1 **Research Recommendations to Better Understand the Potential Health Impacts of**

2 **Microplastics to Humans and Aquatic Ecosystems**

3

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24 **Abstract**

25 To assess the potential risk of microplastic exposure to humans and aquatic ecosystems, reliable

26 toxicity data is needed. This includes a more complete foundational understanding of microplastic

27 toxicity and better characterization of the hazards they may present. To expand this understanding,

28 an international group of experts was convened in 2020-2021 to identify critical thresholds at

29 which microplastics found in drinking and ambient waters present a health risk to humans and

30 aquatic organisms. However, their findings were limited by notable data gaps in the literature.

31 Here, we identify those shortcomings and describe four categories of research recommendations

32 needed to address them: 1) adequate particle characterization and selection for toxicity testing; 2)

33 appropriate experimental study designs that allow for the derivation of dose-response curves; 3)

34 establishment of adverse outcome pathways for microplastics; and 4) a clearer understanding of

35 microplastic exposure, particularly for human health. By addressing these four data gaps,

36 researchers will gain a better understanding of the key drivers of microplastic toxicity and the

37 concentrations at which adverse effects may occur, allowing a better understanding of the potential

38 risk that microplastics exposure might pose to human and aquatic ecosystems.

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40 **Keywords:** microplastic, research recommendations, hazard characterization, aquatic organisms,

41 human health, environmental management

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47 **Introduction**

48 Researchers are finding microplastics almost everywhere they look. Microplastics, defined as

49 solid, polymeric particles with at least three dimensions greater than 1 nm and < 5 mm in size

50 (CSWRCB, 2020), contaminate marine (Wilcox et al., 2020), freshwater (Horton et al., 2017), and

51 terrestrial (Xu et al., 2020) habitats, and more recently, have been detected in drinking water

52 (Koelmans et al., 2019; Pivokonsky et al., 2018), food (Bouwmeester et al., 2015), and the

53 atmosphere (Brahney et al., 2020; Wright et al., 2020). Given their ubiquity, most organisms,

54 including humans, are frequently exposed to microplastics. Studies in aquatic organisms show that

55 microplastics can cause inflammation and tissue damage (Jin et al., 2018; Lu et al., 2018), reduced

56 growth (Zimmerman et al., 2020), altered development (Gardon et al., 2020), and reductions in

57 reproductive success (Cole et al. 2015; Jaikumar et al., 2019). Though the possible effects in

58 humans are less well-defined, initial studies in rodent models suggest that exposure to some forms

59 of microplastics may impact endocrine signaling (Amereh et al., 2019; Amereh et al., 2020; Hou

60 et al., 2021), initiate oxidative stress and inflammation (Li et al., 2020; Xie et al., 2020; Zheng et

61 al., 2021), and reduce gamete viability (An et al., 2021; Hou et al., 2021; Li et al., 2021a).

62 These findings have captured the attention of the public and increased societal concern for

63 ecosystem and human health, prompting legislators, environmental managers, and other

64 organizations to take action to better understand the risks of microplastic exposure. Within the past

65 decade, the United Kingdom Parliament, the European Chemicals Agency, the European Food

66 Safety Authority, and the World Health Organization have all released comprehensive reports and

67 specific recommendations to assess the impact and potential risks of plastic and microplastic

68 pollution (Parliament. House of Commons, 2016; EFSA CONTAM Panel 2016; ECHA 2019;

69 WHO 2019; BfR 2020). Effective management of microplastics requires an understanding of the

70 potential adverse health effects on humans and the environment, as well as the key drivers of

71 toxicity (e.g., particle size, composition, etc.) and concentration thresholds at which these effects

72 begin to manifest. However, developing health-based thresholds for microplastics is challenging

73 because they represent a diverse suite of physical and chemical characteristics (Rochman et al.,

74 2019). Toxicological effects may be initiated via a variety of mechanisms rather than a single

75 molecular initiating event, and some of these mechanisms are poorly elucidated. In support of

76 legislative mandates to develop microplastics management strategies for aquatic habitats and

77 drinking water for human consumption (California Ocean Protection Act 2018; California Safe

78 Water Drinking Act, 2018), the State of California convened a group of international experts in

79 microplastics research to identify and characterize the hazards associated with microplastics.

80 Specifically, experts were tasked with identifying which microplastic characteristics (e.g., size,

81 morphology, polymer, etc.) contribute most to toxicity (Hampton et al., *In Review*) and developing

82 health-based thresholds for both the aquatic environment (Mehinto et al, *Accepted*) and drinking

83 water (Coffin et al.,2022). These efforts were limited by critical gaps in knowledge, or a lack of

84 studies the experts deemed fit for the purpose of risk assessment (Coffin et al., 2022; Gouin et al.,

85 2022; Mehinto et al.,*Accepted*). Here, we identify those shortcomings and the research initiatives

86 needed to address them, which can be grouped into four categories: 1) improved particle selection

87 and characterization for toxicity testing; 2) experimental designs that allow for establishing dose-

88 response curves; 3) the connection of microplastics to established or novel adverse outcome

89 pathways (AOPs); and 4) a clearer understanding of exposure (Figure 1). Each of the four research

90 gaps are discussed in depth below and recommendations for future study designs are postulated

91 (Table 1).

92

I. Improved Particle Characterization and Selection for Hazard Identification and Characterization

Assessing potential microplastic toxicity in aquatic organisms has been achieved primarily via laboratory studies in which biota are exposed to microplastics at a given concentration or concentrations and physiological responses are measured. Most exposure studies have been conducted using a single particle type (e.g., polystyrene spheres of a single size), but if particles are well characterized, these studies can provide important information on the potential hazards of specific microplastic types and characteristics (e.g., size, morphology, polymer type, etc.). In addition, there is also a need for studies in which organisms are exposed to combinations of microplastics as close as possible to what they would be exposed to in the ambient environment. For instance, fibers and spheres respectively make up 52-73% and 1-3% of anthropogenic particles detected in the environmental water samples (Burns and Boxall 2018; Athey and Erdle 2021; Zhu et al., 2021), but roughly only 7% of studies published through 2020 use fibers whereas 62% use spheres (Hampton et al., 2022). Similarly, 82% of studies are conducted with polystyrene or polyethylene polymers, which make up only 5-28% of what is reported in the aquatic environment (Burns and Boxall 2018). Only 12% of aquatic organism tests used weathered particles (Hampton et al., 2022), which are likely to present greater risks to biota due to increased ingestion probability, leachates, biofilm formation, particle roughness, increased surface area, and potentially other mechanisms (Liu et al., 2020; Hariharan et al., 2021; Khosrovyan and Kahru 2021). For studies focused on the potential human health impacts of microplastics, similar biases regarding particle selection were observed as 77% of the rodent *in vivo* studies used polystyrene spheres (Hampton et al., 2022). In addition, microplastic particles were often limited to a single size (69% of studies),

115   and no studies used weathered particles. This lack of particle diversity is also reflected in *in vitro*

116   studies.

117   If particles are comprehensively characterized (see de Ruijter et al., 2020 and Gouin et al.,

118   2022 for guidance on minimum particle characterization), experiments that employ a single

119   particle type may provide insight regarding specific relationships between microplastic

120   characteristics and biological effects. Thus, it is recommended that future toxicity tests address

121   one of two experimental objectives. The first is to determine how specific microplastic particle

122   types (e.g., polyester fibers, tire wear particles) and characteristics (e.g., size, surface area, volume)

123   may present a hazard to aquatic organisms and/or humans. Identification of the most harmful

124   microplastic types is important for the development of monitoring programs, as there are numerous

125   measurement techniques that can be used to quantify microplastics, with some being more

126   appropriate and cost-effective for different sizes, morphologies, and polymer types (De Frond et

127   al., 2022). The second objective is to determine concentrations at which environmentally relevant

128   distributions of microplastics cause adverse effects. The limited particle diversity and incomplete

129   particle characterization in most existing studies are impediments to achieving either objective.

130

131   ***Recommendation 1****: Identify microplastic characteristics that best predict hazards through*

132   *extensive particle characterization and toxicity screening*

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134   Results from studies using singular particle types can be extrapolated to more relevant mixtures of

135   microplastics found in the natural environment so long as particles are extensively characterized,

136   and the relative importance of different particle characteristics to toxicological outcomes are

137   understood (Koelmans et al., 2020). For example, Zimmerman et al. (2020) exposed *Daphnia*

138   *magna* to polyvinyl chloride, polyurethane, or polylactic acid with or without extractable chemical

139   additives. The particle morphology (i.e., fragments) and size (i.e., 20-40 μm) were held constant.

140   Using this experimental design, Zimmerman et al., could discern which effects were driven by

141   polymer type and which were driven by additive chemicals. Perhaps most importantly, the size,

142   polymer composition, and morphology of the microplastics used were all extensively

143   characterized. These findings provide much-needed insight into which microplastic characteristics

144   may cause toxicity. More similarly designed studies are needed for other types of microplastics to

145   identify which particle characteristics and polymer types are of greatest toxicological concern.

146   Study relevance may be further increased by using particle types frequently most detected in the

147   environment.

148   Particle size is a critical factor influencing microplastic toxicokinetics and toxicodynamics

149   (Coffin et al., 2022; Hampton et al., *In Review*). In aquatic organisms, smaller microplastics may

150   be taken up via the gills and ingested, while larger plastics may interfere with motility through

151   entanglement (Galloway et al. 2017; Jâms et al., 2020). Once a particle is ingested, its size also

152   influences the likelihood for translocation beyond the gut or gills to other tissues (McIlwraith et

153   al., 2021; Wang et al., 2021), as well as their retention and excretion (Kinjo et al., 2019). Current

154   evidence indicates that size strongly affects the observed adverse outcomes, including differential

155   effects on growth (Silva et al., 2019), immune function (Li et al., 2021b), oxidative stress (You et

156   al., 2021), and mortality (Gray and Weinstein 2017). Many studies suggest that toxic effects are

157   more likely to be observed following exposure to smaller particles (Bucci et al., 2020), although

158   larger particles may be more harmful to aquatic species in specific scenarios. For example, larger

159   particles take up more volume in the gut once ingested, possibly leading to reduced food

160   assimilation and food dilution (Koelmans et al, 2020; Hampton et al., *In Review*).

161   In humans, size determines the extent to which particles may be taken up and distributed

162   within the body. For instance, particles <10 μm may be inhaled (Porter et al., 1999) and those <1

163   μm may be taken up by cells (Beier and Gerbert 1998; Geiser et al., 2003). As the size of inhaled

164   particles decreases, translocation efficiency increases (Kreyling et al., 2009). Smaller, orally

165   ingested microplastic particles are also expected to translocate from the gut more efficiently

166   (Wright and Kelly 2017). For instance, 50 and 100 nm polystyrene nanospheres were detected in

167   the liver, spleen, blood, and bone marrow of female rats after 10 days of exposure via gavage. In

168   contrast, particles larger than 100 nm were not detected in the bond marrow and those larger than

169   300 mn were not detected in the blood (Jani et al., 1990). Yet despite these observations, there is

170   insufficient data to reliably model the particokinetics of microplastics for humans (Coffin et al.,

171   2022) or other organisms (Mehinto et al., *Accepted*), thus increasing uncertainties of risk

172   assessments.

173   Experiments that disentangle the relative effects of different microplastic morphologies are

174   also needed as particle morphology likely influences retention, translocation, and toxicity. In

175   aquatic organisms, a fiber, defined as having a length to width ratio of three or greater, may be

176   retained in the gut for extended periods of time (Xiong et al., 2019) or more likely to translocate

177   via its smallest dimension (McIlwraith et al., 2021) compared to fragments or spheres with similar

178   particle lengths. Several studies report that fibers or irregularly shaped particles (e.g., fragments)

179   are more toxic than uniform particles such as pellets or spheres (Qiao et al., 2019; Botterel et al.,

180   2020). In some instances, specific morphologies may elicit unique adverse effects as fibers have

181   been shown to cause respiratory stress (Stienbarger et al., 2021). Similar findings have been

182   described in mammalian studies as fibers have been found to persist in airways in humans (Omenn

183   et al., 1986), and fragments were found to induce hemolysis in human-derived cells at rates

184 proportionate to their roughness (Choi et al., 2020). However, mammalian toxicity studies that use

185 diverse particle morphologies are limited, with most ingestion-based studies using spheres, several

186 using fragments, and none using fibers (Coffin et al., 2022).

187   At the interface of size and shape are particle volume and surface area, which were

188 identified as being the primary drivers of food dilution and oxidative stress in aquatic species

189 (Koelmans et al., 2020; Hampton et al., *In Review*) and used as the basis for thresholds in the

190 ambient environment (Mehinto et al., *Accepted*). Though food dilution is not relevant for human

191 health, similar relationships between surface area and oxidative stress and other adverse effects

192 have been detected in mammalian models. For example, Schmid and Stoeger (2016) found that

193 nanoparticle surface area was highly correlated with acute lung inflammation when *in vivo* studies

194 in mice and rats were retroactively analyzed. Surface area also influences the formation of the

195 particle corona, which can include toxicants and antigens which influence both uptake and toxicity

196 in humans and other organisms (Evans et al 2002; Lundqvist et al., 2008). To date, most studies

197 focused on the influence of surface area on toxicokinetics and toxicodynamics use small, spherical

198 particles (typically less than 1 μm). Additional studies are needed to determine if the previously

199 described relationships between surface area and toxicity persist across larger size ranges and other

200 particle types with high surface area to mass ratios (e.g., fragments, fibers).

201   In laboratory studies, volume and surface area particle characteristics may be estimated

202 using equations based upon the shape (e.g., volume of a sphere = $4/3\ \pi r^3$). To estimate these

203 parameters in environmental particles, modelling techniques may be used (Kooi and Koelmans

204 2019). However, particle volume and surface area are not typically measured, estimated, or

205 reported in microplastic occurrence or toxicity studies. Measurement or estimation of such

206 parameters in laboratory studies is crucial to understanding the relevant exposure metric for

207 specific types of toxicological effects. Thus, it is important that studies not only report these

208 characteristics but that they are considered as potential drivers of toxicity in future experiments

209 using aquatic species or rodents.

210   Finally, experiments designed to decouple particle-driven effects from those caused by

211 chemical leachates (i.e., monomers, additive mixtures) and sorbed chemicals (e.g., Zimmerman et

212 al., 2020) are critical to understanding the toxicological drivers of microplastics. Adverse effects

213 have been attributed to chemical additives following the inhalation of nylon fibers (Porter et al,

214 1999; van Dijk et al., 2021) and polyvinyl chloride particles (Xu et al., 2003) in humans; and in

215 aquatic organisms, leachates from tire wear particles (Tian et al., 2020; Tamis et al., 2021) and

216 single-use food packing (Zimmermann et al., 2021) have been demonstrated to be toxic.

217 Disentangling physical and chemical particle characteristics causing toxicity will facilitate more

218 targeted, efficient management and mitigation strategies for reducing environmental and human

219 health risks from microplastics (e.g., prioritizing assessment of alternatives for chemical additives

220 in plastics).

221   Though methods for microplastic analysis and particle characterization are still emerging,

222 techniques for quantifying particles as well as determining size, morphology, and polymer type

223 are readily available for most particle types excluding nanoplastics (< 1 µm) (Brander et al., 2020;

224 Primpke et al., 2020). Microplastics are most often enumerated by manually counting particles via

225 visual light microscopy, which may be facilitated by staining particles with nile red. For smaller

226 particles < 20-50 µm, other microscopic or light scattering techniques (e.g., scanning electron

227 microscopy, transmission electron microscopy, dynamic light scattering) are often preferrable.

228 Particle size is most often assessed by manual measurements via microscopy, but other techniques

229 such as dynamic light scattering can be used to generate size distributions. Polymer confirmation

230 and identification are most commonly achieved via Fourier-transform infrared spectroscopy

231 (FTIR) or Raman spectroscopy, but polymers may also be identified using pyrolysis-gas

232 chromatography/mass spectrometry. Other particle characteristics such as surface area and volume

233 may be estimated in some cases (Koelmans et al., 2020; Kooi et al., 2021), but currently, there are

234 no widely used techniques for gathering empirical data describing these characteristics in

235 microplastics. Future methodological studies should seek to develop methods for better particle

236 characterization, particularly for characteristics hypothesized to drive toxicological effects (e.g.,

237 surface area, volume).

238

239 **Recommendation 2**: *Better characterize microplastic hazards by conducting toxicity tests using*

240 *polydisperse, environmentally relevant distributions of microplastic particles*

241

242 To fully characterize the hazards of microplastics, it is important to understand how

243 environmentally relevant mixtures of particles may cause toxicity (Halden et al., 2021). Assessing

244 the integrated effects of multiple plastic types from exposures conducted with a single type of

245 microplastic is challenging as some evidence suggests organisms respond differently to diverse

246 mixtures of microplastics (i.e., polydisperse) than each type of microplastic alone. For instance,

247 Ziajahromi et al., (2017) found polyester fibers or polyethylene beads to be more toxic to

248 *Ceriodaphnia dubia* when presented alone than when presented as a mixture (Ziajahromi et al.,

249 2017). There are few studies that have tried to mimic naturally occurring mixtures of microplastics

250 by exposing organisms to more than one particle type at the same time (i.e., ~5% of aquatic

251 organism studies, 0% of human health studies; Hampton et al., 2022), but more studies are needed

252 to definitively identify the primary hazards of microplastics.

253   Microplastic distributions vary greatly depending on the environmental matrix (Zhu et al.,

254   2021; Kooi et al., 2021). However, some patterns have emerged from studies aimed at describing

255   mixtures of microplastics in the real-world (Kooi and Koelmans 2019). In drinking water, studies

256   have shown that most samples are typically a mixture of relatively small (<10 μm) fragments and

257   fibers (Pivokonsky et al., 2018; Pivokonsky et al., 2020), whereas in the aquatic environment, most

258   surface water and sediment samples, and thus biota, appear to be dominated by fibers and a diverse

259   array of fragments, films, and foams (Burns and Boxall 2018, Zhu et al., 2021). Toxicity

260   evaluations reflective of these particle distributions would be useful in bridging the gap between

261   laboratory studies and realistic exposures, ultimately leading to better hazard characterization.

262   Predicting microplastic toxicity may be further complicated by the influence of

263   environmental weathering. Most organisms will encounter microplastics that are weathered (ter

264   Halle et al., 2017), fouled with life (including pathogens; Amaral-Zettler et al., 2020), and that

265   include a sorbed mixture of ambient chemical pollutants (organics and metals; Rochman, 2015).

266   There is some evidence that particles aged in the natural environment have different bioavailability

267   and toxicity compared to the effects observed from pristine microplastics (Capo et al., 2021; Bucci

268   et al., 2021). Studies have found enhanced effects from microplastics that had been soaked in ocean

269   or lake water compared to virgin microplastics (Rochman et al., 2013; Bucci et al., 2021); though

270   in some cases, weathering has been shown to decrease toxicity (Schultz et al., 2021). Other studies

271   have found increased chances of translocation (Ramsperger et al., 2020). Despite this, there is a

272   lack of toxicity data for weathered particles as only roughly 12% of studies used microplastics that

273   were collected from the environment or artificially weathered prior to toxicity tests (Hampton et

274   al., 2022). The use of weathered particles in future studies will provide more realistic assessments

275   of microplastic toxicity, though it is important that researchers fully describe approaches used for

276 particle weathering to ensure that studies are representative of environmental conditions and

277 repeatable.

278   Approaches for generating polydisperse, environmentally realistic distributions of

279 microplastics may include the acquisition or generation of the most prevalent particle types

280 typically found within the habitat and matrix of interest. These particles may then be combined in

281 toxicity tests in similar proportions observed in the environment. Artificial weathering can also

282 increase environmental relevance as has been demonstrated in previous studies (Liu et al., 2021a).

283 Alternatively, some studies have also used field-collected microplastics in toxicity testing (Kühn

284 et al., 2018; Bucci et al., 2021; Peters et al., 2022). However, if this approach is taken, it is essential

285 that particles are well-characterized as described in Recommendation 1.

286

287 **II. Inform the Development of Health-Based Thresholds for Microplastics**

288

289 Most microplastic toxicity studies are focused on determining if physiological or behavioral effects

290 can be detected, rather than developing robust dose-response data. Though exploratory,

291 hypothesis-driven studies have supplied the field with a foundational understanding of

292 microplastic toxicity effect mechanisms (i.e., hazard identification), studies which generate robust

293 dose-response data are needed to identify critical concentrations at which those effects manifest

294 (i.e., hazard characterization). Thus, future studies should aim to generate robust dose-response

295 data from which critical effect metrics can be derived. Below, we discuss why this is important

296 and provide specific recommendations for future studies seeking to inform health-based thresholds

297 for aquatic organisms and humans.

298

299   ***Recommendation 3****: Design experiments to generate robust dose-response data for health-based*

300   *threshold development*

301

302   Health-based guidance values are traditionally derived from chronic *in vivo* laboratory studies

303   (Belanger et al., 2017), though in vitro data may also be used for hazard characterization (see

304   section III, recommendation 5). However, it was challenging for the experts in the California

305   Health Effects Workshop to derive health-based thresholds for drinking water and the aquatic

306   environment due to the availability of few fit-for-purpose studies (Mehinto et al., *Accepted*, Coffin

307   et al., 2022). Of the *in vivo* studies in the Toxicity of Microplastics Explorer (ToMEx) database,

308   only 52% of human health studies (n = 14) and 44% aquatic organism studies (n = 73) included

309   three or more exposure concentrations in their experimental design (Hampton et al., 2022). Robust

310   dose-response data is essential to threshold development because it captures the critical points at

311   which contaminant concentrations elicit adverse health effects.

312   To analyze and describe dose-response relationships, different approaches should be used

313   depending on the specific aims of the study. In environmental toxicology, no observed effect

314   concentrations (NOECs) and lowest observed effect concentrations (LOECs) are often used to

315   inform threshold development for the aquatic environment. Here, it is important to consider that

316   NOECs and LOECs are entirely dependent on the dose selection and experimental design of the

317   study from which they are extracted (Landis and Chapman 2011; ECHA 2008). For instance, if

318   the LOEC is also the lowest test concentration, it is possible that even lower concentrations not

319   included in the design will induce an adverse biological response. This could result in an

320   underestimation of risk. Conversely, if no effects are observed in a study, the highest observed

321   effect concentration (HONEC) may overestimate risk. Therefore, approaches that consider the

322   whole dose-response curve such as effect concentrations (e.g., ECX) are preferred. Theoretically,

323   only a minimum of three distinct test concentrations are required to derive lethal or effect

324   concentrations of certain percentages (i.e., LCX or ECX, respectively), but a greater number of

325   test concentrations is strongly recommended when possible to ensure that an adequate dose-

326   response relationship may be observed (OECD 2006). Of the 162 studies in the ToMEx aquatic

327   organisms database, only 16 report ECX or LCX for distinct species, most of which are

328   cladocerans (Hampton et al., 2022). This represents a lack of robust dose-response data for aquatic

329   species, particularly for organism groups of regulatory interest such as bivalves and fish.

330   To understand dose-response relationships for human health, approaches similar to those

331   used for ecological health are often employed. Here, NOEC and LOEC values (referred to as

332   No/Lowest Observed Adverse Effect Levels, NOAELs/LOAELs for human health applications)

333   are often used as a starting point for threshold development (JECFA 2006; USEPA 2012).

334   Alternative approaches like Benchmark Dose (BMD) modelling make use of all the data to

335   describe dose-response relationships for a particular endpoint rather than only using discrete

336   experimental concentrations (USEPA 2012). A significant advantage of the BMD approach is that

337   it provides an estimate of uncertainty via a confidence interval (EFSA 2017). Another major

338   benefit is that *in vitro* data may be incorporated into a BMD analysis, so long as quantitative *in*

339   *vitro* to *in vivo* extrapolation models are available (see section III, recommendation 5). Yet, despite

340   these advantages, only 53% of evaluated *in vivo* studies were identified as having acceptable dose-

341   response data appropriate for BMD modelling (i.e., at least three microplastic treatment groups

342   with a concentration range ≥3, including control accompanied by estimates of uncertainty such as

343   standard deviation) (Coffin et al., 2022). Thus, it is recommended that future studies ensure they

344   use a sufficient number and spacing of exposure concentrations as close as possible to the linear

345 range of the dose-response curve, and adequately report uncertainties associated with effects (e.g.,

346 standard deviation, 95% confidence intervals).

347

348 **III. Increase Understanding of Toxicological Pathways Induced by Microplastics for**

349 **Improved Hazard Characterization**

350

351 Numerous studies have demonstrated the potential for microplastics to cause a wide array of

352 biological effects in aquatic organisms, including oxidative stress (Jeong et al., 2016), reduced

353 growth (Lo et al. 2018, Athey et al. 2020), tissue damage (Jin et al. 2018), reduced reproductive

354 output (Jaikumar et al., 2019; Cole et al., 2015) and behavioral alterations (Lwanga et al., 2017,

355 Gambardella et al., 2017, Choi et al., 2018). In rodents, microplastic exposure has been shown to

356 impact endocrine signaling (Amereh et al., 2019; Amereh et al., 2020; Hou et al., 2021), initiate

357 oxidative stress and inflammation (Li et al., 2020; Xie et al., 2020; Zheng et al., 2021), and

358 negatively affect reproductive potential (An et al., 2021; Hou et al., 2021; Li et al., 2021a). Yet,

359 the specific mechanisms and pathways by which microplastics cause adverse effects are not yet

360 well understood. Many studies provide evidence of altered molecular or cellular-level responses

361 following microplastic exposure (Canesi et al., 2015, Lu et al., 2016, Franzellitti et al., 2019), but

362 it is often unclear if these observations are indicative of adverse effects at the organism or

363 population level, or if they are merely adaptive and healthy responses (e.g., increased levels of

364 antioxidative enzymes) to a stressor with no significant impact on overall health over longer time

365 periods. In addition, it is unknown if effects observed on apical endpoints such as sperm count in

366 male rodents (Xie et al., 2020) are due to general inflammation or more specific mechanisms

367 targeting sensitive tissues such as testis (Coffin et al., 2022). To address these uncertainties, it is

368 recommended that future studies aim to characterize AOPs by assessing endpoints across multiple

369 levels of biological organization in both humans and aquatic species. Initial efforts to identify

370 AOPs for microplastics have indicated a need for further elucidation of mechanisms linking

371 molecular and whole organism adverse effects (Jeong and Choi 2019). This effort may be

372 accelerated using *in vitro* systems and a framework for linking *in vitro* results to *in vivo* effects

373 (Romeo et al., 2020). Development of AOPs may also help to understand the interplay of physical

374 and chemical effects from microplastics but should be based on realistic exposure levels (Jeong

375 and Choi 2020).

376

377 ***Recommendation 4****: Connect microplastics to existing or novel adverse outcome pathways*

378

379 Workshop participants agreed that, while not necessary for developing risk-based regulatory

380 thresholds, scientific confidence in thresholds expands when the mode of action of the microplastic

381 related effects and pathways of effect are understood. Such knowledge would facilitate read across

382 attempts which are of importance for microplastics due to their extreme diversity. AOPs provide

383 a powerful conceptual mechanism for creating this linkage, often starting with a molecular

384 initiating event ultimately leading to an effect at the organism level (Ankley et al., 2010,

385 Villeneuve et al., 2014). For aquatic organisms, there is demonstrable evidence ingestion of

386 microplastics can cause food dilution (Galloway et al., 2017; Koelmans et al., 2020; De Sales-

387 Ribeiro et al., 2020; Walkinshaw et al., 2020) and experts agreed that there is at least partial

388 evidence for the induction of oxidative stress responses following particle translocation (Hampton

389 et al., *In Review*). In turn, these pathways were used to form the basis for thresholds for the aquatic

390 environment (Mehinto et al., *Accepted*). However, experts also agreed that these pathways need

391   further development and experimental validation to increase confidence in the derived thresholds

392   (Mehinto et al., *Accepted*). For human health, biomarkers suggestive of effect mechanisms (e.g.,

393   oxidative stress, inflammation, reactive oxygen species formation, etc.) have been identified at

394   varying levels of biological organization, however confirmatory linkages to apical endpoints (e.g.,

395   sperm reduction in testis) are absent (Coffin et al, this issue).

396   Though most toxicity mechanisms for microplastics are only partially understood or have

397   yet to be explored, some recent studies have proposed partial AOPs or hypothesized which existing

398   AOPs may be applicable to microplastics (Liu et al., 2021b, Hu and Palić 2020, Kim et al., 2020,

399   Jeong and Choi 2019, Jeong and Choi 2020). For instance, following a systematic literature review,

400   Jeong and Choi identified several putative AOPs to which nano- and microplastics could be

401   connected, leading to adverse outcomes on growth, reproduction, and survival following oxidative

402   stress (Jeong and Choi 2019). Similarly, Coffin et al. (this issue) noted that several rodent studies

403   found that microplastic ingestion induced oxidative stress responses in conjunction with impacts

404   to reproductive biomarkers (e.g., Xie et al 2020; An et al., 2021), and that some responses had

405   similarities with key events described in AOPs characterizing generalized inflammatory responses

406   (Villenueve et al., 2018). While it may be reasonable to assume that these observations are directly

407   related, these effects have yet to be linked by distinct key event relationships and experimentally

408   observed within the same network of events. Furthermore, uncertainties with regards to particle

409   characterization (e.g., verification of the absence of chemical additives or impurities) in these

410   studies prevent direct linking of molecular endpoints to apical endpoints (Coffin et al., 2022).

411   Future studies should aim to identify and develop AOPs for microplastics using one or more

412   strategies (summarized by Villeneuve et al., 2014). An example of this might be top-down

413   development where researchers may begin with a well-defined adverse outcome at the organismal

414   level and work their way down biological levels of organization. Researchers should also draw

415   upon existing AOP knowledge, for example by using the AOP-wiki (aopwiki.org) or AOP

416   knowledgebase (aopkb.oecd.org), as these pathways are not contaminant-specific and multiple

417   contaminants may share the same AOP. Thus, it is likely that some existing AOPs may inform

418   microplastic effect mechanisms and require only experimental validation. Even if the primary goal

419   of the study is outside the scope of AOP development, researchers should always strive to describe

420   cascades of specific biological responses and include endpoints across biological levels of

421   organization. This is particularly important for aquatic organisms, and making mechanistic

422   linkages between the cellular, organismal, population, and community levels can be achieved with

423   carefully designed mesocosm or macrocosm approaches.

424

425   **Recommendation 5**: *Increase the relevance of in vitro studies for hazard characterization by*

426   *developing a framework for extrapolating in vitro results to in vivo effects*

427

428   *In vitro* approaches in toxicology have become more widespread as new applications are developed

429   and reductions in animal testing are encouraged (NRC 2007, OECD 2018). However, the use of

430   such data for developing management thresholds is currently limited due to unclear methods for

431   reliably extrapolating *in vitro* results to potential *in vivo* effects for particles (Romeo et al., 2020),

432   though strategies for soluble chemicals have been previously developed (Stadnicka-Michalak et

433   al., 2014). If reliable methods for extrapolating *in vitro* results to *in vivo* effects are established,

434   researchers may take advantage of the cost, resource, and time benefits often provided by *in vitro*

435   systems while generating meaningful data that can be used to characterize the hazards of

436   microplastics. *In vitro* approaches could also be used as part of a tiered system, with the use of cell

437   lines as a screening tool to prioritize which particle sizes, morphologies, etc. should be studied in

438   costlier *in vivo* models. This strategy has been used and is recommended for soluble chemicals

439   such as endocrine disruptors (e.g., Gray et al., 2002). In the United States, development of a

440   quantitative *in vitro* to *in vivo* extrapolation model for microplastics and other contaminants may

441   be necessary to conduct risk assessments due to the mandate phasing out the use of *in vivo* studies

442   by the United States Environmental Protection Agency by 2035 (USEPA, 2019).

443   Microplastics are different from many other contaminants because they are comprised of

444   both chemical and physical constituents and behave as colloid particles that can settle, diffuse, and

445   agglomerate differentially. This presents a challenge in seeking to develop a tool for the

446   extrapolation of *in vitro* data. For instance, buoyant microplastics may rapidly move away from

447   the cell surface in an unagitated system, resulting in an exceedingly low effective concentration

448   (Stock et al., 2019). Thus, in addition to the concentration and exposure duration, particle (e.g.,

449   size, density, buoyancy, surface chemistry) and media characteristics (e.g., viscosity, density,

450   presence of proteins) must be extensively described to fully understand differences between *in*

451   *vitro* and *in vivo* test systems. A second, unique challenge is that the organ partitioning of

452   microplastics *in vivo* is not dictated by hydrophobicity as is the case for many chemical

453   contaminants, but rather the phagocytic capacity of the organ (Praetorious et al., 2014; Deng et al.,

454   2019). There are some interesting developments that may help in addressing the challenges

455   associated with microplastic exposures *in vitro*, including the use of semi-wet (Navabi et al., 2013;

456   Lehner et al., 2020), inverted culture systems (Watson et al., 2016; Stock et al., 2020; Busch et al.,

457   2021), or dynamically flowing systems such as cell-on-a-chip models, which may facilitate cell-

458   particle contact.

459   Novel computation approaches need to be further developed to support the incorporation

460   of *in vitro* data from microplastic studies into risk assessment exercises. This firstly includes the

461   use of dosimetry models, specifically tailored to capture the particle dynamics *in vitro* such as the

462   *In vitro* Sedimentation, Diffusion and Dosimetry, *In vitro* Sedimentation, Diffusion, Dissolution,

463   and Dosimetry, and Distorted Grid models (Hinderliter et al., 2010; DeLoid et al., 2014; DeLoid

464   et al., 2015; Thomas et al., 2018) which provide time-dependent particle and aggregate

465   concentrations at any given height in the media column. Secondly, efforts have also been made to

466   predict *in vivo* microplastic concentrations based on results from *in vitro* studies using

467   Physiologically Based Kinetic (PBK) models which take into account the partitioning of particle-

468   based on phagocytic capacity as described earlier (Li et al., 2010). Thus far, PBK models have

469   been developed for quantum dots (20nm) (Lin et al., 2008), metallic nanoparticles (Bachler et al.,

470   2013), titanium dioxide (15-150nm) (Bachler et al., 2015), nanocrystals and some polymers such

471   as PLGA (50-135nm) (Li et al., 2012, Carlander et al., 2016). In a final step, PBK models can be

472   used in quantitative *in vitro* to *in vivo* extrapolations of observed effects (Punt et al., 2019; Kasteel

473   et al., 2021). Though these efforts demonstrate the possibility of *in vitro* to *in vivo* extrapolation

474   for microplastics, research investments for improving estimates of dosimetry and generating

475   reliable data describing the transport and partitioning of microplastic particles *in vivo* are

476   warranted.

477

478   **IV Improved Exposure Assessment for Microplastics**

479

480   ***Recommendation 6****: Characterize understudied microplastic exposure routes*

481

482 The primary purpose of this working group was hazard identification and characterization for the

483 purposes of health-based threshold development. However, there is also a need for better

484 microplastic exposure assessment to improve future assessments of risk. Most microplastic

485 toxicity studies have focused on a limited number of exposure routes. For aquatic organisms, most

486 studies have added microplastics to water (Bucci et al., 2020; Jacob et al., 2020). However,

487 effective risk assessment and management require a holistic understanding of relative

488 contributions from multiple sources with a similar route of exposure (e.g., ingestion, dermal,

489 inhalation). While this working group largely focused on aqueous exposures, aquatic organisms,

490 particularly those species associated with the benthos, are likely to be exposed to microplastics via

491 the sediment, which generally have higher microplastic concentrations than the water column (Erni

492 Cassola et al., 2019). Thus, it is recommended that future studies aim to evaluate microplastic

493 exposure and toxicity in aquatic organisms in sediment and conduct depth-integrated risk

494 assessments.

495 Here, ingestion-based studies where microplastics were added to food (Li et al., 2020) or

496 drinking water (Hou et al., 2020, An et al., 2021) or administered via oral gavage (e.g., Park et al

497 2020) were the primary focus. However, humans are exposed to microplastics via a wide variety

498 of sources including food, and air (Mohamed Nor et al., 2021). The relative contribution of these

499 sources to microplastic exposure, uptake, and toxicity are not well characterized in humans.

500 Relative source contribution from drinking water was identified as the most sensitive parameter in

501 the derivation of a health-based guidance level for drinking water (Coffin et al this issue). As such,

502 it is recommended that future studies aim to evaluate microplastic exposures such that a

503 comprehensive exposure assessment through all relevant sources may be conducted. Having a

504 comprehensive understanding of exposure for both will allow for more reliable estimations of risks

505 that microplastics may pose to humans.

506

507 **Conclusions**

508 The field of microplastics research has reached the point where there is no longer any doubt of

509 widespread exposure of animals and humans to plastic particles. This has led the management

510 community to seek advice regarding whether there is a need to set limits, and what those limits

511 should be, for microplastics in drinking water, foods, and the natural environment. Research into

512 the bioavailability and effects of microplastics have demonstrated that microplastics can cause

513 harm, but it is often the case that these studies cannot readily inform risk assessments. Here, we

514 have discussed the research gaps that need to be filled to increase our understanding of the risk

515 microplastics pose to biota and humans and best advise managers on setting health-based

516 thresholds in a more accurate and relevant way. Such data are essential for researchers to

517 understand the extent to which microplastics, varying in size, shape, and chemical profile, at

518 environmentally relevant concentrations, and capturing myriad exposure pathways, pose a risk to

519 human health and the health of aquatic species, biodiversity, and ecosystems. With increased

520 understanding, we can adapt management strategies and risk assessments to help effectively and

521 efficiently manage this novel contaminant.

522

523 **List of Abbreviations**

524 • Adverse Outcome Pathway (AOP)

525 • Benchmark Dose (BMD)

526 • Highest observed effect concentration (HONEC)

527 • Lowest observed adverse effect level (LOAEL)

528 • Lowest observed effect concentration (LOEC)

529 • No observed adverse effect level (NOAEL)

530 • No observed effect concentration (NOEC)

531 • Physiologically Based Kinetic (PBK)

532 • Toxicity of Microplastics Explorer (ToMEx)

533

534 **Declarations**

535 **Availability of Data and Materials**

536 The Toxicity of Microplastics Explorer (ToMEx) databases, web applications, and source code

537 may be accessed at https://microplastics.sccwrp.org.

538

539 **Competing Interests**

540 The authors L.M.T.H., S.C., A.C.M., E.M., and S.B.W. declare having no known competing

541 financial interests or professional relationships that could have appeared to influence the work

542 reported in this paper.

543 The following authors declare financial interests/professional relationships which may be

544 considered as potential competing interests:

545 - H.B. is actively involved in research on the safety of nano and microplastics currently in two

546 projects: Momentum (funded by the Dutch research organization ZonMW and Health Holland)

547 and Plasticheal (EU H2020).

548 - S.M.B. reports a relationship with Ocean Science Trust that includes consulting or advisory and

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558   L.M.T.H., H.B., S.M.B., S.C., M.C., L.H., A.C.M., E.M., C.M.R., and S.B.W. all assisted in the

559   conceptualization and preparation of the manuscript.

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