Virgin and UV-weathered polyamide microplastics posed no effect on the survival and reproduction of *Daphnia magna*?

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Although evidence suggests that microplastic (MP) particles pose a risk to organisms, the effects of virgin and weathered MP should be evaluated separately as their effects may be different. In this work, we provide new information on the toxic potential of virgin and UV-weathered polyamide, one of the commonly used plastics worldwide. Polyamide MP particles were subjected to UV-weathering in wet conditions during 26 days in a customized irradiation chamber equipped with UV-C light tubes (15 W each, max. wavelength 254 nm). The toxicity of virgin and UV-weathered polyamide MP (<180 µm in one dimension, 100 and 300 mg L^{-1}) was evaluated by studying *Daphnia magna* reproduction in natural lake water spiked with MP, following the 21-day OECD 211 test guideline. In parallel, a nonionic surfactant Tween 20 (7 mg L^{-1}) was added to the test medium to improve the suspendability of the MP. The results of the tests showed no adverse effects of either virgin or UV-weathered polyamide MP on the reproduction of *D. magna*.

In addition, presence of Tween 20 in the test medium had no effects on the test results. These results bring a new perspective on the potential long-term impact of polyamide particles on aquatic organisms, especially considering that the polyamide has received marginal attention in the ecotoxicological research. However, standard test endpoints (survival and reproduction) may still miss long-term adverse effects of insoluble e.g., plastic particles and additional studies may be necessary.

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10 Abstract

12 Although evidence suggests that microplastic (MP) particles pose a risk to organisms, the effects

13 of virgin and weathered MP should be evaluated separately as their effects may be different. In

14 this work, we provide new information on the toxic potential of virgin and UV-weathered

15 polyamide, one of the commonly used plastics worldwide. Polyamide MP particles were

16 subjected to UV-weathering in wet conditions during 26 days in a customized irradiation

17 chamber equipped with UV-C light tubes (15 W each, max. wavelength 254 nm). The toxicity of

18 virgin and UV-weathered polyamide MP (<180 μm in one dimension, 100 and 300 mg L^{-1}) was

19 evaluated by studying *Daphnia magna* reproduction in natural lake water spiked with MP,

20 following the 21-day OECD 211 test guideline. In parallel, a nonionic surfactant Tween 20 (7

21 mg L^{-1}) was added to the test medium to improve the suspendability of the MP. The results of the

22 tests showed no adverse effects of either virgin or UV-weathered polyamide MP on the

23 reproduction of *D. magna*.

24 In addition, presence of Tween 20 in the test medium had no effects on the test results. These

25 results bring a new perspective on the potential long-term impact of polyamide particles on

26 aquatic organisms, especially considering that the polyamide has received marginal attention in

27 the ecotoxicological research. However, standard test endpoints (survival and reproduction) may

28 still miss long-term adverse effects of insoluble e.g., plastic particles and additional studies may

29 be necessary.

30 Keywords: nylon, polymer, solar radiation, UV-irradiation, natural water, water flea, degradation

31 Introduction

32   Recent ecotoxicological studies on the effects of microplastics (plastic particles < 5mm in

33 one dimension, MP) in aquatic systems have included more freshwater organisms whereas

34 previously marine systems/organisms were the primary focus (Castaneda et al., 2014; Hurley,

35 Woodward & Rothwell, 2018; Khosrovyan & Kahru, 2020; Khosrovyan & Kahru, 2021; Nel,

36 Dalu & Wasserman, 2018; Silva et al., 2021; Wang et al., 2020). Indeed, increased impact of

37 plastic pollution in freshwater systems has been reported in rivers and lakes worldwide (Baztan

38 et al., 2014; Klein, Worch & Knepper, 2015). However, many knowledge gaps still exist. For

39 example, not all widely-used plastic types have been investigated for their potential adverse

40 effects: the available ecotoxicological data is biased towards particular MP types e.g.,

41 polyethylene (PE) ~ 7%, polystyrene (PS) ~ 2%, polypropylene (PP) and polyvinyl chloride

42 (PVC) ~ 1% and polyamide (PA) ~ 0.5% (data from the Web-of-Knowledge topic search

43 performed by the authors using *plastic* (or a particular type of it), test*, toxic*). In addition,

44 research on the potential toxicity of UV-weathered plastics and their leachates is also inadequate,

45 even though photo-oxidation by UV light is the primary cause of plastic degradation. A recent

46 search on the Web-of-Knowledge indicated that fewer than 2 % of the studies on plastics toxicity

47 considered UV-weathered plastics. Photooxidation of the plastics surface following UV

48 irradiation results in the polymer breakdown and embrittlement causing further fragmentation of

49 the plastics by lower energy levels (Huffer, Weniger & Hofmann, 2018) and surface

50 functionalization fostering absorption of not only hydrophobic but also hydrophilic organic

51 pollutants (Liu et al., 2019). UV-weathered PVC beads (50-100 μm) were more toxic to the

52 freshwater algae *Chlamydomonas reinhardtii* than virgin PVC beads, due to the formation of

53 carbonyl groups on the surface of the beads (Wang et al., 2020). Leachate from UV-irradiated

54 plastics of various types released plasticisers at concentrations comparable to those measured in

55 the riverine water in the United States and caused significant vitellogenin induction in Japanese

56 medaka *Oryzias latipes* larvae compared to virgin plastic leachate counterparts (Coffin et al.,

57 2018). Khosrovyan & Kahru (2021) reported significant reduction in the emergence of midges

58 *Chironomus riparius* exposed to UV-weathered polyamide particles compared to virgin particles.

59   Further, the impact of ingested MP particles is not easily predictable. For example,

60 despite a greater number of ingested polyamide MPs at higher concentration (1000 mg kg^{-1} of

61 dry sediment) compared to lower concentration (100 mg kg^{-1} of dry sediment), the number of

62 emerged *C. riparius* adults in both treatments was similar, presumably due to the successful

63 excretion of the particles from their gastrointestinal tract (Khosrovyan, Gabrielyan & Kahru,

64 2020). Localized leaching of monomers and additives in the gastro-intestinal system of

65 organisms may also be responsible for the chemical toxicity of ingested particles (Wright &

66 Kelly, 2017). Several studies have demonstrated that the leachate obtained after 24 h of contact

67 with virgin polyethylene pellets or different plastic products with seawater was toxic to aquatic

68 organisms such as barnacle (*Amphibalanus amphitrite*) nauplii (Li et al., 2016), brown mussel

69 (*Perna perna*) larvae (Gandara e Silva et al., 2016) and sea urchin (*Lytechinus variegatus*) larvae

70 (Nobre et al., 2015).

71   In this study, the potential adverse effects of virgin and UV-weathered polyamide MP of

72   irregular shape and different sizes were evaluated using the OECD 211 *Daphnia magna*

73   reproduction test. *D. magna* is one of the key freshwater organisms filtering water column. Our

74   working hypotheses were as follows: (i) virgin particles are harmful to daphnids over the long-

75   term exposure, (ii) UV-weathering promotes degradation of the particles and/or leaching

76   chemicals from them, and (iii) UV-weathering of the particles augments the hazard of the

77   particles to aquatic biota. A comparison with the effects of virgin and UV-weathered polyamide

78   particles on another key freshwater/sediment organism - *C. riparius* - evaluated using the OECD

79   233 test guideline (Khosrovyan *&* Kahru, 2021) is provided.

80   Material and methods

81   Virgin and UV-weathered microplastics

82   Virgin polyamide particles (PA-MP) were from the batch described in our previous work

83   (Khosrovyan *&* Kahru, 2020; Khosrovyan *&* Kahru, 2021). Specifically, they were bought from

84   a commercial supplier (Abifor AG, Germany). They had irregular shape and size range from 0 to

85   180 μm with 88% of particles being <160 μm. Particles were fluorescent which made their

86   observation in the animal gut under fluorescent microscope possible without any modification.

87   Information on fluorescent dye and additives included in the particles was not provided by the

88   supplier.

89   UV-weathered particles (UV-PA-MP) were obtained by a process of artificially

90   aging/weathering of virgin particles (PA-MP) in a customized temperature-controlled UV-C light

91   irradiation chamber for 26 days. Measures to avoid dusting of the particles inside the chamber

92   were applied: chamber door was kept shut and the chamber was placed in a closed room.

93   Temperature in the chamber during the exposure ranged from 33-35°C. Virgin PA-MPs were

94   placed in a glass bowl filled with dechlorinated tap water and exposed to five new UV-C light

95   tubes (15 W each, max. wavelength 254 nm), mixing the particles in the bowl from time to time

96   to ensure equal water and UV light exposure conditions and thus, uniform aging. This

97   wavelength was selected based on higher electron volt energy compared to UV-A band

98   (http://www.uvresources.com/) and previous reports (Huffer et al., 2018; Liu et al., 2019;

99   Khosrovyan & Kahru, 2021). Before use of the aged particles in the experiments the particles

100   were air-dried.

101   For detecting changes on particle surfaces after UV-C irradiation, surface scans of 20

102   particles of each type (PA-MP or UV-PA-MP) were obtained using a Zeiss Confocal laser

103   scanning microscope LSM 800 (objective Plan-Apochromat 20x/0.8 M27, laser wavelength 404

104   nm, detection wavelength 400-531 nm) and images before and after UV irradiation visually

105   inspected under the microscope and compared.

106   For quantification of the increase of the share of small-sized particles as the result of UV-

107   weathering, particles of each type (virgin and UV-weathered) were placed on the slides of an

108   imaging microscope (Nikon SMZ1270) and those ranging from 10-40 μm size were counted by

109   random application of 1x1 mm frames to each slide (10 frames per type, x3 magnification).

110

111   Test organism and exposure scenarios

112

113   The water flea *D. magna* was obtained from an in-house facility originated from ephippia

114   purchased from Microbiotests, Inc. (Belgium), batch number: DM020421. The *D. magna*

115   were cultured in glass vessels at 20±1°C (16:8 h light:dark period, 1000 lux) in the medium used

116   in the assays. Daphnids were fed with algae *Raphidocelis subcapitata* suspension *ad libitum*.

117   Culturing conditions followed the OECD 211 *D. magna* reproduction test guidelines (OECD,

118   2012). On reaching maturation, several parental daphnids were separated into the mother stock

119   facility for producing offspring to be used in the experiments.

120   Two exposure scenarios in two different media were used: natural water medium (hereafter,

121   water-only) and the same medium spiked with a surfactant (hereafter, surfactant-including).

122   Natural water from Lake Ülemiste (Estonia) was provided by the water company ''Tallinna

123   Vesi''. The water was immediately passed via 0.45 μm Millipore filter and stored for a few days

124   in the dark at 4°C until used in the bioassays. The physico-chemical characterization of Lake

125   Ülemiste water is presented in Table S1. As a non-ionic surfactant, Tween 20 (Sigma-Aldrich)

126   was used.

127

128   Experiments

129   Toxicity tests were conducted according to adapted OECD 211 *D. magna* reproduction test

130   guidelines (OECD, 2012): semi-static test with water renewal every third day at a temperature of

131   20±0.5°C was performed in an Algaetron incubator (Photon Systems Instruments), photoperiod

132   16:8 h light:dark, light intensity 1000 lux, pH within the range 6 - 9. Ten newborn daphnids (<

133   24 h old, not the first brood) were placed individually into ten 50 mL glass vials (one daphnia per

134   vial, 10 replicates for the controls and treatments). Each vial contained 50 mL of exposure

135   medium: Ülemiste water or Ülemiste water spiked with a surfactant. As a surfactant, Tween 20

136   (Sigma-Aldrich) was used at 7 mg L^{-1} (0.0007% v/v). This concentration was not toxic to

137   daphnids and was selected based on the results of a duplicated 48 h acute immobilization test

138   according to OECD 202 guidelines (OECD, 2004) which revealed mortality of 1/10 daphnids in

139   1/10 replicates at 130 mg Tween L^{-1} (in the Ülemiste water). Mortality after 96 hours was 1/10

140   and 4/10 (in the test and its repetition, correspondingly).

141   The medium of each experimental vial was spiked with a nominal PA-MP or UV-PA-MP

142   concentration of 100 or 300 mg particles L^{-1}. In the water-only scenario, to each vial PA-MP or

143   UV-PA-MP particles were added at both concentrations (100 and 300 mg L^{-1}), while in the

144   surfactant-including scenario, only UV-PA-MP at 300 mg L^{-1}. This procedure was repeated at

145   each medium renewal (every third day). Because of frequent medium renewals no additional

146   measurement of exposure concentrations and comparison with the nominal concentrations was

147   performed. As a negative control, filtered natural water was used. An additional surfactant

148   control was run for the surfactant-including test at 7 mg Tween 20 L^{-1}.

149   During the first days each daphnid was fed 55 µl of the same algal suspension as in the culture

150   (*R. subcapitata*) and on reaching the adult age this volume was increased to 98 µL (such a

151   volume corresponded to the daily carbon requirement of 0.1 mg C/*Daphnia* based on optical

152   density measurement conducted daily in our laboratory). Following the guidelines, the exposure

153   medium was regularly renewed every third day during the 21 days of exposure (six times in

154   total). At renewals, daphnids were carefully transferred to new vials filled with aerated fresh

155   medium and/or polyamide particles. The test endpoints were the number of surviving parents at

156   the end of the experiment and the number of offspring per live parent.

157   Daily, animals were monitored for any unusual swimming behavior, parental mortality was

158   recorded, dead animals removed and offspring counted. pH of the medium when renewed was

159   randomly verified during the experiments.

160   The validity of the tests was verified in accordance with the OECD 211 guidelines for the

161   controls: the mortality of parental daphnids should not exceed 20% and the mean number of

162   living offspring per surviving parent should be ≥ 60 at the end of the test.

163   Statistical analysis

164   Significant differences in the reproduction between the control and treated animals (within

165   experiment) and between similar treatments from two independent experiments (between

166   experiments) were analyzed by means of one-way ANOVA ($p < 0.05$) run on R (version 4.1.3

167   (2022-03-10)) using *rstatix* library. Mean and standard deviation were calculated with MS Excel.

168   48-h and 96-h LC50s of Tween 20 dispersed in natural water (Lake Ülemiste) for *D. magna* were

169   calculated with MS Excel macro Regtox (Vindimian, 2016), commonly used in ecotoxicology

170   for estimating the dose-response relationship. The concentration-effect curves were derived

171   using five nominal exposure concentrations (7, 15, 30, 60, 130 mg Tween 20 L^{-1}) vs the

172   mortality of *Daphnia* and LC50s were calculated based on the log-normal model.

173   The number of particles of each plastic type (virgin and UV-weathered, 10-40 μm)

174   counted with the help of an imaging microscope was analyzed for mean difference by applying

175   the independent samples T-test run on R ($p < 0.05$). Normality and homogeneity of variance was

176   verified prior to the T-test.

177   Results

178   Post-aging changes in the MP particles

179   During artificial aging in the UV-C light irradiation chamber, the color of the virgin particles

180   changed from white to light yellowish/brownish (Fig. 1). The buoyancy of particles was also

181   affected: initially fluffy particles floating on the water surface sank to the bottom by the 10th day

182   of irradiation. The particles also became susceptible to breaking into small pieces when smashed

183   manually by a stone pestle: the size range of UV-weathered particles included a significantly

184   larger fraction of smaller particles (10-40 µm) in contrast to virgin PA-MP particles (means: 89

185   and 33.1, respectively; t=-5.4, p-value = 3.636e-05).

186   However, a microscopic examination of in total, 40 particles (20 per type) for surface changes

187   likely caused by UV-C weathering did not reveal any distinguishable changes. For example,

188   surfaces of all particles (before and after weathering) had similar wrinkles, cracks and

189   deepenings and they hosted bunches of smaller particles. Microscope images of the surfaces of

190   four particles of each type (PA-MP or UV-PA-MP) are shown in Fig. S1.

191

192   Life –cycle effects

193   The effects on *D. mag*na caused by polyamide particle ingestion were evaluated using the

194   standard test (OECD 211) endpoints: survival and reproduction. Ingestion of particles was

195   verified via a separate 2-day exposure investigation with *D. magna* as shown in Fig. 2 depicting

196   the particles in the gut of the animal under Zeiss LSM 800 laser scanning microscope LSM 800.

197   The particles were clearly visible in the gut due to their fluorescence (blue particles in Fig. 2).

198   Quantification of ingested, accumulated or eliminated particles from the daphnids was out of

199   scope of this work.

200

201   However, despite the evident ingestion of the particles, no significant differences in the number

202   of offspring produced by daphnids were seen between the treatments and their corresponding

203   controls in both experiments (water-only and surfactant-including). No mortality was observed in

204 the water-only experiment, except for three replicates (10% in each). In the surfactant-including

205 experiment, survival of parental daphnids was 100%. Also, no significant differences were

206 observed between the experiments e.g., between PA-MP treatments from the two experiments

207 (duplicated experiment). The details of the test of difference are presented in Table S2. As no

208 difference was detected between experiments, the data for the corresponding treatments and

209 controls were pooled and averaged, Fig. 3.

210 The initial pH of the natural water was 8.32 and after addition of the surfactant 8.38. Random

211 measurements of pH of the medium at renewals demonstrated negligible variations (8.4±0.03 in

212 the water-only and 8.3±0.1 in the surfactant-mediated experiments), which conformed to the test

213 guidelines: pH within the range 6 – 9 and no variation by more than 1.5 units in any test (OECD,

214 2012).

215 Discussion

216 In this study, newborn daphnids were exposed for 21 days to virgin and UV-weathered PA-MP

217 particles at two concentrations (100 and 300 mg L^{-1}) under two exposure scenarios (with and

218 without surfactant), in accordance with the OECD 211 *D. magna* reproduction test guideline.

219 The conditions for UV weathering of the PA-MP particles (open air and in water environment)

220 were chosen to resemble environmentally relevant ones since discarded plastics often end up in

221 surface water where it undergoes exposure to UV radiation, wind and wave action and abrasion

222 (Wright & Kelly, 2017). UV weathering is accelerated by exposure to the air (Gijsman, Meijers &

223 Vitarelli, 1999) and in humid conditions (Singh & Sharma, 2008). Also, at any given temperature

224 and humidity, the rate of degradation increases with increasing UV flux (Singh & Sharma, 2008).

225 UV weathering occurs due to impurities embedded in the polymer matrix that can absorb

226 photons, which are excited and cleave the polymer chain (Singh & Sharma, 2008). The presence

227 of oxygen initiates photooxidative degradation: a radical-based autoxidative process during

228 which free radicals (formed due to a photolysis reaction of an impurity) propagate by thermal

229 reaction (stimulated by the ease of hydrogen abstraction from the carbon-hydrogen bond of the

230 polymer). At the final stage, peroxy radicals cause further reaction leading to the formation of

231 dialkylperoxides, alcohols and ketones (Gijsman, Meijers & Vitarelli, 1999). Hence, the

232 formation of different substances on the surface of the UV-PA-MP particles used in this study

233 was expected to cause some toxicity to the daphnids upon particle ingestion. In addition,

234 photooxidation of plastic particles makes them brittle and susceptible to degradation into smaller

235 fragments (Jahnke et al., 2017; Wright & Kelly, 2017). Hence, an increase in the fraction of

236 smaller particles in the pool of particles of different sizes due to UV weathering (Fig. 1) was

237 expected to increase their bioavailability and contribute to the toxicity to the daphnids.

238 The particle concentrations chosen for this study were in the range used in testing of the

239 responses of the *D. magna* to MP exposure by previous researchers, e.g., 100 mg L^{-1} PE

240 terephthalate textile fibers (PET) (Jemec et al., 2016) or PE microbeads at 100 mg L^{-1} (Canniff

241 and Hoang, 2018) or at 0.0001 - 10 g L^{-1} (Frydkjær, Iversen & Roslev, 2017) or 100 mg L^{-1}

242 polystyrene (PS) nanoplastics explored in nine bioassays including *D. magna* (Heinlaan et al.,

243 2020). The consensus is that the current environmental concentrations are not harmful e.g., to

244 microalgae (Prata et al., 2019) or as a vector for hydrophobic pollutants (Frydkjær, Iversen &

245 Roslev, 2017). However, the actual concentrations of MP in aquatic environments remain largely

246 unknown. They can vary e.g., from 257-1215 pieces m^{-3} along the south-eastern coastline of

247 South Africa (Nel & Froneman, 2015) to 30 g L^{-1} on the high tide line in Fuerteventura beaches

248 in the Canary Islands in Spain (Baztan et al., 2014). Normally, the MPs sampled from aquatic

249 environments contain particles of different types, shapes and sizes (usually disregarding low

250   micrometer ranges) and the determination of environmental concentrations depends among

251   others on the geomorphological profile of sampling sites, sampling tool, mesh size, duration and

252   depth of sampling, extraction and identification methods. For example, geomorphological

253   conditions (such as rocky shorelines with little sand deposition) and high energy waves can

254   affect the deposition of MP particles on beaches (Baztan et al., 2014) and hence, their availability

255   for sampling. In addition, MP concentrations tend to increase because of the wide applications of

256   microbeads in various spheres and the degradation of the existing plastics. Hence, the effects of

257   the supposedly higher concentrations tested in laboratory conditions worldwide help proactively

258   estimate the future impacts of the plastics on the biota.

259   Nevertheless, the results obtained for the water-only scenario, when daphnids were continuously

260   exposed to virgin and UV-weathered PA-MP particles through their life cycle (21 days), showed

261   no effect on their survival or number of offspring produced (Fig.3), even though the UV-

262   weathered PA-MP particles were more abundant at 10-20 µm (compared to the virgin ones) and

263   small particles can be more easily ingested by non-selective filter-feeders (e.g., Desforges,

264   Galbraith & Ross, 2015; Rehse, Kloas & Zarf, 2016).

265   The addition of Tween 20 to the medium (i.e., surfactant-including scenario) was used to ensure

266   that the plastic particles are well mixed within the medium and will be readily bioavailable to *D.*

267   *magna* throughout the experiments. While *D. magna* can feed from the bottom (authors

268   observations, also Imhof et al., 2013, 2017) and can swim into the particle layer on the water

269   surface (authors observations, also Rehse, Kloas & Zarf, 2016), the addition of the surfactant was

270   expected to improve the contact of particles with water and ensure their homogeneous mixing in

271   the water column and thus availability to the filter-feeding *D. magna*. Frydkjær, Iversen &

272   Roslev (2017) also used Tween 20 at 0.001 vol% to facilitate mixing of PE and water in the

273   ingestion study with *D. magna*. Besides, the scenario with the surfactant possesses high

274   environmental realism as nonionic surfactants have found wide application in various spheres

275   e.g., as detergents, emulsifiers, cosmetic and pharmaceutical components (Ferreira, Bernin &

276   Topgaard, 2013). They can be found in the environment and can modulate the bioavailability of

277   MP (e.g., by improving the contact among MP and animals and dispersing MP in the water

278   column (Renzi, Grazoli & Blaškovic, 2019)), in addition to posing a direct toxicity risk to aquatic

279   animals (Garcia et al., 2007; Ivanković & Hrenović, 2010). For example, according to Renzi,

280   Grazoli & Blaškovic (2019), the addition of a surfactant (Triton X-100) equal to 0.001% v/v to

281   various MP dispersions (PE, PP, PVC, PVC/PE) increased mortality and immobilization of early

282   stages of *D. magna* under both fasting and feeding conditions during 96 h of exposure (MP range

283   size 10-100 µm and concentration 50 mg L^{-1}).

284   The results of 48 and 96-h LC50 values of Tween 20 (> 130 mg L^{-1}) and of the 21-day life cycle

285   experiment at 7 mg Tween 20 L^{-1} (alone and in combination with UV-PA-MP) showed no effect

286   on the survival or number of offspring produced by *D. magna* (Fig.3). Indeed, according to the

287   hazard classification of the European Union legislation 2008 to the aquatic environment

288   (categories I-III), Tween 20 cannot be categorized even as category III (LC50 > 10 to ≤ 100 mg

289   L^{-1}). However, effects of surfactants depend on species and surfactant type and the effect

290   concentrations of nonionic surfactants ranged from 0.3 to 400 mg L^{-1} (Evsyunina et al., 2016;

291   Jahan, Balzer & Mosto, 2008; Liwarska-Bizukojc et al., 2005; Pettersson, Adamsson & Dave,

292   2000).

293   Effects caused by MP particles ingestion may not be restricted to the particles themselves.

294   Leachate of toxic additives embedded in the plastic polymer matrix to impart desired qualities

295   (Bejgarn et al., 2015; Hahladakis et al., 2018) may also pose a risk to the biota. Such toxic

296 additives may be loosely bound to the structure and thus can easily leach out from the polymer.

297 According to the data, the leachate obtained from different plastic products cut into 10x10 cm

298 pieces during 24 h at 28°C (PE, PET, PS, PP, PVC, polycarbonate - PC) significantly lowered

299 survival and settlement of barnacle *Amphibalanus amphitrite* larvae at concentration 0.10 and

300 0.50 m^2 L^{-1} (Li et al., 2016). Leachates from 9/32 plastic products prepared on a rotator for 24 h

301 at ~20°C had 48-h *D. magna* immobilization EC50s ranging from 5 to 80 g of plastic material L^{-1}

302 (for PC, PVC, polyurethane) (Lithner et al., 2009). Furthermore, leachate obtained from PVC

303 and epoxy products (2x2 cm pieces) over 3 days at 50°C had 48-h *D. magna* immobilization

304 EC50s in the range 2-235 g of plastic material L^{-1} (Lithner, Nordensvan & Dave, 2012).

305 Although the PA-MP concentrations used in our experiments were much lower than the above-

306 mentioned estimates of toxicity (24-h and 48-h EC50s), the time for leachate formation was

307 longer in our case (72 hours between medium renewals) and some toxicity of the leachate was

308 expected at least for the earlier life stages of the daphnids. However, insignificant differences in

309 the number of offspring between the controls and treatments in both (water-only and surfactant-

310 including) experiments suggest no toxic potential of the leachates. Apparently, particles did not

311 contain leachable toxic additives or at the selected concentrations 72 hours (in between water

312 renewals) was not sufficient for the formation of toxic leachate.

313   On the other hand, abundant studies indicate low MP effects on *D. magna* where

314 comparable particle size range, type, concentrations, and exposure conditions were tested (e.g.,

315 Aljaibachi and Callaghan, 2018; Jemec et al., 2016). *D. magna* was shown to ingest 63-75 μm

316 PE beads (25, 50, and 100 mg L^{-1}) and although their gut was filled with them, no significant

317 effect on survival and reproduction was seen (Canniff and Hoang, 2018).

318   The particles used in the study were irregularly shaped and thus their egestion from the

319   gut of daphnids could be complicated, compared to regular particles, thus supposedly exerting

320   additional stress on them. Frydkjær, Iversen & Roslev (2017) showed that the full egestion of

321   irregular PE particles (10–75 μm) by *D. magna* was significantly lower than that of spherical PE

322   beads (10–106 μm) after 24 hours of MP ingestion at concentrations 10, 100, 1000 and 5000 mg

323   L^{-1}. Although MP particle morphology can play a role in particle uptake and evacuation from the

324   organism, our results showed no effect of the used irregular shape (virgin and UV-altered)

325   polyamide MP on the reproduction of the *D. magna*. This largely agrees with reported data of no

326   acute toxicity to *D. magna* of irregular shape MPs at < 100 μm size at 100 mg L^{-1} (Kokalj, Kunej

327   & Skalar, 2018) or no effect on reproduction or malformation of juveniles caused by irregular

328   particle mixtures 22-72 μm size at concentrations of 1% of the food particles although with some

329   inconsistent alterations in stress response and gene expression (Imhof et al., 2017). A contrasting

330   variety of experimental outcomes with *D. magna* on MP ingestion (from increased mortality to

331   no effect) was also pointed out by Bosker et al. (2019).

332   When comparing the responses of two key aquatic species (*D. magna* and *C. riparius*) to

333   virgin and UV-weathered polyamide exposure, one can see that the standard endpoint in the

334   midge *C. riparius* (emergence of winged adult midges) was also not affected when the midges

335   were exposed to 100 or even 1000 mg kg^{-1} of virgin PA-MP during life cycle experiments

336   (Khosrovyan & Kahru, 2020; Khosrovyan & Kahru, 2021). In a multi-generation experiment,

337   emergence was reduced after the first generation but recovered during the second and third

338   generations (Khosrovyan & Kahru, 2021). However, in contrast to *D. magna*'s response to

339   exposure to UV-PA-MP (at 300 mg kg^{-1}), significantly lower emergence, compared to the

340   control, was seen in *C. riparius* exposed to UV-PA-MP (1000 mg kg^{-1}) throughout its life cycle.

341   Ingestion of the polyamide particles was verified for both organisms (Khosrovyan, Gabrielyan *&*

342   Kahru, 2020 and Fig. 2). That no toxicity of virgin polyamide particles at the *Daphnia*'s

343   organism level was observed in this study may suggest that they were efficiently removed from

344   the body after being ingested and/or did not contain leachable toxic additives. However, it

345   remains unclear what can account for the adverse effect of UV-weathered particles on *C.*

346   *riparius* and no adverse impact of the same particles on *D. magna*.

347   Responses to virgin or weathered MP may be species-specific, even if the species are standard

348   test organisms. Different dietary habits (e.g., benthic gathering vs filter-feeding) influence the

349   amount and possibility of the uptake of solid particles and further going through the digestive

350   tract of these items. Hence, overgeneralization of response variables may occur if the species-

351   specificity to response or types of exposure (external or ingested) is disregarded (Suckling,

352   2021). However, more sensitive endpoints in microplastic exposure studies must be considered

353   to improve the performance of standard tests.

354   Conclusions

355   Exposure to virgin and UV-weathered polyamide particles at 100 and 300 mg L^{-1} with and

356   without Tween 20 surfactant did not affect the survival or number of offspring produced by the

357   *D. magna.* The no adverse effect of particle ingestion on *D. magna*'s survival and reproduction

358   may suggest that virgin plastic particles lacking toxic additives do not pose a toxicity risk to

359   them. This can also be supported by no long-term adverse effects of virgin particles on *C.*

360   *riparius* in a multi-generation study.

361   However, toxicity of pure MP particles should still not be overlooked because in the

362   environment they adsorb contaminants and/or undergo degradation and this entails

363 corresponding consequences for the biota. Although the impact on the biota may depend on the

364 species and types of exposure, the existing standard tests may not perform well in the case of

365 insoluble particulates such as MP particles and new endpoints could be suggested for detecting

366 MP effects.

367 Low concentrations of Tween 20 (e.g., 7 mg L^{-1}) were nontoxic but sufficient for altering surface

368 tension of polyamide particles (and likely, other polymer) particles without causing confounding

369 effects on the crustacea and it can be recommended for use in plastics toxicity studies.

370

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527

Figure 1

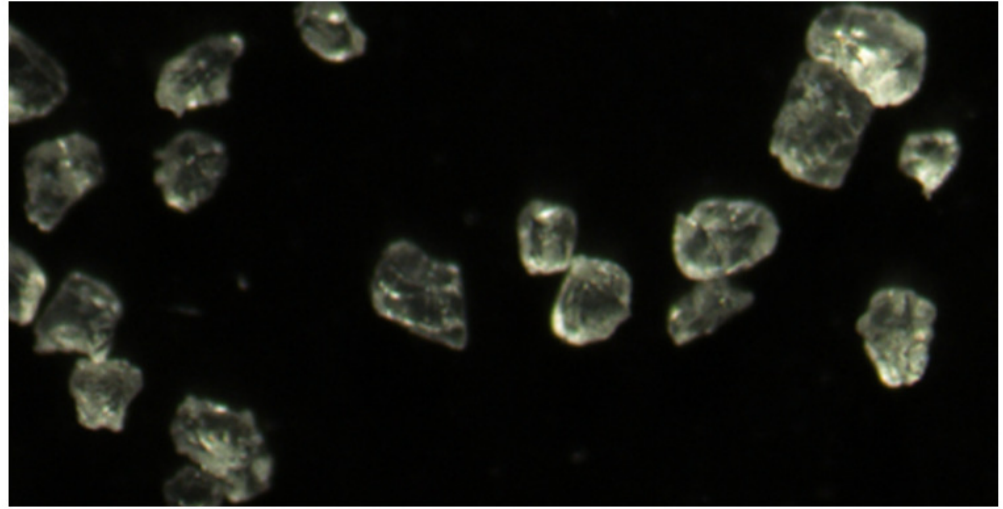
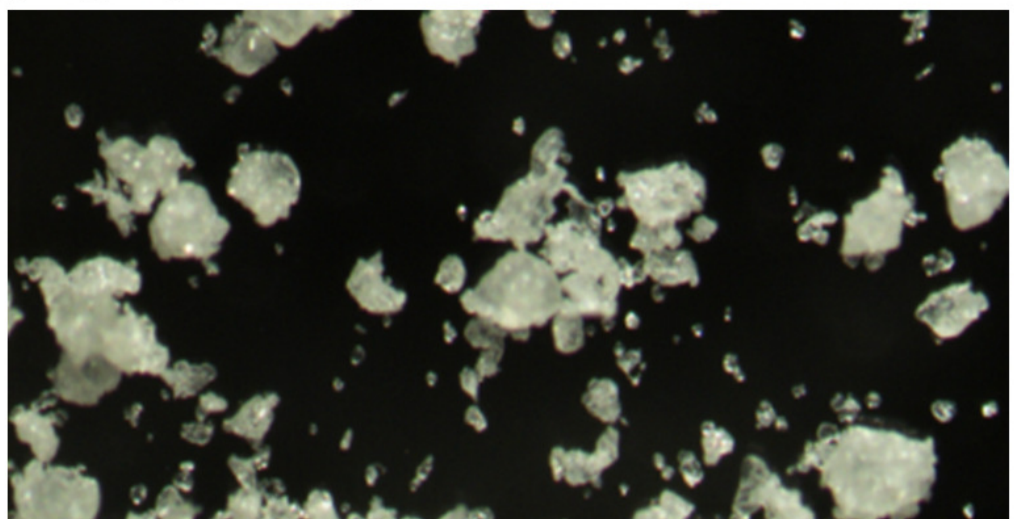
Virgin polyamide particles and the change of colour and increase in the fraction of smaller particles after UV-C weathering.

Virgin polyamide particles

UV-weathered polyamide particles

Figure 2

UV-weathered fluorescent polyamide particles in the gut of *D. magna* (image from the Zeiss confocal laser scanning microscope LSM 800, laser wavelength 405 nm, detection wavelength 400-531 nm).

Arrows point to the gut (up) and polyamide particle (bottom). Particle size is indicated by distance measured between two edges of the particle.

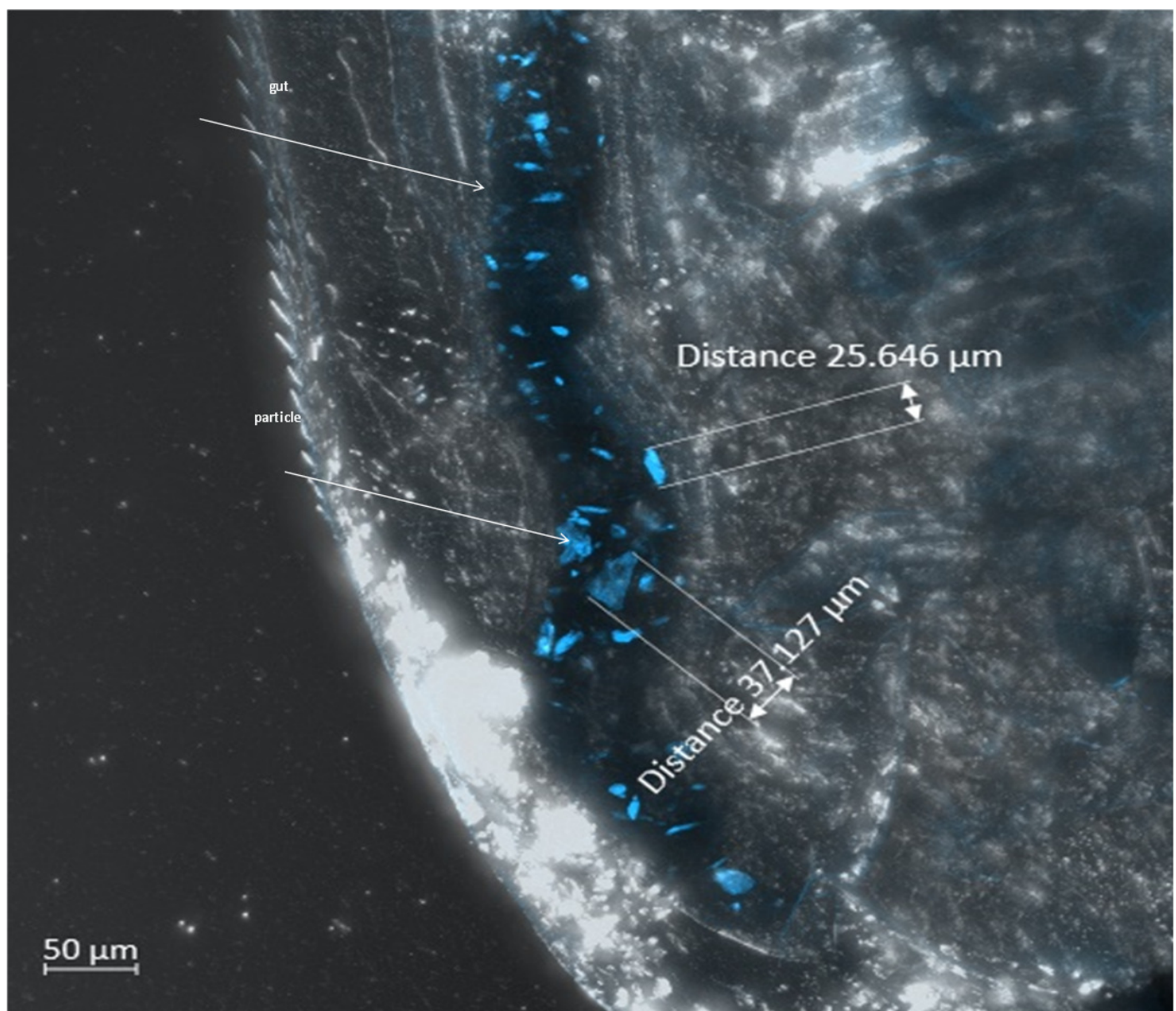



Figure 3

Mean number of offspring per surviving parent *Daphnia* (% from the control) at the end of the 21-day exposure to two types of polyamide: virgin (PA-MP) and UV-weathered (UV-PA-MP).

Number above each data column: the actual percentage. Bars: standard deviation. Treatments: virgin and UV-weathered polyamide at concentrations of 100 and 300 mg L^{-1} with and without surfactant (7 mg Tween 20 L^{-1}). Data are averaged from the duplicated experiments.