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# Acute sensitivity of three Cladoceran species to different types of microplastics in combination with thermal stress

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**Abstract**

Microplastics (<5 mm, MP) are ubiquitously distributed in the environment, causing increasing concern regarding their potential toxicity to organisms. To date, most research has focussed on the impacts of MPs on marine and estuarine organisms, with fewer studies focussing on the effects of microplastics on freshwater ecosystems, especially under different environmental conditions. In the present study, the sensitivity of two temperate Cladoceran species, *Daphnia magna* and *Daphnia pulex,* and a smaller tropical species *Ceriodaphnia dubia*, to primary microplastics (PMP) and secondary (weathered) microplastics (SMP) was assessed. A prolonged acute toxicity assay (up to 72 or 96 h) was performed at 18 º, 22 º, and 26 ºC, to determine the influence of temperature as an additional stressor and survival data were analysed using toxicokinetic-toxicodynamic (TK-TD) model. Acute sensitivity of *D. magna* and *D. pulex* to both PMP and SMP increased sharply with temperature, whereas that of *C. dubia* remained relatively stable across temperatures. *C. dubia* was the most sensitive species at 18 ºC, followed by *D. pulex* and *D. magna,* which were of comparable sensitivity. However, this ranking was reversed at 26 ºC as could be seen from the No Effect Concentration (NEC) estimates of the TK-TD model. In addition, SMP and PMP had a similar effect on *D. magna* and *D. pulex,* but PMP was more toxic to *C. dubia*. Effects on survival were strongly time-dependent and became substantially more severe after the standard 48 h test period. Our results indicate that sensitivity to microplastics may differ between species for different types of microplastics, and could be drastically influenced by temperature albeit at high exposure concentrations.

**Capsule:**

There is a difference in sensitivity among three Cladoceran species when exposed to two types of microplastic (primary or artificially weathered) at different exposure temperatures.

**Keywords:** *Daphnia* spp; *Ceriodaphnia dubia*; primary and secondary microplastics; temperature stress; TK-TD modelling.

## *1. Introduction*

Plastics are a class of synthetic organic polymers with widespread applications (Andrady, 2011; Thompson et al., 2009), resulting in a global production of ~322 million tons in 2015 (PlasticsEurope, 2016). As plastics are discarded after use in large quantities and are largely non-biodegradable, they have been accumulating in the environment (Moore, 2008; Thompson et al., 2004; Teuten et al., 2009). More recently, concerns have risen about the introduction of smaller fragments of plastic, also known as microplastics (<5 mm) into the environment (Thompson et al., 2004). Microplastics are now ubiquitous in the environment (Free et al., 2014; Lechner et al., 2014; Thompson et al., 2004) and have a high variability in physicochemical characteristics, including differences in shape (fibres, microbeads, fragments; Cole et al., 2011; Ivar Do Sul and Costa, 2014; Wright et al., 2013), size (nano- to mm-range; Cole et al., 2015; Costa et al., 2010; Ivar Do Sul and Costa, 2014; Wright et al., 2013) and chemical constituents (polyethylene, polypropylene, polyvinylchloride and polystyrene; Browne et al. 2010, Andrady 2011).

Due to their small size, microplastics are readily ingested, which is well documented for marine organisms (e.g., Murray & Cowie, 2011; Van Cauwenberghe et al., 2015). Experiments under marine and estuarine laboratory conditions have found adverse impacts such as tissue damage (von Moos et al. 2012), teratogenicity (Nobre et al. 2015), and altered feeding behaviour (Bergami et al. 2016) on different species.

Until recently, information on uptake and effects of microplastics in freshwater organisms was limited (Barnes et al., 2009; Eerkes-Medrano et al., 2015; Wagner et al., 2014). However, several recent studies have focused on the impact of microplastics in freshwater organisms. For example, exposure of zebrafish to (5 µm) microplastics resulted in accumulation in gills, liver, and gut, resulting in the inflammation of the liver (Lu et al., 2016). Similarly, polyethylene flakes (<400 µm) were found to accumulate in the gut and reduce

feeding rates of freshwater Cnidarian *Hydra attenuata* (Murphy and Quinn, 2018). In addition, several studies have demonstrated that exposure of planktonic species (an important food source for higher trophic levels) to microplastics can also result in adverse effects. Au et al. (2015) analysed the uptake and effects of microplastics on the freshwater amphipod *Hyalella azteca*, and reported that chronic exposure to 10 µm polyethylene particles significantly decreased growth and reproduction, at relatively high exposure concentrations (5000 particles/mL). A study on *Daphnia magna* reported increased immobilization with dose and time of exposure to 1 µm polyethylene particles, albeit at relatively high concentrations (Rehse et al., 2016) while another study on the same species reports reduced feeding rates during prolonged exposure to (100 nm) polystyrene particles (Rist et al., 2017). Another study on *Ceriodaphnia dubia* during exposure to polyester fibers and polyethylene showed dose-dependent effect on survival and reproduction during acute and chronic exposure respectively (Ziajahromi et al., 2017). However, no studies so far have directly compared the species sensitivity of freshwater zooplanktonic organisms to microplastics. This is of importance as studies with other contaminants, including nanomaterials, have shown marked differences in sensitivity across species (Naddy et al., 2011; Völker et al., 2013, Song et al., 2015). Although there is not a lot of evidence for acute effects due to microplastic exposure under standardized laboratory conditions (Rehse et al., 2016), the inclusion of additional stressors may influence toxic effects observed (Heugens et al., 2001). The general stress framework supports that sensitivity of organisms to contaminants is enhanced by environmental variants like temperature that push organisms out of their optimal performance ranges (Van Straalen, 2003). A recent short-term study has investigated the combined impact of microplastics and additional thermal stress on fish larvae and has reported increased impacts under stress-on-stress conditions as compared to single-stress conditions (Ferreira et al., 2016). However, more research is needed on the interactive effects of microplastics with additional stressors such as temperature for planktonic species.

In addition, microplastics exist as primary and secondary microplastics (Wright et al., 2013). Primary microplastics are intentionally produced as micro-sized pellets or powders for commercial applications, such as in personal care products (Gregory, 1996; Zitko and Hanlon, 1991). Secondary microplastics are formed by the environmental degradation of larger plastic debris (Andrady, 2011), mainly by wave action and abrasion, UV-B radiation and temperature changes (Andrady, 2011; Browne et al., 2007). To date, however, the majority of studies have used primary microplastics to study adverse impacts, although secondary microplastics are more abundant in natural environments (Connors et al., 2017; Phuong et al., 2016; Potthoff et al., 2017). Ogonowski et al. (2016) was the first study to compare the toxicity of primary and secondary microplastics on life history parameters such as feeding, growth and reproductive capacity during chronic exposure to *D. magna.* They reported that exposure to secondary microplastics resulted in a significant reduction in reproductive output of *D. magna*, while primary microplastics had limited impacts.

We adopted a comparable setup, with the objective to investigate the acute toxicity of primary and secondary microplastics on three different Cladoceran species, to determine species sensitivity. All three species are commonly used in toxicity testing. Two of the species under study are temperate in distribution (*Daphnia magna* and *Daphnia pulex*), whereas one is a predominantly tropical species (*Ceriodaphnia dubia*). We exposed all species under a range of temperature conditions to study stress-on-stress effects. The dose-response data from acute tests were analysed using toxicokinetic-toxicodynamic (TK-TD) models that are descriptive of the whole time-course of toxicity. We hypothesized that acute sensitivity is species-specific, dependent on the type of microplastic, and influenced by temperature.

## 2. Materials and methods:

### *2.1. Test species*

Cladocerans are primarily freshwater, small-sized (0.2-6 mm) crustaceans, inhabiting pelagic, littoral and benthic zones (Forró et al., 2008). They are important basal components of food chains that higher trophic levels depend on in freshwater ecosystems; playing an important role in the food web of stagnant waters (Forró et al., 2008).

The three species used in this research have wide distribution ranges and were specifically chosen due to their different sizes but similar life histories, which make comparisons across species possible. The chosen species represent three different size classes, from large to small: *Daphnia magna* (2-5 mm), *Daphnia pulex* (2-3 mm) and *Ceriodaphnia dubia* (< 1.4 mm) (Clare, 2002; Balcer et al., 1984; *Fig 1)*. In addition*, D. magna* and *D. pulex* are temperate species whereas *C. dubia* is a predominantly tropical species (Sarma et al., 2005), although it is also found in some temperate habitats.

### *2.2. Laboratory culture and maintenance of test organisms*

*D. magna* and *D. pulex* originate from Leiden University stock and were maintained in similar conditions as recommended by OECD guideline 211 (OECD, 2012). Stock populations were held in 5-L aquaria with 4 L of Elendt M4 medium. Daphnids were fed with a diet of *Pseudokirchneriella subcapitata* in standard doses ($10^4$ cells/organism/day). Aquaria were aerated and kept in a climate chamber at 22 ± 1 ºC, with 16-8 h day-night cycle and a pH of 7.0 ± 0.5. The aquaria were cleaned weekly with periodic removal of neonates, and cultures were renewed once in four weeks. The sensitivity of the species is tested once in 6 months using the standardized $K_2CrO_7$ chemicals (according to OECD guidelines).

*C. dubia* was maintained in a 26 ± 1 ºC climate chamber according to USEPA guidelines (USEPA, 2012). The organisms were cultured in aerated 3-L aquaria containing 2 L of Elendt

M4 with 16-8 h day-night cycle and a pH of 7.0 ± 0.5. They were fed a diet of yeast, trout chow, and cerophyll extracts (YCT) and *P. subcapitata* (doses as recommended by protocol). The aquaria were cleaned twice every week and neonates were removed. Cultures were renewed once every 10 to 12 days.

*2.3. Preparation of microplastics*

Green fluorescent plastic microspheres of size range 1-5 µm with a density of 1.30 g/cm$^3$ were used as models for primary microplastics (Cospheric LLC, Goleta, USA). These particles were readily brought in suspension. Stock solutions of $10^8$ particles/mL were prepared by the addition of Elendt M4 medium followed by vortexing for 10 seconds. The number of particles was validated and adjusted by direct counts using hemocytometer.

Secondary microplastics were prepared as described by Ogonowski et al. (2016). Briefly, polyethylene spheres of sizes 850-1000 µm and with a density of 0.96 g/cm$^3$ (Cospheric LLC, Goleta, USA) were taken and ground in liquid nitrogen using a Retsch CryoMill (Retsch, Dusseldorf, Germany). The ground particles were then sieved using a 63-µm sieve (Retsch, Dusseldorf, Germany). Due to the irregular and coarse shape of ground particles, only particles of sizes roughly comparable to the primary microplastics (1-10 µm) could pass through. As the ground particles were static, they were subsequently centrifuged in 2-mL eppendorf tubes, with 750 µL of 0.1% solution of surfactant Tween 80 (Sigma-Aldrich) in Milli-Q water. Excess surfactant was discarded and the particles were centrifuged three times serially with Milli-Q water to remove the surfactant. The particles were then brought in suspension by addition of Elendt M4 to make stock suspensions of $10^7$ particles/mL; the number of particles was validated and adjusted by direct count using hemocytometer. By this forced weathering, the secondary particles were oddly shaped (Fig 2).

*2.4 TEM imaging of microplastics*

Transmission electron microscopy (TEM; JEOL 1010, JEOL Ltd., Tokyo, Japan) was used to ascertain the shape and size of PMPs and SMPs (Fig 2). Suspensions of PMP and SMP were centrifuged in 0.1% solution of surfactant Tween 80 and incubated for 1 h, prior to imaging.

*2.5 Acute toxicity test*

Acute toxicity assays were performed for all three species, using both primary and secondary microplastics at three different temperature points: 18 º, 22 º, and 26 ºC. Exposures were conducted using a modified OECD protocol (OECD, 2004), in which tests were conducted for 96 h rather than 48 h. Neonates (<24 h old) were held in 15 mL of M4 medium and exposed to control, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ particles/mL of either PMP or SMP (n=5 neonates per beaker, 4 replicates per treatment, and 8 replicates for controls). Stock suspensions were vortexed for 30 s each time prior to pipetting. To ensure that the microplastics remained in suspension, the test beakers were pipetted from bottom to top twice every day. For each set of experiments, the parent cultures were acclimatised to the exposure temperatures for at least four days prior to the start of the assays.

Every 24 h, the numbers immobilised and dead individuals were recorded. In all cases, control mortality was <10% after 48 h. At 18 ºC, control mortality was also <10% at 96 h, however, exposure at 22 º and 26 ºC resulted in increased mortality in the controls, especially in the two larger species: *D. magna* and *D. pulex*. Therefore, at 72 and 96 h a higher mortality rate ≤15% was considered acceptable.

*2.6. Modelling and Statistical Analyses*

*2.6.1 Toxico-kinetic - Toxico-dynamic modelling*

Survival data were analysed with the survival module of the Dynamic Energy Budget theory (Bedeaux and Kooijman, 1994) using Matlab (DEBtool, version R2016B). This is a toxico-

kinetic toxico-dynamic (TK-TD) model for survival based on the Stochastic Death model, which is accepted by the OECD for survival analysis (OECD 54, 2006).

The model uses four time-independent parameters to describe the whole time course of toxic effects:

- the Blank Mortality Rate (BMR), as a measure of background mortality ($h^{-1}$);
- the No Effect Concentration (NEC), as a sensitivity threshold below which no effects occur for any exposure time (particles/mL);
- the elimination rate ($k_e$), as a toxicokinetic trait that determines the equilibrium between internal and external concentration ($h^{-1}$);
- the killing rate ($k_r$) as a toxicodynamic trait that describes the toxic potency (damage potential) of the stressor ((particles/mL) $^{-1}$ $h^{-1}$ ).

The NEC, BMR, $k_e$ and $k_r$ were estimated using survival data for all three species at 18 º, 22 º and 26 ºC. The actual measured survival was plotted against the model prediction using these parameter values, to obtain survival surfaces for every species, at every temperature point (Figures S3-S5). Further, 48 h and 96 h $LC_{50}$ values were calculated using the time-independent parameter estimates of the model. The NEC was used as a measure for the toxicity of the microplastics. As the NEC is not time-dependent this is an excellent proxy to compare the sensitivity of different species (Jager et al., 2006). Additional information on model application is provided as supplementary information (S1).

## 3. Results

### 3.1. Temperature dependence of toxicity

The NEC estimates for *D. magna* and *D. pulex* during acute exposure to PMP and SMP declined sharply with temperature, indicating a marked increase in sensitivity of the species from 18 º to 26 ºC (Table 1; Fig. 3). For instance, NEC estimates of *D. magna* during exposure to PMP decreased from approximately $10^5$ particles/mL at 18 ºC to approximately 47 particles/mL at 26 ºC (Table 1; Fig. 3). For *D. pulex* the decrease was comparable, going from $10^5$ particles/mL at 18 ºC approximately 8 particles/mL at 26 ºC (Table 1; Fig. 3).

In contrast, the pattern of temperature-dependent increase in sensitivity was less pronounced in the case of *C. dubia* during exposure to both PMP as well as SMP, as NEC estimates did not vary as steeply as for the other two species (Table 1, Fig 3). For instance, the NEC for PMP exposure at 18 ºC was 5 x $10^3$ particles/mL whereas, at 26 ºC, it was approximately 500 particles/mL (Table 1, Fig 3).

### 3.2 Comparison of species sensitivity

Species sensitivity comparisons based on NEC estimates for PMP and SMP suggested that *D. magna* and *D. pulex* were of comparable sensitivity at all three temperatures. For example, the NEC of both species during PMP exposure at 18 ºC was roughly $10^5$ particles/mL. At the lowest temperature of 18 ºC, *C. dubia* was more sensitive than both other species, especially to PMP exposure reflecting in a NEC of 5 x $10^3$ particles/mL. However, the sensitivity of *D. magna* and *D. pulex* exhibited a drastic temperature-dependent increase while that of *C. dubia* showed much less variation across temperatures, as previously highlighted. As a result, at a temperature of 26 ºC the species *D. magna* and *D. pulex* were more sensitive compared to *C. dubia* (Fig 3). NEC values at 26 ºC NEC of PMP for *D. magna* and *D. pulex* were estimated to be 45 particles/mL and 8 particles/mL

240 respectively while that of *C. dubia* was 435 particles/mL.

241 *3.3. MP type influence on toxicity*

242 NEC estimates of *D. magna* and *D. pulex* for both PMP and SMP exposure were comparable
243 across all three temperatures (Table 1), suggesting that both types of microplastic had a
244 comparable toxicological impact on both species (Fig 3). As an example, at 18 ºC, the NEC
245 for *D. magna* and *D. pulex* during exposure to PMP was ~$10^5$ particles/mL, while that of SMP
246 were ~5 x $10^4$ particles/mL and ~$10^5$ particles/mL respectively.

247 In contrast, PMP was generally more toxic than SMP to *C. dubia* at all temperatures, which
248 was observed and fitted by the survival matrices. NEC estimates followed the same pattern,
249 but not at 18 ºC. For example, at 18 ºC the NEC during exposure to SMP was ~$10^5$
250 particles/mL while that of PMP was ~5 x $10^3$ particles/mL.

251 *3.4. Time dependence of toxicity*

252 Acute toxicological responses elicited by PMP and SMP increased with prolongation of time
253 of exposure from 48 h to 96 h for all species and temperatures, as could be seen from the
254 estimates of 48-h and 96-h $LC_{50}$ values of the DEB model, which differed by up to a few
255 orders of magnitude (Table 2). As an example, the 48-h and 96-h DEB $LC_{50}$ values of *D.*
256 *magna* exposed to PMP at 26 ºC were $10^8$ particles/mL and $10^4$ particles/mL, respectively.

257

258 ***4. Discussion***

259 To our best knowledge, this is the first study directly comparing the sensitivity of freshwater
260 species to both primary and secondary microplastics at three different temperatures.
261 Comparison of species sensitivity based on both NEC and $LC_{50}$ values indicated that *D.*
262 *magna* and *D. pulex* were of comparable sensitivities, but were less sensitive in comparison

263 to *C. dubia* at 18 ºC. However, *D. magna* and *D. pulex* showed a marked increase in
264 sensitivity to both PMP and SMP with an increase in temperature, while this had a lesser
265 impact on the acute sensitivity of *C. dubia*, causing the reversal of this trend at 26 ºC. This
266 pattern might relate to the intrinsic temperature tolerance of chosen species as a function of
267 their geographic distribution in natural habitats. *D. magna* and *D. pulex* are predominantly
268 temperate in distribution (Sarma et al., 2005) whereas *C. dubia* is a mainly tropical species
269 (although found in some temperate habitats). Therefore, as *D. magna* and *D. pulex* survive
270 optimally at 18-22 ºC temperatures as compared to *C. dubia*¸ which is more commonly found
271 at higher temperatures, they may be more influenced by the inclusion of temperature as an
272 additional stressor. Thus, interpreting temperature-dependent sensitivity of species in the
273 environment may also require consideration of climate change and the consequent increased
274 likelihood of temperature fluctuations. As the temperature has a major effect on sensitivity,
275 temperature corrections may also be necessary when translating toxicity data from laboratory
276 to the field (Heugens et al., 2003). There have been discussions about the lack biological
277 significance of standard dose-response testing outside of laboratory conditions (Newman &
278 Dixon 1996; Isnard et al., 2001). The sensitivity of organisms to contaminants can be
279 enhanced if organisms are outside or at the limits of their optimal environmental range (Van
280 Straalen, 2003). To understand the risks of PMP and SMP under environmentally relevant
281 conditions, there is therefore a need for multiple-stressor experiments that mimic
282 environmental variations, including changes in salinity, pH, and food availability.

283 These results also concur with a similar study of cadmium toxicity to *D. magna,* which
284 reported lower NEC and higher killing rates at elevated temperatures (Heugens et al., 2003).
285 The temperature dependent increase in sensitivity of *D. magna* and *D. pulex*, which was also
286 observed to a lesser extent in *C. dubia* is often related to the increase in metabolic turnover
287 at higher temperatures, which has been shown to relate to sensitivity (Baas and Kooijman,
288 2015). Higher metabolic rates could also cause faster use of lipid-reserves, resulting in

elevated feeding and ventilation rates (Heugens et al., 2003). This may in turn, cause increased ingestion of microplastics or accelerated clogging of respiratory apparatus by particulate contaminants in exposed organisms. An overall and broad comparison of species sensitivities suggests that acute sensitivity to microplastics decreases with body size at 18º C (*C. dubia*> *D. magna* ≥ *D. pulex)*; however, sensitivity increases with body size at 26º C (*D. pulex* ≥ *D. magna* > *C. dubia*). As energy demands and usage increase with body size (Goulden et al., 1982), the effect of starvation may be magnified for the larger species at elevated temperatures (where metabolic rates are enhanced). Furthermore, a similar study comparing the sensitivity of five Cladoceran species to copper nanoparticles (Song et al., 2015) also reported that *D. magna* and *D. pulex* were less sensitive than *C. dubia* during acute exposures at 20 ºC. Similarly, a study assessing the acute toxicity of silver nitrate reported that *C. dubia* was more sensitive than *D. magna* during 48-h assays in the absence of food (Naddy et al., 2011). These observations confirm that species sensitivities have variable trends and may differ for different compounds, underlining the need for multiple species comparisons during environmental risk assessment of toxicants.

In the present study, both PMP and SMP had comparable toxicological effects on *D. magna* and *D. pulex* during acute exposures at all temperatures, whereas PMP had more adverse effects on *C. dubia* in comparison to SMP. The PMP and SMP used in the current experiments were composed of different polymers. Therefore the observed effects may have been influenced by plastic additives or unbound monomers of particles (Ogonowski et al., 2016). However, this is unlikely as no toxic effects of leachates from plastics have been detected for *D. magna*, even at much higher exposure concentrations than those used in the present study (Lithner et al., 2009). Further, the propensity of microplastics to form aggregates in the gut following ingestion has been previously described and suggested to cause internal abrasions and mechanical damage (Ogonowski et al., 2016). This does raise the question if naturally occurring inert particles such as clay or kaolin, which may be

315 comparable in shape and size but are much more environmentally abundant than
316 microplastics could have similar toxic effects on species under study. Indeed some studies
317 have reported lower survival (Robinson et al., 2010) as well as lower overall growth and
318 fecundity (Kirk, 1992) when exposed to clay suspensions while others report no significant
319 negative effects due to natural minerals (kaolin particles) on Daphnids (Ogonowski et al.,
320 2016). Therefore, the inherent properties causing toxicity of microplastics, as well as their
321 associated mechanisms warrant further investigations.

322 It should be noted that the levels of exposure used in this study exceed reported
323 environmental levels. Despite their ubiquitous presence, enormous variability has been
324 reported in the observed microplastic concentrations in various geographic locations and
325 ecosystems. Aside from geophysical influences like wind, water current and waves (Wright et
326 al., 2013), reported MP concentrations are affected by the lack of standardized sampling
327 techniques, analytical methodologies and units of measurement (Besley et al., 2017, Phuong
328 et al., 2016). For instance, concentrations as high as 9200 particles/$m^3$ were reported in parts
329 of the North-East Pacific Ocean (Desforges et al., 2014) whereas concentrations as low as
330 0.004 particles/$m^3$ were reported in other parts of the North-Pacific ocean (Doyle et al.,
331 2011). Quantitative estimations of environmental microplastics in freshwater ecosystems also
332 reflect similar variability. A recent study of the river sediments in the Shanghai region of
333 China indicated approximately 800 particles/ kg dry weight of sediment (Peng et al., 2018).
334 Importantly, many of these studies focus on larger pieces of microplastics, while the levels of
335 microplastics in the size ranges used in the current experiment are very poorly understood,
336 due to detection difficulties (Huvet et al., 2016).
337
338 However, the acute NEC and $LC_{50}$ estimates for both PMP and SMP, for all species and
339 temperatures are well above the highest reported levels of microplastics found in the
340 environment. This is in line with other acute toxicity studies using microplastics. For example,

a study of the acute toxicity of 1µm polyethylene microspheres to *D. magna* (Rehse et al., 2016) reported a 96-h $LC_{50}$ of 57.43 mg/L (approximately $10^7$ particles/mL). Another study assessing the acute toxic effects of polypropylene microplastic fibers on *Hyalella azteca* reported an $LC_{50}$ of 4.6 x $10^4$ particles/mL after 10 days of exposure (Au et al., 2015). However, it is important to note that the annual increase in plastic production coupled with the minimal capacity of plastics to undergo biological degradation, suggests that concentrations are likely to build up in the coming years (Eerkes-Medrano et al., 2015).

Comparison of 48 h and 96 h $LC_{50}$ values indicated a strong time dependence of toxicity, as has been previously suggested in a study assessing the acute toxicity of polyethylene microspheres to *D. magna* (Rehse et al., 2016). A similar observation was also made in a study investigating the acute exposure effects of nano-materials to *D. magna* (Baumann et al., 2014). The marked increase in toxicity when the exposure time is prolonged to 96 h highlights the need for modifications of existing testing standards, which normally stipulate 48 h of exposure for acute toxicity assays (Rehse et al., 2016).

## 5. Conclusion

The current study presents a comparison of the sensitivity of two temperate and one tropical Cladoceran species, during acute exposure to primary and secondary microplastics, in the presence of temperature as an additional stressor. The acute sensitivity of *D. magna* and *D. pulex* showed a temperature-dependent increase, whereas that of *C. dubia* remained stable across temperatures. *C. dubia* was the most sensitive species during acute exposure at 18 ºC, followed by *D. pulex* and *D. magna*, which were of comparable sensitivities, however, this trend was reversed at 26 ºC. These results suggest that it is important to include multiple stressors to mimic more environmentally relevant conditions of exposure, and that temperature might be an important factor to include in the interpretation of sensitivity of

366 species and toxicity of microplastics. Both PMP and SMP had comparable effects on *D.*
367 *magna*, but PMP had higher levels of toxic effect on *C. dubia* than SMP. Effects on survival
368 were strongly time-dependent and became substantially more severe after the standard 48 h
369 test period. Results of the present study show that acute mortality to microplastics is species-
370 specific, dependent on the type of microplastic exposed, and largely influenced by the
371 temperature of exposure.

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380 **Supplementary information:**

381 **Figure S1:** Survival surface for Ceriodaphnia dubia exposed to PMP at 22 ºC. Actual
382 measured survival ('+') is plotted against model predicted values (smooth lines) using
383 parameter estimates.

384 **Figure S2:** Deviance of best fitting NEC parameter estimates for *Ceriodaphnia dubia*
385 exposed to PMP at 22 ºC.

386 **Figure S3:** Survival surfaces from TK-TD modelling of *Daphnia magna* during acute
387 exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f)
388 SMP at 26 ºC

**Figure S4:** Survival surfaces from TK-TD modelling of *Daphnia pulex* during acute exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26 ºC

**Figure S5:** Survival surfaces from TK-TD modelling of *Ceriodaphnia dubia* during acute exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26 ºC

**Supplementary information 1.** Application of the Toxico-kinetic and Toxico-Dynamic (TK-TD) model

**Reference List**


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## List of table titles:

**Table 1:** Time-independent parameter estimates as log(concentration) ± standard deviation (SD) from Toxicokinetic-Toxicodynamic (DEB) modelling of survival data. Data obtained from 96 h acute toxicity tests performed on *Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia* at 18 °, 22 ° and 26 °C. BMR – Blank Mortality Rate, NEC – No Effect Concentration, $K_e$ – Elimination rate, $K_r$ – Killing rate

Footer 1: * indicates more minima in parameter estimates. Reported parameter estimates obtained by comparisons with independent parameter estimates as well as survival data.

**Table 2:** Estimates log-transformed 48 h $LC_{50}$ and 96 h $LC_{50}$ values (particles/mL) from DEB model for primary (PMP) and secondary (SMP) microplastics during exposure to *Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia* at 18 °, 22 ° and 26 °C.

605 **Table 1:** Time-independent parameter estimates as log(concentration) ± standard deviation
606 (SD) from ToxicoKinetic-ToxicoDynamic (TK-TD) modelling of survival data. Data obtained
607 from 96 h acute toxicity tests performed on *Daphnia magna*, *Daphnia pulex* and
608 *Ceriodaphnia dubia* at 18, 22 and 26 °C. BMR - Blank Mortality Rate, NEC - No Effect
609 Concentration, $K_e$ - Elimination rate, $K_r$ - Killing rate
610

| Species | Type of MP | Temp [°C] | BMR [$(h)^{-1}$] | NEC [log(particles/mL)] | $K_r$ [$(h)^{-1}$] | $K_e$ [$log(particles/mL)^{-1}(h)^{-1}$] |
|---|---|---|---|---|---|---|
| *Daphnia magna* | PMP | 18 | <0.0001±0.0000 | 5.00±2.10 | 0.0006±0.0010 | 0.2000±0.0000 |
| | | 22* | 0.0026±0.0005 | 3.50±0.00 | 0.0400±0.0000 | 0.0150±0.0080 |
| | | 26* | 0.0017±0.0005 | 1.67±0.60 | 0.0400±0.0000 | 0.0100±0.0040 |
| | SMP | 18 | <0.0001±0.0000 | 4.70±0.24 | 0.0064±0.0024 | 0.0520±0.0120 |
| | | 22* | 0.0016±0.0046 | 3.50±0.00 | 0.0400±0.0000 | 0.0150±0.0070 |
| | | 26* | 0.0013±0.0005 | 0.75±0.27 | 0.0400±0.0000 | 0.0070±0.0020 |
| *Daphnia pulex* | PMP | 18 | 0.0002±0.0001 | 5.00±0.00 | 0.0200±0.0000 | 0.0200±0.0000 |
| | | 22* | 0.0003±0.0002 | 0.85±0.29 | 0.0200±0.0000 | 0.0044±0.0013 |
| | | 26* | 0.0021±0.0008 | 0.92±0.43 | 0.0200±0.0000 | 0.0110±0.0040 |
| | SMP | 18 | <0.0001±0.0000 | 5.00±0.90 | 0.0056±0.0037 | 0.2800±0.1800 |
| | | 22* | 0.0002±0.0002 | 1.01±0.36 | 0.0200±0.0000 | 0.0079±0.0025 |
| | | 26* | 0.0016±0.0007 | 1.13±0.47 | 0.0200±0.0000 | 0.0160±0.0015 |
| *Ceriodaphnia dubia* | PMP | 18* | 0.0005±0.0003 | 3.70±0.12 | 0.0220±0.0044 | 0.0890±0.0150 |
| | | 22* | 0.0002±0.0000 | 2.60±0.00 | 0.0160±0.0000 | 0.0500±0.0000 |
| | | 26* | 0.0003±0.0000 | 2.64±0.00 | 0.0150±0.0000 | 0.1100±0.0000 |
| | SMP | 18* | 0.0002±0.0002 | 5.00±0.00 | 0.0038±0.1000 | 0.1100±0.0400 |
| | | 22* | 0.0004±0.0000 | 2.50±0.00 | 0.0230±0.0000 | 0.2500±0.0000 |
| | | 26* | 0.0008±0.0000 | 3.60±0.00 | 0.0060±0.0000 | 0.2000±0.0000 |

611
612
613 *more minima in parameter estimates. Reported parameter estimates obtained by comparisons with independent
614 parameter estimates as well as survival data.
615

**Table 2:** Estimates log-transformed 48 h $LC_{50}$ and 96 h $LC_{50}$ values (particles/mL) from DEB model for primary (PMP) and secondary (SMP) microplastics during exposure to *Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia* at 18 °, 22 ° and 26 °C.

| Type of MP | Temp | *D. magna* | | *D. pulex* | | *C. dubia* | |
|---|---|---|---|---|---|---|---|
| | | 48 h $LC_{50}$ | 96 h $LC_{50}$ | 48 h $LC_{50}$ | 96 h $LC_{50}$ | 48 h $LC_{50}$ | 96 h $LC_{50}$ |
| PMP | 18 | 32.0 | 18.0 | 13.0 | 7.6 | 5.1 | 4.2 |
| | 22 | 10.0 | 5.8 | 15.0 | 5.7 | 5.1 | 3.5 |
| | 26 | 8.0 | 4.0 | 6.8 | 3.0 | 4.2 | 3.3 |
| SMP | 18 | 10.0 | 6.7 | 8.0 | 6.4 | 4.8 | 4.1 |
| | 22 | 10.0 | 5.8 | 9.3 | 3.9 | 9.0 | 5.8 |
| | 26 | 6.5 | 2.8 | 5.5 | 2.6 | 6.6 | 5.0 |

622 **List of Figures:**

623

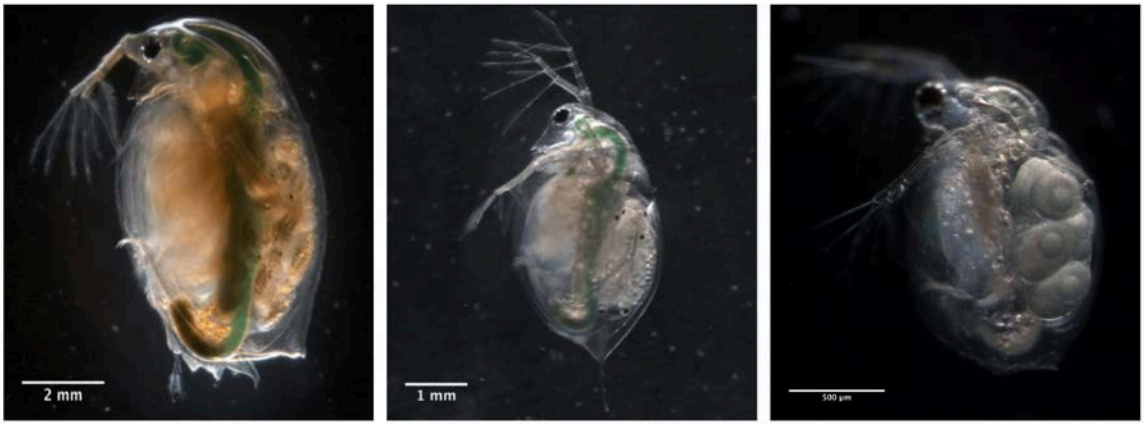
624 **Figure 1:** Species of Cladocerans used in the study: a) *Daphnia magna*, b) *Daphnia pulex*, c)
625 *Ceriodaphnia dubia.*

626

627 **Figure 2:** Transmission Electron Microscopy (TEM) images of microplastics used in the
628 study. a) Primary microplastics of spherical shape and sizes between 1-5 μm. b) Secondary
629 microplastics of irregular shapes and sizes 1-10 μm

630

631 **Figure 3:** The log-transformed No Effect Concentration (NEC) estimates for primary (PMP)
632 and secondary (SMP) microplastics at three different temperatures for *Daphnia magna* (blue,
633 diamond), *Daphnia pulex* (red, triangle) and *Ceriodaphnia dubia* (green, square) based on
634 acute (96 h) exposures. Solid and dashed lines indicate trends for PMP and SMP
635 respectively.

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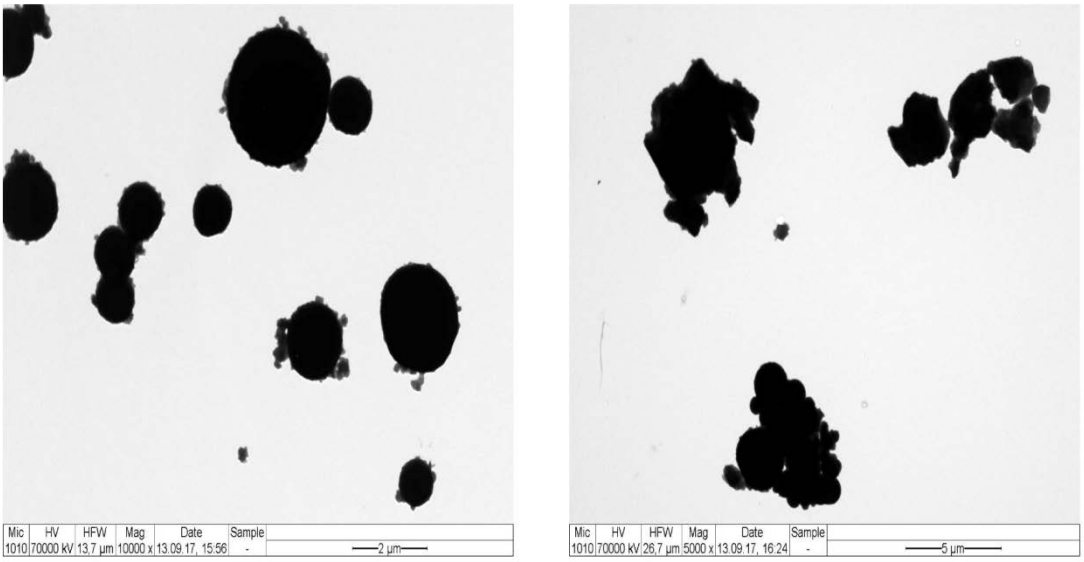
638 *Ceriodaphnia dubia.*

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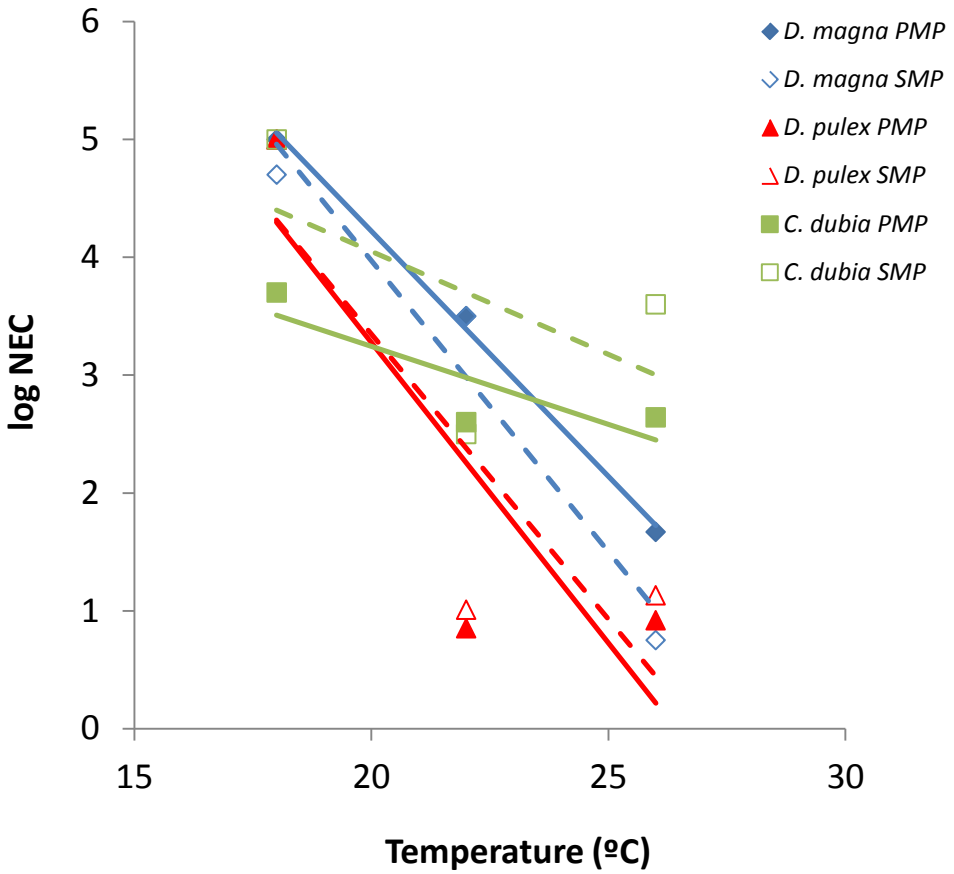



**Figure 3:** The log-transformed No Effect Concentration (NEC) estimates for primary (PMP) and secondary (SMP) microplastics at three different temperatures for *Daphnia magna* (blue, diamond), *Daphnia pulex* (red, triangle) and *Ceriodaphnia dubia* (green, square) based on acute (96 h) exposures. Solid and dashed lines indicate trends for PMP and SMP respectively.

**Supplementary information:**

**Table S1:** Survival matrix for *Ceriodaphnia dubia* exposed to PMP at 22 °C.

**Figure S1:** Survival surface for Ceriodaphnia dubia exposed to PMP at 22 ºC. Actual measured survival ('+') is plotted against model predicted values (smooth lines) using parameter estimates.

**Figure S2:** Deviance of best fitting NEC parameter estimates for *Ceriodaphnia dubia* exposed to PMP at 22 ºC.

**Figure S3:** Survival surfaces from TK-TD modelling of *Daphnia magna* during acute exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26 ºC

**Figure S4:** Survival surfaces from TK-TD modelling of *Daphnia pulex* during acute exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26 ºC

**Figure S5:** Survival surfaces from TK-TD modelling of *Ceriodaphnia dubia* during acute exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26 ºC

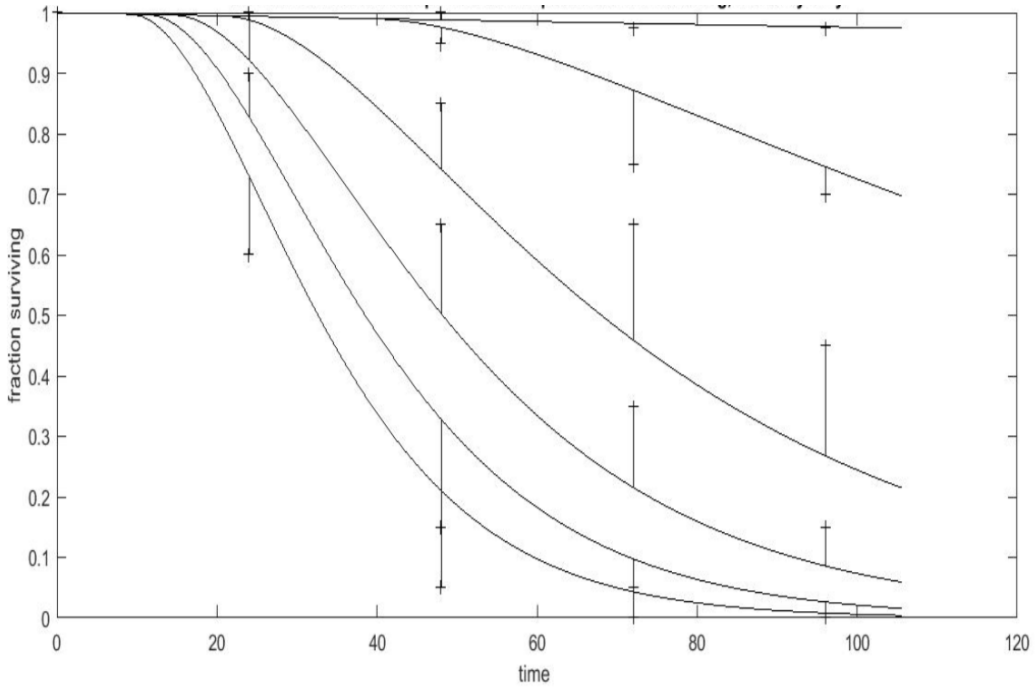
**Supplementary information 1.** Application of the Toxico-kinetic and Toxico-Dynamic (TK-TD) model

671 **Table S1:** Survival matrix for *Ceriodaphnia dubia* exposed to PMP at 22 °C.

| Time | Treatment | | | | | |
|---|---|---|---|---|---|---|
| (hr) | (log (concentration particles/ml)) | | | | | |
| | Control | 3 | 4 | 5 | 6 | 7 |
| 0 | 40 | 20 | 20 | 20 | 20 | 20 |
| 24 | 40 | 20 | 20 | 20 | 18 | 12 |
| 48 | 40 | 19 | 17 | 13 | 3 | 1 |
| 72 | 39 | 15 | 13 | 7 | 1 | 0 |
| 96 | 39 | 14 | 9 | 3 | 0 | 0 |

672

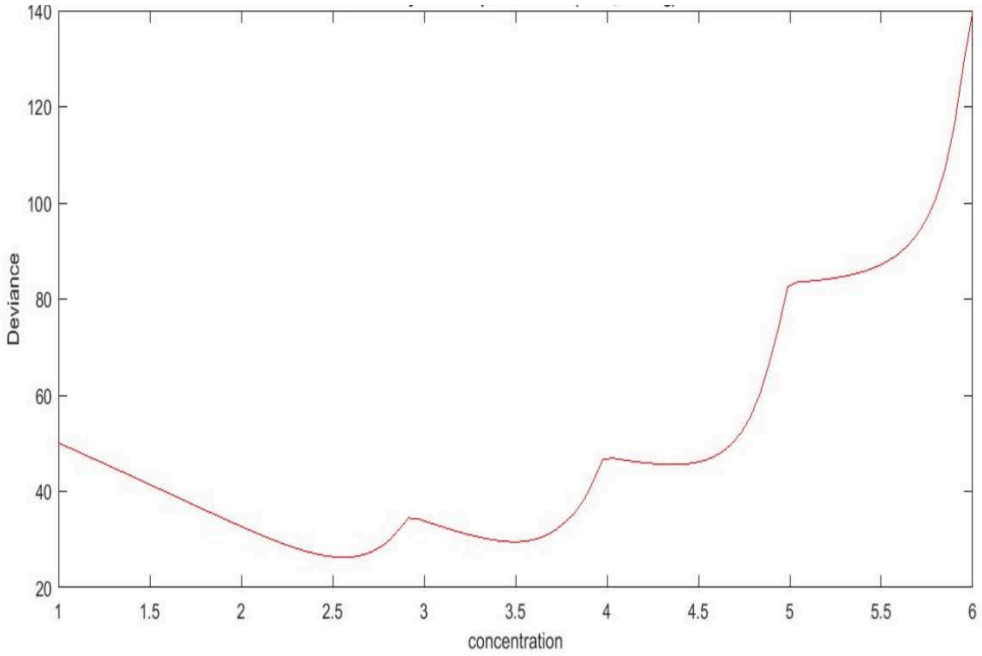
673




674

675 **Figure S1:** Survival surface for *Ceriodaphnia dubia* exposed to PMP at 22 ºC. Actual
676 measured survival ('+') is plotted against model predicted values (smooth lines) using
677 parameter estimates.

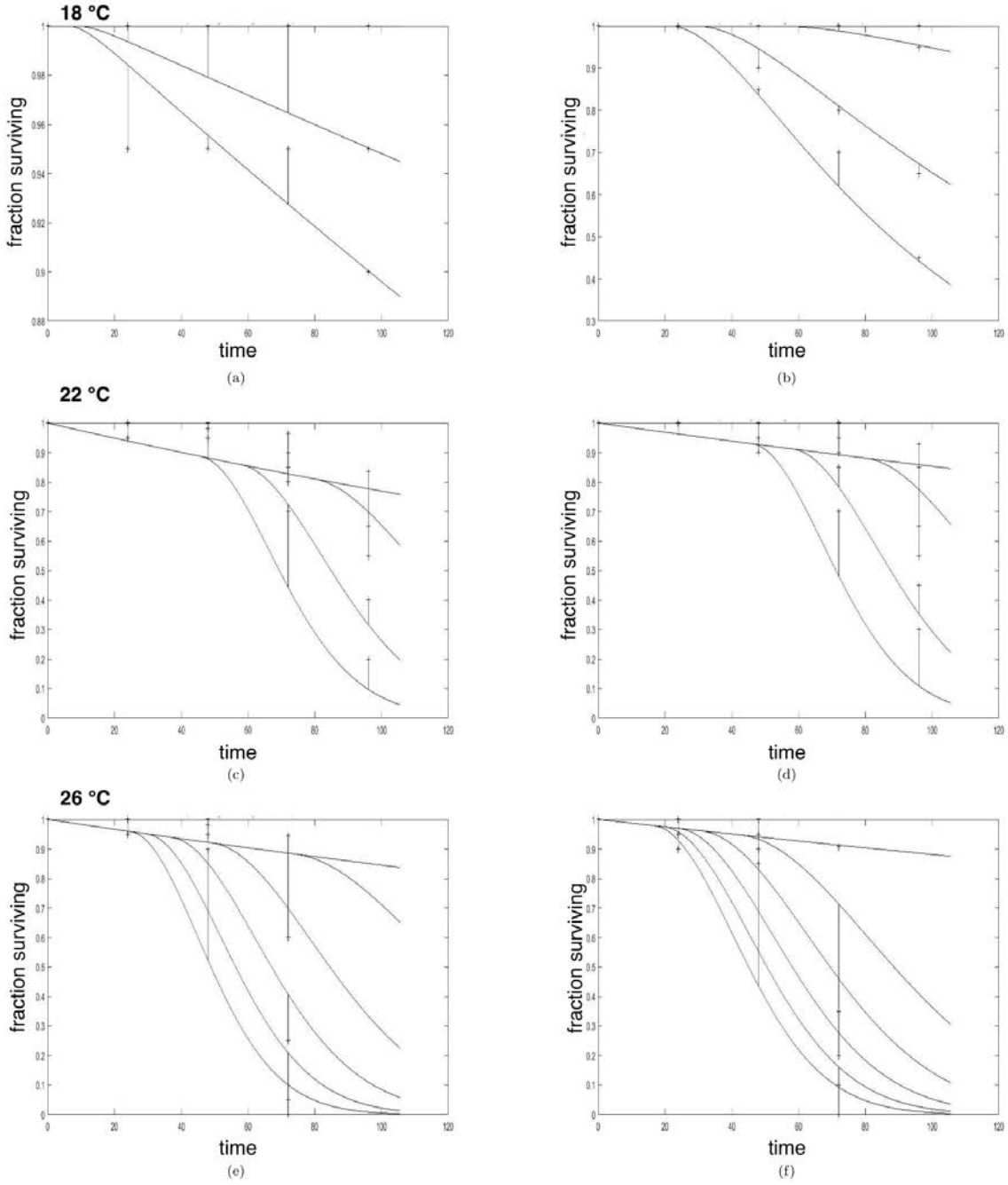
678




679

680 **Figure S2:** Deviance of best fitting NEC parameter estimates for *Ceriodaphnia dubia*
681 exposed to PMP at 22 ºC.

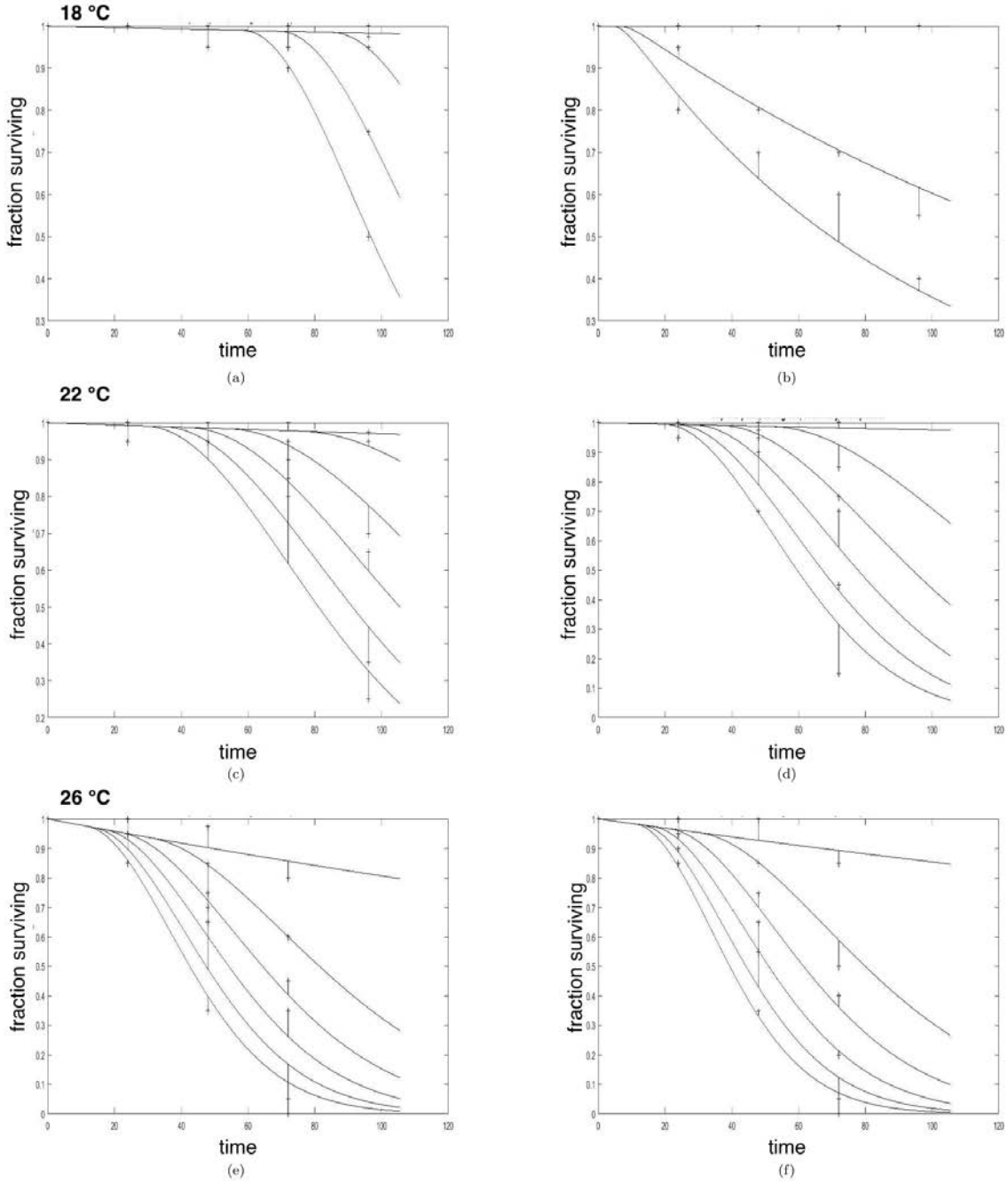
682




683

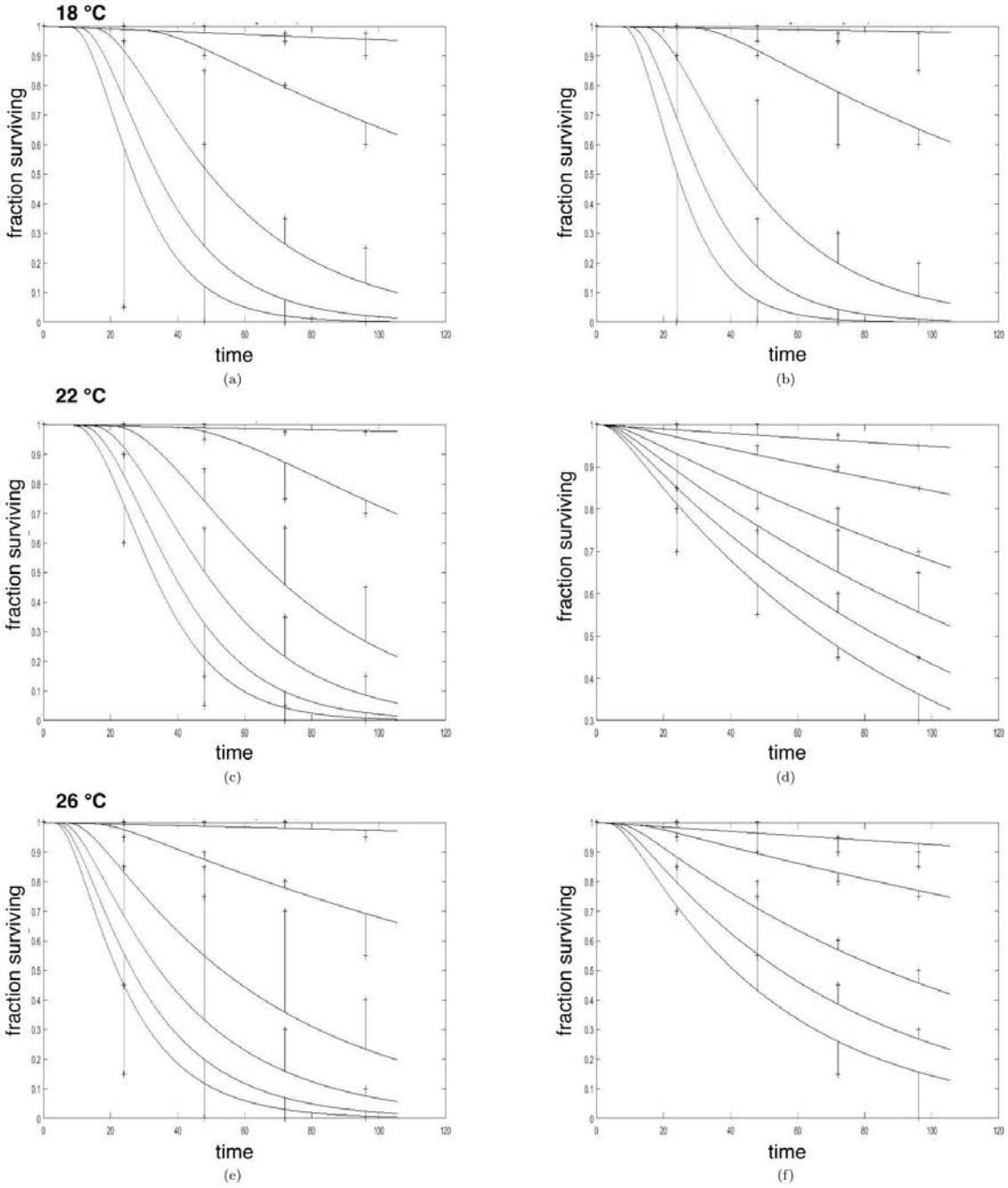
684 **Figure S3:** Survival surfaces from TK-TD modelling of *Daphnia magna* during acute
685 exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f)
686 SMP at 26 ºC

687




688

689 **Figure S4:** Survival surfaces from TK-TD modelling of *Daphnia pulex* during acute exposure
690 to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f) SMP at 26
691 ºC




692

693 **Figure S5:** Survival surfaces from TK-TD modelling of *Ceriodaphnia dubia* during acute
694 exposure to a) PMP at 18 º b) SMP at 18 º c) PMP at 22 º d) SMP at 22 º e) PMP at 26 º f)
695 SMP at 26 ºC

696

**Supplementary information 1.** Application of the Toxico-kinetic and Toxico-Dynamic (TK-TD) model

A Toxico-kinetic and Toxico-Dynamic (TK-TD) model was used for the estimation of parameter values. These parameter values can be interpreted in terms of the sensitivity of the different species to microplastics. To illustrate the application of the model to derive parameter estimates, a randomly chosen example (*Ceriodaphnia dubia* exposed to PMP at 22°C) is given in Table S1.

This gives the following parameter estimates:

- **BMR:** 2.45 * $10^{-4}$ (2.55 *$10^{-4}$)$h^{-1}$
- **NEC:** 2.6 (0.18)
- **$K_r$:** 0.016 (0.0028)
- **$K_e$:** 0.049 (0.0088) $h^{-1}$

In Fig.S1, the actual measured survival (+) is plotted against the model prediction (the lines) with these parameter values.

The best fitting parameter set is shown, however, there is a statistical probability that the effect at 96 h was caused by background control mortality and not by the toxicant. Therefore a second minimum exists at a concentration of ~3.5, which is shown by plotting the deviance against the value of the NEC (see Fig.S2). Each minimum represents a set of parameter values with a good fit. The deepest minimum (in this case at a NEC ~2.6) represents the most likely value.

In this case, there is even a third and fourth minimum around concentrations of ~4.5 and

719 ~5.5 respectively but with decreasing probability. If the NEC is higher this implies that the
720 control mortality and the killing rate should be higher to explain the effect, which was indeed
721 the case. The minimum at a concentration of 3.5 has the following set of parameter values:

722 • **BMR:** 0.0011 ($4.3 * 10^{-4}$) $h^{-1}$

723 • **NEC:** 3.5 (0.18)

724 • **$K_r$:** 0.029 (0.0061)

725 • **$K_e$:** 0.060 (0.0090) $h^{-1}$

726 An independent estimate of the control mortality shows that this is estimated to be $2.45 * 10^{-4}$
727 $h^{-1}$, which is very close to the first estimate of $2.3 * 10^{-4}$E-04 $h^{-1}$ . This gives an independent
728 confirmation of the parameter estimates. Therefore comparisons with independent data,
729 (including an independent estimate of the control mortality) as well as survival data at
730 different temperatures and different species, are important in cases where multiple minima
731 exist in parameter estimates. This enables the determination of the most likely set of
732 parameter values, not only from a statistical point of view but also from a biological point of
733 view.