect happens when the pore spaces of medium are too narrow to allow the particles to pass.

Consequently, gravel had a much higher average diameter than sand, which increased the depth of penetration of MPs (Crittenden et al., 2012), leading to a reduction of retention capacity of VFCWs.

3.4 Influence of earthworm activity on the fate of MPs

VFCW9 and 10 were filled with sand and populated with earthworms. These VFCWs were used to investigate the influence of earthworm activity on the fate of MPs. The distribution of MPs inside the VFCWs was also changed due to the activities of the earthworms. The maximum depth at which MPs were detected in the sand was increased to layer 4 (depth between 10 and 15 cm), and the average total concentration of MPs was 0.27 particles/cm$^3$ (Fig. 4d). MPs were not detected in the sand of deeper layers (15-80 cm) but shown in the effluent, which could be due to the extremely small concentrations of MPs transported by earthworms. However, the MPs concentrations in layer 2 (depth between 3 and 6 cm) and 3 (depth between 6 and 10 cm) also increased a lot compared to VFCW5/6 which were also fed with synthetic
wastewater but without earthworms. The average total MPs concentration of VFCW9/10 in layer 2 was 5.57 particles/cm$^3$, which was about 2 times higher than the average of VFCW5/6. Likewise, the average total concentration of MPs in layer 3 was 1.31 particles/cm$^3$, which was 10 times higher than the average of VFCW5/6. Apparently, the earthworms increased the mobility of MPs considerably. A recent study also reported the transport of MPs by earthworms in soil (Rillig et al., 2017b). They used PE particles for a transportation experiment, and the results showed that earthworms could transport them from the surface down to a depth of 10 cm. In this study, the depth of transport was higher and reached 15 cm. On the one hand, when earthworms move in the CWs, they could create numerous tunnels (Huerta Lwanga et al., 2017; Yang et al., 2019). With the tunnels, the MPs could move down more easily with the infiltrating water. On the other hand, the macroinvertebrates could ingest the MPs and deposit them elsewhere when defecating. Also, the MPs could attach to the body of macroinvertebrates and thus be transported (Rillig et al., 2017b). The distribution of earthworms was different between VFCW9 and 10. In VFCW9, 18, 1, 0, 0, 2, 0, 17 earthworms were found at a depth of 0-3, 3-6, 6-10, 10-15, 15-30, 30-50, 50-80 cm, respectively (Fig. 5). However, no earthworms were found in the bottom layer of CW10. This partial migration of earthworms in CW9 may be caused
by the effect of humidity and oxygen (Chuang and Chen, 2008). During the 50 days
suspension of feeding caused by COVID-19 pandemic, the bottom layer could
provide earthworms with the same moisture and oxygen to survive as the top layer. In
VFCW9, the earthworms were mainly distributed in the first layer and the last layer. It
suggested that they could move throughout the VFCWs from top to bottom. The
results further showed that MPs were present in the excreta of earthworms (Fig. S2),
and the concentrations were 40.00, 68.26, 30.49 and 10.55 particles/g dw at the depths
of 0-3, 3-6, 15-30 and 50-80 cm, respectively. The presence of MPs in the excreta
directly confirmed the ingestion of MPs by earthworms. The concentrations of MPs in
excreta of earthworms in the upper layer were higher than in the lower layer owing to
the high abundance of MPs in the upper layer. According to the distribution of MPs
inside the VFCWs, there was no MP detected in the layer between 50 and 80 cm, but
the MPs in the excreta found in this layer reached 10.55 particles/g dw. This
suggested that earthworms could carry the MPs from the top to the bottom. The
distribution of earthworms was different in VFCW10. There were 15, 8, 10 and 4
earthworms at the depths of 0-3, 3-6, 6-10 and 10-15 cm, respectively. No earthworm
was found in the deeper layers. The MP concentrations in the excreta of earthworms
were 25.30, 18.62, 20.85 and 21.44 particles/g dw. The highest concentration was also
found in the top layer which was due to the high abundance of MPs. Both in VFCW9 and 10, no MPs were detected in the digestion residue of earthworms after egestion for 48 h. This suggested that MPs might be egested completely from the gut of earthworms. Previous studies also confirmed the ingestion and excretion of MPs by earthworms (Huerta Lwanga et al., 2017; Wang et al., 2019), and observed the transportation of MPs. With the presence of earthworms, the distribution of MPs in VFCWs changed considerably when compared to VFCW5/6 without earthworms. Also, the removal efficiency slightly decreased from 100% to 99.8%, which could be caused by the migration of earthworms. This indicates that the bioturbation of earthworms could reduce the MPs removal by VFCWs.

In addition, the total alive earthworms collected at the end of the experiment were 38 and 37 in VFCW9 and 10, respectively. The earthworm population had declined a bit as a few might have escaped from the bottom of the VFCWs. No reproduction occurred. Previous studies also reported that MPs had no significant effects on survival of earthworms (Lumbricus terrestris and Eisenia andrei Bouché) (Huerta Lwanga et al., 2016; Rodriguez-Seijo et al., 2017), but their growth rate was significantly reduced after 60 days of exposure to MPs (28, 45 and 60% dw/dw), and tissue injury was induced (Huerta Lwanga et al., 2016). This is consistent with the
result of this experiment; the survival of earthworms had not been affected. But the
effect on tissue injury and reproduction of earthworms needs to be further
investigated.

The results convincingly showed that activities of earthworms could considerably
influence the distribution of MPs inside VFCWs (p < 0.05), but the MPs removal
efficiency of VFCWs was stable at 99.8%. MPs could reach deeper layer (10-15 cm)
when compared to the same type of VFCWs (5/6) without earthworms. However, the
MPs were still most abundant in the first 10 cm layer, and the concentration of MPs in
the first 10 cm layer was 98.8% of the total concentration inside VFCWs. In this case,
only the first 10 cm layer of VFCWs would be highly polluted with MPs. When
needing to manage the wetland media because of clogging, a special management
would thus be required to avoid the dispersion of MPs from the first 10 cm layer
solids. Also, it has been reported that microorganisms such as bacteria and fungi can
degradate MPs (Yuan et al., 2020), the abundant MPs and organic matter in the top
layer (0-10 cm) could favor degradation.

3.5 MPs balance
Based on the distribution of MPs inside the VFCWs and their respective effluent concentrations, the total amount of MPs of each VFCW was estimated and compared with the total MPs in the influent to ensure the reliability of the data. As shown in Table 3, all the MPs, except the microbeads in sand-filled VFCW9/10 with earthworms, reached a recovery rate more than 89%. The difference may be due to the uneven distribution of MPs in the solids. However, in the sand-filled VFCW9/10 with earthworms, the recovery rate of microbeads only reached 78%, while this rate was 90% in sand-filled VFCW5/6 with biofilm. This decrease could be related to the activities of earthworms. This can be explained as follows. First, the size of microbeads ingested by earthworms was very small (only 45-53 µm), so they could have served as the nuclei for the aggregates in earthworm casts (Shipitalo and Protz, 1989). In this case, the microbeads could be missed in the analysis due to their invisibility. The second possibility is the efficiency of the applied KOH digestion method. The residue after digestion still wrapped this small size microbeads which made it difficult to detect. Overall, the recovery rate of MPs reached a considerable degree, which supports the reliability of the analyzed data.

4. Conclusion
In this study, the effects of biofilm, media type and earthworms on the fate and removal of MPs in unplanted lab-scale VFCWs were investigated over a time span of 45 days. The sand-filled VFCWs had a high removal capacity for MPs. MPs were retained in the first 10 cm, and the removal efficiency was 100%. The growth of biofilm slightly enhanced the filtering capacity of VFCWs, leading to a tiny difference in MPs distribution. When gravel was used to fill the VFCWs, the removal efficiency of MPs was 96%, and the MPs were distributed throughout the VFCWs. In the presence of earthworms, the maximum depth that MPs reached in VFCWs increased from 6-10 cm to 10-15cm, while the MP concentrations at the depth of 3-6 cm and 6-10 cm respectively increased 2 and 10 times compared to the same type VFCWs without earthworms. Although MPs were not found in the sand of deep layers (15-80 cm), MPs were found in the excreta of earthworms present in the bottom layer and also in the effluent, but the removal efficiency of MPs was still 99.8%. Also, the concentration of MPs in the first 10 cm layer was still 98.8% of the total MPs concentration in VFCWs with earthworms. Accumulation of large quantities of MPs in the first 10 cm layer of sand-filled VFCWs could be expected after a long run, which should be properly managed to avoid the dispersion of MPs.
It should be noted that other important factors in full-scale CWs were not considered in this study, such as the vegetation, soil composition, hydraulic loading rate and flow patterns. These are critical parameters in CWs and expected to affect the fate and removal of MPs. Likewise, natural weather (e.g. rain and wind), plant root system and bioturbation from abundant organisms were missing in lab-scale VFCWs. We recommend that further studies could consider more factors from the real-world, there is still a large knowledge gap in real-scale CWs. Also, various shapes and more types of MPs warrant further research in this regard.

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References


Vymazal J. Does clogging affect long-term removal of organics and suspended solids in gravel-based horizontal subsurface flow constructed wetlands? Chemical


1 **TABLES AND FIGURES**

2 Table 1 Characteristics of used MPs. PS, HDPE and PP granules were home-made, the PE spheres were purchased from Cospheric (California, US).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PS</th>
<th>HDPE</th>
<th>PP</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (µm)</td>
<td>75-425</td>
<td>75-425</td>
<td>75-425</td>
<td>45-53</td>
</tr>
<tr>
<td>Color</td>
<td>Red</td>
<td>Blue</td>
<td>Green</td>
<td>Orange</td>
</tr>
<tr>
<td>Morphotype</td>
<td>Granular</td>
<td>Granular</td>
<td>Granular</td>
<td>Sphere</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>1.05</td>
<td>0.95</td>
<td>0.90</td>
<td>1.00</td>
</tr>
</tbody>
</table>

3 Table 2 Experimental design.

<table>
<thead>
<tr>
<th>Number</th>
<th>Media type</th>
<th>Influent</th>
<th>MPs</th>
<th>Earthworms</th>
</tr>
</thead>
<tbody>
<tr>
<td>VFCW1</td>
<td>Sand</td>
<td>Tap water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VFCW2</td>
<td>Gravel</td>
<td>Tap water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VFCW3</td>
<td>Sand</td>
<td>Tap water</td>
<td>PS, PP, HDPE</td>
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</tr>
<tr>
<td>VFCW4</td>
<td>Sand</td>
<td>Tap water</td>
<td>PS, PP, HDPE</td>
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</tr>
<tr>
<td>VFCW5</td>
<td>Sand</td>
<td>Synthetic wastewater</td>
<td>PS, PP, HDPE, Microbeads</td>
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<tr>
<td>VFCW6</td>
<td>Sand</td>
<td>Synthetic wastewater</td>
<td>PS, PP, HDPE, Microbeads</td>
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<tr>
<td>VFCW7</td>
<td>Gravel</td>
<td>Tap water</td>
<td>PS, PP, HDPE</td>
<td>-</td>
</tr>
<tr>
<td>VFCW8</td>
<td>Gravel</td>
<td>Tap water</td>
<td>PS, PP, HDPE</td>
<td>-</td>
</tr>
<tr>
<td>VFCW9</td>
<td>Sand</td>
<td>Synthetic wastewater</td>
<td>PS, PP, HDPE, 40 individuals added Microbeads</td>
<td>-</td>
</tr>
<tr>
<td>VFCW10</td>
<td>Sand</td>
<td>Synthetic wastewater</td>
<td>PS, PP, HDPE, 40 individuals added Microbeads</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3: The average recovery rates (average ± stand error) of different MPs in VFCWs, the recovery rates were estimated by the MPs in the influent, effluent, solids and earthworms.

<table>
<thead>
<tr>
<th>CWs</th>
<th>PS (%)</th>
<th>HDPE (%)</th>
<th>PP (%)</th>
<th>Microbeads (%)</th>
</tr>
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<tbody>
<tr>
<td>Sand-filled VFCWs without biofilm</td>
<td>99.5 ± 0.5</td>
<td>95.0 ± 0.4</td>
<td>90.3 ± 1.0</td>
<td>-</td>
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<tr>
<td>Sand-filled VFCWs with biofilm</td>
<td>101.5 ± 1.2</td>
<td>98.7 ± 1.2</td>
<td>91.4 ± 4.2</td>
<td>90.0 ± 2.3</td>
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<tr>
<td>Gravel-filled VFCWs</td>
<td>98.4 ± 6.1</td>
<td>90.3 ± 9.0</td>
<td>89.1 ± 8.1</td>
<td>-</td>
</tr>
<tr>
<td>Sand-filled VFCWs with earthworms</td>
<td>102.3 ± 2.2</td>
<td>99.4 ± 4.1</td>
<td>94.0 ± 8.3</td>
<td>78.1 ± 4.9</td>
</tr>
</tbody>
</table>

Fig. 1. Stereomicroscopic photographic images of the four MPs used, (a) PS, (b) HDPE, (c) PP, (d) Microbeads.
Fig. 2. (a) COD concentration in the influent and effluent of sand-filled VFCW5/6, (b) COD concentration in the influent and effluent of sand-filled VFCW9/10, (c) MPs concentration in the effluent of gravel-filled VFCW7/8, (d) MPs concentration in the effluent of sand-filled VFCW9/10. The concentration of each kind of MPs in influent was 50 particles/L per day.
Fig. 3. Humidity and Loss on ignition of VFCWs.
<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>PS</th>
<th>PEHD</th>
<th>PP</th>
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<tr>
<td>0</td>
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<td></td>
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<td>80</td>
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</tbody>
</table>

Fig. 4. Distribution of MPs inside VFCWs, (a) sand-filled VFCW3/4 without biofilm, (b) sand-filled VFCW5/6 with biofilm, (c) gravel-filled VFCW7/8, (d) sand-filled VFCW9/10 with earthworms.
Fig. 5. Distribution of earthworms and MPs content in the excreta of earthworms in sand-filled VFCWs with earthworms, (a) VFCW9, (b) VFCW10.
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