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Single and combined toxicity of polystyrene microplastics and pharmaceutical diclofenac to neonate and adult *Daphnia magna*

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Tiivistelmä - Referat – Abstract

Mikromuoveja (MP) löytyy maailmanlaajuisesti suurimmasta osasta ekosysteemejä, ja niiden vaikutusta vesieliöihin on selvitetty monessa tutkimuksessa. Myös lääkeaineita tavataan runsaasti vesiympäristössä. Useiden haitta-aineiden samanaikainen esiintyminen johtaa tarpeeseen ymmärtää niiden yhteenlaskettua myrkyllisyyttä. Se voi poiketa haitta-aineiden itsenäisesti aiheuttamasta myrkyllisyydestä, liittyen esimerkiksi mikromuovien kykyyn toimia vektorina monille saasteille. Haitta-aineiden yhteisvaikutus voi olla additiivinen, synergistinen, jolloin myrkyllisyys on suurempi, tai antagonistinen, jolloin myrkyllisyys on pienempi kuin haitta-aineilla yksistään. Tämän tutkimuksen tavoitteena oli selvittää yleisesti esiintyvän mikromuovin, polystyreenin (PS), sekä yleisen lääkeaineen diklofenaakin (DCF) myrkyllisyyttä *Daphnia magna* -vesikirpulle kahdessa eri kehitysvaiheessa, neonaateille poikasille ja täysikasvuisille vesikirpuille, sekä yksistään että aivan ensimmäisiä kertoja myös yhdistelmänä. Seurattavia parametrejä olivat liikkumattomuus sekä oksidatiiviseen stressiin liittyvät reaktiiviset happiradikaalit (ROS), ja altistuksissa käytetyt PS- ja DCF-pitoisuudet vaihtelivat 0.01 mg/L ja 100 mg/L välillä.

PS- ja DCF-altistukset sekä yksittäin että yhdistelmänä lisäsivät merkitsevästi liikkumattomuutta *D. magnalla*. Yksittäisten altistusten merkitsevä vaikutus havaittiin neonaateissa poikasissa 48 tunnin jälkeen, ja yhteisvaikutus neonaateissa 24 ja 48 tunnin sekä täysikasvuisissa vesikirpuissa 48 tunnin jälkeen. Arvioitu EC_{50} arvo viittasi PS-mikromuovin ja diklofenaakin yhdistelmän synergistiseen vaikutukseen. Selvimmät muutokset ROS-tasossa havaittiin yhteisaltistusten seurauksena, todennäköisesti osoituksena oksidatiivisesta stressistä etenkin neonaateilla vasteena yhdistelmän kasvavaan PS-pitoisuuteen. Tulokset myös viittaavat neonaattien olevan herkempiä tutkituille aineille. Havaitut PS-mikromuovin ja diklofenaakin haitalliset vaikutukset *D. magnaan*, etenkin niiden yhdistelmälle arvioitu synergistinen vaikutus, korostavat aiheen merkitystä ja lisätutkimuksen tarvetta liittyen mikromuovien ja lääkeaineiden sekä muiden ympäristössä esiintyvien haitta-aineiden yhteenlaskettuun myrkyllisyyteen vesieliöille, sekä samalla mahdollisiin laajempiin vaikutuksiin vesiekosysteemeissä.

Avainsanat – Nyckelord Mikromuovi, polystyreeni, diklofenaakki, myrkyllisyys, yhteisvaikutus, *Daphnia magna*, kehitysvaiheet	
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Tiivistelmä - Referat – Abstract

Microplastics (MP) are found globally in most ecosystems, and many studies have investigated their effects on aquatic organisms. Pharmaceuticals are also widely present in the aquatic environment. The co-occurrence of multiple contaminants creates a need to understand their combined toxicity. It can differ from the single toxicity of the contaminants, relating to, for example, the ability of MPs to act as vectors for many other pollutants. The combined effect can be additive, synergistic with higher toxicity, or antagonistic with lower toxicity than that of the contaminants individually. The aim of this study was to investigate the toxicity of a commonly occurring MP, polystyrene (PS), and a common pharmaceutical diclofenac (DCF) to two life stages of *Daphnia magna,* neonates and adults, both individually and, among the very first times, in combination. The endpoints used were immobilization and reactive oxygen species (ROS), related to oxidative stress, exposure concentrations ranging from 0.01 mg/L to 100 mg/L.

A significant decrease in *D. magna* mobility was observed after individual and combined exposures to PS and DCF. A significant individual effect was seen on neonate daphnids after 48 h and a significant combined effect on neonates after 24 and 48 h as well as adults after 48 h. Median effective concentration (EC_{50}) estimation for neonates after 48 h suggested a synergistic effect of the combination of PS and DCF. The clearest changes in ROS levels were observed with combined exposures, probably indicating oxidative stress especially in neonates as a response to increasing PS concentration in the combination. The results also suggest higher sensitivity of neonates for the contaminants studied. The observed adverse effects of PS and DCF on *D. magna*, especially the estimated synergistic effect of their combination, emphasize the importance of the topic and the need for more studies concerning the combined toxicity of MPs and pharmaceuticals and other environmental contaminants to aquatic organisms as well as the possible wider effects on aquatic ecosystems.

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Abbreviations

BSA	Bovine serum albumin
CAT	Catalase
CBZ	Carbamazepine
COX	Cyclooxygenase
DCF	Diclofenac
EC_{50}	Median effective concentration
LD_{50}	Median lethal dose
LOAEC	The lowest observed adverse effect concentration
MP	Microplastic
MDA	Malondialdehyde
NOAEC	No observed adverse effect concentration
NP	Nanoplastic
NSAID	Nonsteroidal anti-inflammatory drug
PE	Polyethylene
PP	Polypropylene
PPCP	Personal care product
PS	Polystyrene
PS-MP	Polystyrene microplastic
ROS	Reactive oxygen species
ROX	Roxithromycin
SOD	Superoxide dismutase
TCS	Triclosan

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1 Introduction

1.1 Microplastic pollution

1.1.1 Global plastic pollution

Global plastic pollution is a growing concern. The use of plastics has changed dramatically since the invention of the first synthetic plastics in the beginning of 20th century (Geyer et al., 2017). The special characteristics of plastic, such as its versatility and durability as well as its low price, led to its use expanding from military purposes to other products as well (Dey et al., 2021; Jansen, 2023). Plastic polymers, most often made from monomers obtained from fossil hydrocarbons (Jansen et al., 2023), are nowadays used for many purposes from packaging to building, consumer products, transportation and electronics (Geyer et al., 2017). In 2017 the annual production was already around 438 million tons (UNEP, 2021), and it has been estimated that a total of 8.3 billion tons of plastic had been produced by 2020 (Dey et al., 2021). Only 10% of all plastic has been recycled or reused since 1950s (UNEP, 2021), and almost 60% of all the plastic produced has been estimated to have ended up to landfills or to the natural environment (Dey et al., 2021), which may result in around 12 billion tons of plastic waste by 2050 (Jansen et al., 2023).

1.1.2 Microplastics

Plastic debris found in nature can be either macro-, meso-, micro- or nanoplastic, depending on its size. The definition of microplastic (MP) size varies between publications, but the classification used by e.g. The European Food Safety Authority (EFSA, 2016) is 0,1-5000 µm for MP. (Hartmann et al., 2019; Pikuda et al, 2023.) MPs can be primary or secondary particles. Primary MPs are manufactured plastic particles, that are used e.g. in cosmetics, pharmaceuticals, and clothing, and that end up in the environment as micro sized particles (Funke et al., 2024; Atugoda et al., 2021). Secondary MPs and further secondary nanoplastics (NPs) form when macro- and mesoplastics degrade in the environment e.g. as a result of photodegradation and other physical, chemical and biological factors causing the fragmentation of the macromolecules (Atugoda et al., 2021; Prasad et al., 2023). Polyethylene (PE), polystyrene (PS) and polypropylene (PP) are the MP polymers most often found in the aquatic environments (Mao et al., 2018).

1.1.3 MP in the environment

MP can be found globally in most ecosystems, involving land, air and aquatic environments (Funke et al., 2024; Samadi et al., 2022). Because of sewage discharge and rainwater scouring, a larger

amount of MP particles ends up in aquatic environments (Funke et al., 2024). MPs have been found in seas both in pelagic and benthic areas, as well as in coasts, estuaries and fresh waters, also including arctic lakes (Atugoda et al., 2021). MP concentrations found in different aquatic environments vary between areas and studies (Table 1). That can be explained by several factors, such as the proximity to anthropogenic activities, but also different sampling methods that can affect the observations (Grbić et al., 2020; Talbot & Chang, 2022). The sampling method affects e.g. when and where the sampling can be performed, as well as what MP particles sizes can be detected (Uurasjärvi et al., 2020). In addition, other factors such as seasonality and precipitation can affect observed MP concentrations. Different sampling methods and other sampling procedures can impact the reported MP concentrations and comparability between studies. (Talbot & Chang, 2022.)

Table 1. *Examples of MP concentrations observed in freshwater and marine environments.*

Body of water	Location information	MP concentration	References
Antuã River	Portugal	5–51.7 mg/m³	Rodrigues et al. (2018)
Baltic Sea	Gulf of Finland	0.2–1.3 particles/m³	Setälä et al. (2016)
Florida Keys lagoon	US	76000 PS particles/L	Badylak et al. (2021)
Lake Kallavesi	Finland	0.27–155 particles/m³	Uurasjärvi et al. (2020)
Lake Taihu	China	3.4–25.8 particles/ L	Su et al. (2016)
Mediterranean Sea	Israeli coast	7.68 ± 2.38 particles/m³	van der Hal et al. (2017)

MP concentration (mg/L) can be estimated from the number of particles per liter if the weight of the average MP particle is known (Esterhuizen et al., 2023). Hwang et al. (2020) have calculated the average weight of 3 µm PS particles to be 0.015 ng. Based on their estimates including the weight and also the annual intake of particles of different sizes, it is possible to propose that the weight of a 100 µm PS-MP particle would be about 0.5 ng. The size of the PS particles in the study by Badylak et al. (2021) ranged from 33 to 190 µm, with an average closer to 100 µm. Based on the estimated values, the 76000 particles in liter reported in Florida Keys could be converted to a concentration in the range of approximately 30-40 µg/L for PS-MPs alone.

1.2 Pharmaceutical pollution

1.2.1 Pharmaceuticals

Increasing attention has also been paid to the prevalence of pharmaceutical compounds in aquatic environments and their potential toxicity (Haap et al., 2008; Parolini, 2020). Medicinal substances

are widely used to treat both humans and animals, and despite of regulations big amounts of pharmaceuticals end up in the environment (Drzymała & Kalka, 2024; Parolini et al., 2020). Urban wastewater is a particularly significant source of pharmaceutical emissions globally. In addition, hospitals, industry, and agriculture, among others, are responsible for pharmaceuticals ending up in the environment (aus der Beek et al., 2016). The efficiency of wastewater treatment plants in removing pharmaceuticals is often low, and pharmaceuticals and their metabolites most typically end up in water systems through them, causing significant pharmaceutical pollution especially in surface waters. Pharmaceuticals consist of different kinds of substances that are intended to cure medical conditions by producing a specific effect on the target organism. (Parolini et al., 2020.)

1.2.2 Nonsteroidal anti-inflammatory drugs and diclofenac

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain and inflammation. They are one of the most commonly occurring pharmaceutical groups in the environment, where they end up both in their original form and as metabolites after excretion. (Du et al., 2016; Parolini, 2020.) The NSAID most often found in the aquatic samples worldwide is diclofenac (DCF), 2-(2-(2,6-dichlorophenyl)amino)phenyl)acetic acid. It is particularly used for diseases such as arthritis, rheumatoid arthritis and osteoarthritis. (aus der Beek et al., 2016; Olaitan et al., 2014; Parolini, 2020.) DCF has been in use since the 1970s, and today it is globally used to a large extent as an over-the-counter medicine for humans, as well as for treating domestic animals (Drzymała & Kalka, 2024; Parolini, 2020; Sathishkumar et al., 2020). Wastewater treatment plants are not able to remove most of the DCF, and its concentrations have been increasing (Sathishkumar et al., 2020). One characteristic of DCF is its susceptibility to photodegradation. Especially in summertime its environmental concentration can be affected by light, but on the other hand photodegradation can also lead to the formation of by-products, some of which may have potentially even more adverse effects on the environment than the parent compound itself. (Boreen et al., 2003; Qutob et al., 2023; Sathishkumar et al., 2020.)

1.2.3 DCF in the environment

Despite the fact that the consumption of many medicines has decreased due to regulation, e.g. in the EU, the use of DCF has continued to rise and the global annual consumption of DCF has increased from an average of 1443 tons in the early 2010s (Acuña et al., 2015; Drzymała & Kalka, 2024) to 2119 tons in 2020 (Acuña et al., 2020). It is estimated that around 75% of the used DCF ends up in the environment. DCF can be found in multiple aquatic environments including surface waters, groundwater, seawater and wastewater. It ends up to surface waters, e.g. in lakes, canals and rivers, especially in densely populated areas or near extensive farming. (Sathishkumar et al., 2020.) Among

the highest environmental concentrations reported for DCF so far are 15.013 µg/L in Erft river in Germany (Jux et al., 2002) and 18.74 µg/L in surface water in western Europe (aus der Beek et al., 2016). Also, an even higher DCF concentration of 57.16 µg/L has been detected in surface water of an irrigation canal in Nigeria (Sathishkumar et al. 2020), while the global average has been estimated to be 0.032 µg/L in surface, ground and drinking waters (aus der Beek et al., 2016). Reported concentrations of DCF and other NSAIDs in the aquatic environment may not seem high, but the increasing discharge and high biological activity of these pharmaceuticals may cause potential harm to non-target organisms (Du et al., 2016; Parolini, 2020).

1.3 Toxic effects of MPs and pharmaceuticals

1.3.1 Effect of PS-MP

There is a large number of research investigating the toxicity of MP to aquatic organisms, especially to the cladocerans *Daphnia* spp. Many studies have concentrated on the species *Daphnia magna*. (Funke et al., 2024; Pikuda et al., 2023.) There is a lot of variation in the MPs used in those studies, including different plastic polymers, sizes, and shapes, and the effect of PS-MP have been investigated in sizes from 0.02 - 150 µm and with concentrations ranging from 0.01 mg/L to 1000 mg/L (Pikuda et al., 2023). Variation of i.e. MP polymers, sizes and shapes can however make the comparison between studies difficult, especially when not all studies provide all this information (Pikuda et al. 2023; Samadi et al, 2022). In addition, MP types used in the studies do not always represent well the MPs found in the environment, for example most studies have used spherical MP particles, when fragmented MP particles are reported to be most commonly found in the aquatic environment (Phuong et al., 2016; Pikuda et al. 2023; Samadi et al, 2022; Wright et al., 2013). In contrast, however, some other studies have identified fibers as the most abundant type among MPs in the aquatic environment (Malla-Pradhan et al., 2023), as well as among MPs and macroplastics together (Bagaev & Chubarenko, 2018). Many kinds of adverse effects of PS-MP have been observed on *D. magna*. Acute and chronic exposures, including also multigenerational studies (Schür et al. 2020), have shown to cause oxidative stress (Esterhuizen et al., 2023), changes in growth, reproduction, and behavior (De Felice et al., 2019; Eltemsah & Bøhn, 2019; Trotter et al., 2021), proteomic changes (Trotter et al., 2021), as well as immobility (Eltemsah & Bøhn, 2019) and mortality on *D. magna* (Schür et al. 2020).

The toxicity of MPs can be explained by chemical or physical toxicity depending e.g. on polymer type. PS is considered as an inert polymer (Esterhuizen et al., 2023), that is composed of styrene monomers (Schellenberg, 2009). Leaching of chemicals, such as styrene oligomers, from the PS-MP,

could potentially affect aquatic organisms like *D. magna* (Mueller et al., 2020). The effect is however dependent on the circumstances, especially on the PS-MP concentration in the environment (Esterhuizen et al., 2023). In addition to potential toxic responses induced from plastic released contaminants, ingestion of MP can lead to accumulation of MP particles in the gut system of filter-feeders and interfere digestion (Rist et al., 2017; Samadi et al., 2022). The break-down rate of the MPs is slow, and it is possible that MPs and the chemicals leaking from them will bioaccumulate in food chain (Pikuda et al., 2023; Smith et al., 2018).

1.3.2 Effect of DCF

As an NSAID, DCF inhibits the synthesis of prostaglandins that leads to inhibition of cyclooxygenase (COX) enzymes (Brausch et al., 2012; Parolini, 2020). This is also known to contribute to oxidative stress by impacting antioxidative responses and increasing production of reactive oxygen species (ROS) (Augello et al., 2025; Gómez-Oliván et al., 2014; Ryan et al., 2008). The toxic effect of DCF has been studied on aquatic organisms including *D. magna*. The DCF concentrations used in toxicity tests have ranged from 0.50 mg/L to 486 mg/L, including both acute and chronic tests on *D. magna*. (Du et al., 2016; Parolini, 2020.) Oxidative stress is one of the adverse effects of DCF observed on *D. magna* (Nkoom et al., 2019; Nkoom et al., 2022). Other effects of DCF observed in studies on *D. magna* so far include impairment of reproduction (Du et al., 2016; Lee et a., 2011; Liu et al., 2017), behavioral changes (Nkoom et al., 2019; Nkoom et al., 2022), changes in gene expression (Liu et al., 2017; Nkoom et al., 2022) and acute toxicity (Du et al., 2016; Ferrari et al., 2004; Haap et al., 2008).

1.3.3 Combined toxicity of PS-MP and DCF

The fact that multiple stressors are present in the environment makes it more complex to assess the toxicity of MP (Funke et al., 2024). Co-occurrence with pharmaceuticals is one notable factor that can influence the effects of MP on aquatic organisms. In aquatic environments organic contaminants, including pharmaceuticals and personal care products (PPCPs), can accumulate to the MP surface resulting in high amounts of contaminants found in MPs. (Atugoda et al., 2021.) As a result, MPs can act as vectors for many pollutants (Du et al., 2021; Yang et al., 2024). The high surface to volume ratio of MPs and their surface properties make the sorption of contaminants efficient, and it is possible to find much higher concentrations of organic contaminants on MPs than in surrounding waters and in the sediments (Atugoda et al., 2021; Hartmann et al., 2017; Yang et al., 2024). Different kinds of factors are affecting the sorption capacity of MPs, including environmental factors, such as pH and ionic strength, and the properties of PPCP and of MP, especially MP polymer type. PS is a weakly polar polymer, and it can interact with neutral molecules of PPCPs through hydrophobic interactions

or through adsorption-based interaction, the pore-filling mechanism. (Atugoda et al., 2021.) In the sorption of DCF or other NSAIDs on PS the π–π interactions are also in a significant role. The π–π interactions result from non-covalent bonds between the aromatic compounds of both PS particles and PPCP compounds. (Zhang et al., 2017.) The environmental factors are shown to affect the sorption of DCF by PS, especially increasing pH has caused decrease in the sorption capacity (Elizalde-Velásquez et al., 2020).

The combined effect of MP and PPCP can be additive, or then synergistic with higher toxic effect than would be expected for MP and PPCP alone, or antagonistic, with lower toxic effect of the combination (Bell, 2005; Lee et al., 2023; Pablos et al., 2015). Factors that are connected to antagonism involve e.g. the large size of MP particles, which can prevent both MPs and their surface-adsorbed PPCP from entering the organism, and the opposite biological effects of the two contaminants in the organism. Synergism may instead result e.g. from increased entry of adsorbed PPCP into the organism together with sufficiently small MP particles, or from complementary biological effects of the compounds. (Yang et al., 2024.)

So far there are no published studies regarding the combined toxicity of PS and DCF on *D. magna*. Some studies of the combined effect of PS and other pharmaceuticals on *D. magna* however exist, involving the combined effect with carbamazepine (CBZ) on reproduction (He et al., 2023), with roxithromycin (ROX) on biological responses (Liu et al., 2022; Zhang et al., 2019), and with triclosan (TCS) on acute toxicity (Pashaei et al., 2023). The combined toxicity of PS-MP or PS-NP and DCF have been studied on other organisms than *D. magna*, including algal species (Ding et al., 2023) and zebra fish (*Danio rerio*) (Kandaswamy et al., 2024).

1.4 Use of *D. magna* in ecotoxicological studies

1.4.1 Daphnia characteristics

Daphnia are planktonic crustaceans belonging to the suborder Cladocera, also known as water flea (Siciliano et al., 2015; Thakur & Kocher, 2018). They are common in freshwater environments such as in lakes and other standing freshwater reservoirs and they have an important ecological role in pelagic ecosystems (Miner et al., 2012; Siciliano et al., 2015; Thakur & Kocher, 2018; Tkaczyk et al., 2021). As efficient grazers of e.g. phytoplankton and as important food source for predatory invertebrates and planktivorous fish, they have a central role both for the primary and secondary productivity in aquatic food webs (Miner et al., 2012; Siciliano et al., 2015).

1.4.2 *D. magna* life stages

D. magna is the largest herbivorous cladoceran, and its size can range from 0.5 mm of youngest daphnids to even more than 6 mm of adult daphnids (Tkaczyk et al., 2021). The life cycle of *D. magna* is usually under two months, and the maturity is reached in 5 to 10 days after hatching (Castro et al., 2020). Daphnids less than or 24 h old are considered neonates (Esterhuizen et al., 2023). It is possible that Daphnia sensitivity to toxicants is age-dependent (Adema, 1978; Traudt et al., 2017). For instance, some studies suggest neonates to be more sensitive to some metals such as cadmium (Cd) (Traudt et al., 2017) and chemicals such as Corexit 9500 (Salehi et al., 2017). Eltemsah & Bøhn (2019) who exposed *D. magna* with PS-MP found that survival, growth and reproduction of neonates were more affected than those of adult daphnids. The different effects of toxicants on the daphnid life stages can be explained by differences in biochemical, physiological and ontogenic characteristics, including the smaller size of neonates compared to adults (Bianchini & Wood, 2008; George-Ares & Clark, 2000). In general, early juvenile stages have been shown to have higher sensitivity than the older ones, e.g. related to various factors such as lower metabolic capability or incomplete development of organs and organ systems, as well as immature enzymatic systems, that can potentially reduce the ability to counteract the effects of toxicants (George-Ares & Clark, 2000; Mohammed, 2013).

1.4.3 *D. magna* as a bioindicator species

D. magna is a commonly used bioindicator species in ecotoxicological studies (Pikuda et al., 2023; Siciliano et al., 2015). As a definition bioindicator species is "a species or group of species that readily reflects the abiotic or biotic state of an environment, represents the impact of environmental change on a habitat, community or ecosystem or is indicative of the diversity of a subset of taxa or the whole diversity within an area" (Gerhardt, 2002). The popularity of *D. magna* as a model organism for ecotoxicological research is explained by its many advantages, including its short life cycle, its ability to reproduce both sexually and asexually through parthenogenesis, and the ease of culturing it in laboratory conditions. *D. magna* is ideal e.g. for immobilization tests because it is easy to detect its mobility, and also many other endpoints can be studied in *D. magna* to assess toxic effect at multiple levels, always from cellular to ecological level. (Castro et al., 2020; Pikuda et al., 2023; Thakur & Kocher, 2018; Tkaczyk et al., 2021.)

1.4.4 Acute toxicity and median effective concentration

Immobilization and mortality are typical endpoints that can be used to assess the effect of toxicants (Siciliano et al., 2015; Tkcaczyk et al., 2021). Many organizations have guidelines for testing acute

toxicity with *D. magna* (Pikuda et al., 2023; Tkaczyk et al., 2021). The widely used test in many countries is the Test No. 202: *Daphnia* sp. Acute Immobilisation Test by Organisation for Economic Co-operation and Development (OECD, 2004; Tkaczyk et al., 2021). Immobilization reveals the impact of the tested substance on the swimming ability of Daphnia. After 48 h of exposure and optionally also after 24 h the median effective concentration (EC_{50}) is determined, which indicates the concentration at which 50% of the daphnids are immobilized. (Du et al., 2016; OECD, 2004; Tkaczyk et al., 2021.) Acute toxicity tests are easy to conduct and replicate and can provide ecologically important information. Dose-response curves can also help predict the effect of concentrations other than those tested. (Hook et al., 2014; Siciliano et al., 2015.) On the other hand, acute toxicity tests also have limitations, including their inability to reveal the sublethal effects of toxic chemicals that may occur with longer exposure times and lower concentrations. (Hamza-Chaffai, 2014; Hook et al., 2014; Tkaczyk et al., 2021.)

1.4.5 ROS and oxidative stress

Biomarkers are a useful tool in assessing the effect of environmental contaminants on environment and organisms (Hamza-Chaffai, 2014), and they can be defined as "a biological response to a chemical or chemicals that gives a measure of exposure and sometimes also of toxic effect" (Depledge & Fossi, 1994). With them it is possible to separate normal biological responses from those that are changed due to xenobiotics, i.e. foreign compounds (Hamza-Chaffai, 2014; Hook et al., 2014; Lushchak, 2011; Tkaczyk et al., 2021). Oxidative stress is a biochemical phenomenon, and associated biomarkers can provide more subtle information about toxicity than the traditional endpoints (Bownik et al., 2023; Hook et al., 2014; Tkaczyk et al., 2021).

ROS are highly reactive chemicals that involve oxygen radicals as well as nonradicals which can act as oxidants or alternatively be converted into free radicals (Halliwell, 2006). ROS can originate from both endogenous sources, such as normal metabolic processes, and exogenous sources, a typical example of which are xenobiotics. High concentrations of ROS are toxic and increased intracellular ROS levels in oxidative stress can lead to destruction of many macromolecules including lipids, proteins and DNA (Bownik et al., 2023; Esterhuizen et al., 2023; Lushchak, 2011; Nkoom et al., 2019). Normally the antioxidant systems can eliminate the excess ROS and decrease the ROS concentration into steady-state levels (Esterhuizen et al., 2023; Lushchak, 2011). When the antioxidative system is efficient enough, the ROS level quickly returns to its normal range – in such a situation, it is a case of acute oxidative stress. In contrast, in chronic oxidative stress, the antioxidative system is unable to reduce the levels sufficiently, and recovery takes longer, sometimes with a new higher level of ROS

remaining. (Lushchak, 2011.) Various oxidative stress-related biomarkers can be used to assess toxicity. Elevated ROS levels can result from pollutants as well as changes in antioxidative response, and intracellular ROS level is one biomarker of toxicity, used for instance in research including aquatic organisms. (Bownik et al., 2023; Esterhuizen et al., 2023; Lushcak, 2011; Rai et al., 2021.)

1.5 Research rational

1.5.1 Aims of this thesis

Because of the existence of multiple stressors in the environment, there is a need for studies that acknowledge the complexity of ecological systems to get a more comprehensive understanding of the potential effects of MP. One important factor to be considered when assessing the effects of MPs is their co-occurrence with pharmaceuticals. (Atugoda et al., 2021; Funke et al., 2024.) PS is one of the most abundant MP polymers (Faull et al., 2024) and DCF is one of the most common pharmaceuticals found in aquatic environments (aus der Beek et al., 2016; Parolini, 2020), and therefore it is important to understand better their combined effects on aquatic organisms and ecosystems. Daphnids, especially *D. magna*, that are typical bioindicator organisms used in ecotoxicological studies because of their ecological importance and sensitivity to contaminants, are also known to be affected by MP. (Castro et al., 2020; Esterhuizen et al., 2023; 2020; Pikuda et al., 2023; Samadi et al., 2022; Thakur & Kocher, 2018.) The effects of contaminants on daphnids have been found to differ between different life stages, which makes it relevant to consider their effect on different life stages (Adema et al., 1978; Eltemsah & Bøhn, 2019; Esterhuizen et al., 2023; Salehi et al., 2017).

1.5.2 Research questions and hypothesis

Only few studies have assessed the combined effects of MPs and pharmaceuticals on *D. magna* (Samadi et al., 2022; Zhang et al., 2019), and to the author's best knowledge there are no published studies of the combined effect of PS-MP and DCF on *D. magna*. The aim of this study is to contribute to filling this research gap. The main research question that this thesis aims to answer is what the toxic effect of PS-MP and DCF is alone and combined at different concentrations on *D. magna*. The aim is also to investigate whether the combinations of these two contaminants have an antagonistic, additive or synergistic effect, i.e., do the contaminants have a stronger toxicity alone or together. The effect is studied using two endpoints: immobilization and ROS activity. The purpose is also to find out if there are differences in sensitivity between two life stages, neonate and adult daphnids.

The hypothesis is that *D. magna* that are exposed with the higher concentrations of PS and DCF will experience a stronger toxic effect, that is indicated by two biomarkers, immobilization and ROS activity. It is also hypothesized that the combined toxicity of PS and DCF is greater than that of the contaminants alone, i.e. that the combination of PS and DCF has a synergistic effect. In addition, it is expected that the life stage of *D. magna* affects sensitivity to the contaminants studied so that neonate daphnids are more sensitive than adults and this is reflected in higher immobilization and higher ROS activity.

To investigate these questions *D. magna* is exposed under laboratory conditions to PS, DCF and their combinations at different concentrations ranging from environmentally relevant concentrations to higher ones more likely to cause toxic effects. The experiment is carried out separately on both neonate and adult daphnids. Immobilization is assessed as the percentage of daphnids immobilized (at 24 and 48 h), and intracellular ROS levels are also quantified after 48-hour exposure. The acute immobilization test and the testing conditions are based on the OECD guidelines (for the Testing of chemicals) that are widely used in toxicological research (OECD, 2004; Tkaczyk et al., 2021).

2 Materials and methods

2.1 Cultivation of *D. magna*

ISO water was prepared according to OECD (2004), and it was used as a medium for *D. magna* cultivations and exposures. *D. magna* ephippia (Microbiotests, Gent, Belgium) were hatched in ISO water with pH 8.08 ± 0.01 at 21°C. They were kept under illumination of 8500 lux, following a 16-to-18-hour cycle between light and dark conditions, and fed daily with *Chlorella vulgaris* powder (Green Water Farm). Hatching took approximately 80 h, after which the neonates were transferred to bigger beakers with new ISO water and fed daily with *C. vulgaris*, also with TetraMin Baby. Neonates were used for the experiment within 24 h of hatching whereas adults were grown for 9-11 days before the start of the experiment.

2.2 Preparation of PS-MP and DCF treatments

The PS-MP used in the experiment consisted of new, jagged shaped fragments of 45 to 63 µm in diameter (confirmed microscopically). DCF was stored and handled in the dark to avoid photodegradation. The required quantities each of PS and DCF were measured on a small piece of foil, that was

placed into a glass beaker. The contents were resolved to 1 L of ISO water after thorough mixing with a vortex mixer. The flasks were covered with foil to avoid light exposure and magnetic stir bars were added to enable mixing with laboratory shakers. Before the start of each exposure the contents of each flask were stirred for 10 minutes and carefully inverted to ensure that the contents were well mixed.

2.3 Experimental setup

During the experiment neonate and adult *D. magna* were exposed to different concentrations of PS, DCF and combinations of PS and DCF for 24 and 48 h respectively. First, clean glass beakers of 20 ml were rinsed with ISO water. For each control, 10 ml of pure ISO water was used. For the exposures 10 ml of treatment with the right concentration was added to the corresponding glass beakers after thorough mixing. Five daphnids were added to each beaker with a pipette. To avoid photodegradation, both the preparation of the exposures and the transferring of daphnids were made in dark conditions. Also, the exposure of *D. magna* was carried out in the dark under a loose foil cover, also to prevent evaporation and entry of dust, and it lasted for 48 h. Daphnids were not fed during the exposure.

Each part of the experiment was conducted four separate times. The experiment started with exposures of the lower concentrations of the contaminants on neonate *D. magna* and followed by exposure of adult *D. magna* exposed with similar treatments. Those treatments included concentrations of 0.01, 0.1 and 1 mg/L of both PS and DCF alone. The treatments with combinations of PS and DCF included following treatments: PS 0.01 + DCF 0.01, PS 0.01 + DCF 0.1, PS 0.01 + DCF1, PS 0.1 + DCF 0.01, PS 0.1 + DCF 0.1, PS 0.1 + DCF 1, PS 1 + DCF 0.01, PS 1 + DCF 0.1 and PS 1 + DCF 1 mg/L. In the second exposure experiment, neonates, followed finally by adults, were exposed to the higher concentrations of PS and DCF alone and their combinations, including concentrations of 50 and 100 mg/L. The treatments with higher concentrations consisted of the following treatments: PS 50 + DCF 50, PS 50 + DCF 100, PS 100 + DCF 50 and PS 100 + DCF 100 mg/L. In addition to that, neonate exposures involved also 10 mg/L PS and DCF alone, as well as the following combinations: PS 10 + DCF 10, PS 10 + DCF 50, PS 10 + DCF100, PS 50 + DCF 10 and PS 100 + DCF 10. All treatments were performed triplicate, including the controls (5 daphnids per replicate, n=3). (Fig. 1.) The lowest concentrations were included to correspond to the environmentally relevant concentrations, and the highest concentrations were included to be able to assess the dose-dependent effects of PS and DCF, according to the recommendations of Samadi et al. (2022). After the exposures, the mobile daphnids were counted to assess immobilization, and EC_{50} for neonates, as detailed below.

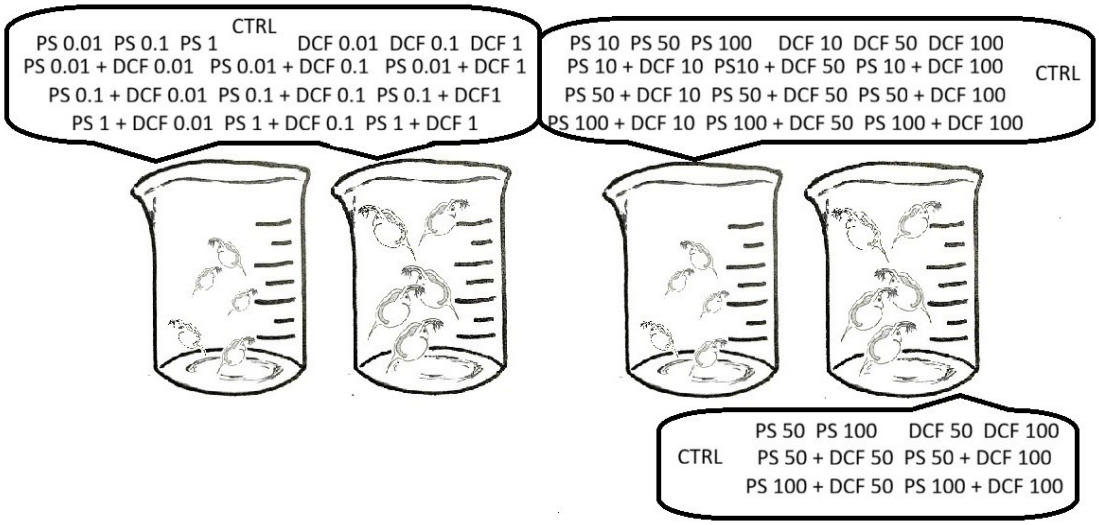


Figure 1. Experimental setup with *D. magna* exposed to different concentrations of PS, DCF and their combination. The experiment included four separate exposures with low concentrations (0.01, 0.1 and 1 mg/L) both for neonate and adult daphnids, and high concentrations (10, 50 and 100 mg/L) for neonates, (50 and 100 mg/L) and adults. One beaker contained 5 daphnids, and all treatments were done in triplicate including controls. Mobile daphnids were counted after 24 and 48 h.

2.4 Acute immobilization test

2.4.1 Immobilization test

The acute immobilization test was conducted based on the OECD Test Guideline 202 (OECD 2004). The test duration was 48 h, and the immobilization of daphnids was observed both after 24 and 48 h. The daphnids were observed carefully and after gently moving the beaker, it was waited for a moment to see if also the stationary daphnids started to move. The daphnids unable to swim within 15 seconds after gentle agitation were counted as immobile.

2.4.2 EC_{50}

EC_{50} as the concentration that causes 50% immobilization of the test animals (Crane & Newman, 2000; Tkaczyk et al., 2021), was calculated according to the Miller and Tainter probit analysis method (1944), in the same way as the median lethal dose (LD_{50}) (Randhawa, 2009; Raj et al., 2013). The EC_{50} values were calculated for PS, DCF and the combination of PS and DCF. The percentage of immobilized daphnids for each concentration was transformed into probits by using Finney's (1952) table (Raj et al., 2013). Before the determination of probits for 0% and 100% immobilization those percentages were corrected by using the following formulas a and b:

$$\text{for Immobilization of 0\%} = 100\left(\frac{0.25}{n}\right) \quad \text{(a)}$$

$$\text{and for Immobilization of 100\%} = 100\left(n - \frac{0.25}{n}\right) \quad \text{(b)}$$

Where n is the number of test organisms.

The probit values obtained were plotted against the logged dose, and the dose corresponding probit 5 was estimated to cause a 50% immobilization. (Randhawa, 2009; Raj et al., 2013.) For calculating the EC_{50} for the combination of PS and DCF the equitoxic concentrations (around 0.05%, 0.5%, 5%, 50%, 250% and 500% of the EC_{50} at 48 h) of PS and DCF were used as combinations (Delahaut et al., 2020). The equitoxic values were estimations from the EC_{50} values obtained for PS and DCF alone, which were very close to each other (20.45 ± 1.34 mg/L). The concentration of both PS and DCF were counted together and treated as a total concentration of combination. The EC_{50} for the combination of PS and DCF was also calculated using the Miller and Tainter method. (Raj et al., 2013.) Whether the effect of the combination was additive, synergistic, or antagonistic, was determined by the ratio between expected and observed EC_{50} for the combination (E/O). The equation of Wadley was used for calculation of the expected EC_{50}:

$$EC_{50exp} = (a + b) / [(a/ EC_{50A} + (b/ EC_{50B})]$$

Where EC_{50A} and EC_{50B} are EC_{50} values obtained for individual components, and a and b are proportions of each component in the mixture. (Gisi, 1996.)

Ratios of 0.5 and lower were considered to indicate antagonism, ratios between 0.5 and 1.5 were interpreted to represent an additive effect and ratios of 1.5 and higher a synergistic effect. (Gisi et al., 1985; Gisi, 1996.)

2.4.3 LOAEC and NOAEC

The lowest observed adverse effect concentration (LOAEC) and no observed adverse effect concentration (NOAEC) for PS, DCF and the combination of PS and DCF were estimated. The lowest tested concentration that had a significant difference compared to control was identified as the LOAEC, and the highest tested concentration that has no significant difference compared to the control was identified as NOAEC (Crane & Newman, 2000).

2.5 ROS determination

The ROS levels were determined from *D. magna* after 48 h PS, DCF and PS + DCF exposures. The determination was done by using Fluorometric Intracellular ROS Assay Kit (Sigma Aldrich Kit MAK144) and by measuring the fluorescence intensity with a Tecan Infinite F Nano+ spectrophotometer. The fluorescence intensities were then normalized against protein concentration obtained for each sample. The ROS level was determined in the same way for daphnids exposed to PS, DCF and their combinations as well as for controls.

After the 48-hour exposure the still mobile daphnids were removed from each sample to 1.5 ml Eppendorf tubes. All remaining ISO medium was removed with a pipette, and the daphnids were moved to -20°C for 45 minutes. NaP buffer (200 µl) (20 mM, pH 7) was added to the tubes with frozen daphnids, and they were homogenized with a plastic pestle. The samples were centrifuged for 3 min at 13500 rpm. From the supernatant, 50 µl of each sample was added to the fluorescent 96-well microplate in duplicate and 50 µl of reagent mix was added to each well. The fluorescence intensity (λex = 540 nm/ λem = 570 nm) was measured after a 45-minute incubation at 37°C. The relative ROS level was calculated as the ratio of the fluorescence intensity obtained in ROS measurement to the protein concentration of each sample, which was determined according to Bradford (1976).

2.6 Determination of protein concentration

2.6.1 Bradford's analysis

Bradford's method was used to determine the protein concentration in *D. magna* samples. The remaining samples were kept at -20°C and used for protein analysis within 24 h. After defrosting, the samples were pipetted to a 96-well transparent Greiner microplate and incubated for 10 minutes. For samples with adult daphnids 4 µl of each sample was used with 196 µl of Bradford's reagent (Sigma Aldrich), and for neonate samples 20 µl of sample and 180 µl of reagent was used. The reagent contains an acidic solution of Coomassie Brilliant Blue G-250, and when binding to protein, its absorbance maximum shifts from 465 nm to 595 nm, which is possible to be detected (Bradford, 1976). The absorbance was measured with Tecan Infinite F Nano+ spectrophotometer.

2.6.2 Standard curves

To be able to estimate the protein concentration for the samples, bovine serum albumin (BSA) was used to prepare a standard curve showing the relationship between absorbance and protein concentration. For the preparation of the related standard curves, a series of different concentrations of BSA (in NaP buffer) was used. The concentrations used to prepare the standard curves related to adults were 1000, 500, 250, 125, 62.5, 31.25 and 0 µg/ml, and the corresponding concentrations for neonates were 1000, 100, 10, 1, 0.1, 0.01 and 0 µg/ml. BSA dilution samples (4 µL for adult and 20 µL to neonate related standard curves) together with Bradford's reagent (196 µL for adult and 180 µL to neonate related standard curves) were added into the microplates, and the absorbance was measured with Tecan Infinite F Nano+.

2.7 Statistical analysis

Microsoft Excel was used to calculate, organize, and graphically represent the data, and also for probit analysis. Data analysis was done in IBM SPSS Statistics 28. Normality was tested with Shapiro-Wilk test and homogeneity with Levene's test. The data are presented as the mean values ± standard deviation (n = 3). For the data that were not normally distributed and not homogenous, a non-parametric test was employed. The individual effects of PS and DCF were tested with the Kruskal-Wallis test, which is a popular non-parametric rank-based test, and Bonferroni correction was used for adjustment of significance values ($p < 0.05$) (Ostertagová et al., 2014; Kruskal & Wallis, 1952). Two-way analysis of variance (ANOVA) was applied to analyze the combined effect of PS and DCF. As a moderately conservative test that is widely used when comparing many groups, the Tukey's HSD (Honest significant difference) test was chosen as the post-hoc test (Midway et al., 2020; Tukey, 1949). The available post hoc tests do not show a comparison between all possible combinations, as this would easily lead to an overwhelming number of results (pers. comm. Jukka Siren, Statistician, 2025). Therefore, data for combined exposures were visualized as mean values of different concentrations of PS and DCF in the combinations, in the same way as in the graphs obtained from Tukey's test in SPSS showing estimated marginal means, which practically corresponded to the observed means. Dunnett's test as a post-analysis of variance (ANOVA) multiple comparison test was used to estimate LOAEC and NOAEC. Paired-samples T-test was used for comparison of ROS levels between neonate and adult daphnids. The significance level used was $p < 0.05$.

3 Results

3.1 Immobilization

Neonate and adult *D. magna* (n = 5) were exposed to varying PS and DCF concentrations (0.01, 0.1, 1, 10, 50 and 100 mg/L) individually as well as combined. Mobility counts were performed after 24 and 48 h from the start of the exposures and expressed as a percentage (%) of mobile daphnids (Fig. 2 and 3).

When comparing the effect of PS in neonate daphnids after 48 h of exposure (Fig. 2A), a corresponding decrease in mobility was observed as a result of increasing PS exposure concentration. The decrease in mobility was significant for neonates exposed to PS concentration of 100 mg/L compared to the control (Kruskal-Wallis p = 0,027). Compared to the control, the mobility percentage in neonates exposed to the highest PS concentration (100 mg/L) was 3.4-fold lower after 48 h. No significant effects were observed in neonates after 24 h of exposure (Fig. 2A), nor in adults exposed to any of the PS concentrations (Fig. 2B).

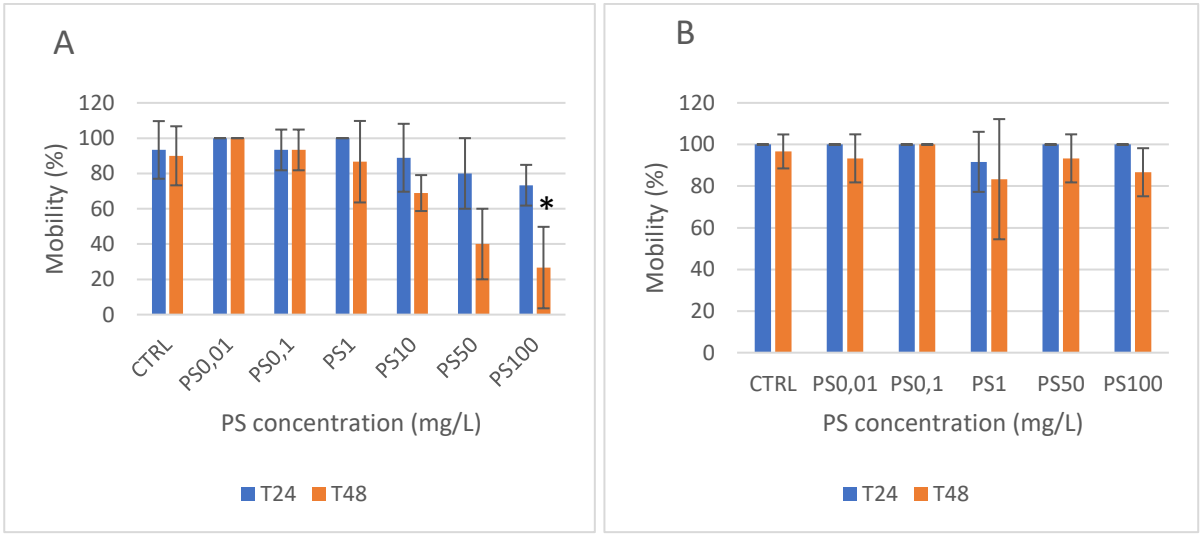


Figure 2. Mobility (%) of *D. magna* after 24 h (T24) and 48 h (T48) when exposed to different concentrations of PS (0, 0.01, 0.1, 1, 10, 50 and 100 mg/L) for neonates (A) and for adults (B). The error bars represent average percentage of mobility (%) ± standard deviation (SD) (n = 5). Significant difference ($p < 0.05$) compared to the control is marked with an asterisk (*).

There was a decrease in the average percentage of mobility of neonate daphnids after 48 h with increasing DCF concentration (Fig. 3A), with a statistically significant difference between neonates exposed with DCF concentration of 100 mg/L and control (Kruskal-Wallis p = 0.033). The mobility

changed from 90% for control treatment to 0% for treatment with the highest DCF concentration (100 mg/L). However, for adult daphnids (Fig. 3B), the same trend was not observed. The percentage of mobile daphnids did not differ significantly with any of the DCF treatments after 24 h, neither the neonates nor adults (Figs. 2A & B).

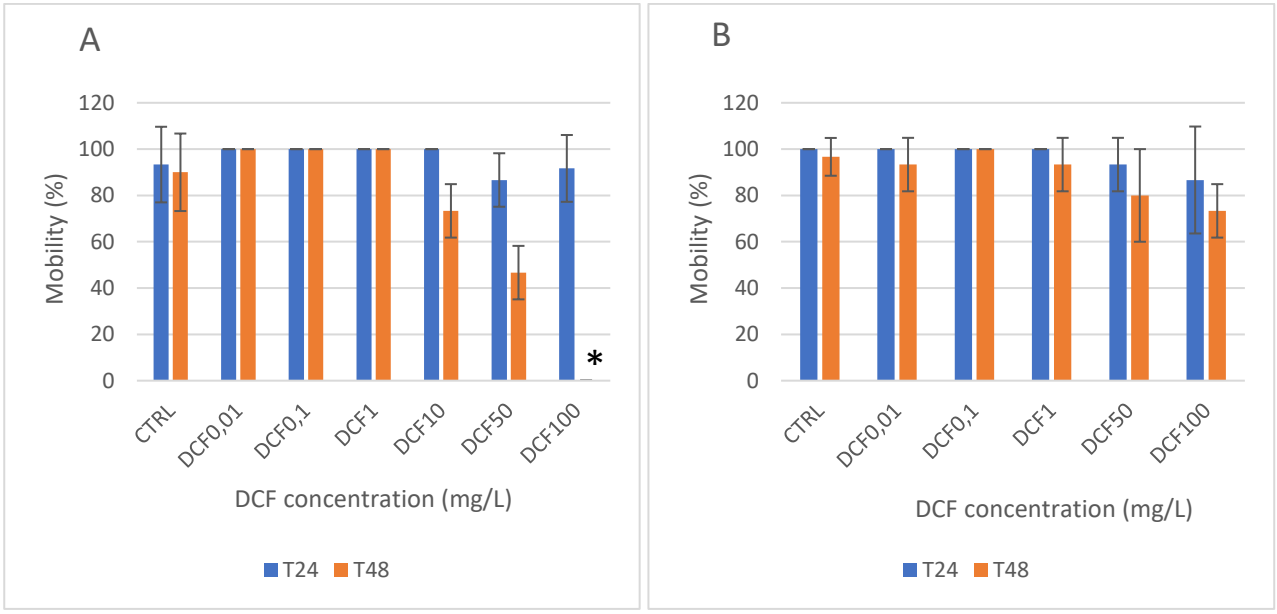


Figure 3. Mobility (%) of *D. magna* after 24 h (T24) and 48 h (T48) when exposed to different concentrations of DCF (0, 0.01, 0.1, 1, 10, 50 and 100 mg/L) for neonates (A) and for adults (B). The error bars represent average percentage of mobility (%) ± SD (n = 5). Significant difference ($p < 0.05$) compared to the control is marked with an asterisk (*).

The combination of PS and DCF had a significant effect on the neonates' average mobility percentage after 24 h (two-way ANOVA $p = 0.022$) and after 48 h (two-way ANOVA $p = 0.018$), as well as in adults after 48 h (two-way ANOVA $p = 0.021$), but not for adults after 24 h (Fig. 4A-D). In adult daphnids after 24 h, significant differences between treatments were explained by DCF but not by combination of PS and DCF (Fig. 4C1&2). In most cases higher concentrations of PS and DCF in the combination led to lower mobility. In neonate daphnids after 24 h, PS concentrations of 50 mg/L and 100 mg/L resulted in highly significantly lower mobility compared to PS concentrations of 0 mg/L and 10 mg/L, when combined with DCF concentrations 10, 50 and 100 mg/L (two-way ANOVA $p < 0.001$) (Fig. 4A1). When combined with PS concentration of 10 mg/L, the mobility was significantly lower when comparing DCF concentration of 100 mg/L with 0 mg/L DCF (two-way ANOVA $p = 0.001$) and 50 mg/L and 100 mg/L DCF with 10 mg/L DCF (two-way ANOVA $p = 0.004$, $p < 0.001$, respectively). In combination with PS concentration of 50 mg/L, the following DCF concentrations resulted in lower mobility when compared together: 10 mg/L, 50 mg/L and 100 mg/L DCF when compared to 0 mg/L DCF (two-way ANOVA $p = 0.027$, $p < 0.001$ and $p < 0.001$, respectively), and

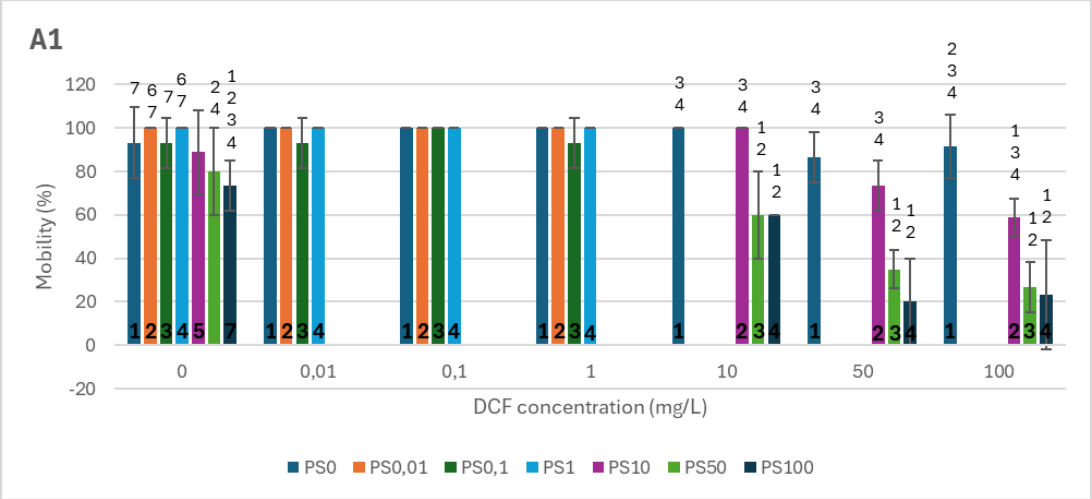
50 and 100 mg/L DCF compared to 10 mg/L DCF (two-way ANOVA $p = 0.006$ and $p < 0.001$, respectively). PS concentration of 100 mg/L combined with DCF concentrations 50 and 100 mg/L resulted in highly significantly lower mobility compared to both 0 and 10 mg/L DCF (two-way ANOVA $p < 0.001$). (Fig. 4A2.)

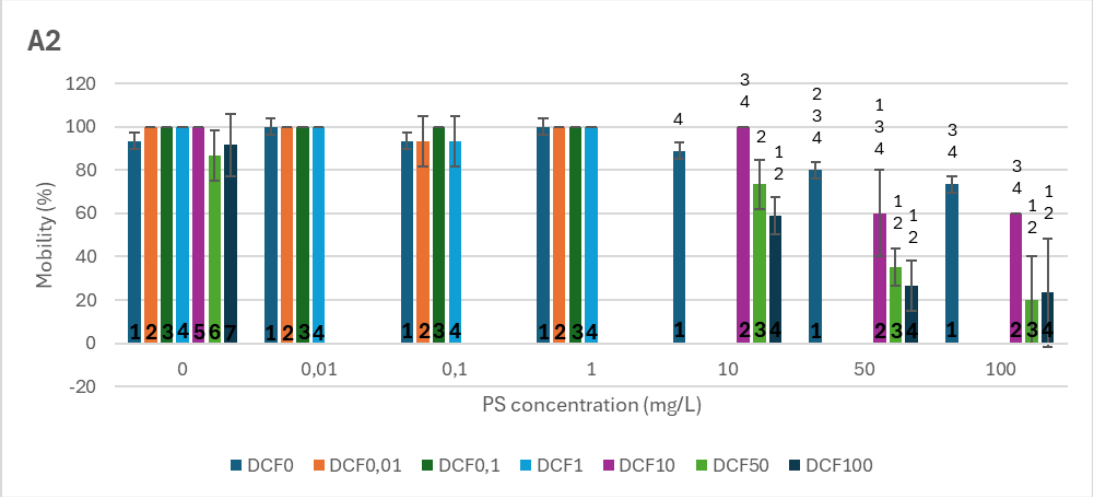
In neonate daphnids, after 48 h, PS concentrations of 50 mg/L and 100 mg/L resulted in highly significantly lower mobility compared to PS concentration of 0 mg/L and 10 mg/L when combined with DCF concentrations of 10 and 50 mg/L (two-way ANOVA $p < 0.001$). However, when combined with DCF concentration of 100 mg/L, PS concentrations 0, 50 and 100 mg/L were significantly lower in comparison between PS concentration of 10 mg/L (two-way ANOVA $p = 0.013$). There was also a significantly lower mobility in the comparison between PS concentration of 0.1 mg/L and PS concentrations 0 and 0.01 mg/L when combined with DCF concentration of 1 mg/L (two-way ANOVA $p = 0.019$). (Fig. 4B1.) When combined with PS concentration of 10 mg/L, DCF concentrations 50 mg/L and 100 mg/L led in significantly lower mobility compared to 0 mg/L DCF (two-way ANOVA $p = 0.049$ and $p < 0.001$, respectively), as well as when comparing 100 mg/L DCF with 10 mg/L DCF (two-way ANOVA $p < 0.001$). Also, in combination with PS concentration of 50 mg/L, DCF concentration of 50 and 100 mg/L led to lower mobility compared to 0 mg/L DCF (two-way ANOVA $p = 0.004$, $p < 0.001$, respectively), as well as DCF concentration of 100 mg/L compared to DCF concentration of 10 mg/L (two-way ANOVA $p = 0.019$). PS concentration of 100 mg/L combined with 100 mg/L DCF resulted in significantly lower mobility in comparison with DCF concentrations 0 and 10 mg/L, also PS concentration of 0.1 mg/L combined with 1 mg/L DCF led to lower mobility when compared to combination with 0.1 mg/L DCF (two-way ANOVA $p = 0.019$). (Fig. 4B2.)

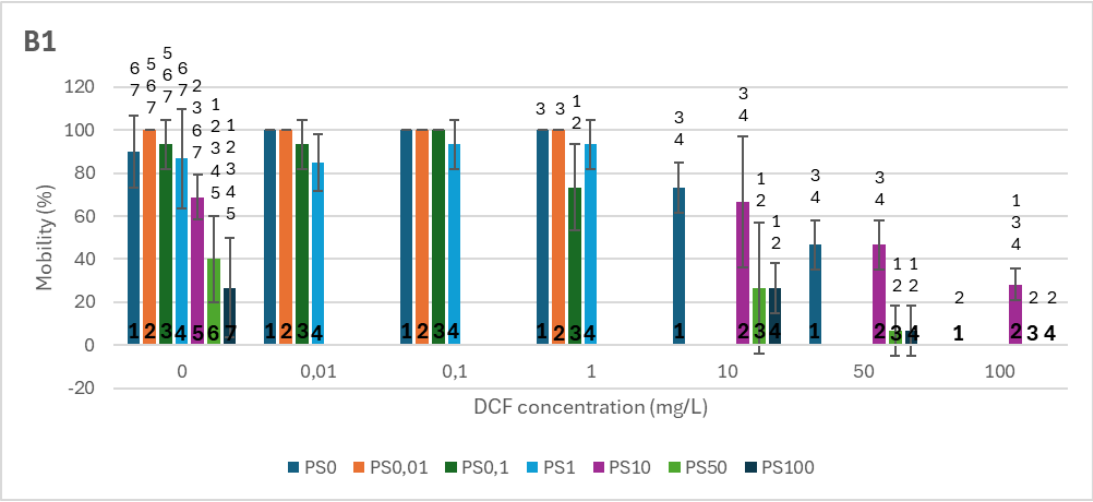
In adult daphnids after 48 h DCF concentration of 100 mg/L combined with PS concentration of 100 mg/L resulted in significantly lower mobility compared to PS concentrations 0 and 50 mg/L (two-way ANOVA $p < 0.001$ and $p = 0.025$, respectively), also when combined with 50 mg/L PS compared to 0 mg/L PS (two-way ANOVA $p < 0.001$). When combined with 0.01 mg/L DCF, PS concentration of 1 mg/L resulted in significantly lower mobility in comparison with 0.01 mg/L PS (two-way ANOVA $p = 0.017$). (Fig. 4D1.) Mobility was also significantly lower when comparing DCF concentration of 0.01 mg/L to 0.1 mg/L DCF when combined with PS concentration of 1 mg/L (two-way ANOVA $p = 0.017$), and highly significantly lower when comparing DCF concentration of 100 mg/L to 0 and 50 mg/L DCF when combined with PS concentration of 50 mg/L, and when comparing 100 mg/L DCF to 0 and 50 mg/L DCF in combination with PS concentration of 100 mg/L (two-way ANOVA $p < 0.001$). (Fig 3D2.) Since the interaction effect of PS and DCF was significant, it is not

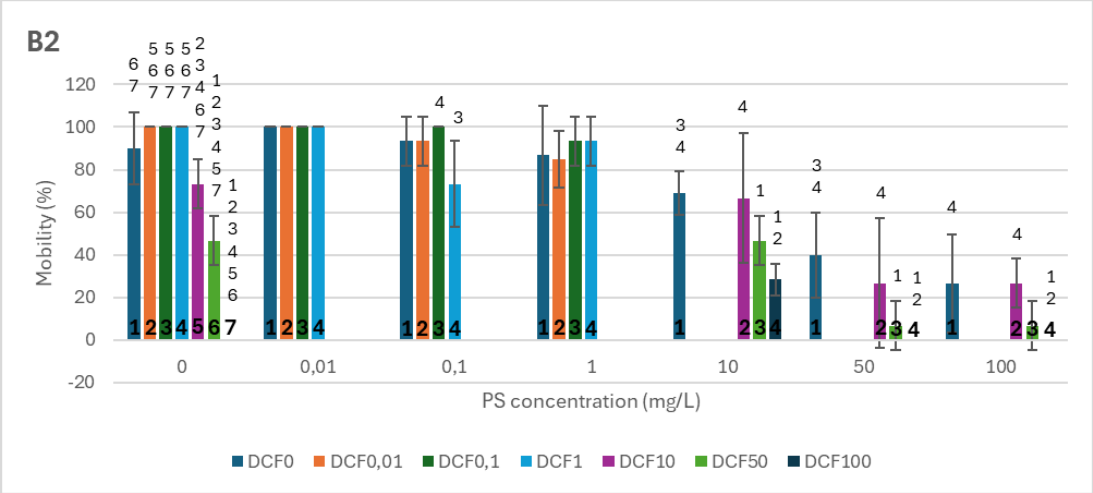
recommended to interpret the main effects of individual exposures (i.e. combinations with DCF concentrations of 0 mg/L) with the same test, which is why these differences are not further examined here but have been tested separately (Fig. 2 & 3) (Pallant, 2011).

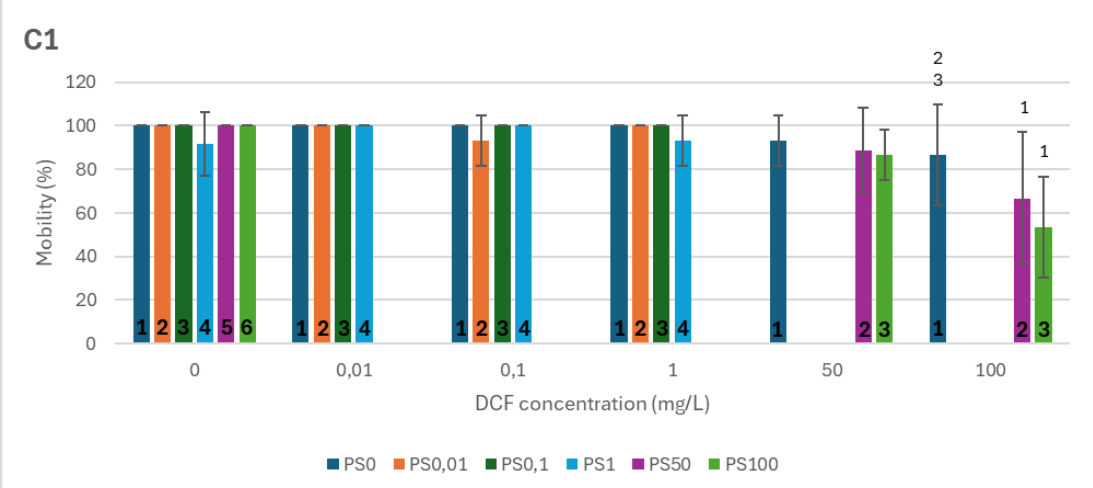
After 48 h, the average percentage of mobility decreased in neonates from 90% and in adults from 96,7% (control) to 0% with highest combined concentrations PS 100 + DCF 100 mg/L. For neonates after 24 h there was a four-fold decrease with exposure to PS 100 + DCF 100 mg/L (23,3%) compared to control (93,3%). For neonate *D. magna*, both after 24 and 48 h, the effect of PS and DCF was highly significant (two-way ANOVA $p < 0.001$) within combinations. For adults, after 48 h, DCF had a highly significant effect (two-way ANOVA $p < 0.001$) and PS had a significant effect (two-way ANOVA $p = 0.001$) when comparing combinations.






B1
Mobility (%)
DCF concentration (mg/L)
PS0
PS0,01
PS0,1
PS1
PS10
PS50
PS100


B2
Mobility (%)
PS concentration (mg/L)
DCF0
DCF0,01
DCF0,1
DCF1
DCF10
DCF50
DCF100


C1
Mobility (%)
DCF concentration (mg/L)
PS0
PS0,01
PS0,1
PS1
PS50
PS100

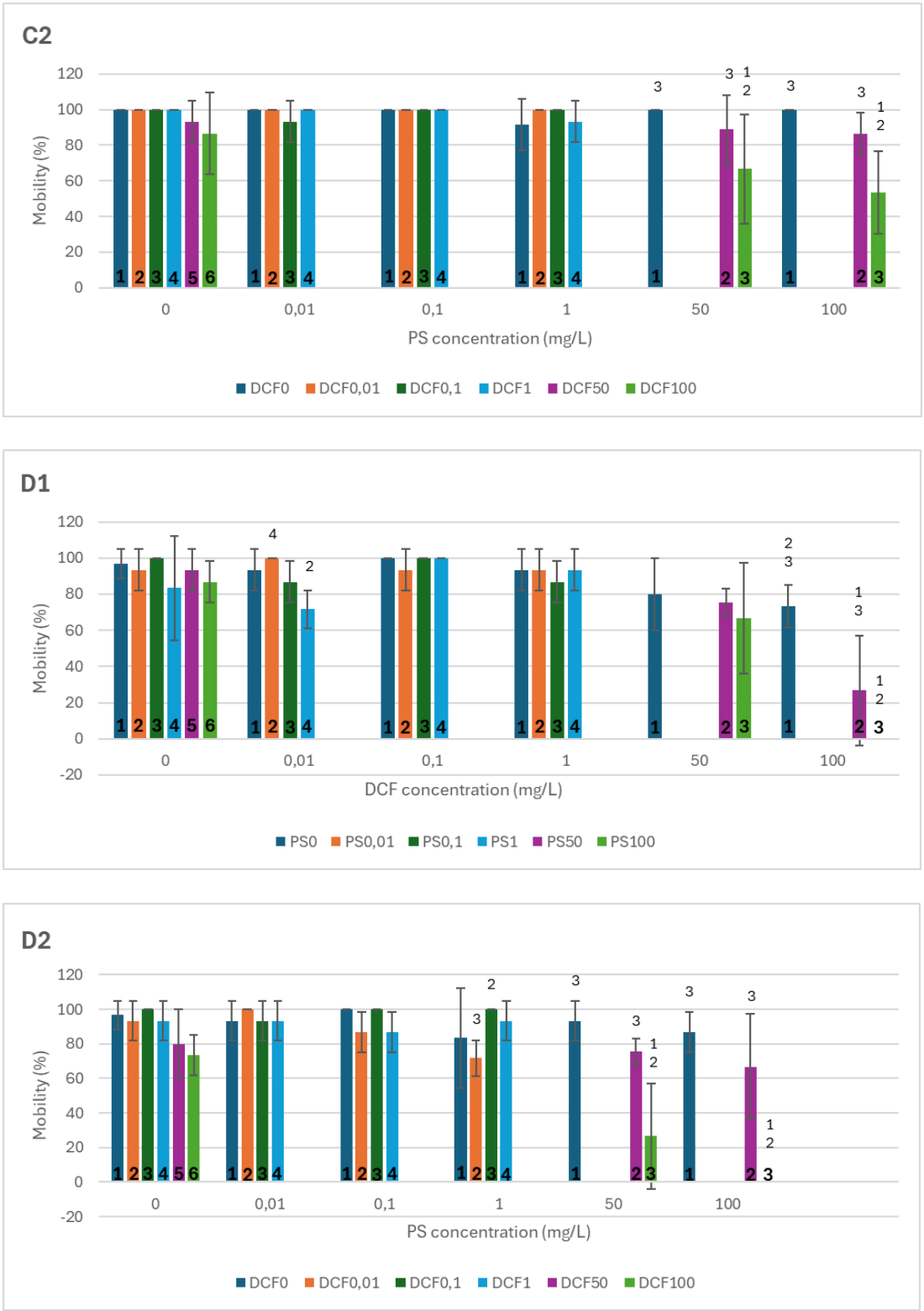


Figure 4. Average mobility (%) of *D. magna* exposed to combinations of PS and DCF with different concentrations. Significant differences are presented separately between different concentrations of PS combined with a specific DCF concentration (1), and between different concentrations of DCF combined with a specific PS concentration (2), for neonates after 24 h (A1&2) and 48 h (B1&2), and for adults after 24 h (C1&2) and 48 h (D1&2). Included exposures both for neonates and adults involve control exposure PS 0 + DCF 0, individual exposures PS 0 + DCF 0.01, PS 0 + DCF 0.1, PS 0 + DCF 1, PS 0 + DCF 10, PS 0 + DCF 50, PS 0 + DCF 100, PS 0.01 + DCF 0, PS 0.1 + DCF 0, PS 1 + DCF 0, PS

50 + DCF 0, PS 100 + DCF 0, and combined exposures PS 0.01 + DCF 0.01, PS 0.01 + DCF 0.1, PS 0.01 + DCF 1, PS 0.1 + DCF 0.01, PS 0.1 + DCF 0.1, PS 0.1 + DCF 1, PS 1 + DCF 0.01, PS 1 + DCF 0.1, PS1 + DCF 1, PS 50 + DCF 50, PS 50 + DCF 100, PS 100 + DCF 50, PS 100 + DCF 100 mg/L. In addition, for neonates also following exposures are involved: PS 0 + DCF 10, PS 10 + DCF 0, PS 10 + DCF 10, PS 10 + DCF 50, PS 10 + DCF 100, PS 50 + DCF 10, PS 100 + DCF 10. The error bars represent average percentage of mobility (%) ± SD (n = 5). Significant difference ($p < 0.05$) compared to other exposure is marked with the corresponding number.

The magnitude of the effect of treatments with PS, DCF and their combinations on daphnid mobility varied between neonates and adults (Fig. 2–4A & B), being significantly smaller for adults after 24 h (Kruskal-Wallis $p = 0.022$), but the difference was not significant after 48 h. However, when comparing only the highest concentrations of PS and DCF and their mixtures (50 and 100 mg/L) there was a highly significant difference (Kruskal-Wallis $p < 0.001$) between the average mobility percentage of neonate and adult daphnids after 48 h. With the lowest PS and DCF concentrations (0.01–1 mg/L), there was no significant difference between neonates and adults.

3.2 Oxidative stress and ROS

The physiological effect of PS and DCF exposures was determined by assessing the cellular ROS levels as indicators of oxidative stress. The relative ROS levels were calculated as a ratio of fluorescence measurement and protein concentration (µg/ml) of a sample after a 48-hour exposure. Because of the smaller number of daphnids surviving with exposure to the higher PS and DCF concentrations (10, 50 and 100 mg/L) for ROS determination, and because of different relative ROS levels of controls between high and low concentration exposures, the relative ROS levels were calculated only for daphnids treated with lower PS and DCF concentrations (0.01, 0.1, and 1 mg/L). For neonates (Fig. 5A), the average relative ROS levels were significantly higher than the control both for daphnids treated with 0.01 and 1 mg/L PS (Kruskal-Wallis $p = 0.042$ and 0.005, respectively). For adult daphnids (Fig. 5B) the PS treatments of 0.01 and 0.1 mg/L resulted to higher ROS levels than the PS treatment of 1 mg/L, and they were significantly higher than the control (Kruskal-Wallis $p = 0.009$ and $p = 0.013$, respectively). Daphnids treated with PS had higher relative ROS levels than the untreated controls, for neonates on average 2.4 times the level of control (87.2 ± 12.7 fluorescence/ µg/ml) and for adults on average 1.6 times the level of control (38.3 ± 2.2 fluorescence/ µg/ml).

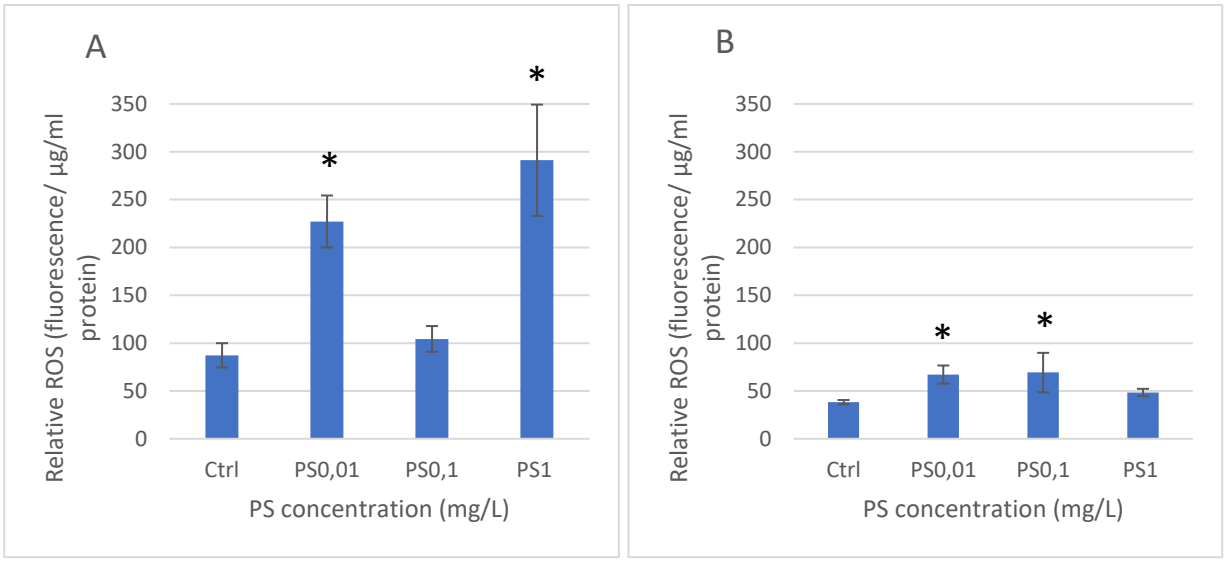


Figure 5. Relative ROS (fluorescence/ µg/ml) for daphnids exposed with PS concentrations 0.01, 0.1, and 1 mg/L. A) For neonate daphnids and B) for adult daphnids. The error bars represent average relative ROS (fluorescence/ µg/ml protein) ± SD (n = 5). Significant difference ($p < 0.05$) compared to the control is marked with an asterisk (*).

For the neonate daphnids (Fig. 6A), DCF exposures did not cause a significant effect on relative ROS level compared to the control. For the adult daphnids (Fig. 6B) exposed to DCF along, the concentrations of 0.01 and 0.1 mg/L led to significantly higher ROS levels compared to the control (Kruskal-Wallis $p = 0.002$ and 0.042, respectively). In adult daphnids, DCF treatments increased the ROS level on average 1,8 times compared to the control, and the highest average ROS level was recorded with exposure to 0.01 mg/L DCF (85,7 ± 2,3 fluorescence/ µg/ml).

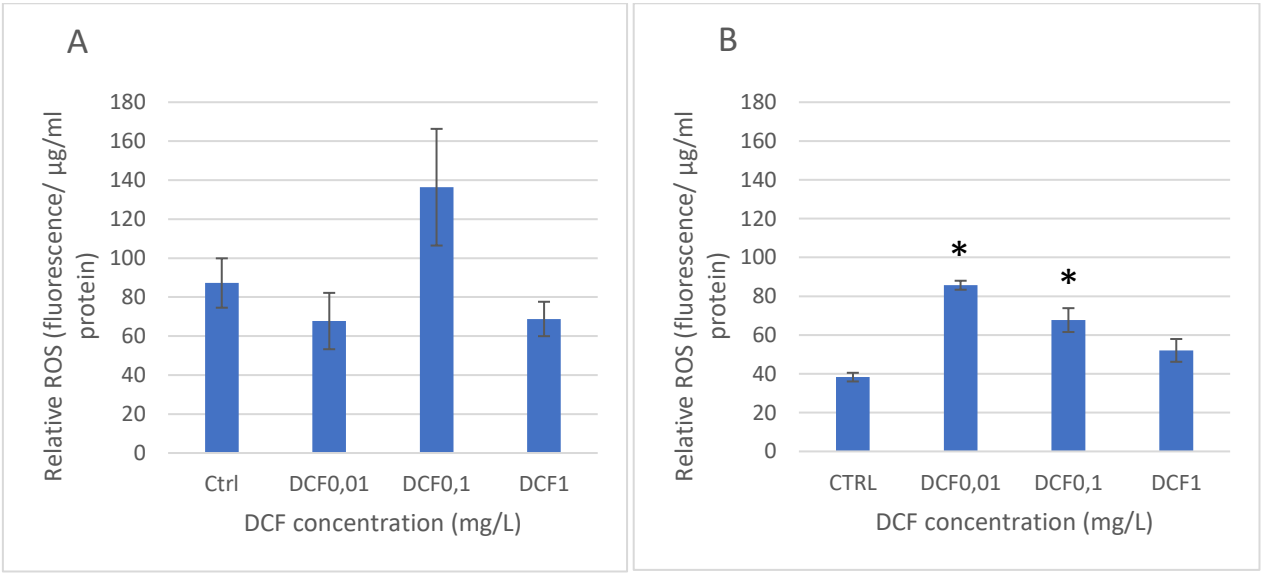


Figure 6. Relative ROS (fluorescence/ µg/ml) for daphnids exposed with DCF concentrations 0.01, 0.1, and 1 mg/L. A) For neonate daphnids and B) for adult daphnids. The error bars represent average relative ROS (fluorescence/ µg/ml protein) ± SD (n = 5). Significant difference ($p < 0.05$) compared to the control is marked with an asterisk (*).

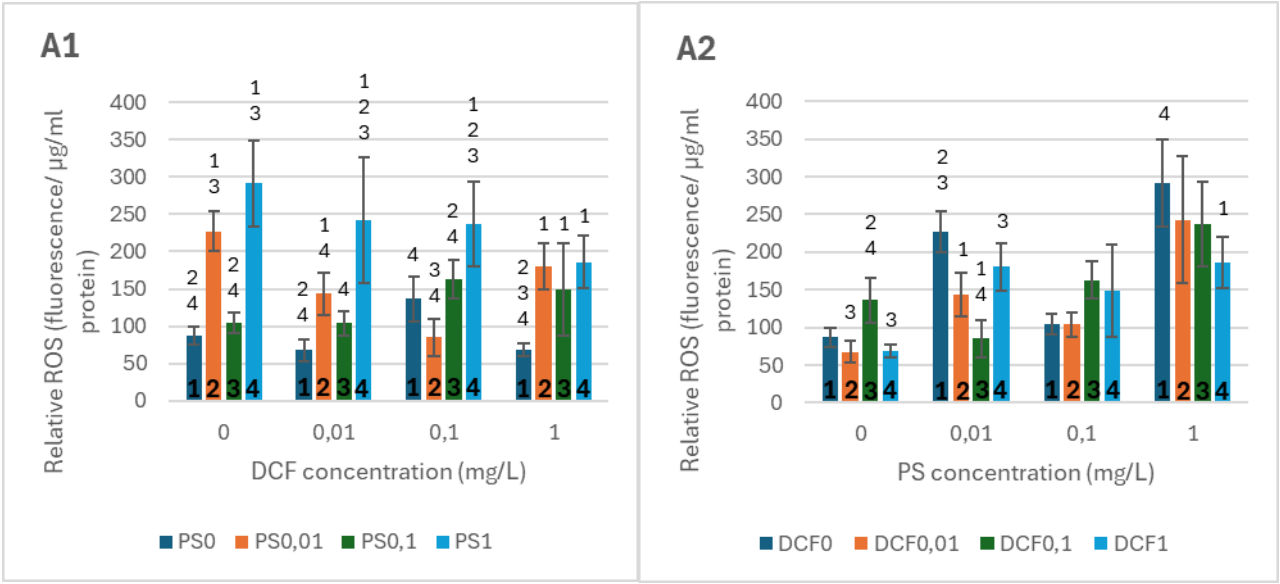
A significant combined effect of combination of PS and DCF on relative ROS level was observed in neonate daphnids (two-way ANOVA $p = 0.001$) and a highly significant combined effect in adult daphnids (two-way ANOVA $p < 0.001$) (Fig. 7A-B).

When comparing the combined exposures of different concentrations of PS with a given concentration of DCF, a positive trend in the relative ROS levels was observed in neonates. In combination with DCF concentration of 0.01 mg/L, a significantly higher ROS level was observed when comparing 0.01 and 1 mg/L PS to 0 mg/L PS (two-way ANOVA $p = 0.023$ and $p < 0.01$) and 1 mg/L PS to 0.01 and 0.1 mg/L PS (two-way ANOVA $p = 0.004$, $p < 0.01$). When combined with 0.1 mg/L DCF, a significantly lower ROS level was observed for 0, 0.01 and 0.1 mg/L PS in comparison with 1 mg/L PS (two-way ANOVA $p = 0.003$, $p < 0.001$ and $p = 0.026$, respectively), and also when comparing 0.01 mg/L PS to 0.1 mg/L PS (two-way ANOVA $p = 0.020$). In combination with 1 mg/L DCF, 0 mg/L PS resulted in significantly lower ROS in comparison with 0.01, 0.1 and 1 mg/L PS (two-way ANOVA $p = 0.001$, $p = 0.016$ and $p < 0.001$, respectively). (Fig. 7A1.) However, when different concentrations of DCF were compared in combination with a given concentration of PS, a similar pattern in neonate daphnids was not observed. The relative ROS level was significantly lower when 0.01 mg/L PS was combined with 0.01 or 0.1 mg/L DCF than with 0 mg/L DCF (two-way ANOVA $p = 0.013$ and $p < 0.001$), and also when combined with 0.1 mg/L DCF than with 1 mg/L DCF (two-way ANOVA $p = 0.005$). When the concentration of PS in the combination was 1 mg/L, a significantly lower relative ROS level was observed with 1 mg/L DCF than with 0 mg/L DCF (two-way ANOVA $p = 0.002$). (Fig. 7A2.)

In adult daphnids, increasing level of both PS and DCF in the combination resulted in a decrease in the relative ROS level. Coupled with DCF concentration of 0.01 mg/L, PS concentrations of 0.01, 0.1 and 1 mg/L resulted in significantly lower relative ROS level compared to 0 mg/L PS (two-way ANOVA $p < 0.001$) as well as PS concentration of 0.01 mg/L when compared to 1 mg/L PS (two-way ANOVA $p = 0.044$). PS concentrations of 0.01, 0.1 and 1 mg/L resulted in significantly lower relative ROS levels compared to 0 mg/L PS in combination with both 0.1 mg/L DCF (two-way ANOVA $p < 0.001$) and 1 mg/L DCF (two-way ANOVA $p = 0.002$, $p = 0.001$ and $p = 0.014$, respectively). (Fig. 7B1.) When combined with 0.01 mg/L PS, DCF concentrations of 0.01, 0.1 and 1 mg/L led to significantly lower relative ROS level than 0 mg/L PS (two-way ANOVA $p = 0.025$, $p < 0.001$ and $p < 0.001$, respectively), and 0.1 and 1 mg/L DCF resulted in significantly lower ROS level than 0.01 mg/L PS (two-way ANOVA $p = 0.011$). A significantly lower relative ROS level was also observed with the combination of PS concentration of 0.1 mg/L when comparing DCF

concentrations of 0.01, 0.1 and 1 mg/L to 0 mg/L DCF (two-way ANOVA $p = 0.002$, $p < 0.001$ and $p = 0.001$, respectively), as well as DCF concentration of 1 mg/L to 0.01 mg/L DCF (two-way ANOVA $p = 0.048$). Also, when comparing DCF concentrations of 0.01, 0.1 and 1 mg/L with 0 mg/L DCF in combination with 1 mg/L PS, a significantly lower relative ROS level was observed (two-way ANOVA $p = 0.020$, $p < 0.001$ and $p = 0.035$) (Fig. 7B2). As recommended by Pallant et al. (2011), the individual effects of PS and DCF are not further examined because of the significant interaction effect, but they have been tested with their own test and the results discussed in their own section (Fig. 5 & 6).

The difference in ROS levels between treatments with DCF alone and combinations of PS and DCF was highly significant for adult daphnids (Kruskal-Wallis $p < 0.001$) and significant for neonate daphnids (Kruskal-Wallis $p = 0.003$). Between treatments with PS alone and combination of PS and DCF a highly significant difference (Kruskal-Wallis $p < 0.001$) was found for adults, but no significant difference was found in neonates. In neonates, the combined exposures resulted in an average of 1.8 times higher relative ROS levels compared to exposures with DCF only. For adults PS exposures led on average to 2.4 times higher and DCF exposures 2.6 times higher relative ROS levels than the combined exposures.



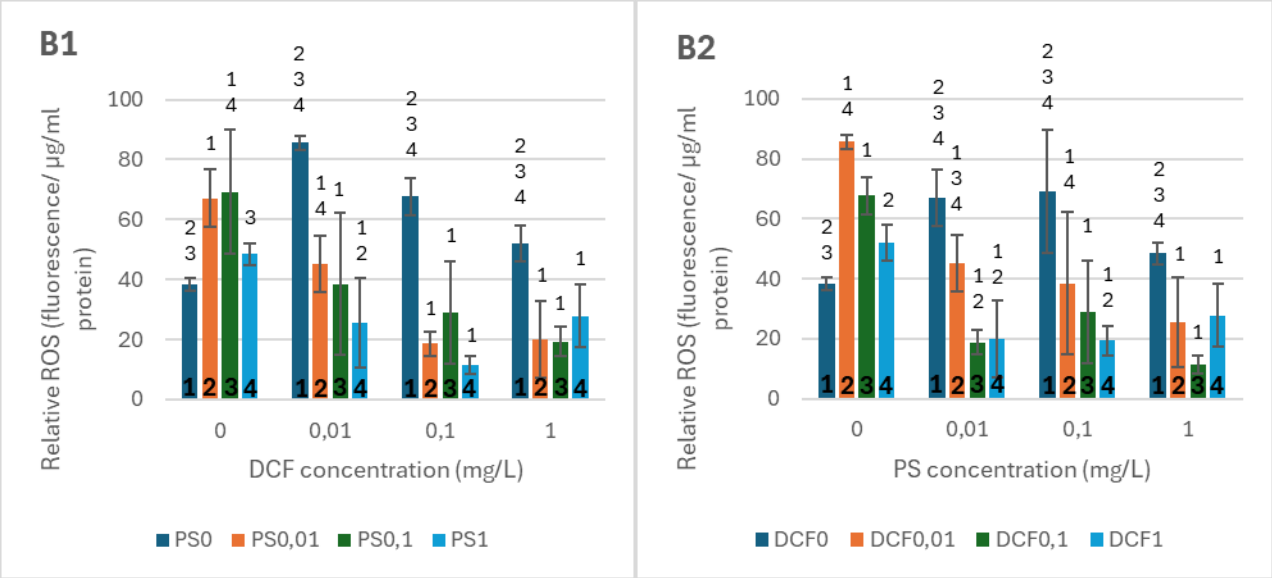


Figure 7. Relative ROS (fluorescence/ µg/ml) in *D. magna* exposed with different combinations of PS and DCF concentrations. Significant differences are presented separately between different concentrations of PS combined with a specific DCF concentration (1), and between different concentrations of DCF combined with a specific PS concentration (2), for neonates (A1&2) and adults (B1&2). Included exposures involve control exposure PS 0 + DCF 0, individual exposures PS 0 + DCF 0.01, PS 0 + DCF 0.1, PS 0 + DCF 1, PS 0.01 + DCF 0, PS 0.1 + DCF 0, PS 1 + DCF 0, and combined exposures PS 0.01 + DCF 0.01, PS 0.01 + DCF 0.1, PS 0.01 + DCF 1, PS 0.1 + DCF 0.01, PS 0.1 + DCF 0.1, PS 0.1 + DCF 1, PS 1 + DCF 0.01, PS 1 + DCF 0.1 and PS1 + DCF 1mg/L. The error bars represent average relative ROS (fluorescence/ µg/ml protein) ± SD (n = 5). Significant difference ($p < 0.05$) compared to other exposure is marked with the corresponding number.

The relative ROS levels were higher in neonates than in adult daphnids, and the difference between life stages was highly significant (paired-samples *t*-test $p < 0.001$) (Fig. 8). The difference between the two life stages was mainly explained by the differences in relative ROS levels of PS treated neonate and adult daphnids (paired-samples *t*-test $p = 0.002$) as well as those treated with combination of PS and DCF (paired-samples *t*-test $p < 0.001$).

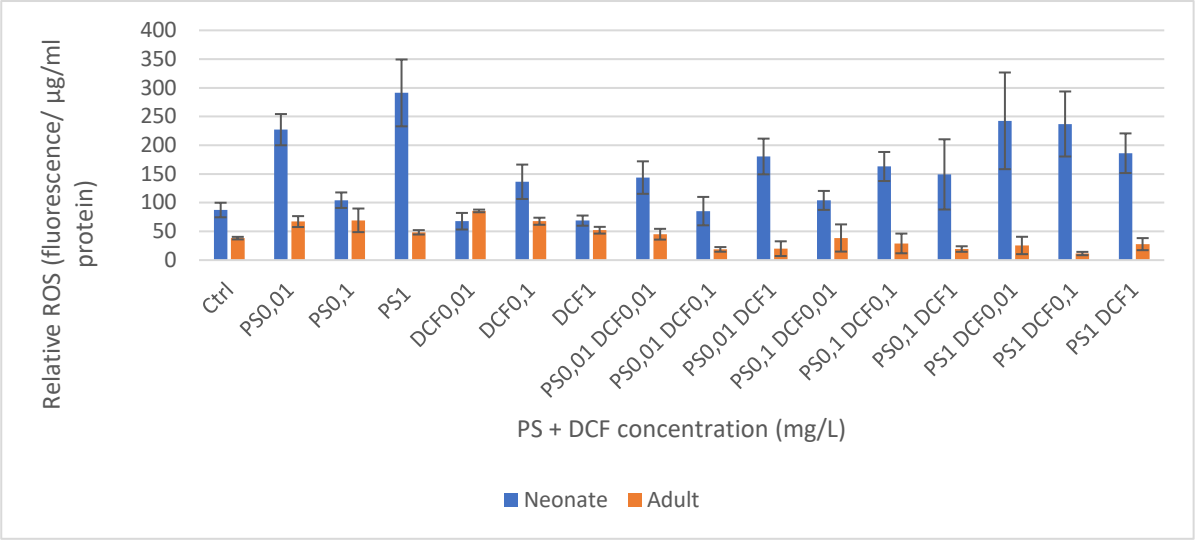


Figure 8. Relative ROS (fluorescence/ µg/ml) for neonate and adult daphnids treated with PS (0.01, 0.1, and 1 mg/L), DCF (0.01, 0.1, and 1 mg/L) and combination of PS and DCF (PS 0.01 + DCF 0.01, PS 0.01 + DCF 0.1, PS 0.01 + DCF 1, PS 0.1 + DCF 0.01, PS 0.1 + DCF 0.1, PS 0.1 + DCF 1, PS 1 + DCF 0.01, PS 1 + DCF 0.1 and PS1 + DCF 1 mg/L. The error bars represent the average relative ROS (fluorescence/ µg/ml protein) ± SD (n = 5).

3.3 EC_{50} response analysis

EC_{50} was calculated for each contaminant individually and combined, and it is shown in table 2. For neonate *D. magna,* the EC_{50}s after 48 h were determined as following: for PS EC_{50} was 21.4 mg/L and for DCF EC_{50} was 19.5 mg/L. For determination of the combined effect of PS and DCF, equitoxic concentrations were used in previous studies. Because the EC_{50} of PS and DCF obtained for neonates were close to each other, the combinations used were PS 0.01 + DCF 0.01, PS0.1 + DCF 0.1, PS 1 + DCF 1, PS 10 + DCF 10, PS 50 + DCF 50 and PS 100 + DCF 100 mg/L. Those were considered double concentrations in probit analysis, and the EC_{50} obtained for PS + DCF was 12.3 mg/L. The ratio between the expected EC_{50} for PS + DCF (20.4 mg/L) as well as the observed one (12.3 mg/L) was 1.66, which could be interpreted as a synergistic effect. Because of the high mobility percentage also with the highest exposure concentrations, the calculation of the EC_{50} is considered unreliable for adult *D. magna*. (Table 2.)

NOAEC was obtained as the lowest concentration that had no significant difference compared to control, and for neonate daphnids after 48 h, this was determined as 10 mg/L for PS alone and DCF alone, and 2 mg/L for PS and DCF combined. LOAEC was the lowest tested concentration that had a significant difference compared to control, and it was expected to be around 50 mg/L for PS alone and DCF alone, and 20 mg/L for PS and DCF combined for neonate daphnids after 48 h. (Table 2.)

Table 2. *Dose descriptors for PS, DCF and PS + DCF for neonate D. magna after 48 hours. EC_{50} = median effective concentration, NOAEC = no observed adverse effect concentration, LOAEC = the lowest observed adverse effect concentration.*

Dose descriptor	PS	DCF	PS + DCF
EC_{50}	21.4 mg/L	19.5 mg/L	12.3 mg/L
NOAEC	10 mg/L	10 mg/L	2 mg/L
LOAEC	50 mg/L	50 mg/L	20 mg/L

4 Discussion

4.1 Individual Toxicity

In this study, a significant decrease in *D. magna* mobility was observed in neonate daphnids after 48 h individual exposures to PS and DCF, aligned with the hypothesis that the toxicity of those contaminants increases with concentration (Fig. 2A & 3A). The concentrations tested included both environmentally relevant concentrations and concentrations much higher than those observed in aquatic environments. However, the lowest concentrations did not cause significant decrease in mobility, and neither did the higher test concentrations in neonate daphnids after 24 h and adult daphnids after 24 and 48 h (Fig. 2A&B, 3A&B). The results indicate that only the higher than environmentally relevant concentrations of PS and DCF affect *D. magna* mobility after 48 h, and that exposure time and life stage also have a significant impact on the immobilization response. In EC_{50} estimation for neonates after 48 h, a value of 21.4 mg/L was obtained for PS (Table 2), which is very close to the LC_{50} value obtained for 5 µm PS-MPs by He et al. (2023), which is 21.66 mg/L. However, there is a lot of variation in the values obtained in the 48-hour acute toxicity test of PS on *D. magna*, for example Yin et al. (2020) estimated an EC_{50} of 36.5 mg/L and Ma et al. (2016) found no immobilization with the highest test concentration of 100 mg/L, both using spherical PS particles around 5 µm in size.

The characteristics of PS particles can affect their toxicity, including i.e. PS particle shape and size (Lee et al., 2025; Li et al., 2025; Samadi et al., 2022; Yin et al., 2020). The study by Lee et al. (2025) detected a clear difference in the acute toxicity of PS to *D. magna* related to the PS particle shape, fragmented particles being more toxic than bead-shaped ones. Also, in the present study PS particles were fragmented, which can be one reason explaining a low EC_{50} value. The acute toxicity of PS to *D. magna* has been found to be greater for smaller particles (Pikuda et al., 2023) e.g. in a study by Li

et al. (2025), that observed a difference in toxicity between 1 and 50 µm particles. However, not all studies suggest smaller PS particles to have the greatest toxic effect. In the study by Esterhuizen et al. (2023), that used i.e. ROS levels as endpoint, only the medium sized, 45-63 µm, and large, 100-200 µm, particles caused oxidative stress indicating changes in adult daphnids. The authors argued that the toxic effect could have been explained by additives derived from PS particles because of the size of the largest particles. If that had been the case also in this study, the changes observed in mobility would not have been related to consumption of PS by *D. magna*. However, it can be assumed that the PS particles of 45-63 µm used in this study could have been consumed by daphnids, that are able to feed on particles ranging from 0.2 to 70 µm (Rosenkranz et al., 2009). Also, both fragmented and larger MP particles can be more difficult to be egested than the round shaped and smaller sized particles within the food size spectrum of *D. magna* (Chen et al., 2022; Frydkjaer et al., 2017), suggesting that these characteristics of PS particles used in this study could have increased PS toxicity.

The EC_{50} estimated for DCF, 19.5 mg/L (Table 2), was close to the EC_{50} values found for DCF in previous studies, involving 18.1 mg/L obtained by Du et al. (2016) and 22.4 mg/L by Ferrari et al. (2004). However, de Oliveira et al. (2016) obtained a much higher EC_{50} of 123.3 mg/L. The EC_{50} values estimated for DCF are multiples compared to the DCF concentrations detected in the environment (de Oliveira et al., 2016; Parolini, 2020). It is argued that some factors may increase the toxic risks of DCF and other NSAIDS on species such as *D. magna*, including their directed impact i.e. on gastrointestinal tract, which in *D. magna* is mainly responsible for feeding (Du et al., 2016), as well as the potentially higher toxicity of biotransformation products and related effects on bioaccumulation (Fu et al., 2020; Miller et al., 2018).

A biochemical marker of oxidative stress, ROS is a more subtle endpoint than mobility (Hook et al., 2014), and in the present study the analysis of ROS was done only with the lowest PS and DCF concentrations (0.01-1 mg/L), because of high mortality at the highest concentrations would have caused difficulty in comparison while dead daphnids were excluded from the ROS samples. In neonate daphnids the results of individual exposures seemed to be inconsistent with the hypothesis for the most part. For PS a significant difference was observed but increase in ROS level in comparison with control only applied to PS concentrations 0.01 and 1 mg/L (Fig. 5A). The pattern seemed opposite to that observed with DCF, however no significant differences between DCF treatments and control were observed (Fig. 6A). The drop in ROS level in the middle concentration is difficult to explain, and a possible error in the measurement cannot be excluded. However, an example of nonlinear effects of different concentrations is found at least for DCF in the study by Nkoom et al. (2019), where

the malondialdehyde (MDA) content of *D. magna*, related to over production of ROS, was significantly highest after exposure to the medium test concentration of DCF, 15 µg/L, tested concentrations ranging from 5 µg/L to 100 µg/L. In a study conducted by Esterhuizen et al. (2023) 0.5 mg/L PS-MP did not affect ROS levels in neonate *D. magna* after 48 h. That result is different from the significant effect of PS on ROS in neonates observed in the present study, but on the other hand the concentration they used is closest to the concentration 0.1 mg/L, also with no effect on ROS levels in the present study.

Neither the results for adult daphnids were not totally aligned with the hypothesis: both exposures with PS or DCF alone led to significant increase in ROS for the two lowest concentrations 0.01 and 0.1 mg/L, but not for 1 mg/L compared to control (Fig. 5A&B). Similar pattern observed for both PS and DCF exposures could however suggest that there could be some factor decreasing ROS level in adult daphnids when the concentration of those contaminants increases enough, i.e. related to activation of the antioxidative system. However, that is not possible to conclude here, especially without knowing the responses of the antioxidative system. For example, measuring activity of the typical enzymes involved in the antioxidative response counteracting elevated ROS levels, such as superoxide dismutase (SOD) and catalase (CAT), could contribute to a better understanding of the explanations for the observed ROS levels (Esterhuizen et al., 2023). In the study by Esterhuizen et al. (2023) activation of antioxidant defense system was a probable explanation behind the decrease of ROS levels to control level after 48 h of 0.5 mg/L PS-MP exposure in adult *D. magna.* Elevated ROS levels had been observed after 24 h indicating oxidative stress experienced by daphnids, whereas the return of ROS to control levels after 48 h was probably explained by the increase in SOD and CAT activity, possibly helping to regain redox homeostasis in that case. ROS levels after 24 h are not included in the present study, so the ROS levels can be examined only after 48-hour time. The possible explanation for low ROS levels observed in adults exposed to 1 mg/L PS or DCF can be related i.e. to the activation of antioxidant defense system, but the result can also indicate that the exposure concentrations in question do not affect ROS levels and thus are not causing significant oxidative stress in adult *D. magna*.

4.2 Combined toxicity

A significant combined effect for both PS and DCF was observed in neonate daphnid mobility after 24 h and in neonate and adult daphnids after 48 h, with higher concentrations of both contaminants in combination leading to lower mobility (Fig. 5A1&2, B1&2 and D1&2). This was according to the

hypothesis, as was the estimated synergistic effect of the combination of PS and DCF on neonates after 48 h indicated by the ratio between expected and observed EC_{50} values, with the observed value being 12.3 mg/L (Table 2). Also, statistical analysis found a significant effect for both PS and DCF in combinations both for neonate and adult daphnids, suggesting they both play an important role in toxicity of the combination. The estimated synergistic effect suggests a possibility that PS-MPs may have acted as vectors to DCF (Du et al., 2021; Yang et al., 2024). This could possibly have resulted from DCF sorption on PS particles e.g. through the π–π interactions or hydrophobic interactions (Atugoda et al., 2021; Zhang et al., 2017) and thus increased DCF concentration on PS particle surface that would have been carried inside daphnids with the PS particles (Pei et al., 2025; Rehse et al., 2016). DCF accumulated on PS particles could have been further released within daphnids, enhanced also by different conditions present in medium and inside the organisms (Rehse et al., 2016; Bakir et al., 2014). It is probable that PS particle size used in this study could have allowed a synergistic effect, while particles have been small enough to be consumed by daphnids (Rosenkranz et al., 2009). This may have allowed DCF, possibly bound to PS particles, to end up inside daphnids instead of remaining outside them, as would happen if the particle size was too big to enter the organism, likely leading in an antagonistic direction in that case (Yang et al., 2024). Also, among other possibilities behind synergistic effect are for example complementary biological effects of the two contaminants in the organism (Yang et al., 2024), as well as leaching of additives from MP, affecting MP toxicity and the final combined effect with the sorbed contaminants (Samadi et al., 2022; Pikuda et al., 2023). The toxicity of combined exposure can thus be increased in comparison to single exposure related e.g. to bioavailability and lead to oxidative stress and immobilization responses in daphnids, suggesting also a possibility for wider ecological implications, related to the ecological importance of *D. magna* as a key species (Bosker et al., 2019; Pei et al., 2025; Pikuda et al., 2023).

As hypothesized, the combination of PS and DCF led to significantly higher ROS levels in neonate daphnids when comparing different PS concentrations with each other in the combinations with DCF, probably indicating increased oxidative stress (Fig. 7A1). However, increasing DCF level in the combination did not similarly contribute to elevated ROS level, in line with the low ROS levels observed in neonates as response to DCF alone (Fig. 7A2). In adult daphnids, however, the higher concentrations of both PS and DCF resulted in lower ROS levels (Fig. 7B1&2). One possible explanation behind the different results between ROS levels found in neonates and adults after co-exposure to PS and DCF could be related to different biochemical responses between the two life stages. Esterhuizen et al. (2023) found increase in SOD and CAT activity in adult daphnids as well as a subsequent drop in ROS levels after 48 h exposure, probably resulting from the antioxidant enzyme activation. Similar

increase in SOD and CAT was not observed in neonate daphnids, leading to a conclusion that in neonate daphnids nonenzymatic activities, rather than enzymatic ones, could be involved in elimination of excess ROS. If that would be the case also in the present study, the lower ROS levels observed in adult daphnids as a response to combination of PS and DCF could result from more efficient antioxidant defense systems in comparison to neonates, helping the recovery from possible oxidative stress. Another possible explanation is, of course, that the low ROS levels indicate that no significant oxidative stress was caused by the combined exposures.

Previous peer reviewed studies on the combined effect of PS and DCF on *D. magna* do not exist so far to the author's best knowledge, however, some research has investigated the combined effect of PS and other pharmaceuticals on *D. magna*. He et al. (2023), who exposed *D. magna* with PS and CBZ, found different LC_{50} values depending on PS particle size, so that the largest PS particles tested, 5 µm, had an increasing effect on CBZ toxicity. Zhang et al. (2019) on the other hand observed the strongest oxidative stress in response to combined exposure to ROX and the smallest tested PS-MPs of 1 µm. In a study conducted by Pashaei et al. (2023) PS-MP and PS-NP size as well as concentration of both PS and TCS affected the mortality rates of *D. magna*, suggesting the complex nature of interactions between multiple contaminants. The combined effects of PS-MP and pharmaceuticals have also been seen after long-term exposure and in next generations when reproductive parameters in *D. magna* have been exposed to PS-MP and CBZ (He et al., 2023) or ROX (Liu et al., 2022). Those results suggest i.e. that factors, such as PS particle size, can affect the combined toxicity of PS-MP and pharmaceuticals on *D. magna*, but since different pharmaceuticals have been used in the studies in question, little can be concluded from their results regarding the present study. The situation is similar for the research concerning the combined effect of PS and DCF studied so far on other organisms than *D. magna*. Kandaswamy et al. (2024) found synergistic effects of PS-NP and DCF on zebra fish (*Danio rerio*), including effects to e.g. embryonic development and adult mortality, as well as higher ROS levels of fish larvae. By contrast, a study with two algal species**,** *Phaeodactylum tricornutum* and *Euglena* sp., found that the combination of PS-MP and DCF resulted in lower toxicity than the contaminants alone, i.e. in terms of growth rate and oxidative stress (Ding et al., 2023). However, one master's thesis has previously addressed the combined effect of PS-MP and DCF on adult *D. magna* (Honkanen, 2023). No effect on acute toxicity was observed, but changes in oxidative stress related antioxidative enzyme responses suggested rather an antagonistic effect of the combination of PS and DCF. The contrasting results from the current study may be explained at least partly by shorter exposure time of 24 h combined with lower exposure concentrations of 0.5 mg/L for PS, DCF and their combinations. The concentration of that magnitude did not affect daphnid mobility in the present

study, but an impact on ROS levels in adult daphnids was observed, however after a 48-hour exposure. Unlike the study by Esterhuizen et al. (2023), no elevated ROS levels were observed after 24 h in the study by Honkanen (2023). The increase in ROS levels observed by Esterhuizen et al. (2023) only applied to the particle size 45-63 µm in the case of adult daphnids, which is the same size range used in the current study, whereas PS particles used by Honkanen (2023) had a wider scale, ranging from 25 to 100 µm in size. PS particle size is one factor shown to affect PS toxicity towards *D. magna* (Li et al., 2025), also in combination with pharmaceuticals (He et al., 2023; Zhang et al., 2019).

4.3 Overall assessment of toxicity to *D. magna*

The EC_{50} values estimated for PS and DCF in this study were very close to each other, 21.4 and 19.5 mg/L, respectively, whereas the EC_{50} estimation for the combination of the two contaminants was clearly lower, indicating greater toxicity of the combination (Table 2). Based on the EU classification in European Directive EC 93/67/EEC (European Commission, 1993; Grabarczyk et al., 2020) EC_{50} values obtained both for the toxicants alone and in combination fall between 11-100 mg/L, which is classified as "harmful to aquatic organisms". The NOAEC and LOAEC estimations of this study are only indicative and estimated to be between the values obtained: from 10 to 50 mg/L for PS and DCF alone, and from 2 to 20 mg/L for the combination, not providing much additional information (Table 2). In addition to testing acute toxicity, the more subtle endpoints can detect impact of the contaminants also at lower concentrations (Hook et al., 2014; Tkaczyk et al., 2021), that may also have significant adverse effects on aquatic organisms, especially with long-term exposures and when affecting important life stage events, e.g. concerning reproduction. (Du et al., 2016.) In the present study the lower test concentrations, 0.01-1 mg/L, did have impact on ROS levels in *D. magna*, the effect being most clearly interpretable as an indication of oxidative stress for neonate daphnids exposed to combination of PS and DCF. Even though the EC_{50} values obtained for PS, DCF and their combination are much higher than the environmental concentrations, the ROS results suggest possible adverse effects on *D. magna* in response to concentrations of the same magnitude, also after a 48 h exposure.

When comparing the different responses in mobility, neonate daphnids were significantly more sensitive to PS, DCF and their combination after a 24-hour exposure than adult daphnids, but the difference between the life stages was no longer significant after 48 h, when the adult mobility has decreased closer to the neonate mobility percentages. However, if considering only the highest concentrations, practically 50 and 100 mg/L, a significant difference between the two life stages was observed also after 48 h. The results from mobility do support the hypothesis of higher sensitivity of

neonates compared to adults. The ROS results are not that simple to compare, also because of the possibility that the two life stages may have different antioxidative defense systems (Esterhuizen et al., 2023). However at least the ROS levels observed in neonate daphnids in response to the combined exposure are higher than those observed in adult daphnids. It is not possible to say how the ROS levels have reacted i.e. after 24 h, but it is obvious that the ROS levels in neonates were higher than the ROS levels in adults when measured at 48 h. It is possible that neonate daphnids were not, at least before that point, able to combat the excess ROS, perhaps due to differences in antioxidant defense systems or other sensitivities (Esterhuizen et al., 2023; Traudt et al., 2017).

4.4 Reliability and restrictions of the study

Attention has been drawn to the fact that the MP concentrations used in studies are usually much higher than the environmentally relevant concentrations (Samadi et al., 2022). When considering the highest concentrations detected in the aquatic environment, estimated here to be around 0.03–0.04 mg/L for PS from the 76000 PS-MP particles per liter observed by Badylak et al. (2021), and 0.015 or 0.019 mg/L for DCF (Jux et al., 2002; aus der Beek et al., 2016), the lowest test concentrations used in the present study, 0.01 mg/L are of the same order of magnitude. The highest concentrations included, 100 mg/L, indeed are far from the concentrations found in the environment. However, including them is important for EC_{50} estimation, and in fact even higher concentrations would have been required especially for EC_{50} estimation for adult daphnids. While the irregular shape is assumed to be the most common in MPs found in the aquatic environment (Phuong et al., 2016; Wright et al., 2013), and 20-100 µm was the most abundant MP size found in a study by Uurasjärvi et al. (2020), however, recognizing the difficulty of determining the most abundant MP sizes, the PS-MPs used in this study can be considered quite representative of the MP particles found in the aquatic environments in terms of their shape and size. Also, some factors present in the environment may affect the behavior of MP and pharmaceuticals and their combined effect in ways that are not considered in the estimations only based on concentrations. For example, biofilm that forms on MP surface can change the density of MP particles and further lead to different probability of encounter of MPs and Daphnia. (Funke et al., 2024) Aging of MP particles and biofilm formation is also one factor that can affect the interaction between MP particles and chemicals, potentially leading to enhanced adsorption of chemicals to MPs as well as increased toxicity towards Daphnia (Funke et al., 2024: Qi et al., 2021).

A possible factor that can impact DCF concentrations also in laboratory conditions, is its susceptibility to photodegradation (Boreen et al., 2003; Qutob et al., 2023). All the steps in the study with DCF

present were carried out in dark conditions to prevent photodegradation, however DCF concentration was not confirmed by measurement during the test exposures. It is also possible that the test conditions can alter i.e. Daphnia behavior. For example, the fact that during the 48-hour exposure time daphnids were not fed anymore may have potentially impacted the intake of MP by daphnids, as it is possible that daphnids may selectively avoid plastics when algae are present (Aljaibachi & Callaghan, 2018). Also, visible PS adhesion to daphnids external appendages was observed with the exposures containing 100 mg/L PS, in line with previous observations of MP adhesion into body surfaces on *D. magna* (Eltemsah & Bøhn, 2019) as well as on copepods (Cole et al., 2013). In the present study similar impact was not observed in daphnids exposed to lower concentrations of PS, so the effect was probably result of the high PS concentration, however the possibility exists that some factors in the experimental setup could have had an impact as well.

Regarding the research conditions, a problem occurred with adult daphnids while they were grown for the last experiment. Daphnids started to die, probably due to automatic feeder used for practical reasons, possibly leading to accumulation of excess food in the media and possible subsequent problems in hygiene. The experiment was repeated with normal feeding procedure, with no problems observed at that time. The test concentrations in use allowed the estimation of LOAEC and NOAEC only at a very indicative level and the results show a very wide range within which these values lie. Also, although the comparison of EC_{50} estimations for neonate and adult daphnids would have been a nice addition, calculating EC_{50} for adults was not possible because of the low immobilization rate even at high concentrations. However, according to the OECD instructions EC_{50} testing is intended to be performed on neonates, and so the results are comparable to other studies (OECD, 2004).

4.5 Thoughts for future research

The importance of research on the effects of MP is further emphasized when considering possible future prospects and their wider impact on ecosystems. The environmental MP concentrations are still increasing if nothing is done to prevent it – for example in the report of INC-5.2 by UNEP (2025) it is estimated that the amount of plastic waste will increase over threefold from the present situation by 2050. Also, multiple stressors present at the same time, involving global change related phenomena, such as increasing temperature and eutrophication, as well as presence of multiple contaminants need to be considered (Cambronero et al., 2018; Hook et al., 2016; Pashaei et al., 2023). As an important key species, that has a role both as a grazer of phytoplankton and a food source for species at higher trophic levels, the adverse effects of MP on *D. magna* populations could further affect the

entire freshwater ecosystems (Bosker et al., 2019; Pikuda et al., 2023). Also, other parts of food web may be influenced by the MP ingested by daphnids as a result of bioaccumulation and biomagnification (Pikuda et al., 2023). In fact, trophic transfer of MP has been observed to occur widely in aquatic food web, starting from zooplankton (Suman et al., 2021).

Longer exposure times as well as more versatile endpoints can be recommended for future studies considering the individual and combined effects of MP and pharmaceuticals. The results of acute toxicity tests alone do not take into account the fact that in natural environments aquatic organisms can be affected by contaminants throughout their lifetime (Parolini et al., 2020) and the effects can also be transgenerational (He et al., 2023; Liu et al., 2022). The role of chronic exposures as well as sublethal effects should not be underestimated, and thus the potential adverse effects of lower concentrations of contaminants than those determined in acute toxicity tests are also worth considering (Du et al., 2016; Parolini et al., 2020). Investigating the effects of multiple stressors is challenging but it is important for evaluating the interactions between them and understanding the complex responses (Cambronero et al., 2018, Hook et al., 2016; Pashaei et al., 2023). For a wider understanding of the effects of MP on aquatic ecosystems more research concerning MP and their combined effects with different chemicals, involving PS-MP and DCF as well as other types of MPs and pharmaceuticals, is required. More knowledge is needed of *D. magna* responses as well as wider ecological impacts related to the simultaneous exposure to MP and different pharmaceuticals and other chemicals, as well as other stressors, to be able to assess the risks involved and consider effective ways to protect the aquatic ecosystems. (Cambronero et al., 2018, Pashaei et al., 2023.)

5 Conclusions

This study provides one of the very first insights into the combined effect of PS-MP and DCF on *D. magna.* The results support the hypotheses of the toxicity of these contaminants, both individually and in combination, to *D. magna*, indicated by changes in mobility and ROS levels. There also seems to be differences in the responses between the two life stages, while the effects on neonate daphnids were more pronounced. Aligned with the hypothesis of greater combined toxicity, EC_{50} estimation suggests a synergistic effect of the combination of PS and DCF on *D. magna*. Using EU classification, PS, DCF and their combination can be referred as harmful to aquatic organisms based on the estimated EC_{50} values, which however all are much higher than the environmental concentrations. Instead, concentrations used in ROS analysis are lower and more environmentally relevant. Based on

ROS results, it can be assumed that oxidative stress may have been caused, especially in neonates as a response to increasing PS concentrations in combined exposure. All the factors that can affect the toxicity of MP and pharmaceuticals in natural conditions are not possible to take into account in laboratory experiments. However, in future studies it is good to consider particularly different, including more subtle, endpoints, longer exposure times, different exposure concentrations, life stages and contaminants. With predicted increase in the amount of plastic in the aquatic environment, and presence of mixture of other contaminants as well as other environmental change related stressors, the need of evaluating the combined toxicity is still increasing, especially when the research on the subject is still limited. The results of this study contribute to knowledge about combined toxicity of MPs and pharmaceuticals, and the possible increased adverse effects of their combination on aquatic organisms and ecosystems compared to the individual effects of the contaminants. The co-occurrence of multiple contaminants and the potential enhanced toxicity related to their interactions is good to be considered in environmental risk assessment frameworks and environmental policy. Highlighting the importance of efficient management and mitigation of both plastics and pharmaceuticals is essential, including, for example, measures to reduce the excess use of these substances as well as their release into the environment, i.e. by replacing plastics with other materials and by improving wastewater management.

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References

Acuña, V., Ginebreda, A., Mor, J. R., Petrovic, M., Sabater, S., Sumpter, J., & Barceló, D. (2015). Balancing the health benefits and environmental risks of pharmaceuticals: Diclofenac as an example. *Environment International*, *85*, 327–333. https://doi.org/10.1016/j.envint.2015.09.023

Acuña, V., Bregoli, F., Font, C., Barceló, D., Corominas, L., Ginebreda, A., Petrovic, M., Rodríguez-Roda, I., Sabater, S., & Marcé, R. (2020). Management actions to mitigate the occurrence of pharmaceuticals in river networks in a global change context. *Environment International*, *143*, 105993-. https://doi.org/10.1016/j.envint.2020.105993

Adema, D. M. M. (1978). Daphnia magna as a test animal in acute and chronic toxicity tests. *Hydrobiologia*, *59*(2), 125–134. https://doi.org/10.1007/bf00020773

Aljaibachi, R., & Callaghan, A. (2018). Impact of polystyrene microplastics on Daphnia magna mortality and reproduction in relation to food availability. *PeerJ (San Francisco, CA)*, *6*, Article e4601. https://doi.org/10.7717/peerj.4601

Atugoda, T., Vithanage, M., Wijesekara, H., Bolan, N., Sarmah, A. K., Bank, M. S., You, S., & Ok, Y. S. (2021). Interactions between microplastics, pharmaceuticals and personal care products: Implications for vector transport. *Environment International*, *149*, 106367-. https://doi.org/10.1016/j.envint.2020.106367

Augello, F. R., Lombardi, F., Ciummo, V., Ciafarone, A., Cifone, M. G., Cinque, B., & Palumbo, P. (2025). COX-2 Inhibition in Glioblastoma Cells Counteracts Resistance to Temozolomide by Inducing Oxidative Stress. *Antioxidants*, *14*(4), 459. https://doi.org/10.3390/antiox14040459

Badylak, S., Phlips, E., Batich, C., Jackson, M., & Wachnicka, A. (2021). Polystyrene microplastic contamination versus microplankton abundances in two lagoons of the Florida Keys. *Scientific Reports*, *11*(1), Article 6029. https://doi.org/10.1038/s41598-021-85388-y

Bagaev, A., Khatmullina, L., & Chubarenko, I. (2018). Anthropogenic microlitter in the Baltic Sea water column. *Marine Pollution Bulletin*, *129*(2), 918–923. https://doi.org/10.1016/j.marpolbul.2017.10.049

Bakir, A., Rowland, S. J., & Thompson, R. C. (2014). Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution (1987)*, *185*, 16–23. https://doi.org/10.1016/j.envpol.2013.10.007

aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., & Küster, A. (2016). Pharmaceuticals in the environment—Global occurrences and perspectives. *Environmental Toxicology and Chemistry*, *35*(4), 823–835. https://doi.org/10.1002/etc.3339

Bell, A. (2005). Antimalarial drug synergism and antagonism: Mechanistic and clinical significance. *FEMS Microbiology Letters*, *253*(2), 171–184. https://doi.org/10.1016/j.femsle.2005.09.035

Bianchini, A., & Wood, C. M. (2008). Sodium uptake in different life stages of crustaceans: the water flea Daphnia magna Strauss. *Journal of Experimental Biology*, *211*(Pt 4), 539–547. https://doi.org/10.1242/jeb.009175

Boreen, A. L., Arnold, W. A., & McNeill, K. (2003). Photodegradation of pharmaceuticals in the aquatic environment: A review. *Aquatic Sciences*, *65*(4), 320–341. https://doi.org/10.1007/s00027-003-0672-7

Bosker, T., Olthof, G., Vijver, M. G., Baas, J., & Barmentlo, S. H. (2019). Significant decline of Daphnia magna population biomass due to microplastic exposure. *Environmental Pollution (1987)*, *250*, 669–675. https://doi.org/10.1016/j.envpol.2019.04.067

Bownik, A., Adamczuk, M., & Skowrońska, B. P. (2023). Effects of cyanobacterial metabolites: Aeruginosin 98A, microginin-FR1, anabaenopeptin-A, cylindrospermopsin in binary and quadruple mixtures on the survival and oxidative stress biomarkers of Daphnia magna. *Toxicon (Oxford)*, *229*, 107137–107137. https://doi.org/10.1016/j.toxicon.2023.107137

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brausch, J. M., Connors, K. A., Brooks, B. W., & Rand, G. M. (2012). Human Pharmaceuticals in the Aquatic Environment: A Review of Recent Toxicological Studies and Considerations for Toxicity Testing. *Reviews of Environmental Contamination and Toxicology Volume 218*, *218*, 1–99. https://doi.org/10.1007/978-1-4614-3137-4_1

Cambronero, C., M., Marshall, H., De Meester, L., Davidson, T. A., Beckerman, A. P., & Orsini, L. (2018). Predictability of the impact of multiple stressors on the keystone species Daphnia. *Scientific Reports*, *8*(1), Article 17572. https://doi.org/10.1038/s41598-018-35861-y

Castro, G. B., Bernegossi, A. C., Felipe, M. C., & Corbi, J. J. (2020). Is the development of Daphnia magna neonates affected by short-term exposure to polyethylene microplastics? *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, *55*(8), 935–946. https://doi.org/10.1080/10934529.2020.1756656

Chen, C. C., Shi, Y., Zhu, Y., Zeng, J., Qian, W., Zhou, S., Ma, J., Pan, K., Jiang, Y., Tao, Y., & Zhu, X. (2022). Combined toxicity of polystyrene microplastics and ammonium perfluorooctanoate to Daphnia magna: Mediation of intestinal blockage. *Water Research (Oxford)*, *219*, Article 118536. https://doi.org/10.1016/j.watres.2022.118536

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., & Galloway, T. S. (2013). Microplastic Ingestion by Zooplankton. *Environmental Science & Technology*, *47*(12), 6646–6655. https://doi.org/10.1021/es400663f

Crane, M., & Newman, M. C. (2000). What level of effect is a no observed effect? *Environmental Toxicology and Chemistry*, *19*(2), 516–519. https://doi.org/10.1002/etc.5620190234

De Felice, B., Sabatini, V., Antenucci, S., Gattoni, G., Santo, N., Bacchetta, R., Ortenzi, M. A., & Parolini, M. (2019). Polystyrene microplastics ingestion induced behavioral effects to the cladoceran Daphnia magna. *Chemosphere (Oxford)*, *231*, 423–431. https://doi.org/10.1016/j.chemosphere.2019.05.115

Delahaut, V., Rašković, B., Salvado, M. S., Bervoets, L., Blust, R., & De Boeck, G. (2020). Toxicity and bioaccumulation of Cadmium, Copper and Zinc in a direct comparison at equitoxic concentrations in common carp (Cyprinus carpio) juveniles. *PloS One*, *15*(4), e0220485-. https://doi.org/10.1371/journal.pone.0220485

Depledge, M. H., & Fossi, M. C. (1994). The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology (London)*, *3*(3), 161–172. https://doi.org/10.1007/BF00117081

Dey, A., Dhumal, C. V., Sengupta, P., Kumar, A., Pramanik, N. K., & Alam, T. (2021). Challenges and possible solutions to mitigate the problems of single-use plastics used for packaging food items: a review. *Journal of Food Science and Technology*, *58*(9), 3251–3269. https://doi.org/10.1007/s13197-020-04885-6

Ding, T., Huang, X., Wei, L., & Li, J. (2023). Size-dependent effect of microplastics on toxicity and fate of diclofenac in two algae. *Journal of Hazardous Materials*, *451*, 131071–131071. https://doi.org/10.1016/j.jhazmat.2023.131071

Drzymała, J., & Kalka, J. (2024). Assessment of genotoxicity, mutagenicity, and cytotoxicity of diclofenac and sulfamethoxazole at environmental concentrations on Vicia faba. *International Journal of Environmental Science and Technology (Tehran)*, *21*(4), 3633–3648. https://doi.org/10.1007/s13762-023-05238-4

Du, J., Mei, C.-F., Ying, G.-G., & Xu, M.-Y. (2016). Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea Daphnia magna. *Bulletin of Environmental Contamination and Toxicology*, *97*(1), 84–90. https://doi.org/10.1007/s00128-016-1806-7

Du, J., Zhou, Q., Li, H., Xu, S., Wang, C., Fu, L., & Tang, J. (2021). Environmental distribution, transport and ecotoxicity of microplastics: A review. *Journal of Applied Toxicology*, *41*(1), 52–64. https://doi.org/10.1002/jat.4034

EFSA Panel on Contaminants in the Food Chain. Presence of microplastics and nanoplastics in food, with particular focus on seafood. EFSA J. 2016, 14 (6), 4501. https://doi.org/10.2903/j.efsa.2016.4501

Elizalde-Velázquez, A., Subbiah, S., Anderson, T. A., Green, M. J., Zhao, X., & Cañas-Carrell, J. E. (2020). Sorption of three common nonsteroidal anti-inflammatory drugs (NSAIDs) to microplastics. *The Science of the Total Environment*, *715*, 136974–136974. https://doi.org/10.1016/j.scitotenv.2020.136974

Eltemsah, Y. S., & Bøhn, T. (2019). Acute and chronic effects of polystyrene microplastics on juvenile and adult Daphnia magna. *Environmental Pollution (1987)*, *254*(Pt A), 112919-. https://doi.org/10.1016/j.envpol.2019.07.087

Esterhuizen, M., Lee, S.-A., Järvinen, R., Kim, Y., Pflugmacher, S., & Kim, Y. J. (2023). Ecotoxicology of Polystyrene Microplastic Fragments: Oxidative Stress Effects in Neonate Versus Adult Daphnia magna. *Water, Air, and Soil Pollution*, *234*(11), 713-. https://doi.org/10.1007/s11270-023-06741-7

European Commission (1993) Commission Directive 93/67/EEC of 20 July 1993 laying down the principles for assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC

Faull, M., L. E., Zaliznyak, T., & Taylor, G. T. (2024). From the Caribbean to the Arctic, the most abundant microplastic particles in the ocean have escaped detection. *Marine Pollution Bulletin*, *202*, 116338–116338. https://doi.org/10.1016/j.marpolbul.2024.116338

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxēaus, N., Giudice, R. L., Pollio, A., & Garric, J. (2004). Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environmental Toxicology and Chemistry*, *23*(5), 1344–1354. https://doi.org/10.1897/03-246

Finney, D. J. (1952). Probit Analysis, 2nd Ed., p. 131. Cambridge Univ. Press, Cambridge, England.

Frydkjær, C. K., Iversen, N., & Roslev, P. (2017). Ingestion and Egestion of Microplastics by the Cladoceran Daphnia magna: Effects of Regular and Irregular Shaped Plastic and Sorbed Phenanthrene. *Bulletin of Environmental Contamination and Toxicology*, *99*(6), 655–661. https://doi.org/10.1007/s00128-017-2186-3

Fu, Q., Fedrizzi, D., Kosfeld, V., Schlechtriem, C., Ganz, V., Derrer, S., Rentsch, D., & Hollender, J. (2020). Biotransformation Changes Bioaccumulation and Toxicity of Diclofenac in Aquatic Organisms. *Environmental Science & Technology*, *54*(7), 4400–4408. https://doi.org/10.1021/acs.est.9b07127

Funke, E., Webb, L., & Wolinska, J. (2024). The effect of microplastics on Daphnia fitness – Systematic review and meta-analysis. *Freshwater Biology*, *69*(4), 461–476. https://doi.org/10.1111/fwb.14228

George-Ares, A., & Clark, J. R. (2000). Aquatic toxicity of two Corexit ® dispersants. *Chemosphere (Oxford)*, *40*(8), 897–906. https://doi.org/10.1016/S0045-6535(99)00498-1

Gerhardt, A. (2002). Bioindicator species and their use in biomonitoring. *Environmental monitoring*, *1*, 77-123.

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782–e1700782. https://doi.org/10.1126/sciadv.1700782

Gisi, U., Binder, H., & Rimbach, E. (1985). Synergistic interactions of fungicides with different modes of action. *Transactions of the British Mycological Society*, *85*(2), 299–306. https://doi.org/10.1016/S0007-1536(85)80192-3

Gisi, U. (1996). Synergistic interaction of fungicides in mixtures. *Phytopathology*, *86*(11), 1273-1279.

Gómez-Oliván, L. M., Galar-Martínez, M., García-Medina, S., Valdés-Alanís, A., Islas-Flores, H., & Neri-Cruz, N. (2014). Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen in Daphnia magna. *Drug and Chemical Toxicology (New York, N.Y. 1978)*, *37*(4), 391–399. https://doi.org/10.3109/01480545.2013.870191

Grabarczyk, Ł., Mulkiewicz, E., Stolte, S., Puckowski, A., Pazda, M., Stepnowski, P., & Białk-Bielińska, A. (2020). Ecotoxicity screening evaluation of selected pharmaceuticals and their transformation products towards various organisms. *Environmental Science and Pollution Research International*, *27*(21), 26103–26114. https://doi.org/10.1007/s11356-020-08881-3

Grbić, J., Helm, P., Athey, S., & Rochman, C. M. (2020). Microplastics entering northwestern Lake Ontario are diverse and linked to urban sources. *Water Research (Oxford)*, *174*, Article 115623. https://doi.org/10.1016/j.watres.2020.115623

Haap, T., Triebskorn, R., & Köhler, H.-R. (2008). Acute effects of diclofenac and DMSO to Daphnia magna: Immobilisation and hsp70-induction. *Chemosphere (Oxford)*, *73*(3), 353–359. https://doi.org/10.1016/j.chemosphere.2008.05.062

van der Hal, N., Ariel, A., & Angel, D. L. (2017). Exceptionally high abundances of microplastics in the oligotrophic Israeli Mediterranean coastal waters. *Marine Pollution Bulletin*, *116*(1–2), 151–155. https://doi.org/10.1016/j.marpolbul.2016.12.052

Halliwell, B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. *Plant Physiology (Bethesda)*, *141*(2), 312–322. https://doi.org/10.1104/pp.106.077073

Hamza-Chaffai, A. (2014). Usefulness of bioindicators and biomarkers in pollution biomonitoring. *International Journal of Biotechnology for Wellness Industries*, *3*(1), 19-26.

Hartmann, N. B., Rist, S., Bodin, J., Jensen, L. H., Schmidt, S. N., Mayer, P., Meibom, A., & Baun, A. (2017). Microplastics as vectors for environmental contaminants: Exploring sorption, desorption, and transfer to biota. *Integrated Environmental Assessment and Management*, *13*(3), 488–493. https://doi.org/10.1002/ieam.1904

Hartmann, N. B., Hüffer, T., Thompson, R. C., Hassellöv, M., Verschoor, A., Daugaard, A. E., Rist, S., Karlsson, T., Brennholt, N., Cole, M., Herrling, M. P., Hess, M. C., Ivleva, N. P., Lusher, A. L., & Wagner, M. (2019). Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris. *Environmental Science & Technology*, *53*(3), 1039–1047. https://doi.org/10.1021/acs.est.8b05297

He, Y., Zhang, Y., Zhou, W., Freitas, R., Zhang, Y., & Zhang, Y. (2023). Combined exposure of polystyrene microplastics and carbamazepine induced transgenerational effects on the reproduction of Daphnia magna. *Environmental Science and Pollution Research International*, *30*(25), 67596–67607. https://doi.org/10.1007/s11356-023-27270-0

Honkanen, J. (2023). *Combined toxicity of polystyrene microplastic and diclofenac in Daphnia magna: Immobility, oxidative stress status, and antioxidant response* (Master's thesis, University of Helsinki). Available: http://hdl.handle.net/10138/568448

Hook, S. E., Gallagher, E. P., & Batley, G. E. (2014). The role of biomarkers in the assessment of aquatic ecosystem health. *Integrated Environmental Assessment and Management*, *10*(3), 327–341. https://doi.org/10.1002/ieam.1530

Hwang, J., Choi, D., Han, S., Jung, S. Y., Choi, J., & Hong, J. (2020). Potential toxicity of polystyrene microplastic particles. *Scientific Reports*, *10*(1), Article 7391. https://doi.org/10.1038/s41598-020-64464-9

Jansen, V. (2023). *The Fate of Synthetic Polymers-an Analysis on the Future of Plastic Waste* (No. 9594). EasyChair.

Jux, U., Baginski, R. M., Arnold, H.-G., Krönke, M., & Seng, P. N. (2002). Detection of pharmaceutical contaminations of river, pond, and tap water from Cologne (Germany) and surroundings. *International Journal of Hygiene and Environmental Health*, *205*(5), 393–398. https://doi.org/10.1078/1438-4639-00166

Kandaswamy, K., Guru, A., Panda, S. P., Antonyraj, A. P. M., Kari, Z. A., Giri, J., Almutairi, B. O., Arokiyaraj, S., Malafaia, G., & Arockiaraj, J. (2024). Polystyrene nanoplastics synergistically exacerbate diclofenac toxicity in embryonic development and the health of adult zebrafish. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology*, *281*, 109926–109926. https://doi.org/10.1016/j.cbpc.2024.109926

Kruskal, W. H., & Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*, *47*(260), 583–621. https://doi.org/10.1080/01621459.1952.10483441

Lee, J., Ji, K., Lim Kho, Y., Kim, P., & Choi, K. (2011). Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. *Ecotoxicology and Environmental Safety*, *74*(5), 1216–1225. https://doi.org/10.1016/j.ecoenv.2011.03.014

Lee, J.-S., Oh, Y., Park, H. E., Lee, J.-S., & Kim, H. S. (2023). Synergistic toxic mechanisms of microplastics and triclosan via multixenobiotic resistance (MXR) inhibition–mediated autophagy in the freshwater water flea Daphnia magna. *The Science of the Total Environment*, *896*, 165214–165214. https://doi.org/10.1016/j.scitotenv.2023.165214

Lee, S.-A., Esterhuizen, M., Kim, Y., Kim, M., & Kim, Y. J. (2025). Assessing the acute differential toxicity of polystyrene microplastic particles and comparing the impacts of bead-shaped versus fragmented particles on Daphnia magna. *Applied Biological Chemistry*, *68*(1), Article 34. https://doi.org/10.1186/s13765-025-01012-x

Li, W., Zu, B., Li, J., Chen, W., Wang, M., & Li, J. (2025). Adverse Effects of Polystyrene Microplastic Exposure on Daphnia magna: a Comprehensive Assessment of Acute Toxicity, Behavioral Changes, and Oxidative Stress. *Water, Air, and Soil Pollution*, *236*(7), Article 456. https://doi.org/10.1007/s11270-025-08017-8

Liu, Y., Wang, L., Pan, B., Wang, C., Bao, S., & Nie, X. (2017). Toxic effects of diclofenac on life history parameters and the expression of detoxification-related genes in Daphnia magna. *Aquatic Toxicology*, *183*, 104–113. https://doi.org/10.1016/j.aquatox.2016.12.020

Liu, J., Yang, H., Meng, Q., Feng, Q., Yan, Z., Liu, J., Liu, Z., & Zhou, Z. (2022). Intergenerational and biological effects of roxithromycin and polystyrene microplastics to Daphnia magna. *Aquatic Toxicology*, *248*, Article 106192. https://doi.org/10.1016/j.aquatox.2022.106192

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, *101*(1), 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Ma, Y., Huang, A., Cao, S., Sun, F., Wang, L., Guo, H., & Ji, R. (2016). Effects of nanoplastics and microplastics on toxicity, bioaccumulation, and environmental fate of phenanthrene in fresh water. *Environmental Pollution (1987)*, *219*, 166–173. https://doi.org/10.1016/j.envpol.2016.10.061

Malla-Pradhan, R., Pradhan, B. L., Phoungthong, K., & Joshi, T. P. (2023). Microplastic in Freshwater Environment: A Review on Techniques and Abundance for Microplastic Detection in Lake Water. *Trends in Sciences*, *20*(8), 5202. https://doi.org/10.48048/tis.2023.5202

Mao, Y., Ai, H., Chen, Y., Zhang, Z., Zeng, P., Kang, L., Li, W., Gu, W., He, Q., & Li, H. (2018). Phytoplankton response to polystyrene microplastics: Perspective from an entire growth period. *Chemosphere (Oxford)*, *208*, 59–68. https://doi.org/10.1016/j.chemosphere.2018.05.170

Midway, S., Robertson, M., Flinn, S., & Kaller, M. (2020). Comparing multiple comparisons: practical guidance for choosing the best multiple comparisons test. *PeerJ (San Francisco, CA)*, *8*, e10387–e10387. https://doi.org/10.7717/peerj.10387

Miller, T. H., Bury, N. R., Owen, S. F., MacRae, J. I., & Barron, L. P. (2018). A review of the pharmaceutical exposome in aquatic fauna. *Environmental Pollution (1987)*, *239*, 129–146. https://doi.org/10.1016/j.envpol.2018.04.012

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W., & Hairston, N. G. (2012). Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society. B, Biological Sciences*, *279*(1735), 1873–1882. https://doi.org/10.1098/rspb.2011.2404

Mohammed, A. (2013). *Why are Early Life Stages of Aquatic Organisms more Sensitive to Toxicants than Adults?* IntechOpen. https://doi.org/10.5772/55187

Mueller, M.-T., Fueser, H., Trac, L. N., Mayer, P., Traunspurger, W., & Höss, S. (2020). Surface-Related Toxicity of Polystyrene Beads to Nematodes and the Role of Food Availability. *Environmental Science & Technology*, *54*(3), 1790–1798. https://doi.org/10.1021/acs.est.9b06583

Nkoom, M., Lu, G., Liu, J., Dong, H., & Yang, H. (2019). Bioconcentration, behavioral, and biochemical effects of the non-steroidal anti-inflammatory drug diclofenac in Daphnia magna. *Environmental Science and Pollution Research International*, *26*(6), 5704–5712. https://doi.org/10.1007/s11356-018-04072-3

Nkoom, M., Lu, G., & Liu, J. (2022). Chronic toxicity of diclofenac, carbamazepine and their mixture to Daphnia magna: a comparative two-generational study. *Environmental Science and Pollution Research International*, *29*(39), 58963–58979. https://doi.org/10.1007/s11356-022-19463-w

OECD, 2004. Test No. 202: Daphnia sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264069947-en.

Olaitan, O. J., Anyakora, C., Bamiro, T., & Tella, A. T. (2014). Determination of pharmaceutical compounds in surface and underground water by solid phase extraction-liquid chromatography. *Journal of Environmental Chemistry and Ecotoxicology*, *6*(3), 20-26.

de Oliveira, L. L. D., Antunes, S. C., Gonçalves, F., Rocha, O., & Nunes, B. (2016). Acute and chronic ecotoxicological effects of four pharmaceuticals drugs on cladoceran Daphnia magna. *Drug and Chemical Toxicology (New York, N.Y. 1978)*, *39*(1), 13–21. https://doi.org/10.3109/01480545.2015.1029048

Ostertagova, E., Ostertag, O., & Kovac, J. (2014). Methodology and Application of the Kruskal-Wallis Test. *Applied Mechanics and Materials*, *611*, 115. https://doi.org/10.4028/www.scientific.net/AMM.611.115

Pablos, M. V., García-Hortigüela, P., & Fernández, C. (2015). Acute and chronic toxicity of emerging contaminants, alone or in combination, in Chlorella vulgaris and Daphnia magna. *Environmental Science and Pollution Research International*, *22*(7), 5417–5424. https://doi.org/10.1007/s11356-015-4119-1

Pallant, J. (2011). Survival manual. A step by step guide to data analysis using SPSS, 4(4).

Parolini, M. (2020). Toxicity of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen towards freshwater invertebrates: A review. *The Science of the Total Environment*, *740*, 140043–140043. https://doi.org/10.1016/j.scitotenv.2020.140043

Pashaei, R., Dzingelevičienė, R., Putna-Nimane, I., Overlinge, D., Błaszczyk, A., & Walker, T. R. (2023). Acute toxicity of triclosan, caffeine, nanoplastics, microplastics, and their mixtures on Daphnia magna. *Marine Pollution Bulletin*, *192*, Article 115113. https://doi.org/10.1016/j.marpolbul.2023.115113

Pei, M., Fan, J., Zhang, C., Xu, J., Yang, Y., Wei, H., Zhang, C., Zhu, L., & Gao, P. (2025). Antibiotic and microplastic co-exposure: Effects on Daphnia magna and implications for ecological risk assessment. *Critical Reviews in Environmental Science and Technology*, *55*(5), 287–309. https://doi.org/10.1080/10643389.2024.2406575

Phuong, N. N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C., & Lagarde, F. (2016). Is there any consistency between the microplastics found in the field and those used in laboratory experiments? *Environmental Pollution (1987)*, *211*, 111–123. https://doi.org/10.1016/j.envpol.2015.12.035

Pikuda, O., Roubeau Dumont, E., Chen, Q., Macairan, J.-R., Robinson, S. A., Berk, D., & Tufenkji, N. (2023). Toxicity of microplastics and nanoplastics to Daphnia magna: Current status, knowledge gaps and future directions. *TrAC, Trends in Analytical Chemistry (Regular Ed.)*, *167*, 117208-. https://doi.org/10.1016/j.trac.2023.117208

Prasad, G., Mohanty, S., Nayak, S. K., Bharat, G. K., & Chakraborty, P. (2023). A Scientific Approach to the Occurrence, Isolation, and Characterization of Existing Microplastic Pollution in the Marine Environment—a Review. *Water, Air, and Soil Pollution*, *234*(7), 480-. https://doi.org/10.1007/s11270-023-06494-3

Qi, K., Lu, N., Zhang, S., Wang, W., Wang, Z., & Guan, J. (2021). Uptake of Pb(II) onto microplastic-associated biofilms in freshwater: Adsorption and combined toxicity in comparison to natural solid substrates. *Journal of Hazardous Materials*, *411*, Article 125115. https://doi.org/10.1016/j.jhazmat.2021.125115

Qutob, M., Alshehri, S., Shakeel, F., Alam, P., & Rafatullah, M. (2023). Insight into Photodegradation of Diclofenac: Mechanism, Efficiency, Role of Parameters, Toxicity Assessment and Catalyst Stability. *Reviews of Environmental Contamination and Toxicology*, *261*(1), 27-. https://doi.org/10.1007/s44169-023-00052-y

Rai, P. K., Lee, J., Brown, R. J. C., & Kim, K.-H. (2021). Environmental fate, ecotoxicity biomarkers, and potential health effects of micro- and nano-scale plastic contamination. *Journal of Hazardous Materials*, *403*, Article 123910. https://doi.org/10.1016/j.jhazmat.2020.123910

Raj, J., Chandra, M., Dogra, T., Pahuja, M., & Raina, A. (2013). Determination of median lethal dose of combination of endosulfan and cypermethrin in wistar rat. *Toxicology International*, *20*(1), 1–5. https://doi.org/10.4103/0971-6580.111531

Randhawa, M. A. (2009). Calculation of LD50 values from the method of Miller and Tainter, 1944. *J Ayub Med Coll Abbottabad*, *21*(3), 184-185.

Rehse, S., Kloas, W., & Zarfl, C. (2016). Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of Daphnia magna. *Chemosphere (Oxford)*, *153*, 91–99. https://doi.org/10.1016/j.chemosphere.2016.02.133

Rist, S., Baun, A., & Hartmann, N. B. (2017). Ingestion of micro- and nanoplastics in Daphnia magna – Quantification of body burdens and assessment of feeding rates and reproduction. *Environmental Pollution (1987)*, *228*, 398–407. https://doi.org/10.1016/j.envpol.2017.05.048

Rodrigues, M. O., Abrantes, N., Gonçalves, F. J. M., Nogueira, H., Marques, J. C., & Gonçalves, A. M. M. (2018). Spatial and temporal distribution of microplastics in water and sediments of a freshwater system (Antuã River, Portugal). *The Science of the Total Environment*, *633*, 1549–1559. https://doi.org/10.1016/j.scitotenv.2018.03.233

Rosenkranz, P., Chaudhry, Q., Stone, V., & Fernandes, T. F. (2009). Comparison of Nanoparticle and Fine Particle Uptake by Daphnia Magna. *Environmental Toxicology and Chemistry*, *28*(10), 2142–2149. https://doi.org/10.1897/08-559.1

Ryan, E. P., Bushnell, T. P., Friedman, A. E., Rahman, I., & Phipps, R. P. (2008). Cyclooxygenase-2 independent effects of cyclooxygenase-2 inhibitors on oxidative stress and intracellular glutathione content in normal and malignant human B-cells. *Cancer Immunology, Immunotherapy*, *57*(3), 347–358. https://doi.org/10.1007/s00262-007-0374-4

Salehi, M., Rodriguez, R., Boettcher, A., Powers, S., Geitner, N., Ladner, D. A., Rikard, S., & Whelton, A. J. (2017). Impact of dispersant on early life stages of the water flea Daphnia magna and the eastern oyster Crassostrea virginica. *Journal of Applied Toxicology*, *37*(12), 1464–1470. https://doi.org/10.1002/jat.3494

Samadi, A., Kim, Y., Lee, S., Kim, Y. J., & Esterhuizen, M. (2022). Review on the ecotoxicological impacts of plastic pollution on the freshwater invertebrate Daphnia. *Environmental Toxicology*, *37*(11), 2615–2638. https://doi.org/10.1002/tox.23623

Sathishkumar, P., Meena, R. A. A., Palanisami, T., Ashokkumar, V., Palvannan, T., & Gu, F. L. (2020). Occurrence, interactive effects and ecological risk of diclofenac in environmental compartments and biota - a review. *The Science of the Total Environment*, *698*, 134057-. https://doi.org/10.1016/j.scitotenv.2019.134057

Schellenberg, J. (2009). *Syndiotactic polystyrene: synthesis, characterization, processing, and applications* (1st ed.). Wiley. https://doi.org/10.1002/9780470557006

Schür, C., Zipp, S., Thalau, T., & Wagner, M. (2020). Microplastics but not natural particles induce multigenerational effects in Daphnia magna. *Environmental Pollution (1987)*, *260*, 113904–113904. https://doi.org/10.1016/j.envpol.2019.113904

Setälä, O., Magnusson, K., Lehtiniemi, M., & Norén, F. (2016). Distribution and abundance of surface water microlitter in the Baltic Sea: A comparison of two sampling methods. *Marine Pollution Bulletin*, *110*(1), 177–183. https://doi.org/10.1016/j.marpolbul.2016.06.065

Siciliano, A., Gesuele, R., Pagano, G., & Guida, M. (2015). How Daphnia (Cladocera) assays may be used as bioindicators of health effects. *Journal of Biodiversity & Endangered Species*, *1*(005), 1-6.

Smith, M., Love, D. C., Rochman, C. M., & Neff, R. A. (2018). Microplastics in Seafood and the Implications for Human Health. *Current Environmental Health Reports*, *5*(3), 375–386. https://doi.org/10.1007/s40572-018-0206-z

Su, L., Xue, Y., Li, L., Yang, D., Kolandhasamy, P., Li, D., & Shi, H. (2016). Microplastics in Taihu Lake, China. *Environmental Pollution (1987)*, *216*, 711–719. https://doi.org/10.1016/j.envpol.2016.06.036

Suman, K. H., Haque, M. N., Uddin, M. J., Begum, M. S., & Sikder, M. H. (2021). Toxicity and biomarkers of micro-plastic in aquatic environment: a review. *Biomarkers*, *26*(1), 13–25. https://doi.org/10.1080/1354750X.2020.1863470

Talbot, R., & Chang, H. (2022). Microplastics in freshwater: A global review of factors affecting spatial and temporal variations. *Environmental Pollution (1987)*, *292*, 118393–118393. https://doi.org/10.1016/j.envpol.2021.118393

Thakur, A., & Kocher, D. K. (2018). Laboratory studies on developmental stages and life cycle of Daphnia magna. *Int. J. Fauna Biol. Stud., 5*(4):04-08.

Tkaczyk, A., Bownik, A., Dudka, J., Kowal, K., & Ślaska, B. (2021). Daphnia magna model in the toxicity assessment of pharmaceuticals: A review. *The Science of the Total Environment*, *763*, 143038-. https://doi.org/10.1016/j.scitotenv.2020.143038

Traudt, E. M., Ranville, J. F., & Meyer, J. S. (2017). Age-related differences in sensitivity to metals can matter for Daphnia magna neonates. *Integrated Environmental Assessment and Management*, *13*(1), 208–210. https://doi.org/10.1002/ieam.1858

Trotter, B., Wilde, M. V., Brehm, J., Dafni, E., Aliu, A., Arnold, G. J., Fröhlich, T., & Laforsch, C. (2021). Long-term exposure of Daphnia magna to polystyrene microplastic (PS-MP) leads to alterations of the proteome, morphology and life-history. *The Science of the Total Environment*, *795*, Article 148822. https://doi.org/10.1016/j.scitotenv.2021.148822

Tukey, J. W. (1949). Comparing Individual Means in the Analysis of Variance. *Biometrics*, *5*(2), 99–114. https://doi.org/10.2307/3001913

UNEP. (2021). Drowning in Plastics – Marine Litter and Plastic Waste Vital Graphics. United Nations Environment Programme (UNEP), Secretariats of the Basel, Rotterdam and Stockholm Conventions (BRS) and GRID-Arendal.

UNEP (2025). 2nd Part of the 5th Session of the Intergovernmental Negotiating Committee to Develop an International Legally Binding Instrument on Plastic Pollution, Including in the Marine Environment (INC-5.2). *Earth Negotiations Bulletin, 36*(43), 1–13. https://enb.iisd.org/plastic-pollution-marine-environment-negotiating-committee-inc5.2

Uurasjärvi, E., Hartikainen, S., Setälä, O., Lehtiniemi, M., & Koistinen, A. (2020). Microplastic concentrations, size distribution, and polymer types in the surface waters of a northern European lake. *Water Environment Research*, *92*(1), 149–156. https://doi.org/10.1002/wer.1229

Wright, S. L., Thompson, R. C., & Galloway, T. S. (2013). The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution (1987)*, *178*, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031

Yang, W., Zhang, H., Yang, S., Xiao, Y., Ye, K., He, R., Liu, Y., Hu, Z., Guo, W., Zhang, Q., Qu, H., & Mao, Y. (2024). Combined effects of microplastics and pharmaceutical and personal care products on algae: A critical review. *Environmental Pollution (1987)*, *358*, Article 124478. https://doi.org/10.1016/j.envpol.2024.124478

Yin, C., Yang, X., Zhao, T., Watson, P., Yang, F., & Liu, H. (2020). Changes of the acute and chronic toxicity of three antimicrobial agents to Daphnia magna in the presence/absence of micro-polystyrene. *Environmental Pollution (1987)*, *263*(Pt A), Article 114551. https://doi.org/10.1016/j.envpol.2020.114551

Zhang, Y., Price, G. W., Jamieson, R., Burton, D., & Khosravi, K. (2017). Sorption and desorption of selected non-steroidal anti-inflammatory drugs in an agricultural loam-textured soil. *Chemosphere (Oxford)*, *174*, 628–637. https://doi.org/10.1016/j.chemosphere.2017.02.027

Zhang, P., Yan, Z., Lu, G., & Ji, Y. (2019). Single and combined effects of microplastics and roxithromycin on Daphnia magna. *Environmental Science and Pollution Research International*, *26*(17), 17010–17020. https://doi.org/10.1007/s11356-019-05031-2