An exploratory ecotoxicity study of primary microplastics versus aged in natural waters and wastewaters

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ABSTRACT:

Current understanding of how environmental aging of microplastics contributes to their ecotoxicity is low. We investigated whether incubation of microplastics in waters with different organic load and toxic potential alters the toxicity of microplastics to crustacean *Daphnia magna*, fish embryos *Danio rerio* and plant *Lemna minor*. Polyethylene microplastics; specifically microbeads from facial scrub; were subjected to 3-weeks incubation in low affected spring water, river water, effluent from the municipal wastewater treatment plant (WWTP) and municipal landfill leachate. Primary microplastics had no acute effect on *D. magna* mobility and *D. rerio* embryos development. While high organic load wastewaters; WWTP effluent and landfill leachate; showed evident toxicity for *D. magna* and *D. rerio* embryos, microplastics aged in these wastewaters had no effect. This suggests that adsorption of pollutants from wastewaters to microplastic particles was not high enough to induce acute toxicity to *D. magna* and *D. rerio*. On the contrary, primary microplastics affected the root growth of *L. minor*. Interestingly, aging of microplastics in low organic-load waters mitigated the toxicity of microplastics for *L. minor*, while microplastics aged in high-organic load waters had the same adverse effect as primary microplastics. Partly, these effects can be explained by different extent of coating on microplastics in different water samples. This study suggests that aging of microplastics in wastewaters and natural waters did not significantly enhance the toxicity to selected test species, but further studies on plants may be of interest.

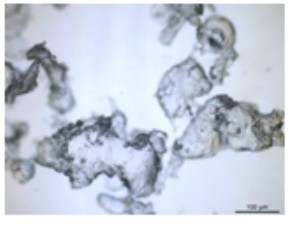
KEYWORDS: aquatic toxicity; plant; polyethylene microbeads; zebrafish; wastewater

Capsule: *Microplastics aged in wastewaters and natural waters are not more toxic as primary microplastics*

- Coating of aged microplastics in natural waters and wastewaters differs from primary microplastics
- Aged microplastics have different sinking properties
- Aging of microplastics does not increase the toxicity to daphnids, duckweed and zebrafish
- Primary microplastics affect only duckweed root length
- Aging in some waters may mitigate toxic effects of microplastics for roots

Different coating
in water samples

no
Increase in toxicity



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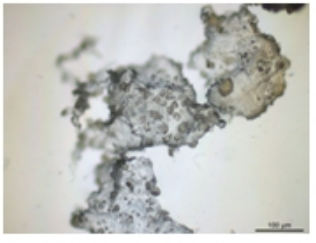

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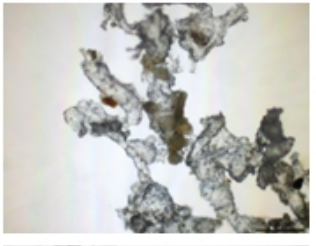

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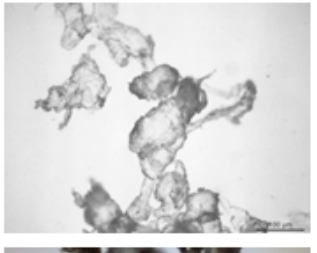
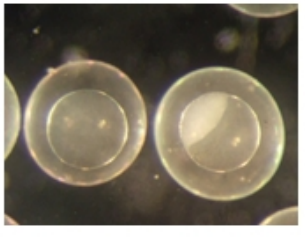


LANDFILL LEACHATE

BUT



1 INTRODUCTION

In recent years numerous reports of microplastics pollution in aquatic environment have emerged (Burns and Boxall, 2018; Horton et al., 2017). As a result, there is increasing scientific, public and regulatory interest in knowing potential adverse effects that microplastics pollution may have on aquatic wildlife. It has been recently pointed out that most of the existing data are not linked to an environmental context (Koelmans et al., 2016; Ogonowski et al., 2018; Jahnke et al., 2017; Burns and Boxall, 2018; Phuong et al., 2016; Connors et al., 2017). For example, microplastics studies lack environmental relevance in terms of the size, shape, and concentration of tested microplastics (Connors et al., 2017). There is also little overlap between the type of microplastics tested in the laboratory and those actually found in the field (Phuong et al., 2016).

An additional aspect is the environmental transformation of microplastics or so called “aging” (Vroom et al., 2017). Plastic has high adsorptive properties and it is coated by a layer of organic and inorganic substances upon entering the environment (Rummel et al., 2017). This phenomenon has been described in the case of nanomaterials and termed as “adsorbsome” (Walkey and Chan, 2012) or “eco-corona” (Lynch et al., 2014). Similarly, microplastics also represent an important sorbent of inorganic and hydrophobic organic pollutants (Bakir et al., 2012; Teuten et al., 2009) suggesting that microplastics can act as a source of these pollutants to organisms (Koelmans et al., 2013). After this initial conditioning of microplastics further colonisation by organisms, like bacteria, algae, protozoa and fungi occurs. This results in biofilm formation (Rummel et al., 2017) which has also been termed as “plastisphere” (Zettler et al., 2013). In addition, microplastics can be colonised by phytoplankton and small invertebrates. In parallel, weathering of plastic including physical stress caused by wave action, abrasion by sand, temperature fluctuations, and UV-initiated degradation of microplastics occurs (Jahnke et al., 2017, Rummel et al., 2017) resulting in surface mechanical deformations like cracking and pitting. Microplastics environmental transformation therefore involves multilateral processes.

There are currently only few studies that addressed aged microplastics (Kowalski et al., 2016; Vroom et al., 2017). It has been shown that biofouling (Kooi et al., 2017) and weathering (Kowalski et al., 2016) affect the vertical transport of microplastics in water column. Biofouling increases the density of microplastics which affects their fate in the environment, and can enhance the uptake of plastic particles into the food web and slow both leaching of chemicals from the plastic and sorption of chemicals from ambient water (reviewed in Jahnke et al., 2017; Weinstein et al., 2016). All these transformations may result in altered fate of microplastics in the environment and the interaction with biological systems. It has been shown that organisms may ingest aged microplastics at higher

rates as primary microplastics (Vroom et al., 2017). However, to our knowledge there is currently no study that would address the contribution of microplastics aging to aquatic ecotoxicity.

The aim of this study was to compare acute toxicity of aged versus primary polyethylene microplastics (cosmetic microbeads) for selected aquatic organisms. For this purpose, microplastics were aged for 3 weeks in four different water samples according to their composition and load of organic and inorganic components: low affected spring water, high affected river water, effluent from the municipal wastewater treatment plant (WWTP) and municipal landfill leachate sample. We hypothesised that those microplastics that were aged in presumably more polluted waters (WWTP, landfill leachate and high affected river water) would provoke higher effects in comparison to low affected spring water aged- and primary microplastics due to the expected adsorption of water pollutants. Aquatic toxicity was assessed with three commonly used standard test organisms: crustacean *Daphnia magna*, fish embryos *Danio rerio* and plant *Lemna minor*.

2 MATERIALS AND METHODS

2.1 Extraction and characterisation of microplastics

Polyethylene microplastics were extracted from facial scrub product purchased in a local store. The extraction was previously described in detail (Kalčikova et al., 2017). Briefly, the product was dissolved in deionized water, and then filtered through Whatman™ filter paper (pore size 4-12 µm). Retained microplastics were washed several times by successive filtration of deionized water through the filter paper to remove the remaining ingredients of cosmetic products.

The size and shape of microplastics were inspected under a field emission scanning electron microscope (FE-SEM, Zeiss ULTRA plus, Carl Zeiss, Germany), at an accelerating voltage of 2 kV and 30 µm aperture size. The micrographs were captured by a secondary electrons (SE) detector. Particles were sputtered with a thin platinum layer and were fixed on an aluminium holder using double sided adhesive carbon tape. The number and volume particle size distributions of microplastics were measured using a Microtrac S3500 Bluewave laser diffraction particle size analyser. Analysis of particles size distributions were carried out on dry powder of microplastics.

Aged microplastics were inspected under light microscope (Zeiss Option, Axioskop, West Germany, camera Leica DFC290 HD) to observe the biofilm formation and adsorption of other inorganic and organic material from the waters.

2.2 Aging of microplastics

Incubation of microplastics in water samples was carried out in 250 mL Erlenmeyer flasks. 150 ml of each water sample was mixed with 150 mg of microplastics resulting in a microplastics concentration of 1000 mg/L. Eight Erlenmeyer flasks per water sample were prepared. Flasks were shaken throughout the duration of incubation, which took place for 3 weeks at room temperature (23 ± 2 °C) under daylight fluorescent lamps with the photoperiod 16/8h (light/dark). At the beginning of each week the water sample with microplastics was filtered (0.45 µm pores; HAWG047S6 Merck) and obtained microplastics were added to a new fresh sample of water. After 3 weeks of incubation microplastics were filtered (Millipore membrane filter, 0.45 µm pore size), grouped from all 8 flasks and stored at -20°C for further analysis.

2.3 Sampling and characterisation of water samples

Microplastics were incubated in water samples from four different sources: at the spring of river Ljubljanica (River source), river Ljubljanica in the Ljubljana city centre (River city), effluent from a municipal wastewater treatment plant (WWTP effluent) and landfill leachate from a regional municipal landfill site (Landfill leachate) (**Table 1**). Spring and river water were sampled by a grab sampling method, treated wastewater was collected by an automatic water sampler as a 24-h combined sample and leachate was sampled from a collection basin by a grab sampling method. Sampling occurred on three successive weeks. Each time a fresh water sample was added to microplastics and the physico-chemical analysis were done on fresh samples. A part of each sample was stored at -20 ± 2 °C for the toxicity analysis.

Table 1: Water samples used in the study.

Sample ID	Sample description	Dates of sampling (all 2017)
River source	Source of river Ljubljanica (Vrhnika; 45°57'16.8''S 14°17'33.4''V; Slovenia)	6.11., 13.11., 20.11.
River city	River Ljubljanica city centre (Prule; 46°02'31.1''S 14°30'29.6''V; Slovenia)	6.11., 13.11., 20.11.
WWTP effluent	Effluent from a municipal wastewater treatment plant (Slovenia)*	29.11., 6.12., 13.12.
Landfill leachate	Landfill leachate from a regional municipal landfill site (Slovenia)*	29.11., 6.12., 13.12.

*exact location cannot be revealed.

2.3.1 Physico-chemical analysis

Each water sample was analysed for concentration of ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$) and nitrite ($N\text{-}NO_2^-$) nitrogen, orthophosphates ($P\text{-}PO_4^{3-}$), chlorides (Cl^-), total organic carbon (TOC, TOC 5000A, Shimadzu) and a number of microorganisms expressed as colony-forming units (CFU, the spread plate method). Each analysis was performed in duplicates or triplicates and standard deviation (SD) was calculated. The samples were not filtered prior to analysis. All measurements were validated by using reference materials and performed according to established protocols (APHA, 2012).

2.3.2 Aquatic toxicity

In addition to chemical analysis, we also checked the toxicity of water samples for water flea *Daphnia magna*, zebrafish *Danio rerio* embryos and duckweed *Lemna minor*. We did not test each of the 3 sub-samples from certain water type (sampled in three successive weeks) but only a combined sample. This was because microplastics were actually incubated in all three samples during the three weeks of incubation. The combined sample was prepared as a joint mixture of the three sub-samples in volume ratio = 1:1:1. An additional reason for testing a combined sample was that it was not feasible to test each of the three sub-samples because all chemical analysis and replacement of new water in incubation flasks needed to be done on fresh samples. Toxicity testing was done in exactly the same way as described for microplastics (Chapter 2.4). Samples River source, River city, and WWTP effluent were tested only at 100% (v/v), since no effect was found. A range of test concentrations was tested for the landfill leachate in zebrafish and duckweed (**Table S1, Fig S3**).

2.4 Microplastics toxicity testing

We tested the toxicity of primary microplastics isolated from the facial scrub (hereafter as MP), and microplastics incubated in the four water samples, hereafter named as MP+river source, MP+river city, MP+WWTP, and MP+landfill. In each of the toxicity tests, the test concentration was 100 mg/L. It was chosen based on recent studies by Jemec Kokalj et al. (2016; 2018), where the authors found no effects up to this high concentration testing similar microplastics from facial scrub. Also, Jemec Kokalj et al. (2018) described in detail a technical challenge with MP toxicity testing because it is difficult to prepare suspensions with lower test concentrations in a reliable manner. In all cases, microplastics samples were weighted, added to the test medium and stirred on a magnetic stirrer without additional dispersion protocol. Care was taken to avoid absorption of microplastics onto

pipette tips and magnet during stirring. Each of the following tests included at least 3 replicates of the same exposure concentration and each experiment was repeated at least twice.

Toxicity tests with *D. magna* were carried out according to ISO 6341:2012 using Daphtoxkit F™ magna and as described in Jemec Kokalj et al. (2018). Daphnids were fed with algae (*Desmodesmus subspicatus*) prior to microplastics exposure for 1.5 h. 24 h old daphnids were exposed for 48 h at 21 ± 1 °C under a 16 : 8 h light/dark regime. Controls containing only ISO 6341:2012 test medium were included in all experiments. Daphnids were exposed in 6-well microplates (TPP®, Switzerland). Each exposure included 4 replicates with 5 daphnids. Hence, altogether 20 daphnids per experimental group and control were exposed. A positive control with the reference chemical potassium dichromate was also tested and the results were in line with ISO 6341:2012. After 48 h of exposure daphnids were inspected for immobility and their mortality was confirmed by the absence of heartbeat.

Toxicity test with *D. rerio* embryos was carried out according to Jemec et al. (2012). Adult zebrafish were bred in a temperature-controlled room in aquarium (60 × 30 × 30 cm) containing 45 L of dechlorinated tap water with constant temperature (22 °C) and controlled photoperiod conditions (12 h light : 12 h dark). A day before breeding a plastic spawning box covered with stainless steel mesh was placed in the breeding tank. On the following day, one hour after the light cycle started, the spawning plastic box was removed from the tank and eggs were collected. Two eggs in developmental stage between 4-128 cells (ISO 15088:2007) were placed into test well with 1 mL of test medium (24–well microplate; TPP®, Switzerland). At each exposure (microplastics and control) 10 replicates (wells) containing 2 eggs were prepared. Therefore, in each test, 20 eggs per control containing only ISO medium (ISO 15088:2007) and 20 eggs per each microplastic sample were exposed. A positive control with the reference chemical 3,4-dichloroaniline was also tested and the results were in accordance with ISO 15088:2007. The embryos and hatching of embryos were followed until 96 h post fertilisation. Mortality and developmental malformations were checked using a stereoscopic microscope (Leica MZ FLIII, Germany).

The tests with *L. minor* were done according to Kalčikova et al (2017). Duckweed has been continuously cultivated in Steinberg medium (ISO 20079: 2005) under controlled conditions (temperature 23 ± 2 °C, photoperiod 16/8 h). For each experiment 100 mL glass beakers were used and each beaker contained 50 mL of Steinberg medium with 100 mg/L of various microplastics and 10 initial duckweed fronds. Controls were always included and contained only Steinberg medium. All experiments were performed in a climate test chamber at temperature 24 ± 2 °C and high humidity (>70%). All treatments were illuminated by daylight fluorescent lamps with the photoperiod 16/8h (light/dark) at a light intensity 6358±1077 lux (mean ± SD, n=10) at the plant level. Each experiment

proceeded for seven days and at the end of the experiment the number of fronds was counted. Afterwards, the duckweed was gently collected and the root length was measured.

2.5 Data analysis

The average specific *L. minor* leaf growth rate for the period of seven days was calculated according to ISO 20079 (2005) as follows: $\mu=(ln(Nj)-ln(Ni))/t;$ where μ (d^{-1}) is the average specific growth rate, Nj (/) is the number of fronds at the end of the experiment, Ni (/) is the number of fronds at the beginning of the experiment and t (d) is a period of time (seven days). The statistical significances of the differences between the control and exposed groups were assessed by the Kruskal–Wallis one-way analysis of variance followed by Mann-Whitney U-test, where differences were considered significant if $p < 0.05$ using the OriginPro 8.0 software (OriginLab Corp., Northampton, MA, USA). 48 EC50 was calculated with Regtox_EV 7.0.7. Macro in Microsoft Excel.

3 RESULTS

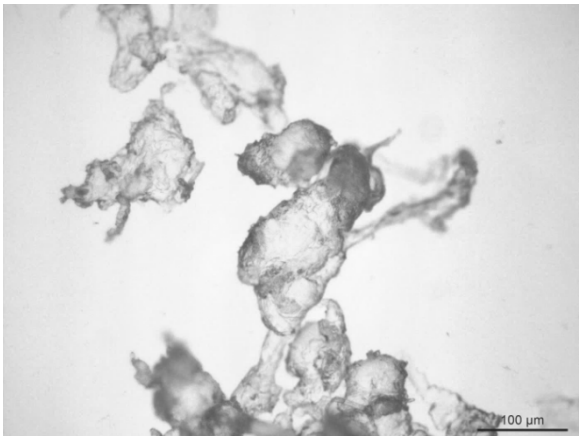
3.1 Characteristics of microplastics

Microplastics extracted from a cosmetic product (primary microplastics) were in a form of fine, white powder. Microplastic particles were of irregular shape with sharp edges (**Figure S1**). According to particle size distribution the majority of particles were rather small (a mean value 140.6 ± 80.0 μm) and their size was maximally up to 1000 μm.

Microplastics after three weeks of aging in various waters and wastewaters were inspected under light microscope to check whether there is evident coating formation on the surface. We observed the attachment of some organic/inorganic material and possibly microorganisms on microplastics which was the most evident in the case of MP+landfill, followed by MP+river city, MP+river source and MP+WWTP, respectively. However, we did not observe any evident growth of green algal biofilm on the micropalstics (**Figure 1A-E**).

Images of microplastics in ISO 6341:2012 test medium for *D. magna* and *D. rerio* test were taken immediately after the preparation of the suspension and after 24h. As inspected by naked eye evident differences in microplastics distribution in test medium were seen already after preparation: primary MP and MP+WWTP were mostly floating on the surface of the medium; MP+river source and MP+river city were distributed vertically throughout the test medium column, while MP+landfill has mostly sunk to the bottom of the vessel. The distribution was not evidently different after 24h (**Figure 1F).** More detailed images are available in **Figure S2**.

A)



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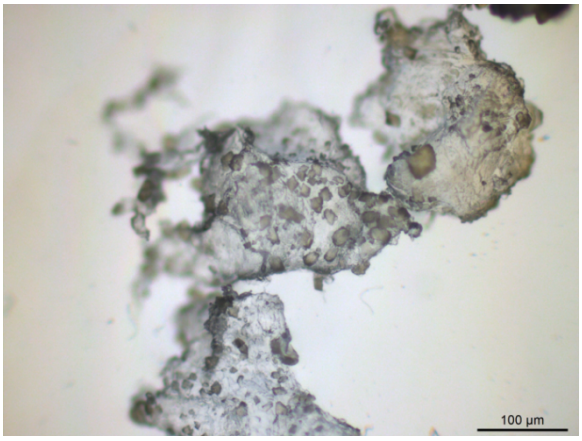

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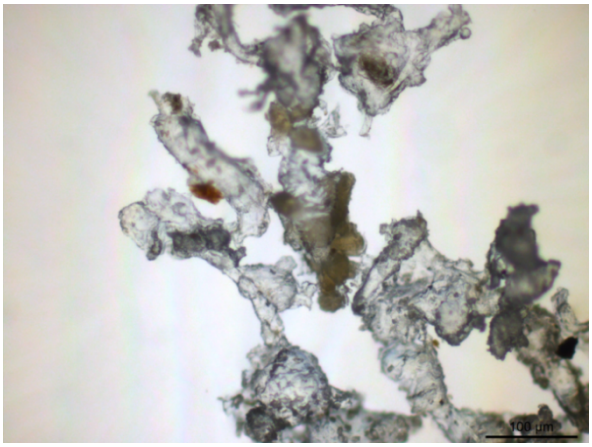

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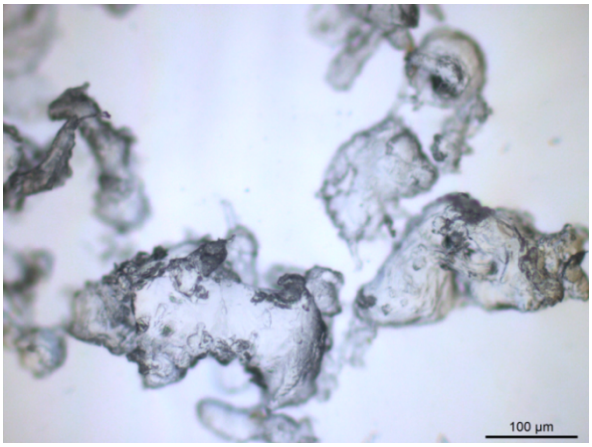

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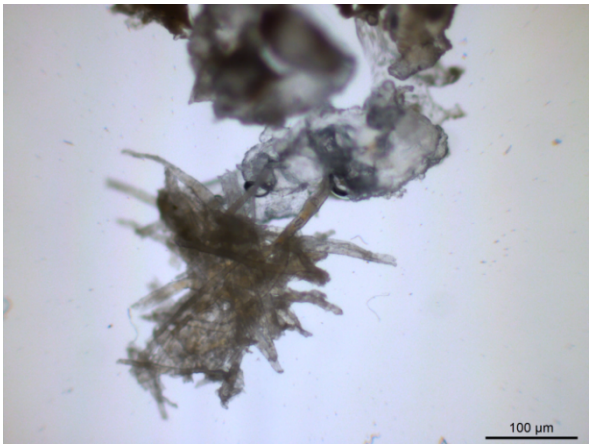


F)

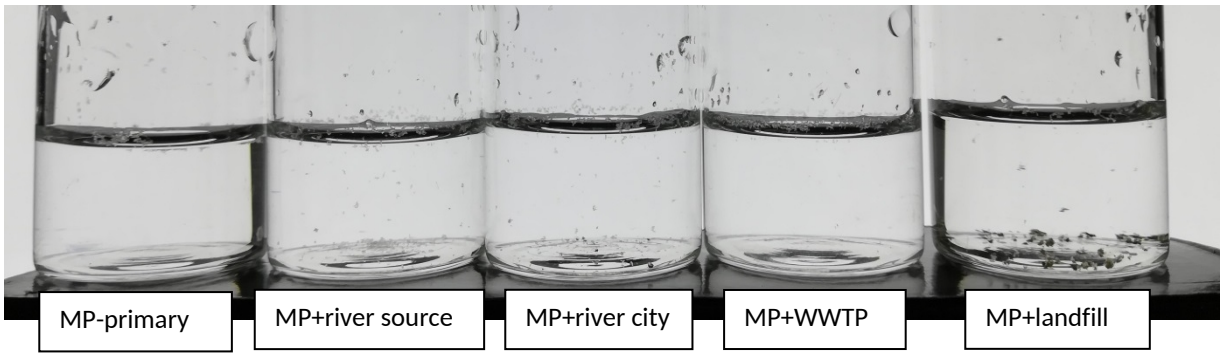


Figure 1. Microplastics inspected under light microscope: (A) primary microplastics, (B) microplastics incubated in water from the source of river Ljubljanica (MP+river source), (C) microplastics incubated in water from river Ljubljanica in the Ljubljana centre (MP+river city), (D) microplastics incubated in effluent from a municipal WWTP (MP+WWTP), (E) microplastics incubated in landfill leachate (MP+landfill). **Distribution of microplastics in ISO 6341:2012 test medium** (F).

3.2 Characteristics of water samples

Results of waters and wastewaters characteristics are given in **Table 2.** Each type of water and wastewater was sampled three times within a 3-week period. Water samples (River source and River water) did not show a high variability among sampling dates, while wastewater samples (WWTP effluent and landfill leachate) did. It was caused by dilution of wastewaters due to a heavy rain fall a few days before the last sampling event (13.12.2017, **Table 1**). As expected the River source samples expressed the lowest level of water contamination due to the remote location of the spring. All physico-chemical parameters increased in the River city samples, however they were not typical for urban areas but rather comparable to low affected rivers in other parts of Slovenia (Zidar et al., 2018). WWTP effluent samples contained high concentrations of $N\text{-}NH_4^+$, which signalizes low nitrification rate within the WWTP (Siripong and Rittmann, 2007). Landfill leachate samples were the most loaded by organic and inorganic components; high concentrations of $N\text{-}NH_4^+$ and lower TOC contents indicating that landfill where leachates were sampled is already in the stabilized phase (Kalčikova et al., 2016). Despite the variability in chemical composition of waters and wastewaters, all samples contained high concentration of viable microorganisms **(Table 2**).

River source and river city centre samples were not acutely toxic for *D. magna*, *L. minor* and *D. rerio*. Effluent from WWTP also did not have any effect on *D. magna* and *L. minor* but significantly decreased the hatching of *D. rerio* embryos after 96 h at 100 % (v/v). Nevertheless, no effect on zebrafish was found at concentrations ≤ 75% (v/v) WWTP effluent. The sample from landfill leachate

was toxic to all three organisms; the highest effect was found in *D. rerio* embryo 96h test followed by *L. minor* and *D. magna* **(Table 2; Table S1, Figure S3**).

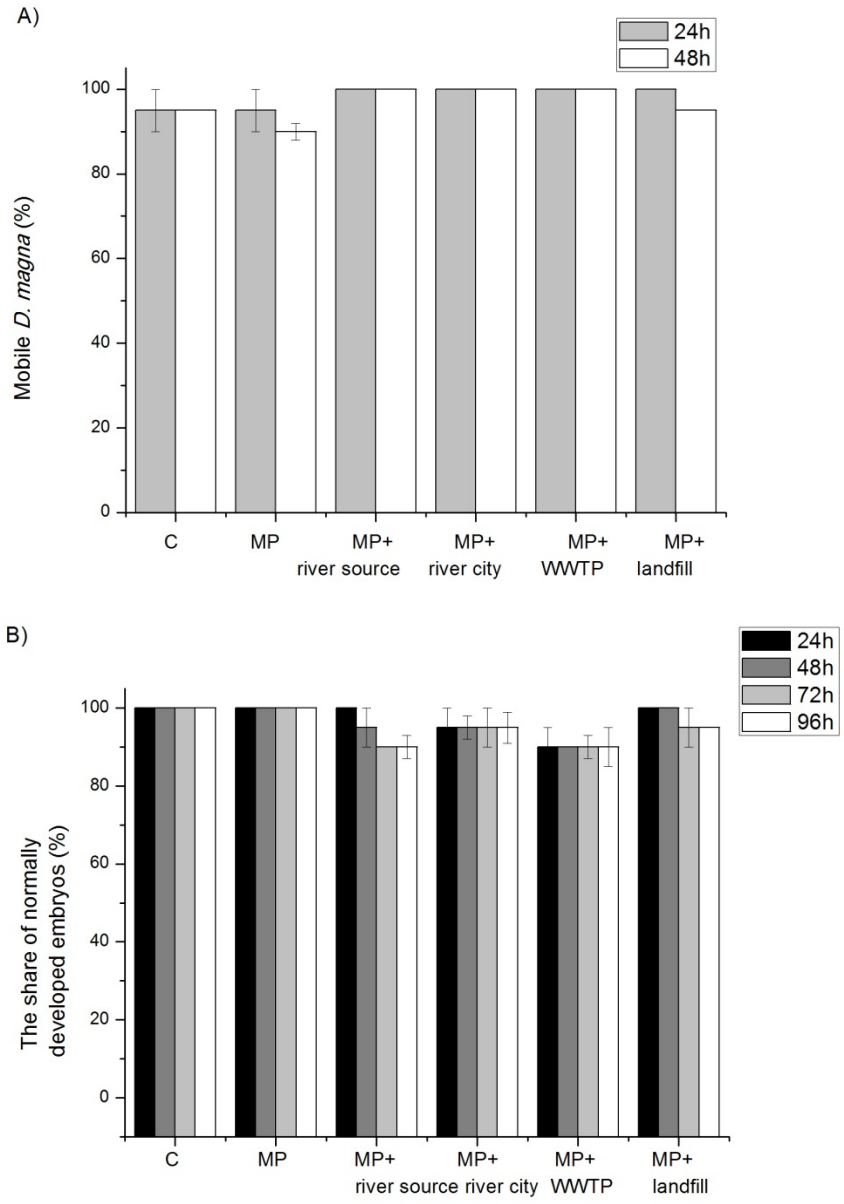
Table 2. Analysis of water and wastewater samples. Each water/wastewater type was sampled on three different occasions within a 3-week period, results are expressed as an average of all three samplings with standard deviations (mean ± SD). For each of the sample chemical properties ($N\text{-}NH_4^+$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, Cl^-, TOC), number of microorganism (MO) expressed as colony forming units (CFU), and toxicity for *Daphnia magna*, *Danio rerio* and *Lemna minor* are given.

Measured parameters								
mg/L]	$N\text{-}NO_2^-$ [mg/L]	$N\text{-}NO_3^-$ [mg/L]	$P\text{-}PO_4^-$ [mg/L]	Cl^- [mg/L]	TOC [mg/L]	MO [CFU]	48h toxicity *D. magna*	168h toxicity *L. minor* (root length)
0.01	< 0.01	1.0 ± 0.1	0.01 ± 0.00	4.4 ± 0.8	< 1	1066 ± 1006	No effect[c]	No effect[c]
0.1	0.02 ± 0.02	1.2 ± 0.3	0.06 ± 0.05	5.12± 0.7	11 ± 1.5	2190[a]	No effect[c]	No effect[c]
24	0.8 ± 1.0	9.8 ± 1.1	1.1 ± 0.6	108.3 ± 31.2	7.4 ± 1.8	4767 ± 2406	No effect[c]	No effect[c]
246	0.1 ± 0.1	16.4 ± 6.6	1.7± 0.6	583.3 ± 235.7	194.8 ± 77.4	99 489 ± 123 043	48h EC50 = 12.57% [b]	168h EC50 < 12.5%[e]

[a]parameter determined only once; [b]95% confidence interval (10.30-13.00%); [c]at 100% (v/v); [d]no effect ≤ 75% v/v ; [e]100% inhibition of root length ≥12.5% (v/v)

3.3 *Effects of primary and aged microplastics on organisms*

Primary microplastics had no acute effects on the development of *D. rerio* as well as mobility of *D. magna*. Only up to 10 % inhibition of *D. magna* mobility was found but this cannot be considered as significant since such inhibition is considered valid for controls (ISO 6341:2012). Contrary to our expectations, also no effects were found when these two organisms were exposed to 100 mg/L of all aged microplastics in different types of waters (maximum 10% inhibition in the case of *D. rerio*) (**Figure 2**).



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277

278 **Figure 2. The effects of microplastics on *Daphnia magna* (A) and *Danio rerio* (B).** Microplastics were
279 aged in: water from the source of river Ljubljanica (MP+river source), water from river Ljubljanica in
280 the Ljubljana centre (MP+river city), effluent from municipal waste water treatment plant
281 (MP+WWTP), and landfill leachate waste water (MP+landfill). MP - primary microplastics. Mean
282 values and standard errors are shown.

283

284 Specific growth rate of *L. minor* was not statistically affected in any of the treatments in comparison
285 to control. A slight not significant trend of growth inhibition by 7%, 12% and 7% in the case of
286 MP+river city; MP+WWTP and MP+landfill, respectively was observed (**Figure 3A**). On the other
287 hand, primary microplastics significantly affected the root length of *L. minor* by 18% in comparison to

control. Similar significant root length inhibition was observed for MP+WWTP and MP+landfill (16% and 19%, respectively) while MP+river source and MP+river city caused only 4% and 12% non-significant inhibition, respectively (**Figure 3B)**.

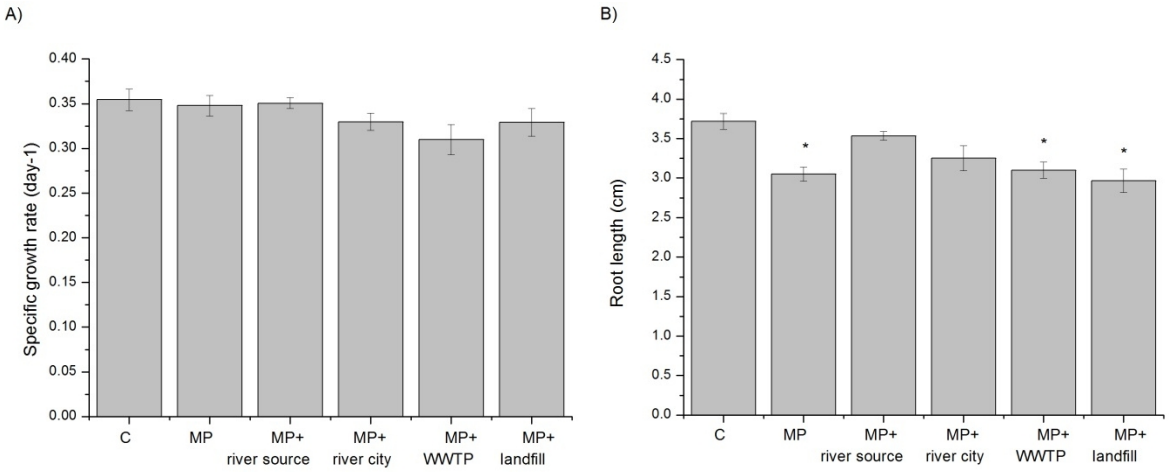

Figure 3: The effects of microplastics on *Lemna minor* specific growth rate (A) and root length (B). Microplastics were aged in: water from the source of river Ljubljanica (MP+river source), water from river Ljubljanica in the Ljubljana centre (MP+river city), effluent from municipal waste water treatment plant (MP+WWTP), and landfill leachate waste water (MP+landfill). Mean values and standard errors are shown. MP - primary microplastics.

4 DISCUSSION

This study aimed to investigate whether 3-weeks incubation of polyethylene microplastics in waters with different organic load and toxic potential alters the aquatic toxicity of microplastics. Contrary to our expectations, aging of microplastics in wastewaters with high organic load and evident aquatic toxicity (WWTP effluent, landfill leachate) did not increase the toxicity of microplastics for *D. magna*, *D. rerio and L. minor*. This suggests that either adsorption of pollutants from wastewaters to microplastics was too low and/or bioavailability of adsorbed pollutants to organisms was not high enough to induce acute toxicity to organisms. Similarly, Batel et al (2018) have shown that exposure to polyethylene microplastics (1-5 and 10-20 µm) coated with benzo[a]pyrene had no teratogenic effects on zebrafish embryos. These findings are in line with recent evaluations that the relative contribution of pollutants derived from ingested microplastics is not likely to increase the exposure to pollutants in comparison to other more significant sources, like contaminated prey or contaminated environment (Koelmans et al., 2016; Burns and Boxall, 2018). However, some studies did show that a combination of microplastics and chemical contaminants can

increase the adverse effects produced by plastic or contaminants individually (Rainieri et al., 2018; Luis et al., 2015). It is possible that longer incubation of microplastics in waters would result in toxic effects. We choose 3 weeks of incubation as an arbitrary period previously selected for aging of microplastics (Vroom et al. 2017). Although this period seems short it is actually rarely the case that microplastics in natural environment would be exposed to the same type of water for such a period due to transport of microplastics along the water current. Additionally, the WWTP effluent and landfill leachate are diluted under real environmental conditions upon release to the environment, while concentrated samples were used in this study. Therefore, the exposure scenario in this study could actually be considered as a worse-case scenario for a certain type of water.

Primary microplastics significantly decreased the root length of *L. minor*. Similar finding was reported by Kalčikova et al. (2017) where polyethylene microplastics extracted from facial scrubs (>10 mg/L) decreased the root lengths of *L. minor*, but the number of roots, the specific growth rate and leaf photosynthetic pigments (chlorophyll a and b) were not affected. These authors suggested that the effect on root growth may be on the account of physical action, since higher effect was induced by sharp edges polyethylene microplastics in comparison to smooth surface ones. Also attachment of microplastics to roots was evidenced. The contribution of potential chemicals leached from primary microplastics to *L. minor* toxicity was ruled out (Kalčikova et al., 2017). Interestingly, the aging of microplastics in low organic-load waters decreased the toxicity of microplastics for *L. minor* root length. We could speculate that coating formed on the MP+river source and MP+river city microplastics mitigates physical effects resulting in lower root length inhibition This is also supported by observed effect of MP+WWTP since these microplastics were the least coated with material resembling primary microplastics,(**Figure 1**). However, this does not explain observed toxicity for roots in the case of MP+landfill leachate which was highly coated. This implies that the contribution of coating to toxicity of reduction needs to be further investigated. Further studies with leaches from these aged microplastics could confirm the reason for observed toxicity of MP+landfill. Nevertheless, toxicity of MP+landfill leachate was not higher as primary MP indicating that aging does not significantly increase the toxicity of microplastics for *L. minor*.

There are only few other studies in the literature dealing with microplastics effects on aquatic plants. Weert et al. (2019) reported that very high concentrations of polystyrene microplastics (20–500 µm, up to 10% dry weight of sediment) affected the main shoot length of *Myriophyllum spicatum*. Some other growth parameters (relative growth rate; shoot to root ratio) were also affected in *M. spicatum* and *Elodea sp.* but they were not dose-dependent (Weert et al., 2019). These data suggest that microplastics have the potential to affect aquatic plants suggesting a need of further studies in this field.

Primary polyethylene microplastics did not affect *D. magna*. Similarly, we previously found no acute effects of polyethylene microplastics extracted from other brands of facial scrubs (roughly the same size range and shape as in this study) on *D. magna* (Jemec Kokalj et al., 2018). Also Rehse et al. (2016) found no acute effects to *D. magna* after exposure to 100 µm polyethylene microplastics (400 mg/L) and 2-5 µm polyethylene microplastics had no effect on the reproduction of *D. magna* (Ogonowski et al., 2016). However, *D. magna* immobility was affected in the case of 1 µm polyethylene particles (72 h; 200 mg/L) (Rehse et al., 2016).

Very few studies have addressed the effects of microplastics on *D. rerio* embryos (Batel et al., 2018), while the majority of studies have investigated the effects on their larvae (Sleight et al, 2017; Karami et al., 2017) and adults (Lu et al., 2016; Khan et al., 2015; Lei et al., 2018; Rainieri et al., 2018; Chen et al., 2017). Larvae and adults can ingest microplastics, but embryos are protected by a chorion which is permeated by chorionic pores (diameter 0.5-0.7 µm) potentially allowing the passage of only small nanoplastics (Lee et al., 2007; Pitt et al., 2018). It has been shown that after hatching, very few particles (10–20 µm polyethylene) were found on the zebrafish larval bodies and some occasional single plastic particles were visible inside the gastrointestinal tract but the exposure did not result in teratogenic effects (Batel et al 2018). Microplastics on the other hand can attach to the chorion (Batel et al., 2018) and potentially induce physical damage of the chorion. We expected that aged microplastics would release adsorbed toxic chemicals which would pass the chorion and affect the embryos, as was the case when we exposed embryos to landfill leachate and WWTP effluent alone. However, this was not the case. Also, we did not observe any microplastics attachment onto zebrafish chorion.

We observed major differences in water column distribution between primary and MP+river source, MP+river city and MP+ landfill . As based on naked eye observations primary microplastics were retained mostly on the surface of the test medium, MP+river source and MP+river city were distributed vertically throughout the test medium column, while MP+ landfill has immediately sunk to the bottom of the vessel. An exception was MP+WWTP which were also retained on the surface as primary microplastics (**Figure 1; Figure S2**). This distribution corresponds to the extent of attachment of organic material on microplastics the highest being for MP+landfill, followed by MP+river city, MP+river source and MP+WWTP which were the least coated (**Figure 1**). This implies that organisms were exposed to primary and aged microplastics in different extent, but this did not significantly affect the outcome of the toxicity test. For example, zebrafish embryos had the largest contact with MP+landfill on the bottom of the test vessel but no effect on their development was found. Daphnids swim along the vertical column of the test vessel and were thus the most exposed to MP+river source and MP+river city, but these daphnids were not affected more as in the case of other MPs. We did not inspect the guts of daphnids for the presence of microplastics but our previous study

using the same type of microplastics and the same exposure set-up has shown that daphnids ingest microplastics of similar size range (Jemec Kokalj et al., 2018) as well as larger plastic fibres (Jemec Kokalj et al., 2016). Duckweeds on the other hand float on the water surface thus being in the highest contact with primary microplastics and MP-WWTP. These two MPs affected the root growth, but so did MP+landfill which had less direct contact with duckweed. However, in this latter case contributing effect of leaching from MP+landfill cannot be ruled out as explained above.

This study was focused on irregularly shaped polyethylene microplastics that were extracted from facial scrub. It has been estimated that these cosmetic products are likely to be an important source of microplastics (commonly called microbeads) contamination, due to the quantity of plastic used in cosmetic products (Napper et al., 2015). The use of microbeads in wash-off cosmetic products is now already banned in some countries, like USA and UK (Microbeads-Free Waters Act of 2015; USA and The Environmental Protection (Microbeads) (England) Regulations 2017). In January 2018, ECHA announced examining the need for an EU-wide restriction on the placing on the market or use of 'intentionally-added' microplastics in products. Despite these efforts it is a fact that microbeads have been used for a long time (Leslie, 2015) and most probably are still used in many countries. Besides microbeads a variety of other morphology and polymer composition types of microplastics enter the environment. It is known that physical and chemical properties of materials, such as topography, roughness and hydrophobicity greatly influence the biofilm attachment (Renner and Weibel; Nauendorf et al., 2016) as well as sorption of pollutants (Hüffer et al., 2018). It is therefore possible that other types/shapes of microplastics aged in water would result in different effect as described in the current study. Environmental transformation of microplastics and the resulting fate and effects therefore remain a point of interest.

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CONFLICT OF INTEREST STATEMENT

An exploratory ecotoxicity study of primary microplastics versus aged in natural waters and wastewaters

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The manuscript has not been previously published, in whole or in part, and that it is not under consideration by any other journal. All authors are aware of, and accept responsibility for, the manuscript. Authors have no competing interests to declare.

SUPPLEMENTARY INFORMATION

An exploratory ecotoxicity study of primary microplastics versus aged in natural waters and wastewaters

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Figure S1

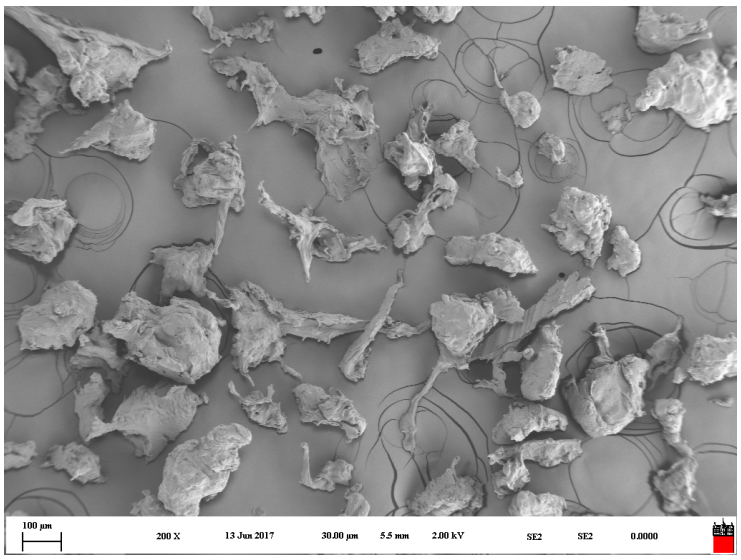

Figure S1: Scanning electron microscopy image of microplastics.

Figure S2

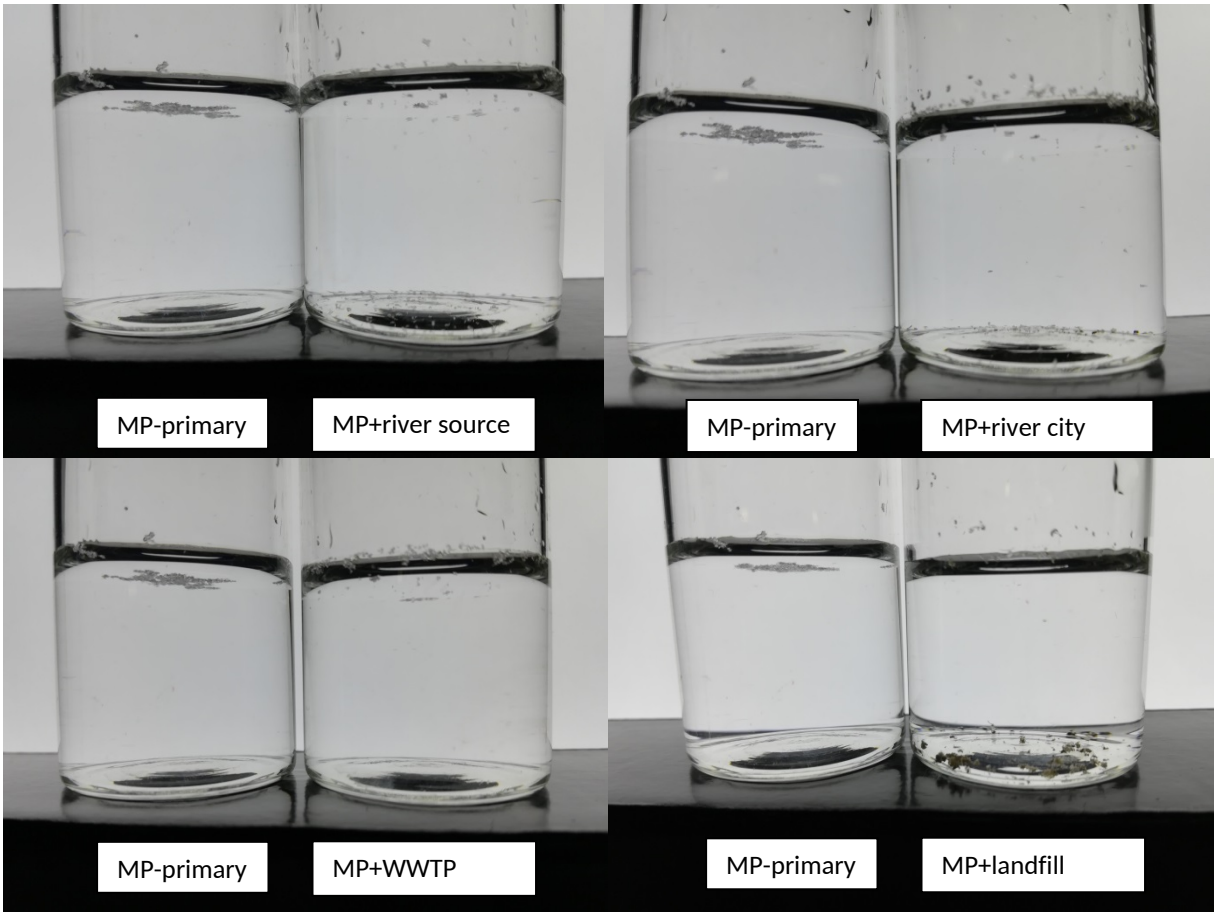

Figure S2. Distribution of microplastics in ISO 6341:2012 test medium (*Daphnia magna and Danio rerio* test**).** Microplastics were aged in: water from the source of river Ljubljanica (MP+river source), water from river Ljubljanica in the Ljubljana centre (MP+river city), effluent from municipal waste water treatment plant (MP+WWTP), and landfill leachate waste water (MP+landfill). Evident difference in microplastics distribution in test medium is seen: primary MP is floating on the surface of the medium; MP-river source and MP-river city are distributed vertically throughout the test medium column, MP-WWTP is floating on the surface of the test medium, while MP- landfill leachate has sunk to the bottom of the vessel.

Figure S3

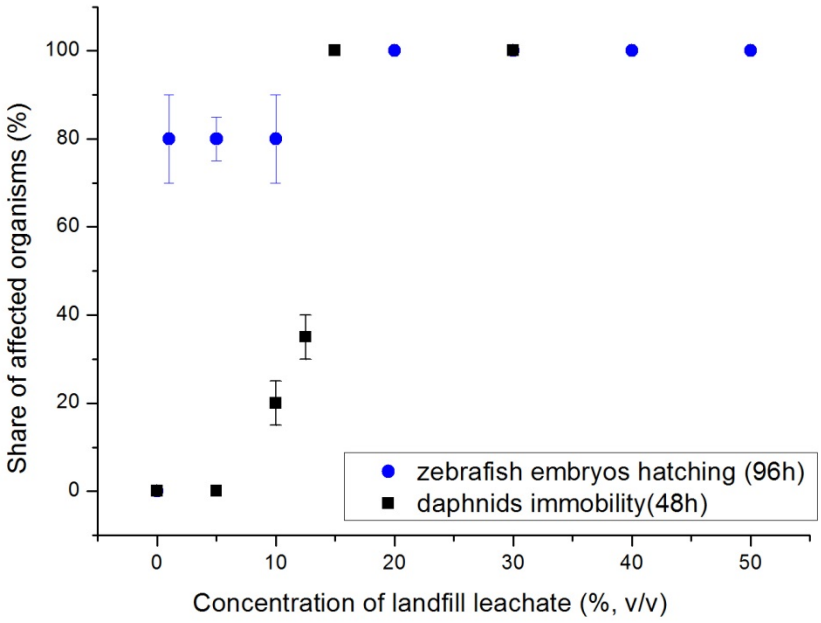

Figure S3. Share of **i**mmobile daphnids and deformed zebrafish embryos exposed to municipal landfill leachate for 48h and 96h, respectively. Mean values ± SE are shown.

Table S1. The effect of water and wastewater samples on *Lemna minor* specific growth and root length after 7 days exposure. River source, River city and WWTP effluent were tested at 100% v/v, municipal landfill leachate was tested at: 12.5; 25; 50 and 100%.

	Control	River source	River city	WWTP effluent	Landfill leachate
Specific growth rate (d^{-1})					
Average ± SE	0.319 ± 0.0053	0.246 ± 0.00988	0.244 ± 0.020189	0.347 ± 0.0064	0
Inhibition (%)	/	22.9	23.9	0	100%[c]
Root lenght (cm)					
Average ± SE	3.4367 ± 0.2167	3.067 ± 0.034	3.27667 ± 0.034	3.36 ± 0.0988	0
Inhibition (%)	/	< 10	< 10	0	100%[c]

[c] ≥ 12.5% v/v