Bridging the gap between standardized laboratory tests and environmental outcomes: investigations of organic ultraviolet filter toxicity to freshwater invertebrates *Daphnia magna* and *Daphnia pulex*

by

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Abstract

The impacts of anthropogenic activities have been disrupting natural environments at a rate exceeding the capacity of researchers to understand their potential consequences. Standardized research models have become widely adopted to allow for rapid data collection in response to contaminants of emerging concern, including organic ultraviolet filters (UVFs) such as avobenzone, octocrylene and oxybenzone. Through their use in skin care products such as sunscreen, they have been found to ubiquitously contaminate freshwater and marine ecosystems and have consequently been found to cause toxicity to a variety of invertebrate species. To further our understanding of the long-term consequences of contamination in freshwater environments, the toxicity of UVFs were assessed in the model invertebrate, *Daphnia magna*. Acute 48 h exposures impaired phototactic behaviours and caused latent mortality up to 5 days post-exposure, with recovery possible in surviving organisms. Chronic 21 d exposures resulted in minimal sublethal effects at lower tested concentrations, but complete mortality at higher tested concentrations, suggesting overall that *D. magna* is among the most sensitive species to these UVFs.

To assess the long-term effects in an exposed population, the standard 21 d chronic toxicity test was repeated to maintain continuous UVF exposures for 5 generations. Mortality and decreased reproduction occurred in the F0 and F1 generations of exposure, with normal physiological function regained by the F3 and F4 generations. Decreased protein abundance was observed in many metabolism and immune functions in the F0 generation, while these same processes were predominantly enriched in the F3 generation, suggesting that physiological adaptations played an important role in the acclimation of *D. magna* populations. These results

suggest that it is incorrect to assume that data generated within a single generation exposure will correlate to long-term outcomes within an exposed population.

During this study, it was found that culturing *Daphnia* in groups can alter their baseline state compared to individual organisms maintained with the same ratio of food and water, including delaying the onset of reproduction and causing the generation of males. While the overall conclusions of toxicity for each UVF were unaffected by the exposure type, subtle changes in development, reproduction, and baseline mortality existed in proportion to the control daphnids across all 5 generations, indicating the importance of considering the context of data when comparing results across studies.

Most studies measure UVF toxicity to understand the effects of sunscreen pollution, as the inactive ingredients within sunscreen products enter the environment through the same processes as the UVFs. To address the lack of studies comparing the toxicity of UVFs in isolation to those within sunscreen mixtures, five off-the-shelf sunscreens were assessed over 21 d exposures, revealing that sunscreen toxicity was far lower than expected based on the concentration of UVFs within the mixture, indicating that antagonistic interactions between the different components of sunscreens may serve to minimize the toxicity of these complex mixtures. By investigating UVF toxicity in isolation, many studies may overestimate the impacts of sunscreen pollution in natural environments.

Much biological research is conducted using model species cultured in a laboratory environment for generations to model outcomes in wild populations. Several studies have found differences in stress responses of lab and wild populations; however, many of these studies tested wild organisms in laboratory water without determining if changing the culture water would impact the outcome of the experiments. The toxicity of UVFs was assessed in lab-reared and wild

caught *Daphnia pulex* populations cultured in both laboratory and lake water. It was found that both lab and wild *D. pulex* were severely weakened when cultured in non-ancestral waters for 3 generations, failing to meet the validity criteria for standard toxicity test guidelines. When cultured in ancestral waters, lab *D. pulex* were more sensitive to AVO and OXY, while wild daphnids were more sensitive to OCT. These results indicate that culture water can greatly influence the performance of aquatic organisms during experimentation, and that lab populations can deviate from wild counterparts after decades of culturing in a laboratory environment.

Overall, this thesis highlights several shortcomings of applying standardized research to natural environments and highlights the necessity to understand how methodological decisions impact the data obtained and conclusions drawn in any avenue of biological research.

Preface

This thesis is an original work by Aaron Boyd. Several chapters of this thesis have been previously published or are submitted for peer review. Biographical details for each data chapter are listed below and indicated at the beginning of each chapter. The roles of all authors for each chapter are described in brief below.

Chapter 2:

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Aaron Boyd: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization. Christina Lummer: Investigation, Formal analysis, Writing – review & editing. Devang Mehta: Investigation, Formal analysis, Writing – review & editing. R. Glen Uhrig: Resources, Writing – review & editing, Supervision. Tamzin A. Blewett: Conceptualization, Writing – review & editing, Supervision.

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List of Symbols, Nomenclature and Abbreviations

α	Alpha
Å	Angstrom
°C	Degrees Celsius
γ	Gamma
%	Percent
AB	Alberta
Al	Aluminum
APCI	Atmospheric pressure chemical ionization
ARO	Aquatic Research Organisms
As	Arsenic
AVO	Avobenzone
BDL	Below detection limits
BP	Biological process
Ca	Calcium
$CaCl_2$	Calcium chloride
Cal	Calibration solution
CI	Confidence interval

Cl	Chloride
cm	Centimetre
COI	Cytochrome c oxidase subunit I
Cu	Copper
d	Day
Da	Dalton
DMSO	Dimethyl sulfoxide
DOC	Dissolved organic carbon
dw	Dry weight
EC50	Median effect concentration
e.g.	*exempli gratia*
EHS	Octisalate
EPA	Environmental Protection Agency
ERα	Estrogen receptor alpha
EtOH	Ethanol
eV	Electron-volts
F[0-5]	Filial generation [0-5]
FDA	Food and Drug Administration

FDR	False discovery rate
et al.	*et alia*
Fe	Iron
g	Gram
g/L	Gram per litre
GO	Gene ontology
h	Hour
H	Hydrogen
HESI	Heated electrospray ionization
HMS	Homosalate
HNO_3	Nitric acid
HPLC-tMS^2	High performance liquid chromatography-tandem mass spectrometry
i.e.	*id est*
ISO	International Organization for Standardization
K	Potassium
KCl	Potassium chloride
km	Kilometre
K_{ow}	Octanol-water partitioning coefficient

L	Litre
LC50	Median lethal concentration
LC-MS	Liquid chromatography mass spectrometry
LC-QQQMS	Liquid chromatography – triple quadrupole mass spectrometry
lm	Lumen
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
m	Metre
MeOH	Methanol
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
mg/mL	Milligrams per millilitre
$mgO_2\ L^{-1}$	Milligrams of oxygen per litre
$mgO_2L^{-1}h^{-1}$	Milligrams of oxygen per litre per hour
$mgO_2L^{-1}h^{-1}mg^{-1}$	Milligrams of oxygen per litre per hour per milligram
$MgSO_4$	Magnesium sulfate

min	Minute
mL	Millilitre
mL/min	Millilitre per minute
Mn	Manganese
MS^2	Tandem mass spectrometry
m/z	Mass to charge ratio
n	Replicate number
N_2	Molecular nitrogen
Na	Sodium
$NaHCO_3$	Sodium bicarbonate
NCE	Normalized collision energy
ng/g	Nanograms per gram
ng/L	Nanograms per litre
ng/mL	Nanograms per millilitre
Ni	Nickel
nL/min	Nanolitres per minute
nm	Nanometre
OCT	Octocrylene

OECD	Organisation for Economic Co-operation and Development
OXY	Oxybenzone
Pb	Lead
POM	Particulate organic matter
PPARγ	peroxisome proliferator-activated receptor-gamma
ppm	Parts per million
QA	Quality assurance
QC	Quality control
r	Intrinsic rate of population increase
R^2	Coefficient of determination
RF	Radio frequency
s	Seconds
SD	Standard deviation
SEM	Standard error of the mean
Se	Selenium
sp.	Species
SPF	Sun protection factor
µA	Micro ampere

µg	Microgram
µg/L	Micrograms per litre
µg/mL	Micrograms per millilitre
UHPLC	Ultra high-performance liquid chromatography
µL	Microlitre
µL/L	Microlitres per litre
µL/mL	Microlitres per millilitre
US	United States
UV	Ultraviolet
UVF	Ultraviolet filter
V	Volts
v/v	Volume per volume
WWTP	Wastewater treatment plant
YCT	Yeast, cereal leaf, trout chow

Chapter 1: Introduction

Anthropogenic impacts on natural environments have steadily increased since the Industrial Revolution, causing widespread disruptions to ecosystems resulting in habitat degradation and loss (Gabrielli et al., 2020; Yang et al., 2022). The manufacture and use of the many products that have been developed to enable the high quality of life expected of the modern lifestyle often results in the release of many unknown and untested contaminants into the environment as a consequence (aus der Beek et al., 2016; Nunes et al., 2023). While there is a desire to minimize anthropogenic impacts to the environment, the rate at which new contaminants are created and subsequently released far outstrips the capacity of researchers to perform high quality toxicological studies and environmental monitoring that assess long-term outcomes, consider environmental factors that may impact chemical toxicity, and are supported by robust data spanning several levels of biological organization. This is further compounded by a general tendency to conduct assessments reactively as contamination issues are discovered (Kosnik et al., 2022; Noguera-Oviedo and Aga, 2016; Zhanyun Wang et al., 2020). The complexity of the natural environment further compounds society's limited research capabilities, as many factors such as predation, disease, inter- and intraspecific competition, and seasonal changes can all influence how an organism would respond to any stressor, including chemical stress (Coors and De Meester, 2008; Gust et al., 2016; Laskowski et al., 2010). Thus, researchers are faced with a trade-off between performing a small number of high-complexity studies that can directly characterize these dynamic interactions, or performing a large number of standardized, simplified assessments in surrogate model systems to allow for consistent and rapid data generation on a broad array of interests.

1.1 Standardized research methods

While both of these approaches are utilized to varying extents, the use of simplified assessments has become widely adopted in many biological disciplines, including microbiology, physiology, ecology, and toxicology (Laskowski et al., 2010; Van de Perre et al., 2018). In many cases, a high research throughput has been facilitated through the development of standardized test guidelines and

standard operating procedures that allow for independent research teams to simplify the complexity of the natural world in a consistent manner so that the resulting data may be more reproducible (Busquet et al., 2014; Henke et al., 2024). These standardizations manage complexity by either treating many environmental variables as constants (e.g., temperature, light cycle), or removing them from consideration altogether (e.g., disease, species interactions) so that specific mechanisms driving observed outcomes can be understood in greater detail (Laskowski et al., 2010; Romero-Blanco and Alonso, 2022). Research standardization can extend to all aspects of a study, including experimental design, methodology, data analysis, and result validity criteria.

Much toxicology research is conducted within a laboratory environment to facilitate the control of the many biotic and abiotic variables that can influence biological experiments (Romero-Blanco and Alonso, 2022; Shaw et al., 2008). This has led to the generation of laboratory cultured model species such as zebrafish (*Danio rerio*), fathead minnows (*Pimephales promelas*), *Daphnia magna*, and *Daphnia pulex*, which are often intentionally inbred to maintain a small genetic pool amongst the various subpopulations cultured within individual laboratories (Brown et al., 2012; Igawa et al., 2015; Lagisz et al., 2011; Suurväli et al., 2020). The high stability and low variability of the genetics and epigenetics of these lab populations has improved the replicability and reliability of research using these model species, particularly aiding mechanistic studies that seek to understand the foundational processes that drive various biological phenomena (Crabbe et al., 1990; Festing, 1975).

1.1.1 Daphnia *as a standard model species*

Of the many model organisms used in toxicology research, the freshwater crustaceans *Daphnia magna* and *Daphnia pulex* are among the most commonly used (Kim et al., 2015; Reilly et al., 2023). These closely related organisms share many characteristics that make them well suited for laboratory-based culturing and research, including a rapid lifecycle which allows reproduction to occur in 6 – 10 days, as well as a preferential cyclic parthenogenic reproductive strategy (Ebert, 2005). These traits

allow researchers to quickly acclimatize *Daphnia* populations to new culturing conditions, supporting the rapid generation of chronic toxicity data while minimizing biological variation through the production of clonal offspring (Koch et al., 2009). In the context of environmental toxicology research, *Daphnia* can be considered to be a more "environmentally relevant" species due to their cosmopolitan distribution in freshwater lakes across the Northern Hemisphere (Benzie, 2005; Patalas et al., 1994) in comparison to other models such as zebrafish (*Danio rerio*), which have a very narrow geographical distribution (C. J. Lee et al., 2020). In addition, both *D. magna* and *D. pulex* have similar sensitivities to a variety of toxicants (Lilius et al., 1995), often ranking among the most sensitive known species and serving as indicator species of overall ecosystem health (Tkaczyk et al., 2021).

Due to the popularity of *Daphnia* as model species, much effort has been made to standardize the methods used to culture these organisms in the laboratory as well as to assess the acute (Environment and Climate Change Canada, 2016; OECD, 2004; United States Environmental Protection Agency, 2016a) and chronic toxicity of prospective toxicants (OECD, 2012; United States Environmental Protection Agency, 2016b). Regardless of which governing agency's guidelines are used, they recommend highly similar study conditions, including the age of exposed organisms (< 24 h), the exposure duration (48 h or 21 d), the culturing conditions used (e.g., temperature, photoperiod, water renewal frequency, nutrition), and the endpoints observed (e.g., survival, reproduction, growth). These guidelines were developed as the result of international efforts to rigorously validate the methods used to ensure the culturing of organisms in good health and the repeatability of data from independent laboratories using these methods (Grandy, 1997).

1.1.2 Assumptions required by standardized test methods

The approach of standardizing and simplifying research methodologies requires assumptions to be made so that the resulting data can be applied to the natural environments that researchers intend to simulate (Laskowski et al., 2010; McCarty, 2012; Romero-Blanco and Alonso, 2022). Assumptions can

be made of the validity of methodology used with respect to the test organism's needs, such as ensuring that study organisms are cultured in the appropriate conditions to be maintained in desirable health prior to experimentation, or that the results obtained are not influenced by specific factors of the methodology such as the use of solvents when working with difficult substances (Birk et al., 2012; Goulden et al., 1982). These procedural assumptions are often directly tested within each individual study through the inclusion of specific control groups (e.g., control organisms exposed to solvent only) that validate the methods used. Further assumptions are made with respect to the applicability of data to the environment, specifically that the results obtained using simplified models are representative to some degree of what would occur in the natural environment (Romero-Blanco and Alonso, 2022); however, these assumptions are not frequently tested due to the increased complexity and cost of performing such research.

There are many assumptions made by researchers following the *Daphnia* toxicity test guidelines, and many of these are applicable to other methods in toxicology or other fields of research. For example, the guidelines assume that conducting a 21-d test of a single exposed generation is sufficient to model outcomes within exposed populations across generations. This is based upon the principle that the toxicity of a contaminant to an organism typically increases with longer duration exposures, reaching a plateau that is representative of the long-term effects (Newman and Newman, 2014). Populations of exposed organisms may be capable of changes on a scale beyond what individual organisms are capable of due to mechanisms such as epigenetic inheritance or selective pressures, which can subsequently allow toxicity within a population to change across generations. For example, the accumulation of damage within germline cells can result in a decrease in offspring quality, resulting in increased toxicity to the population in subsequent generations (Y. Zhang et al., 2023; Zhao et al., 2023). Alternately, phenotypic plasticity through epigenetic changes (Burgess and Marshall, 2014; Chatterjee et al., 2019; Song et al., 2022), or the selection of resistant phenotypes can result in the

eventual acclimation of a population to a stressor (Leblanc, 1982; Poulsen et al., 2021), decreasing toxicity in later generations. As these population level changes can be a matter of classifying toxic chemicals as non-toxic or vice versa, it is important to understand how the data derived from single generation tests scales to long-term outcomes within a population.

The test guidelines also suggest the use of specific *Daphnia* clones that have been maintained in laboratory environments for several decades. Due to many fundamental differences between laboratory and natural environments, challenges arise when applying lab generated data to the natural world. Natural environments are in a constant state of change, in some cases predictable by the organism (e.g., seasonality), and in other cases stochastic (e.g., weather; Burgess and Marshall, 2014). Wild populations endure a myriad of stressors such as disease, competition, and predation that arise from the constant variability of the environments they live in, promoting a greater genetic and epigenetic variability to increase the probability that some individuals will be successful in carrying the population forward regardless of what environmental changes may occur (Hughes et al., 2008; Vogt, 2017). While efforts are made to maintain lab populations in a state of genetic stasis, the genetics of wild populations are expected to constantly change in response to selective pressures (Reid et al., 2016; Vogt, 2017), which can manifest as large phenotypic deviations between lab and wild populations (Carline and Machung, 2001; Heaton et al., 2022; Hirakawa and Salinas, 2020). As the last common ancestor between lab and wild populations becomes more distant over time, each population can further diverge (Brekke et al., 2018), as even subcultures of model species raised in different laboratories exhibit signs of genetic divergence due to their isolation (Suurväli et al., 2020).

It is well understood that many species change in response to their local environment, with many postulating that the stability and predictability of the laboratory environment encourages the selection of highly specialized individuals, while wild populations may be more generalist in function (Barata et al., 2000; Hirakawa and Salinas, 2020). Test methods can further select for “high-

performing" phenotypes, as validity criteria may create an artificial selective pressure for individuals that prioritize reproduction in favour of other biological processes that may affect that organism's performance in the presence of stressors during experimentation (Crabbe et al., 1990). Maintaining domesticated laboratory populations can also cause many changes to an organism's behaviour by limiting the physical space of their environment, removing exposure to predators, and guaranteeing access to a set quantity of resources (Kohane and Parsons, 1988). By culturing model species within a laboratory environment, assumptions must be made that removing these organisms from their normal environments has not altered their behaviour, physiology, or genetics to such an extent that they are no longer valid representations of the biological phenomena under investigation.

While test guidelines do allow for the testing of complex mixtures, the desire to understand the effects of stressors in isolation, and the typical regulation of water quality guidelines on an individual stressor basis (Altenburger et al., 2019; Cedergreen, 2014; Kortenkamp et al., 2019) often results in the use of these guidelines to test individual chemicals or environmental stressors in isolation. This is further compounded by the desire to be able to test representative toxicants as a method of simplifying research on chemically complex solutions (Krewski et al., 2010; Philibert et al., 2021), precluding the interactions that can occur between biotic and abiotic influences upon the stressor. For example, agonism or antagonism between chemicals within a heterogeneous solution can alter the toxicity of individual components by several orders of magnitude (Drzymała and Kalka, 2020; Godoy and Kummrow, 2017; Salomão et al., 2014), which can also occur through interactions of individual chemicals that require the use of solvents during testing (Wang et al., 2022). Changes in temperature, lighting, or biological degradation processes can alter a toxicant's half-life or activity (Cancelli and Gobas, 2022; Patra et al., 2015; Zhang et al., 2018), while other solution characteristics such as the presence of dissolved organic matter can shift toxicants from the aqueous phase into the particulate phase (Day, 1991; Haitzer et al., 1998; Ruotsalainen et al., 2010; Trenfield et al., 2012), all of which

can alter the concentrations of chemical available for organisms to be exposed to. No stressor affects the natural environment in isolation from all of the other factors discussed above, nor do chemical exposures typically occur to isolated toxicants due to the complex contamination profiles reported around the globe (Goksøyr et al., 2009; Kasprzyk-Hordern et al., 2008; Liu et al., 2009; McCance et al., 2018). The ability to assess individual toxicants and stressors as representative driving factors of larger environmental interactions is highly desirable to increase the effectiveness of limited scientific resources; however, the ability to do so cannot be assumed and must be supported through scientific justifications.

1.1.3 Applying laboratory data to the environment

The high effort invested to develop the scientific justification of the *Daphnia* toxicity testing guidelines did not necessarily extend towards assessing the environmental relevance of the resulting data. This was due to the understanding that the differences that naturally arise between laboratory generated data and outcomes in natural environments could be accounted for during the application of data (Krewski et al., 2010). When applying the data from these model systems, it is assumed that any errors that arise can be sufficiently accommodated by applying conservative assessment factors to toxicity thresholds when estimating environmental risks. Several meta-analyses have concluded that any inaccuracies within laboratory-produced data can be mitigated through the application of sufficiently large datasets spanning multiple species to set water quality criteria at levels that protect 95% of species (Hose and Van Den Brink, 2004; Selck et al., 2002; Versteeg et al., 1999). Indeed, the use of weight of evidence approaches such as species sensitivity distributions are the currently accepted best practice in addressing environmental issues, as data-rich statistical assessments are designed to account for the possibility of more sensitive species than those currently assessed (European Chemicals Bureau, 2003; Government of Canada, 2024). Addressing environmental issues through weight of evidence approaches is always desirable; however, access to data can be limited when investigating

novel stressors, local/regional interests, or when access to scientific resources and/or funding supports are minimal. In these cases where actionable policy decisions must be made in the absence of sufficient data, assessment factors are used that account for the uncertainty of extrapolating acute data to chronic outcomes, or data from a single species to a larger ecosystem (Government of Canada, 2024).

Efforts have been made to increase the accuracy of the application of limited data to the environment by testing assumptions such as if chemical classes of varying mechanisms of action can be governed by the same assessment factor (Zhen Wang et al., 2020), or that risk models are unaffected by variation in species' sensitivity (Sorgog and Kamo, 2019). Assumptions regarding data applicability have been tested *post hoc* through investigations from individual research teams (e.g., Cairns Jr, 1994; Chapman, 2000; McCarty, 2012; Zhen Wang et al., 2020), resulting in knowledge gaps on how these data can be applied to the natural world. Understanding all aspects of standard methodologies as widely used as the *Daphnia* toxicity tests is paramount to the effective application of these models; therefore, under-investigated assumptions made regarding how the resulting data relates to the natural environment must be assessed. By understanding the applicability of data generated from current methodologies, our limited research capabilities can be better utilized to assess environmental threats more accurately. Improving the applicability of limited datasets does not preclude the use of species sensitivity distributions as the best practice for environmental risk assessment but can instead compliment them by refining how these statistical models approach data uncertainty. Increasing the accuracy of assessment models can allow for better delineation of the most severe threats so that scientific and political resources are not consumed by inaccurately labelling a particular stressor as an emergent threat. Thus, it is important to understand how methodologies influence results by testing research assumptions so that the resulting data can be applied in the appropriate contexts.

1.2 Organic ultraviolet filters

Ultraviolet filters (UVFs) commonly serve as the active ingredients in a wide variety of consumer cosmetics such as lip balms, skin creams, and sunscreens due to their ability to absorb and protect the skin from harmful ultraviolet (UV) radiation in the UV-A (315 – 400 nm) and UV-B (280 – 315 nm) wavelength spectrums (Butkovskyi et al., 2016; Manová et al., 2013). Organic UVFs such as avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY) are currently among the most commonly used UVFs (Matta et al., 2019), and work by absorbing energy from UV radiation and converting it into thermal energy, while inorganic compounds such as titanium dioxide and zinc oxide serve as physical UV blockers by scattering and reflection UV radiation (Mikkelsen, 2015). Organic UVFs are preferentially used within sunscreens and other cosmetics over physical UV blockers (Rastogi, 2002), primarily due to their ease of application through sprays and desirable aesthetic properties, as inorganic UVFs tend to form a visible, white residue on the skin (Nery et al., 2020) and pose a risk of inhalation toxicity to the users of spray products (Schneider and Lim, 2019a). Although regulatory guidelines limit the concentration of individual chemicals within consumer products, UVFs such as AVO, OCT and OXY are often used in combination to offer broad spectrum protection that benefits from the ability of certain combinations of UVFs to stabilize each other, increasing product effectiveness (Berardesca et al., 2019; Kim and Choi, 2014; Klotz et al., 2019).

UVFs are also used in manufacturing processes to improve the durability of many outdoor and environment-facing products such as rubbers, cements and plastics by reducing the rates of photo-oxidation and photo-degradation (Gago-Ferrero et al., 2013b; Muncke, 2011). UVFs from manufacturing effluents are collected into wastewaters and ultimately introduced into the environment due to their incomplete removal from wastewater treatment plant (WWTP) effluents (Emnet et al., 2015; Kasprzyk-Hordern et al., 2009; Langford et al., 2015; Lindo-Atichati et al., 2019; Wick et al., 2010). Outdoor bathing and recreational activities can directly contaminate the environment as UVFs

present on the skin through sunscreen application can leach into the surrounding water. (Giokas et al., 2007; Labille et al., 2020; Peng et al., 2017b; Pintado-Herrera et al., 2017). Through these sources, UVFs have been reported at concentrations of 0.01 μg/L to 10 μg/L in freshwater and marine environments around the world (Figure 1.1; Mitchelmore et al., 2021; National Academies of Sciences, Engineering, and Medicine, 2022; Tovar-Sánchez et al., 2020), with rare reports of concentrations of 43 – 1,395 μg/L measured in extreme contamination scenarios (Downs et al., 2016; Kasprzyk-Hordern et al., 2009; Vila et al., 2017). The ubiquitous contamination of aquatic environments has led to the classification of UVFs as contaminants of emerging concern, warranting further research into their impacts on the environment.

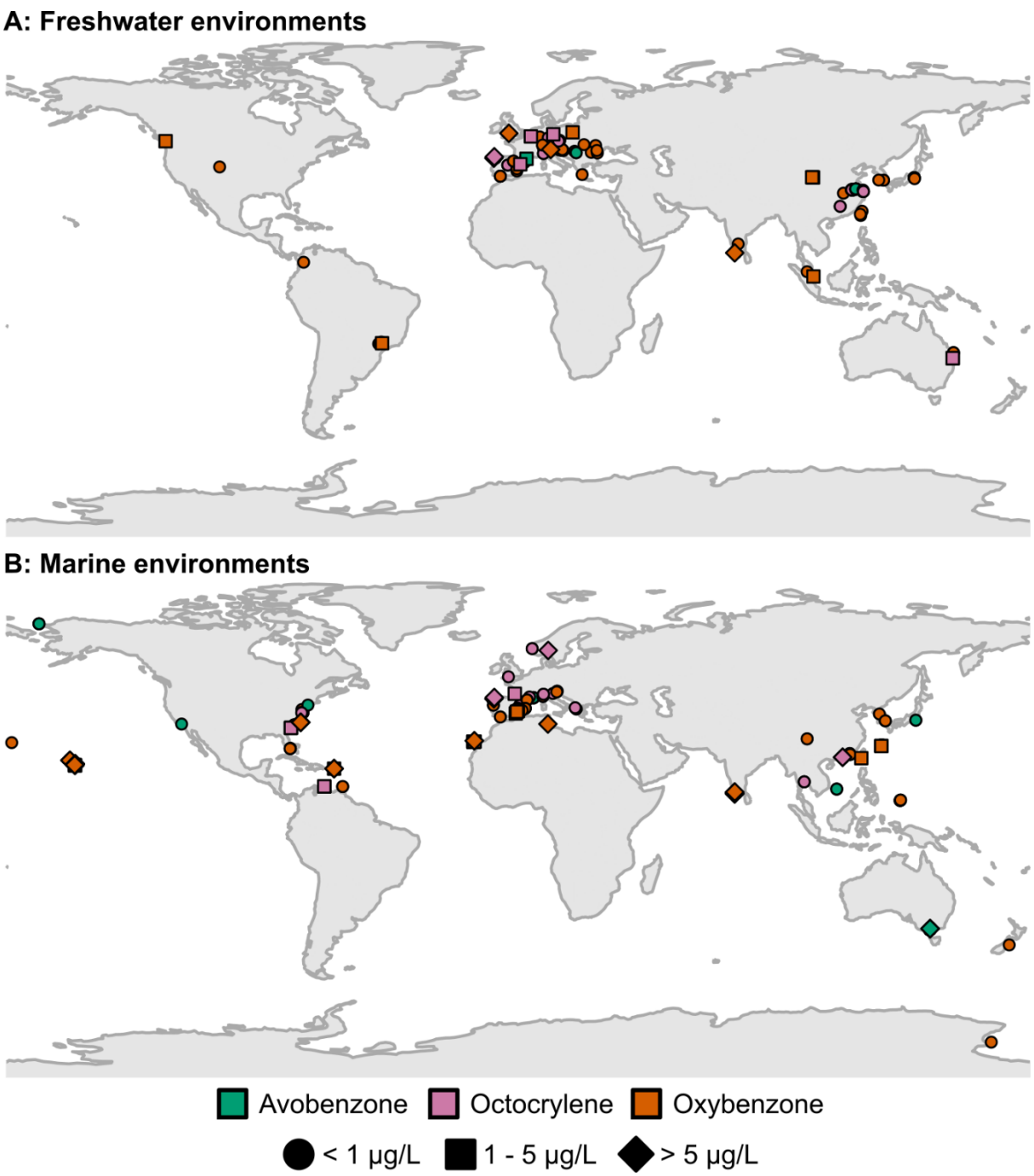

Figure 1.1. Reported locations of UVFs in freshwater (A) and marine (B) environments. Data was compiled from a review of 170 articles in the Web of Science and Google Scholar databases reporting environmental surveys. Concentrations below detection limits were excluded from the visualization.

1.2.1 Sources

A 2001 assessment of the use of sunscreens in the Danish market revealed that AVO was present in 44% all sunscreen products, while OCT and OXY were used in 22.7% and 18.7% each (Rastogi, 2002). By 2014, the market share of OXY declined to 3% of all skin care products in Denmark, while AVO and OCT increased to 60% and 42%, respectively (Mikkelsen, 2015), with similar market distributions reported in Switzerland in 2011 (Manová et al., 2013) and France in 2017 (Labille et al., 2020). While data on the exact market share of each UVF in the United States is considered proprietary, AVO, OCT and OXY were all ranked within the top 5 most commonly used UVFs in United States sunscreen products in 2016, with estimated annual consumption of 29,500, 28,500, and 23,300 tonnes, respectively (National Academies of Sciences, Engineering, and Medicine, 2022). The usage of UVFs varies across regions according to local market conditions and public policy priorities, particularly as data is generated to further our understanding of the environmental impacts of each chemical. In recent years, several bans of specific UVFs have been implemented, including 2020 bans of OXY in Aruba (Homan and Martinus, 2021), and OCT and OXY in Palau (Republic of Palau, 2018), followed by 2021 bans of OXY in Bonaire (Bonaire Pros, 2018), Hawaii (Hawaii State Legislature, 2018), and the US Virgin Islands (USVI Legislature, 2019). Many of the bills instituting these bans in tropical regions cited concerns regarding UVF toxicity to corals, which is discussed in greater detail in section 1.3 below.

Recreational activities are considered to be the primary driver of environmental contamination by UVFs (Balmer et al., 2005; Ekpeghere et al., 2016; Poiger et al., 2004), with multiple studies observing that aquatic concentrations are greatest near local tourism hotspots such as beaches (Downs et al., 2016; Ekpeghere et al., 2016). Measured hourly concentrations correlate strongly with times of peak swimming activity, reaching peaks up to 3-fold greater levels than the overnight minimum (O'Malley et al., 2021; Reed et al., 2017). This is driven by the high usage of sunscreens at beaches, as

approximately 70% of French beachgoers indicated that they apply sun care products 2.6 times per visit, the majority of which applied product to their whole body (Labille et al., 2020). It is estimated that 40 – 70% of applied UVFs are washed off into local waters while swimming, depending on product formulation (Gonzalez et al., 2006; Lambropoulou et al., 2002; Stokes and Diffey, 1999), leading to an estimated annual discharge of 16.8 t per major beach with an approximate attendance of > 3,000 people/day (Labille et al., 2020).

Similar mechanisms lead to UVF discharge into sewage water, allowing for sunscreens used in non-aquatic activities, as well as cosmetics to be washed into shower drains (Alfiya et al., 2018; Butkovskyi et al., 2016). In addition, UVFs are also capable of absorbing through the skin into the bloodstream, where they will then be metabolized and excreted via urine, further increasing the quantities present in domestic wastewaters as well as introducing an array of biological metabolites (Bury et al., 2019; Hiller et al., 2019; Matta et al., 2019). Concentrations in wastewater treatment plant (WWTP) influent are commonly in the range of 0.5 - 10 μg/L (Balmer et al., 2005; Li et al., 2007; Rodil et al., 2008; Tran and Gin, 2017; Wick et al., 2010), but can exceed 1,000 μg/L on occasion (Kasprzyk-Hordern et al., 2009). Removal efficiency can widely vary across treatment techniques, WWTPs and also UVFs; however, removal efficiency in modern facilities is typically in the range of 70 - 99% (Balmer et al., 2005; Magi et al., 2012; Negreira et al., 2009; Pedrouzo et al., 2009), particularly those that use advanced treatment processes such as trickling filter beds (Kasprzyk-Hordern et al., 2009), membrane bioreactors (Tran and Gin, 2017) and ozonation (Li et al., 2007).

Post-treatment concentrations in final effluent are an order of magnitude less than influent levels, at 0.05 – 1 μg/L (Gómez et al., 2009; Magi et al., 2013; Pintado-Herrera et al., 2014, 2013; Rodil and Moeder, 2008), with an estimated daily release of 1.2 – 41 g of any particular UVF per 10,000 persons into receiving waters (Balmer et al., 2005). These concentrations will be further diluted in receiving bodies of water by 5 – 23-fold 2 km downstream in rivers and tend to be greatest during

dry periods of low rainfall, as receiving waters will be at a reduced volume and combined storm and wastewater systems will receive less diluting water to the flow of effluent (Kasprzyk-Hordern et al., 2009). Indirect contamination can occur in areas that rely on septic tanks for waste treatment, as these systems are known to leak, introducing a variety of pollutants into local waters (Aminot et al., 2019), and notably causing contamination of a "pristine" control lake in one study (Bell et al., 2017). Although leakages have also been known to occur in urban sewer pipes, urban groundwater concentrations have been measured to be typically < 10 ng/L, suggesting that leakages are a negligible source in comparison to groundwater recharge from rivers (Cabeza et al., 2012; Jurado et al., 2014).

1.2.2 Environmental fates

The half lives of these UVFs are variable depending on environmental conditions such as sunlight, dissolved organic carbon, redox conditions, and the presence of stabilizing compounds (National Academies of Sciences, Engineering, and Medicine, 2022); however, half-lives are estimated to be ≤ 14 d in many of these degradation conditions (He et al., 2019a; Liu et al., 2013; O'Malley et al., 2021; Semones et al., 2017; Vione et al., 2013). Despite being relatively short-lived in the environment compared to other pharmaceutical and personal care product contaminants, UVFs may still be considered to be a pseudo-persistent contamination threat due to their high usage and continual input into the environment (Martínez Bueno et al., 2012). As a consequence of their UV absorbing nature, these chemicals undergo rapid photodegradation in the presence of UV spectrum light, producing a large variety of degradation products of varying physical and chemical properties (Berenbeim et al., 2020; Jentzsch et al., 2019; Kotnik et al., 2016; Li et al., 2017; Sayre et al., 2007). The diversity of degradation products (discussed in section 1.2.3 below) makes it difficult to include them in quantification of environmental and toxicological water samples, resulting in the vast majority of studies relying on quantification of only the parent molecules to characterize their study waters.

The stability of these chemicals is sufficient to allow them to disperse throughout the water column and away from their sources, although they tend to be most concentrated at the surface microlayer (Labille et al., 2020; Schaap and Slijkerman, 2018; Tovar-Sánchez et al., 2013). Estuarian modeling predicts that UVFs rapidly disperse up to 2 km away from sources within 6 hours, greatly expanding the area affected by tourism and recreation (Lindo-Atichati et al., 2019). Tides and ocean currents can further spread contaminants throughout the ocean, as evidenced by the 2006 *Tangarosa* balsa raft expedition, which detected trace quantities of OXY in remote regions of the Pacific ocean, thousands of km removed from pollution sources (Goksøyr et al., 2009). A similar outcome has been observed in coastal sediments, as contamination levels in the protected waters of Słowiński National Park, Poland (where bathing is banned) were comparable to the levels detected at other Polish beaches, suggesting that longshore littoral drift is another factor driving UVF distribution (Astel et al., 2020). Despite these transport mechanisms, the majority of UVF contamination exists locally, with contamination profiles in waters shown to closely resemble the distribution of local UVF usage in terms of location and the dominant type of UVF(s) used in local sunscreen products (Tashiro and Kameda, 2013). The highest concentrations of UVFs have been observed < 100 m from major sources (Arukwe et al., 2012; Downs et al., 2016), and tend to rapidly decrease with distance from their source.

Concentrations of UVFs in all environments correlate strongly with seasons and local weather, with peaks commonly observed midday during fair weather conditions (Alfiya et al., 2018; Balmer et al., 2005). Usage continues during winter months to a lesser degree, with concentrations up to half of the summertime maximum reported (Alfiya et al., 2018; Astel et al., 2020). Overall contamination levels tend to be higher in marine environments, with median concentrations of 0.312 µg/L, 0.100 µg/L, and 0.136 µg/L for AVO, OCT and OXY, respectively, while median freshwater concentrations are 0.0135 µg/L, 0.035 µg/L, and 0.038 µg/L (Figure 1.2). The majority of environmental surveys report concentrations ± 1 order of magnitude of these medians; however, a small number of highly

contaminated locations have been identified for each UVF. The highest freshwater concentration reported for AVO to date was 1.13 µg/L in a reservoir in Brisbane, Australia (O'Malley et al., 2021), and a marine concentration of 70.97 µg/L in coastal waters near beach showers at Kahalu'u Beach, Hawaii, USA (Downs et al., 2022b). It is important to note that far fewer studies have quantified AVO compared to the research effort present for OCT and OXY; therefore, the lower maximum concentrations of AVO in freshwater could be an artifact of reduced sampling effort. For OCT, 171 µg/L was reported off the Spanish coast, and 323 µg/L in a Spanish river within the same study; however, the authors elected to not disclose their sampling locations (Vila et al., 2016). 44 µg/L of OXY was detected in River Taff, UK (Kasprzyk-Hordern et al., 2009), and 1,395 µg/L of OXY reported in the coastal waters of Trunk Bay in the US Virgin Islands in 2007 (Downs et al., 2016), which is the highest concentration reported in any environment for these UVFs. It should be noted that this extreme level of contamination is an outlier with respect to the next most contaminated locations of Hawksnest Bay, Hawaii (95 µg/L; Downs et al., 2016), and Gabes Bay, Tunisia (36.6 µg/L; Fenni et al., 2022), as well as a later assessment of Trunk Bay in June 2014 (6.07 µg/L; (Bargar et al., 2015); however, it is important to note that the very high concentration detected in 2007 was accompanied by a report of 310 people on the beach or swimming within 100 m of the sample location (Downs et al., 2016). Additionally, the number of visitors to the region was limited to 2000 people/day beginning in 2009, possibly accounting for the reduced concentrations reported in subsequent years.

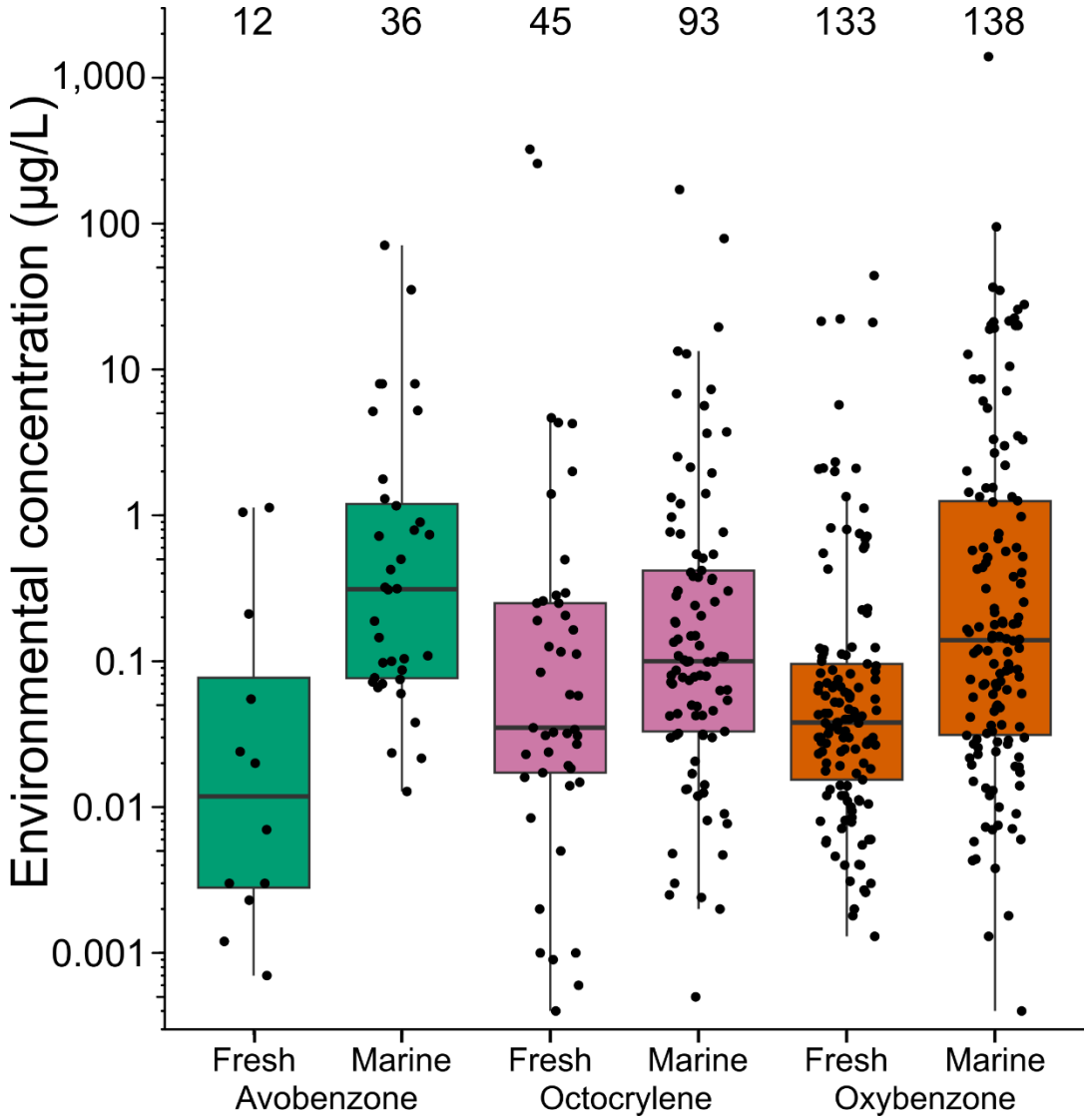

Figure 1.2. Environmental concentrations of UVFs in freshwater and marine environments. Boxes depict median and quartiles, dots depict individual locations assessed. Data was compiled from a review of 170 articles in the Web of Science and Google Scholar databases reporting environmental surveys. The number above each box indicates the number of locations assessed. Concentrations below detection limits were excluded from the visualization but are included in the number of locations assessed.

Many studies have reported difficulties accurately quantifying UVF concentrations due to their high lipophilicity, as the octanol-water partitioning coefficient (log K_{ow}) of AVO and OCT is 6.1, while OXY has a log K_{ow} of 3.45 (National Academies of Sciences, Engineering, and Medicine, 2022). Upwards of 50% of total UVF content within individual samples have been reported to be adsorbed to container surfaces and suspended particles in the particulate fraction of solutions (Cormier et al., 2019; Fel et al., 2019; O'Malley et al., 2021), and can disperse back into the aqueous fraction over time as the initial water-soluble fraction decays (Benedé et al., 2014). These challenges, accompanied with the lack of a standard methodology to quantify UVFs due to their status as an emerging contaminant (National Academies of Sciences, Engineering, and Medicine, 2022), may result in many of the concentrations reported in environmental surveys to be underestimates of the total quantity of UVFs present. The lipophilicity of these UVFs also influence their fate within the environment, resulting in large quantities of UVFs stored within sediments (Díaz-Cruz et al., 2019; Gago-Ferrero et al., 2011; Mizukawa et al., 2017; Vila et al., 2018b), with sediment concentrations increasing along with lipophilicity of each compound (Mitchelmore et al., 2019). OXY has been frequently reported in the range of 1 – 100 ng/g, while OCT can range from 1 – 1,000 ng/g (National Academies of Sciences, Engineering, and Medicine, 2022). Limited data is available for AVO within sediments, but concentrations of 0.6 – 65 ng/g have been reported (Tsui et al., 2015; Yang et al., 2020).

Similarly, these UVFs are expected to bioaccumulate, with many studies reporting UVFs at 1 – 100 ng/g in fish (Cunha et al., 2018; Horricks et al., 2019; Molins-Delgado et al., 2018; Pawlowski et al., 2019; Peng et al., 2017a; Saunders et al., 2019), 50 – 300 ng/g in bivalves (Castro et al., 2018; Falfushynska et al., 2021; Gomez et al., 2012; Rodil et al., 2019), and 5 – 300 ng/g in corals (Mitchelmore et al., 2019; Tsui et al., 2017). Considerable variation in bioaccumulation has been reported across species, with the greatest bioaccumulation factors reported in detritivores that interact frequently with contaminated sediments (Peng et al., 2020), and filter feeding organisms such as

mussels (Bachelot et al., 2012; Vidal-Liñán et al., 2018). The biological half-lives of OCT and OXY have been reported to be < 48 h in fish (Peng et al., 2020) and corals (He et al., 2019a), while AVO is estimated to have a half life of 84 h in fish (ECHA, 2024), indicating that the accumulation of UVFs within biota is limited by biotransformation processes (discussed in section 1.2.3 below), and that observed tissue accumulation is representative of recent UVF exposure. As a result, biomagnification factors < 0.001 have been reported in *Oncorhynchus mykiss* for OCT (Pawlowski et al., 2019; Saunders et al., 2020); however, few direct measurements of biomagnification have been made to date.

1.2.3 Abiotic and biotic transformation products

It is understood that UVFs can undergo degradation through photolysis, oxidation, and biotransformation; however, the specific products formed are not fully understood and are subject to ongoing investigations. Photolysis is the primary outcome for all three UVFs when they absorb light directly, typically resulting in the separation of both aromatic groups (Berenbeim et al., 2020; Dunkelberger et al., 2015), but photoisomerization has also been reported in AVO (Berenbeim et al., 2020; Németh et al., 2023; Trossini et al., 2015) and OXY (Abid et al., 2017; Baker et al., 2015). Many of these products have higher photostability than their respective parent molecules (Moi et al., 2021), but the reactivity of each new chemical species is largely unknown (Jentzsch et al., 2019).

AVO and OXY can be readily oxidized by dissolved organic matter (Li et al., 2016; Semones et al., 2017; Vione et al., 2013) as well as halogens such as chorine and bromine (Wang et al., 2017; Zhang et al., 2016), and can also be stabilized with the addition of antioxidants such as ubiquinone (Afonso et al., 2014). OCT has high reported stability due to presence of cyano and ester functional groups which protect against electrophilic attacks (Manasfi et al., 2017). Between three (OXY; Yang et al., 2018; Zhang et al., 2016) and 60 (AVO; Lebedev et al., 2020; Trebše et al., 2016) unique halogenation products have been identified per UVF, resulting in a large diversity of degradation products that could exist in water released from managed environments such as swimming pools and

wastewater treatment plants. Chlorinated AVO products (e.g., chloro-avobenzone) are stable and can be considered to be persistent pollutants (Trebše et al., 2016; Wang et al., 2017), while OXY products have been demonstrated to have a higher cytotoxicity than the parent molecule (Sherwood et al., 2012).

Biodegradation has been observed for all three UVFs (Chou et al., 2024; Jou-Claus et al., 2024; Liu et al., 2012), with mineralization under aerobic conditions reducing UVF concentrations by 20% over 10 – 60 days (Fagervold and Lebaron, 2022; Suleiman et al., 2019). Biotransformation is believed to occur for all three UVFs; however, it is poorly understood for OCT and particularly AVO. OCT is believed to primarily undergo phase I processing via carboxylesterases, as virtually all depletion of the parent molecule in rainbow trout (*Oncorhynchus mykiss*) liver fractions occurred in the absence of phase II cofactors (Saunders et al., 2019), while metabolomic profiling of corals (*Pocillopora damicornis*) reported a variety of OCT fatty acid conjugates with a high bioaccumulation potential due to their lipophilicity (Stien et al., 2019), which may also be the products of carboxylesterase activity (Ansari et al., 1995). Mussels (*Mytilus edulis*) similarly demonstrated predominantly phase I activity in response to OCT; however, glutathione-S-transferase activity was also reported (Falfushynska et al., 2021), with a single glutathione conjugate produced by human liver microsomes (Guesmi et al., 2020), indicating that some species may additionally rely upon phase II conjugation reactions to metabolize OCT.

In contrast, OXY is believed to undergo extensive processing from both phase I and phase II reactions. Hydrolysis via cytochrome P-450 enzymes has been observed in humans (Guesmi et al., 2020), rats (Okereke et al., 1993), fish (Ziarrusta et al., 2018a), and plants (Chen et al., 2017), producing several demethylated and/or hydroxylated products including 2,2-dihydroxy-4-methoxybenzophenone, which has a higher estrogenic activity than OXY and could therefore have a higher toxicity (Fediuk et al., 2012; Watanabe et al., 2015), serving as a possible case of bioactivation. Conjugation with glutathione and glucuronide has also been reported, resulting in at least 20 different

transformation product reported to date (Guesmi et al., 2020; Wang and Kannan, 2013; Ziarrusta et al., 2018a). AVO biotransformation has been poorly studied to date; however, it is suspected to undergo similar transformation processes as OXY due to the structural similarity of both molecules, with one report of similar demethylation and glutathione conjugation products as OXY (Guesmi et al., 2020).

1.3 Ultraviolet filter toxicity

Studies of UVF toxicity have been increasing rapidly in recent years due to the frequency of environmental contamination reported, with approximately 3-fold more studies focusing on OXY than OCT, while few have investigated AVO. Studies have been conducted in standard species such as *Danio rerio, Daphnia magna*, and non-standard species such as corals, with the majority reporting toxicity at concentrations in excess of the solubility limit for AVO (27 µg/L), OCT (40 µg/L) and OXY (6,000 µg/L; National Academies of Sciences, Engineering, and Medicine, 2022). Generating reliable toxicity data for these UVFs can be challenging due to the low solubility of AVO and OCT, as well as the propensity of all three chemicals to adsorb to test vessel walls and rapidly degrade when exposed to UV spectrum light (Rodil et al., 2009), causing large deviations between nominal and measured exposure doses. In addition, their status as emerging contaminants means that the development of UVF quantification methods has been developed in parallel with toxicology studies and has yet to be standardized (National Academies of Sciences, Engineering, and Medicine, 2022). As a result, there is a diversity of data generated from studies relying on nominal concentrations or quantified using methods of varying sensitivity, presenting several challenges to assessing the level of risk UVFs pose to the environment. Further complications arise from the typical study of the effects of individual UVFs, as these chemicals are used in complex mixtures and environmental exposures do not occur in isolation (Berardesca et al., 2019; Kim and Choi, 2014; Klotz et al., 2019). Overall, current data suggests that lower trophic level species such as aquatic invertebrates (corals, sea urchins, mussels, and

Daphnia) and algae are particularly sensitive to UVFs, while few reports of toxicity at environmentally relevant concentrations exist for vertebrate species.

1.3.1 Avobenzone toxicity

AVO toxicity data is extremely limited, with no impairments observed in ecologically relevant endpoints (e.g., behaviour, growth, reproduction, survivorship) occurring at concentrations below the 27 µg/L solubility limit during single generation exposures. Nominal growth median effect concentrations (EC50s) have been reported at 9.45 mg/L after 72 h in the diatom *Phaeodactylum tricornutum* and 9.89 mg/L after 48 h in tubeworm *Ficopomatus enigmaticus* (Vieira Sanches et al., 2021), and immobilization 48 h EC50s of 1.89 mg/L (de Paula et al., 2022) and 1.95 mg/L in *Daphnia magna* (Park et al., 2017). In corals, no effects were reported in *Acropora sp.* nominally exposed to 33 µL/L for 24 h, nor after 35 d exposure to 516 µg/L in *Stylophora pistillata* (Fel et al., 2019). Nominal sediment toxicity studies have reported no impairments to reproductive characteristics of benthic invertebrates *Chironomus riparius, Lumbriculus variegatus, Melanoides tuberculata,* and *Potamopyrgus antipodarum* at 50 mg/kg dw over 28 – 56 d exposures, nor zebrafish (*Danio rerio*) embryo mortality after 48 h exposure to 1000 mg/kg dw (Kaiser et al., 2012).

The greatest reported sensitivity in a vertebrate was an approximately 30% reduction in acetylcholine esterase activity observed over 21 – 28 d exposures of crucian carp (*Carassius Carassius*) at 36 µg/L; however, no impairments of overall organism function were reported (Ma et al., 2017). Delayed maturation age and reduced reproduction rates were reported in the second generation of *D. magna* continuously exposed to 4.4 µg/L over 21 d (de Paula et al., 2022), serving as the lowest toxic concentration reported to date. These effects occurred at concentrations ~2.5 fold greater than the maximum reported environmental concentrations to date (O'Malley et al., 2021; Sánchez Rodríguez et al., 2015).

1.3.2 Octocrylene toxicity

Studies of OCT toxicity are primarily limited to acute exposures, with few reports of toxicity in ecologically relevant endpoints below the 40 µg/L solubility limit. Nominal EC50s for impairment of growth, hatching or phototaxis have been reported at concentrations > 10 mg/L in tubeworms (*F. enigmaticus*), diatoms (*P. tricornutum*; Vieira Sanches et al., 2021), barnacles (*Balanus amphitrite*; Tsui et al., 2019), brine shrimp (*Artemia salina*), green algae (*Desmodesmus subspicatus*; de Paula et al., 2022), or midge fly larvae (*C. riparius*; Ozáez et al., 2013). Nominal 48 h immobilization EC50s in *D. magna* were reported to be 2.57 mg/L (de Paula et al., 2022) and 3.18 mg/L (Park et al., 2017). No bleaching, changes to zooxanthellae density, or impairments of photosynthesis were reported at concentrations up to 1,000 mg/L in several species of corals over 1 d (Danovaro et al., 2008), 7 d (He et al., 2019a), and 35 d exposures (Fel et al., 2019). *D. magna* exposed to 4.4 µg/L for 21 d across two generations were not developmentally or reproductively impaired (de Paula et al., 2022). No toxicity has been reported in studies of sediment exposures at concentrations > 1 mg/kg dw in *C. riparius* (Campos et al., 2017; Kaiser et al., 2012), or freshwater snails *Melanoides tuberculate* and *Potomopyrgus antipodarum* (Kaiser et al., 2012).

Toxicity at concentrations below the maximum water solubility of OCT are limited to the reported lowest observed effect concentrations (LOEC) of 40 µg/L after 48 h exposures for mussel larvae development (*Mytilus galloprovincialis*) and sea urchin growth (*Paracentrotus lividus*; Giraldo et al., 2017). These impairments occurred at concentrations that have been reported in the most contaminated environments (Vila et al., 2017).

1.3.3 Oxybenzone toxicity

OXY is currently the most well-studied UVF, possibly due to its higher solubility in water (6,000 µg/L) and an early report of toxicity to corals *Acropora sp* and *Acropora pulchra*, with

bleaching and zooxanthellae release observed after 24 h exposure to 33 – 50 µL/L (Danovaro et al., 2008). Downs et al. (2016) reported a 24 h EC50 for planulae deformation in *Stylophora pistillata* of 49 µg/L, as well as 4 h median lethal concentrations (LC50s) to primary cell cultures of 6 different Indo-Pacific and Caribbean-Atlantic coral species of 8 – 74 µg/L. In other taxa, several acute studies have reported effects at concentrations below the solubility limit, including nominal 24 – 48 h LC & EC50s of 1.09 – 3.03 mg/L in *D. magna* (Du et al., 2017; Jang et al., 2016; Sieratowicz et al., 2011), 48 h growth EC50s of 4.04 mg/L in *F. enigmaticus*, and 2.42 mg/L in *P. tricornutum* (Vieira Sanches et al., 2021), and 48 h growth impairment beginning at 100 µg/L in marine bacteria *Epibacterium mobile* and *Pelagibacterium halotolerans* (Lozano, 2020). 72 – 96 h growth inhibition EC50s in algae are typically reported to be 0.67 – 8.54 mg/L (Du et al., 2017; Esperanza et al., 2019; S. H. Lee et al., 2020; Yongfu Li et al., 2024; Sieratowicz et al., 2011), with the exception of one study reporting a 72 h EC50 of 13.9 µg/L for *Isochrysis galbana* (Paredes et al., 2014). Lower acute toxicity has been reported in *Danio rerio*, with 72 – 120 h EC50s for developmental deformities and hatch rates of 6.73 – 12.4 mg/L (Balázs, 2016), while immobilization assays reported no effects after 24 h exposures of barnacles (*B. amphitrite*; Tsui et al., 2019) or midge fly larvae (*C. riparius*) at concentrations up to 10 mg/L (Ozáez et al., 2013).

Chronic toxicity has been reported in several species at low concentrations, including impaired growth over 7 d at 1 µg/L in the algae *Chlamydomonas reinhardtii* (Mao et al., 2017), and decreased reproduction and delayed maturity in the second generation of *D. magna* exposed to 0.17 µg/L for 21 d (de Paula et al., 2022). Studies in vertebrates have reported decreased egg production and hatching rates in Japanese medaka (*Oryzias latipes*) over 21 d at 620 µg/L (Coronado et al., 2008), and 28 d at 26 µg/L (Kim et al., 2014). These chronic effects occurred at concentrations that have been reported in several environments (Bargar et al., 2015; Downs et al., 2016; Kasprzyk-Hordern et al., 2009).

1.3.4 Mechanisms of action

The toxicity studies described above focus on ecologically relevant impairments that could impact an organism's fitness. Further toxic effects at the sub-organism level have been assessed for each UVF, offering insight into their mechanisms of action. It is important to note that in most cases, ecologically relevant effects and mechanisms of action have been assessed through separate studies, with few researchers establishing direct links between proposed mechanisms and larger scale adverse effects on development, reproduction, or other endpoints relevant to populations. No definitive conclusions can be drawn regarding the mechanism(s) of action for these UVFs, as current knowledge relies upon data drawn from studies of differing methodologies across various taxa which could each be subject to differing mechanisms of action.

Several studies have reported endocrine disrupting capabilities in various human, yeast, and fish cell lines for each of these UVFs, reporting strong glucocorticoid-, thyroid hormone-, anti-androgen-like activities for AVO (Klopčič and Dolenc, 2017), and anti-estrogen- and anti-androgenic-like activities for OCT (Kunz and Fent, 2006a) and OXY (Kunz and Fent, 2006b, 2006a; Molina-Molina et al., 2008; Nashev et al., 2010; Zhang et al., 2017). These activities have been attributed towards interactions of each UVF with estrogen receptor- α (ERα) (Kunz and Fent, 2006a; Lee et al., 2022; Majhi et al., 2020), with separate studies of OXY reporting subsequent increases in testosterone (Kim et al., 2014) and impaired gonad development (Kinnberg et al., 2015), but mixed results on vitellogenin, with some studies reporting increases (Coronado et al., 2008; Kim et al., 2014; Rodríguez-Fuentes et al., 2015) while others found no effect (Blüthgen et al., 2012; Kunz and Fent, 2006b; Zhang et al., 2017).

Recent studies have also demonstrated peroxisome proliferator-activated receptor-γ (PPARγ) binding for OCT and OXY, causing dysregulation of adipose tissue development and cell metabolism, indicating that these chemicals are obesogens (Ko et al., 2022; Shin et al., 2020). Interestingly, AVO

demonstrated similar disruptions of adipogenesis by inducing an upregulation of PPARγ, but did not directly bind to PPARγ, leaving the exact mechanism of action unknown (Ahn et al., 2019). These results are consistent with reports of disruptions to fatty acid metabolism for OCT (Stien et al., 2019), and glycerophospholipid (Simons et al., 2022), fatty acid (Kopp et al., 2017; Y.-K. Zhang et al., 2023; Ziarrusta et al., 2018b) and amino acid metabolism for OXY (Ziarrusta et al., 2018b). OXY has also been reported to exert toxicity to corals by increasing their susceptibility to viral infections (Danovaro et al., 2008); and decreasing immunoglobulin M levels in goldfish (*Carassius auratus*; Zhang et al., 2020); however, the exact mechanism causing these immune impairments is currently unknown.

Oxidative stress is believed to be another contributing factor in UVF toxicity, with upregulation of relevant response genes reported for AVO (de Paula et al., 2022; Liu et al., 2021), OCT (Falfushynska et al., 2021; Gayathri et al., 2023), and OXY (Cocci et al., 2020; Rodríguez-Fuentes et al., 2015). Assays in bacterial and human fibroblast cells have also suggested that AVO (Vatan et al., 2023), OCT (Falfushynska et al., 2021; Vatan et al., 2023), and OXY (Zhang et al., 2017) can exert genotoxic effects. OXY has been further linked to Hirschsprung disease in humans (Huo et al., 2016), causing a decreased number of enteric neurons in *D. rerio* (Wang et al., 2021), and a decrease in acetylcholinesterase activity in *Carassius Carassius* (Ma et al., 2017), although no effects on acetylcholinesterase were found in *Sericostoma vittatum* (Campos, 2017) or *Chironomus riparius* (Campos et al., 2017), leaving the overall neurotoxicity of OXY unclear.

1.3.5 Current knowledge on the environmental application of UVF data

Studies that attempt to incorporate additional environmental relevance into the testing of UVF toxicity are largely limited to *in situ* investigations of wild populations, studies of mixture toxicity or the effects of lighting. An *in situ* investigation by Danovaro et al. (2008) in wild corals allowed for temperature and light fluctuations to occur during the exposure period; however, the specific effects of these incubation conditions on UVF toxicity cannot be determined due to the experiment's design. The

authors also assessed the effects of sunscreens vs isolated UVFs, proposing that the UVF component of sunscreen mixtures appears to be responsible for the coral bleaching that occurs due to sunscreen contamination. Several other studies have investigated the combined effects of UVFs, reporting antagonism in most toxicity assessments, (Du et al., 2017; Li et al., 2018; Park et al., 2017), but also synergism of estrogenic activity (Kunz and Fent, 2006a). Others tested the toxicity of sunscreen mixtures but did not attribute the observed effects to specific product components (Araújo et al., 2020; He et al., 2019a; McCoshum et al., 2016; Tovar-Sánchez et al., 2013), while two other studies attributed reduced sunscreen toxicity to a lack of specific preservatives or polymers (Corinaldesi et al., 2017; Varrella et al., 2022).

Investigations of photoperiod effects resulted in an approximate order of magnitude decrease in OXY toxicity when corals were exposed in darkness instead of daylight (Downs et al., 2016), with a smaller reduction in toxicity reported for several species of marine bacteria exposed in darkness (Lozano, 2020); however, UV radiation increased OXY toxicity to sea anemones (*Aiptasia*; (Vuckovic et al., 2022). No change in AVO toxicity was reported in a mouse cell assay due to UV irradiation (Butt and Christensen, 2000), but increased toxicity to *D. magna* was reported via higher immobilization and reduced growth rate (Kim et al., 2023). The effects of alternate exposure routes have also been investigated via a dietary exposure of *Oncorhynchus mykiss* to OCT which resulted in bioaccumulation (Pawlowski et al., 2019), and co-exposure of AVO with microplastics in *Daphnia magna*, which increased daphnid immobilization (Kim et al., 2023). While not direct investigations of toxicity, an investigation of wild-caught loggerhead turtles (*Caretta caretta*) assessed correlations between accumulated UVFs and various gene markers of stress within wild-caught animals (Cocci et al., 2020), while Peng et al. (2017a) studied relationships between bioaccumulation, trophic level, and feeding behaviour in many estuarian fish, cephalopods, and crustaceans, reporting higher uptake in detritus feeding fish. Finally, a study conducting a continuous exposure across two generations observed that

AVO and OXY did not impair *Daphnia magna* survival or reproduction until the second generation of exposure (de Paula et al., 2022). It is clear that there are several shortcomings in our current knowledge regarding how environmental factors may influence the toxicity of UVFs; therefore, further investigations are warranted.

1.4 Thesis aims

It is important that any assumptions made when conducting research are tested so that study models can be applied effectively and within the appropriate contexts. Considering the debate regarding the level of threat UVFs pose to the most sensitive species, the overall assessment of this particular class of contaminants is particularly vulnerable to small deviations in toxicity outcomes that can arise from the assumptions made during standardized laboratory testing. These deviations can be the difference between solidifying UVFs as a threat to be addressed, or providing confidence that periodic monitoring of contamination levels is sufficient to protect the most vulnerable species and environments.

The objectives of this thesis were to test common toxicology assumptions while investigating UVF toxicity so that we can better understand how accurate current predictions of UVF environmental toxicity are while also providing valuable data to clarify the limitations of conducting environmental risk assessments using laboratory-based studies in standard test species. There are many more assumptions made in the use of standardized research than can be assessed within a single thesis; therefore, more common and/or broadly spanning assumptions were prioritized for assessment. To establish a baseline understanding of toxicity and to inform investigations in later studies, Chapter 2 investigated the acute and chronic toxicity of AVO, OCT and OXY in *Daphnia magna*, as well as the 21-d recovery potential after a 48-h exposure. This served to provide a perspective of the conclusions that could be drawn by performing standardized toxicity assessments in a model species in the absence of further environmentally relevant factors.

Chapters 3 – 5 build on of this foundation by investigating the changes that can occur in exposed populations across multiple generations to test the common toxicology assumption that the long-term effects within an exposed population can be understood through sufficiently long exposures within a single generation. Chapter 3 examines the relationship between various physiological endpoints at the individual level to determine the possible nature of observed changes in toxic response are derived from selective pressures or plasticity mechanisms. Chapter 4 provides further investigations of physiological endpoints at the population level and also investigates the underlying mechanisms of toxicity and subsequent changes in toxic response through proteomic alterations. Chapter 5 then tests the assumption that toxicity outcomes are not impacted when conducting exposures of test organisms individually or in groups, leveraging the differing methodologies of Chapters 3 & 4 to compare the physiological outcomes of UVF exposure across 5 generations.

Chapter 6 addresses the assumption that studies investigating UVF toxicity as isolated chemicals or mixtures of UVFs can be used to understand the impacts of sunscreen pollution. The toxicity of commercially available sunscreens was compared to the results obtained when testing each UVF in the sunscreen mixture in isolation. Relationships between sunscreen toxicity and UVF content were investigated to determine if the impacts of sunscreen pollution can be predicted based on studies of individual UVF ingredients.

Chapter 7 then investigates an assumption made by all of the previous chapters; that data generated through tests of laboratory cultured model organisms are representative of wild populations. This was done by comparing UVF toxicity between a laboratory culture of *Daphnia pulex* and a wild-caught population. This study further tests an assumption made by previous lab and wild animal comparisons that wild-caught animals can be appropriately studied when cultured in laboratory water by determining how culturing laboratory *D. pulex* in lake water or wild *D. pulex* in laboratory water impacts their performance during experimentation.

These collective findings are summarized in Chapter 8 to draw major conclusions and identify research that may be necessary to further understand how standardized data can be applied to the natural environment. Recommendations are provided on how to approach the testing of further research assumptions so that the limitations of data obtained from any type of standardized or lab-based research can be understood so that the results and conclusions generated can be understood within the appropriate contexts. The implications of this thesis research may serve to inform future updates to standardized methodology guidelines in an effort to improve both the applicability of and replicability of biological research.

Chapter 2: A burning issue: The effect of organic ultraviolet filter exposure on the behaviour and physiology of *Daphnia magna*

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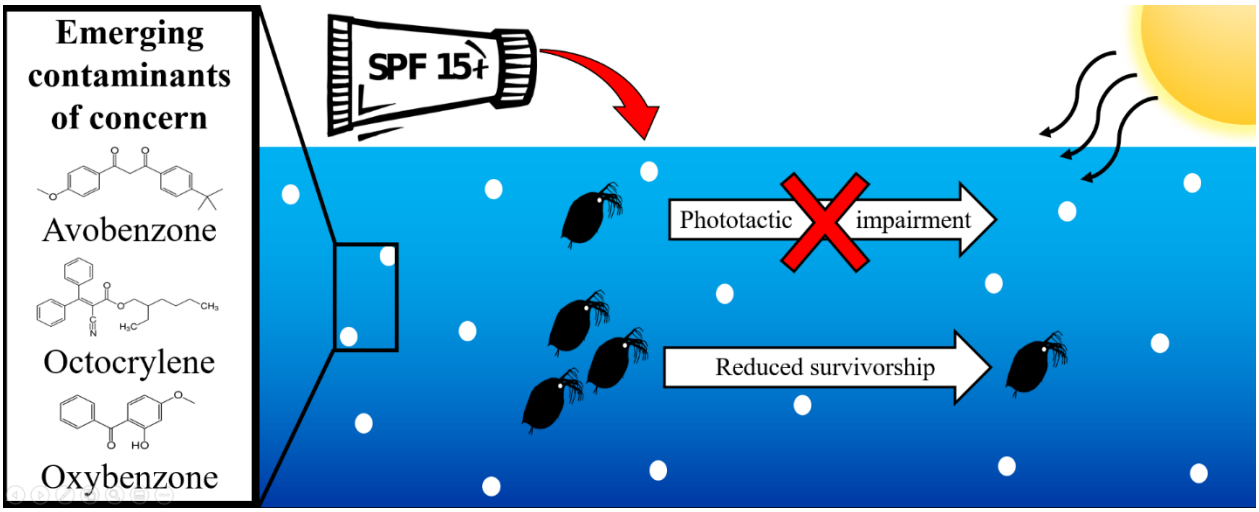

2.1 Introduction

Organic ultraviolet filters (UVFs) such as oxybenzone, avobenzone and octocrylene are emerging as contaminants of concern in aquatic environments (Downs et al., 2016). These compounds are utilized in hundreds of pharmaceutical and personal care products, often in tandem due to their properties of synergistic stabilization of other UVFs, and serve primarily as the active ingredients in sunscreen to protect skin against damage from UV-A and UV-B radiation (Berardesca et al., 2019; Committee for Human Medicinal Products, 2015; Gomez et al., 2012; Manová et al., 2013; Vione et al., 2013). There are two primary modes of entry for UVFs into the aquatic environment; 1) aquatic recreational activity promoting the leaching of UVFs from the skin (Labille et al., 2020; Sánchez Rodríguez et al., 2015; Schaap and Slijkerman, 2018), and 2) point sources such as wastewater treatment plant (WWTP) effluent due to the incomplete removal of these substances from manufacturing process water (Gago-Ferrero et al., 2013b; Semones et al., 2017; Wang and Wang, 2016). As a result, the measured concentrations near beaches fluctuate with recreational activity, peaking at 7.3 μg/L in coastal areas (Bargar et al., 2015; Horricks et al., 2019; Langford and Thomas, 2008; Sánchez Rodríguez et al., 2015), 10-44 μg/L in rivers, and reaching upwards of 200 μg/L in WWTP effluent (Kasprzyk-Hordern et al., 2009).

Recent studies have suggested that UVFs are potentially toxic in many different species, warranting further study into identifying their mechanisms of action. Oxybenzone, octocrylene and avobenzone all systematically absorb into human blood plasma at concentrations up to 210 ng/mL, 8 ng/mL, and 4 ng/mL, respectively (Matta et al., 2019). These concentrations exceed the current US Food and Drug Administration (FDA) guidelines for toxicity testing of 0.5 ng/mL for unknown toxicants, ultimately leading to an FDA request for further research into these active ingredients (Committee for Human Medicinal Products, 2015; US Food and Drug Administration, 2016). Indeed, oxybenzone is a known cause of photobleaching in corals at concentrations as low as 2 μg/L (Downs et

al., 2016), and induces reproductive inhibition in marine algae (S. H. Lee et al., 2020; Rodil et al., 2009). Octocrylene effect concentrations for several marine invertebrate species fall within the range of measured environmental concentrations (see above) (Giraldo et al., 2017). Currently, the potential mechanism of action for these UVFs is thought to be endocrine disruption, exhibiting anti-androgen-like activities and disrupting estrogen receptor signalling (Downs et al., 2016; Klopčič and Dolenc, 2017). However, our current knowledge of the toxicity of these UVFs in freshwater and marine environments is insufficient, and given the high lipophilicity of these compounds, the potential risk of bioaccumulation is high (Cocci et al., 2020; Gago-Ferrero et al., 2013a; He et al., 2019b; Horricks et al., 2019; S. H. Lee et al., 2020; Martín et al., 2020). Environmental contamination poses a potential threat to aquatic organisms, as little time has passed to allow for evolutionary adaptation to occur for these novel toxicants (Tierney and Kennedy, 2008). It is clear that further studies in freshwater environments are required in an effort to comprehensively understand the potential effects of UVF contamination.

Daphnia magna are a promising candidate organism to model the effects of environmental UVF exposure in freshwater invertebrates. The well-documented sensitivity of daphnids to environmental stressors, particularly through reproductive and migratory behaviours, allow for the identification of subtle, yet ecologically relevant changes in addition to more traditional physiological endpoints such as mortality and direct measurements of metabolic processes (Altshuler et al., 2011). Additionally, *Daphnia* serve as an important transitional species in the food web (Hartnett, 2019), linking primary producers to higher trophic levels that may be more susceptible to UVF-mediated environmental disturbances. This includes biomagnification through zooplankton bioaccumulation (Shi et al., 2020; Wang et al., 2019) or a lack of prey species availability as a result of population collapse due to reproductive inhibition or overt mortality (Wathne et al., 2020).

Overall, this study aimed to combine physiological and ecological endpoints in an effort to understand the environmental relevance of the UVFs avobenzone, octocrylene and oxybenzone in a key invertebrate species (*D. magna*). Our objectives were to:

1) Determine the concentration necessary for severe physiological impairment in acute exposures.
2) Assess the extent to which behavioural and physiological recovery is possible at environmentally relevant concentrations.
3) Assess the effects of chronic UVF exposure throughout the *D. magna* lifespan.

By combining acute and chronic developmental exposures, a more complete assessment of the risks of UVFs to freshwater invertebrates can be made. These data are crucial to inform policy regarding the use of these compounds, particularly as several jurisdictions consider implementing restrictions or outright bans of several UVFs (Schneider and Lim, 2019b).

2.2 Materials and methods

2.2.1 Daphnia colony maintenance

Daphnia magna obtained from Aquatic Research Organisms (US, September 2019) were used to culture a colony at the University of Alberta Biological Sciences department. *Daphnia* were housed in 1 L of dechlorinated City of Edmonton water (pH ≈ 7.6) in 2 L glass beakers, prepared according to Organization for Economic Co-operation and Development (OECD) guidelines (294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$, 5.80 mg/L KCl) (OECD, 2012). The complete water chemistry of dechlorinated City of Edmonton water has been previously described in (Delompré et al., 2019b). Colonies were maintained at 20 ± 1°C on a 12h light:12h dark photoperiod and fed 5 mL once daily of both freshwater green algae (*Raphidocelis subcapitata*) and YCT Mix (yeast, cereal leaf, trout chow) supplied by Aquatic Research Organisms (US). The diets were supplemented once weekly with 100 µL

of Roti-Rich invertebrate food (VWR, Edmonton, Alberta, Canada). 100% water changes were performed every 2-3 days.

2.2.2 Exposure solutions

UV filters oxybenzone (OXY), avobenzone (AVO) and octocrylene (OCT) were dissolved in dimethyl sulfoxide (DMSO) to produce working stock solutions (MilliporeSigma, Canada; Figures and tables). These stocks were further diluted into OECD water for treatment exposures. Treatment groups consisted of individual UV filters, or a 1:1:1 mixture of AVO, OXY and OCT. Mixture concentrations are labelled throughout as the concentration of any individual UV filter component (e.g., 200 µg/L each of AVO, OCT and OXY is listed as 200 µg/L mixture). Two controls were run, the first as OECD water without additions, the second as 1.0 µL/mL (0.1% v/v) DMSO, an equivalent concentration to the solvent used in all treatments as a vehicle control. Prior to exposures, all glassware was submerged in 10% EtOH for > 12 hours then rinsed with distilled water.

2.2.3 Analytical methods

The stock solutions previously described in section 2.2.2 were analyzed via ultra-performance liquid chromatography – quadrupole time-of-flight mass spectrometry (Xevo G2-S, Waters) and the mass range of 200-300 (m/z). The electrospray ionization source was operated in positive ion mode. Chromatographic separation was achieved using an Acquity UPLC BEH C18, 50x2.1 mm column, at 40°C with an injection volume of 10 µL. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), and the flow rate was set at 0.4 mL/min. The elution gradient was at 0-5 min, solvent B was increased from 30% to 50%, 5-8 min solvent B was increased to 95% and held at 95% for 1.5 min. Data acquisition was controlled using MassLynx (Waters) and data extraction was performed using TargetLynx (Waters). The criteria for the limit of

detection was 3 times the signal to noise ratio. The detection limit of each compound was determined through a series of dilutions to determine the lowest concentration yielding a signal to noise ratio of 3.

2.2.4 EC50 and LC50

OECD guidelines were followed for a determination of acute 48-hour median effect concentrations (EC50), as well as lethal concentration (LC50) in < 24 h old neonates. The chosen effect for EC50 determination was immobilization, defined as an inability of a neonate to move after a gentle agitation over an observation period of 15 s (OECD, 2004). Daphnids were viewed under a dissecting microscope to differentiate mortality and immobilization as the cause of a lack of mobility by observing the presence of gill motion during respiration. Five neonates housed in 10 mL of each solution and fasted for the entire duration of the experiment were used for each concentration tested, replicated 6 times.

2.2.5 Acute exposures

21-day reproductive tests were performed on daphnids following OECD test 211 guidelines, with modifications (OECD, 2012). < 24 h old neonates were exposed for 48-hours in treatment solutions prior to being raised an additional 19 days in OECD water to an age of 21 days total. Treatment concentrations were 0.2, 2, 20, and 200 µg/L of AVO, OCT, OXY or the UVF mixture. 30 neonates were exposed to each UVF treatment and housed individually in 20 mL glass scintillation vials. In addition, 15 daphnids were each exposed to DMSO and OECD control solutions alongside each concentration of UVF exposure. All exposure solutions were refreshed every 2 days for the duration of the test and *Daphnia* were fed 100 µL of both green algae and YCT mix daily unless otherwise specified. Each daphnid was checked daily for mortality, molting and any release of broods. At the end of the 2-day acute exposure but prior to transferring neonates to vials of fresh OECD water, a phototaxis assay was performed, outlined in Delompré et al. (2019a). Briefly, neonates were

transferred to the centre of a 34 cm long, 3 cm diameter clear polycarbonate tube containing 175 mL of fresh OECD water, housed inside of a closed, opaque dark box. The outer 5 cm of one end of each tube protruded from the container above an illuminated lightbox (approximately 170 lumens) in an otherwise dark room, and the time elapsed for daphnids to move into the illuminated tube section was recorded. Daphnids were allowed a maximum of 5 minutes from the moment the tubes were illuminated to complete the assay, otherwise the trial was considered incomplete. This phototaxis assay was repeated on the same daphnids at the end of experimental day 21.

2.2.6 Chronic exposures

Chronic exposures were performed following the procedures described in section 2.2.5, with modifications. A first group consisted of UVF exposures at approximately 10% of the estimated EC50 concentration (AVO = 150 µg/L, OCT = 7.5 µg/L, OXY = 100 µg/L), followed by a second exposure group at concentrations approximately an order of magnitude lower (AVO = 20 µg/L, OCT = 0.5 µg/L, OXY = 2 µg/L). Both groups were exposed alongside OECD control solutions. Due to a lack of unique results in both of the previous experiments, mixture exposures were not included in the chronic exposures. Daphnids were fasted for the final 48 hours of exposure, prior to measuring metabolic rate at the end of the 21-day experimental period. Metabolic rate was measured by pairing 2 daphnids per 200 µL well filled with aerated OECD water in a 24-well glass optical fluorescence respirometry microplate (Loligo Systems, Denmark). The dissolved oxygen concentration ($mgO_2\ L^{-1}$) was logged over 1.5 hours, and the per daphnid respiration rate was calculated by dividing the slope (change in oxygen concentration over time, in hours) by the number of daphnids per well (2).

2.2.7 Statistics

All statistical analyses were performed using R version 3.6.2 (R Core Team, 2024). EC50 and LC50 determination was performed using the “ecotox” package. Tests of normality and

homoscedasticity (Shapiro-Wilk and Levene's tests, respectively) were performed on all data prior to testing via one-way ANOVA. Data that failed assumption tests and could not be successfully transformed were compared by Kruskal-Wallis test followed by post-hoc Dunn's test. Significance was determined at $\alpha = 0.05$. Values are reported as mean ± standard error of the mean.

2.3 Results

2.3.1 Exposure solution chemistry

A summary of nominal and measured concentrations of AVO, OCT and OXY stock solutions as well as the detection limits of each compound are included in Figures and tables. All measured UVF concentrations were consistent with their respective target nominal concentrations (AVO, OXY: 10 ± 0.1 g/L, OCT: 100 ± 0.5 g/L, DMSO control: all UVFs below detectable limits).

2.3.2 EC50 and LC50

The EC50 concentration was found to be far lower with OCT at 0.03 mg/L (95% CI 0.02-0.04) than with either OXY (EC50 = 1.2 mg/L; 95% CI 0.89-1.6) or AVO (EC50 = 1.2 mg/L; 95% CI 0.91-1.6; Figure 2.1 A-C).

All LC50's were determined to be within 1 order of magnitude in the low mg/L range, the lowest of which was the 1:1:1 UVF mixture at 0.99 mg/L (95% CI 0.82-1.2), followed by OXY (1.7 mg/L; 95% CI 0.74-5.0), OCT (3.6 mg/L; 95% CI 2.5-5.2), and AVO at 6.8 mg/L (95% CI 4.8-9.4; Figure 2.1 D-G). The calculated LC_{50} for the DMSO control was 2.3% v/v in OECD water (95% CI 1.5-4.2), and the no effect concentration was 1.0% v/v, well above the solvent concentration of 0.1% v/v used for all test exposures (Figure 2.1 H).

2.3.3 Acute developmental exposures

Overall, no behavioural impairment was observed after a 48-h exposure to UV filters (Kruskal-Wallis, $p > 0.07$); however, *post-hoc* analysis revealed that 200 µg/L of all treatments excluding OXY yielded an increased phototactic response latency, to a maximum of 87 ± 19 s in daphnids exposed to AVO, representing a 35% increase over OECD controls (Dunn's, $p < 0.01$; Figure 2.2 A). OCT demonstrated a log-linear relationship with dose, to a maximum impairment of 74 ± 15 s (Dunn's, $p < 0.01$). 200 µg/L UVF mixtures produced results similar to their component chemicals, increasing response time by 62 ± 19 s in comparison to the OECD control (Dunn's, $p > 0.10$). All differences in behavioural responses to light stimuli were absent following 19 days of recovery in OECD water (Kruskal-Wallis, $p > 0.10$; Figure 2.2 B).

Physiological effects of UVF exposure were not detected in any of the traditional endpoints measured with 21-day OECD 211 experiments. The number of neonates produced by reproductive daphnids did not vary from either OECD or DMSO controls by more than 10% in AVO and OXY treatments (Kruskal-Wallis, $p > 0.05$; Figure 2.3 A). *Post hoc* analysis revealed two statistically significant exceptions, as marginally more neonates were produced in 200 µg/L OCT exposures and fewer neonates in 20 µg/L mixture exposures (Kruskal-Wallis, $p < 0.01$). The first brood of neonates was released within ± 1 day for every treatment, a minimum of 11 days to a maximum of 13 days (Figure 2.3 B). Molting behaviour was also unaffected by every treatment, as each daphnid molted once every 2-3 days for an average of 8.9 ± 1.1 molts (OECD control) over the duration of the experiment (Figure 2.3 C). It should be noted that statistical significance was detected in AVO treatments, as 200 µg/L treatments molted 9.4 ± 1.4 times (Kruskal-Wallis, $p < 0.01$; Figure 2.3 C).

The mortality of acutely exposed daphnids was unaffected by any treatment group at concentrations ≤ 20 µg/L over 21 days ($p > 0.70$; Figure 2.4 A-C). No significant mortality was observed in any treatment at 200 µg/L for the duration of the 48-h exposure (Kruskal-Wallis, $p > 0.10$;

Figure 2.4 D). However, a sharp increase in mortality rate was observed in daphnids exposed to AVO, OCT and UVF mixtures from days 3-7, with the greatest decline observed on day 4, followed by a return to normal rates on day 8 (Kruskal-Wallis, $p < 0.05$). Daphnid populations stabilized with a survivorship proportion of 0.63 ± 0.03 for AVO, 0.13 ± 0.07 for OCT and 0.13 ± 0.13 for mixtures over the remainder of the observation period. Acute exposure to OXY at 200 µg/L did not adversely impact daphnid survivorship in comparison to both control groups at any point over the 21-day period ($p > 0.40$).

2.3.4 Chronic developmental exposures

Chronic exposures to AVO, OCT and OXY at approximately 10% of EC50 concentrations did not greatly impact the mortality of daphnids until day 5, at which point mortality reached approximately 50% for AVO (150 µg/L) and OCT (7.5 µg/L) exposed groups (Figure 2.5). 100% mortality was reached on day 7 in both cases. 100 µg/L OXY increased the mortality proportion to 0.33 on day 9, and all remaining daphnids were deceased on day 10. A second batch of chronic exposures to lower concentrations of UVFs did not affect mortality with respect to the OECD control group at any point over the 21-day exposure (AVO = 20 µg/L, OCT = 0.5 µg/L, OXY = 2 µg/L).

The time to produce the first brood was unaffected by all UVFs in the second, lower concentration batch of chronic exposures (Kruskal-Wallis, $p < 0.05$; Figure 2.6 A), as well as molting behaviour ($p > 0.20$; Figure 2.6 E). 20 µg/L AVO increased the average brood size per daphnid by nearly 1.5-fold over OECD controls to 13 ± 0.85 neonates/brood (Kruskal-Wallis, $p < 0.01$; Figure 2.6 B). This is similarly reflected in a 20% increase in the proportion of daphnids reproducing at any point over the 21-day exposure compared to controls (Figure 2.6 C). Overall, the average number of neonates produced per reproductive daphnid increased in AVO-exposed individuals by 40% (Kruskal-Wallis, $p < 0.01$; Figure 2.6 D). Metabolic rate increased by 25% (4.8 ± 0.49 $mgO_2L^{-1}h^{-1}$) in 0.5 µg/L OXY treated

daphnids and decreased by 25% in 20 µg/L AVO treatments; however, these differences were not statistically significant (Kruskal-Wallis, $p > 0.10$; Figure 2.6 F).

2.4 Discussion

These results represent the first investigation into the effects of UVF exposure on the behaviour and physiology of *Daphnia magna*, as well as the persisting effects over a 21-day period following both acute and chronic exposures to AVO and OCT. Short-term, developmental exposure impaired the phototactic response of neonates towards light stimuli, in addition to inducing an increased mortality rate up to 7 days post-exposure. These physiological effects are generally recoverable over a two-week period in surviving daphnids. Chronic developmental exposures prove to be more disruptive to *Daphnia* reproduction, particularly to environmentally relevant concentrations of AVO. OCT exposure was shown to be highly lethal over a 1-week period at environmentally relevant concentrations. Interestingly, OXY, the most studied UV filter, was shown to generally be the least toxic of the studied compounds due to effects occurring only at higher concentrations relative to AVO and OCT, indicating that the primary focus of research effort should be expanded to include other compounds such as AVO and OCT.

2.4.1 48-hour acute toxicity

Of the studied compounds, AVO and OXY were equivalently toxic, inducing 25% immobilization at concentrations < 1 mg/L, and complete immobilization at approximately 10 mg/L. OCT immobilized all daphnids at 0.2 mg/L, indicating a higher level of toxicity to developing *Daphnia* than AVO and OXY (Figure 2.1 A-C). The determined OCT EC50 is two orders of magnitude less than that determined by (Park et al., 2017), at 3.2 mg/L; however, this is the only known literature value for this compound at the time of writing. The difference in observed toxicity could be a result of water chemistry differences (City of Edmonton, Canada derived OECD water vs Saarbrücken, Germany

filtered recirculating tap water), or differential toxicant sensitivity across separate daphnid cultures (Reproductive adults sourced from Aquatic Research Organisms, US vs ephippia sourced from MicroBioTest Inc, Belgium). The AVO EC50 is comparable to the value presented in this study, at 2.0 mg/L, as well as the variety of published values for OXY-exposed *Daphnia*, ranging from 1.2-2.2 mg/L (Jang et al., 2016; Molins-Delgado et al., 2016; Sieratowicz et al., 2011). The acute toxicity of OCT is noteworthy due to its proximity to previously measured environmental concentrations of approximately 7 µg/L in surface waters, as EC50 measurements are a more ecologically sensitive measure of toxicity, and immobility can lead to organism death over a longer period of time due to predation (Blewett et al., 2018; Bratkovics et al., 2015; Langford and Thomas, 2008; Schaap and Slijkerman, 2018).

Short-term exposure to any of the tested UVF solutions did not prove to be immediately lethal, as all LC50 doses were determined to be at concentrations above what has been measured in the environment. The calculated LC50 doses for AVO, OCT and OXY were 6.8, 3.6, and 1.7 mg/L respectively, and 0.99 mg/L for an equivalent ratio mixture of these compounds (Figure 2.1 D-G). These results are comparable to previously published research on OXY, ranging from 1.6-1.9 mg/L (Fent et al., 2010; Mikkelsen, 2015; Montes-Grajales et al., 2017). The 48-hour LC50 determined for AVO and OCT are the first to be reported in *D. magna*, and are similar to doses predicted by toxicity modelling (Brooke et al., 2008). It is important to note that due to the high lipophilicity of organic UVFs, the assessed LC50 doses are highly unlikely to occur under normal environmental conditions, requiring a solvent such as DMSO to maintain consistent high concentrations. The solubility of AVO and OCT in water are 0.027 and 0.040 mg/L respectively, and the maximum solubility for OXY is above the median lethal concentration, at 6 mg/L (Giraldo et al., 2017; Molins-Delgado et al., 2016; National Center for Biotechnology Information, 2020).

2.4.2 Physiological effects

Acute 48-hour UVF exposure did not alter *Daphnia* reproductive or molting behaviour over the post-exposure period (Figure 2.3). In a previous experiment by (Sieratowicz et al., 2011), the authors showed that chronic OXY exposures < 0.5 mg/L did not impact the reproductive capabilities of daphnids, suggesting that very high doses are required to observe a physiological response after a 48-hour exposure. Chronic exposure to 20 µg/L AVO increased the reproductive output of daphnids by 1.4-fold, driven by a higher proportion of reproducing adults and a larger average brood size (Figure 2.6 B-D). *Daphnia* have been shown to increase in reproductive rate when influenced by many environmental and chemical stressors, this phenomena may be in accordance with the "spawn or die" hypothesis (Blewett et al., 2017; Giraudo et al., 2019; Im et al., 2020a). Investing a greater proportion of resources into reproduction can ensure the continued survival of populations exposed to potentially lethal substances. Producing more neonates per brood and releasing a higher total number of neonates per period of time provides greater opportunity for phenotypic adaptation, particularly in species with short generation times (Chatterjee et al., 2019). Furthermore, the observed AVO-induced increase in reproductive output suggests that AVO is indeed an endocrine disrupting compound, displaying increases in total neonate production similar to daphnids exposed to 10 µg/L estradiol (Xu et al., 2019). Several studies have estimated the estrogenic potencies of benzophenone and other UVFs to be on a similar scale as known xenoestrogens (Witorsch and Thomas, 2010). It is important to note that the reproductive effects of UVF exposure in the present study may not directly translate to potential effects observed in a sexually reproducing species. *D. magna* are a parthenogenic species; therefore, differences in toxicity may arise due to the different processes governing the two methods of reproduction (Altshuler et al., 2011).

Alterations to metabolic rate by 25% were observed in chronically exposed daphnids to AVO (negative) and OXY (positive), approaching significance (Figure 2.6 F). A previous study by (Ahn et

al., 2019) suggested that AVO is a metabolically-disrupting obesogen, upregulating genes associated with lipid metabolism, cholesterol biosynthesis and metabolic processing of progesterone, in addition to down-regulating an equivalent number of genes involving epidermal differentiation and growth regulation. The authors suggest that metabolic disruption should be considered a major outcome of AVO toxicity, a conclusion that is consistent with the observed decrease in AVO-exposed metabolic rate. Metabolic disruption has also been noted after exposure to environmental levels of OXY; oxidative stress increased via a reduction of glutathione and an increase in catalase, consistent with the increased generation of free oxygen radicals that would be associated with the increased metabolic rate observed in chronic OXY treatment (Gao et al., 2013).

Understanding the metabolic pathways mediating biotransformation of UVFs is crucial to properly assess the environmental risk posed by contamination, as the mean time to removal as well as the toxicity of metabolic intermediates are important factors influencing overall toxicity. Phase I biotransformation of OXY yields 2,2-dihydroxy-4-methoxybenzophenone, a compound with greater estrogenic ability than OXY (Chen et al., 2017). Little information in this regard is available for OCT and particularly AVO; however, OCT has been shown to undergo carboxylesterase-mediated hydrolysis at a high rate (Saunders et al., 2019). Further research is required to more precisely identify the mechanisms causing the observed physiological responses to UVF exposure, as well as the methods of toxicant removal after uptake.

2.4.3 Impact on daphnid behaviour

Significant behavioural impairment was observed in daphnids subjected to 200 µg/L AVO or OCT (Figure 2.2 A). A similar extent of impairment was also observed in the mixture treatments. A previous study on *Daphnia* phototaxis theorized that the two leading causes of a decreased phototactic response are either an impairment of detection through decreased light sensitivity, or a lack of physical capability to respond to said cues (Delompré et al., 2019a). Only 20% of daphnids would be expected

to experience a loss of mobility at this concentration of AVO, therefore it is more likely that this substance reduced the ability of *Daphnia* to detect the stimuli in a timely manner. Due to the complete immobilization observed in 200 µg/L OCT treatments during EC50 determinations (Figure 2.1 B), it is probable that a lack of mobility was the cause of the observed behavioural impairment. However, approximately 25% of daphnids in this treatment group proved capable of mobility by completing the phototactic assay within the allotted 5 minutes, therefore we speculate that partial, immediate recovery of mobility is possible upon removal of UVF contaminants, as the assay was performed in OECD water. Indeed, rapid behavioural recovery has been noted post-exposure for a variety of contaminants, several of which occurred on the scale of minutes to hours (Jüttner et al., 2010; McWilliam and Baird, 2002). Additionally, complete long-term recovery of behavioural functions has been demonstrated in all tested compounds and concentrations in the present study over a 19-day post-exposure period (Figure 2.2 B). Continuous exposure is possibly a requirement to maintain phototactic inhibition.

Maintaining the ability to properly detect and efficiently respond to light stimuli is of utmost importance to wild daphnids (Brausch et al., 2011). *Daphnia* rely on diel vertical migratory behaviours to navigate water columns throughout the day, a delicate balance of predator avoidance and resource gathering, driven by environmental cues such as light stimuli (Glaholt et al., 2016). Any inability to detect, interpret or respond to these cues increases the risk of an individual being left exposed to predators, and subsequently eliminated from the population (Brausch et al., 2011). Extrapolated to the population level, the observed capability of AVO and OCT to disrupt phototactic behaviour through immobilization and other means poses a threat to populations exposed to UVF contaminants, especially OCT due to the low observed EC50 immobilization concentration. Any impairments that lead to untimely death through either behavioural perturbation or other means are the most toxic of all outcomes.

2.4.4 Impact on survivorship

None of the tested UVF treatments increased mortality in *Daphnia* neonates during a 48 h developmental exposure at environmental concentrations (Figure 2.4). However, long-term effects are clearly present post-exposure to 200 µg/L AVO, OCT and UVF mixtures, as daphnid survivorship was reduced to 0.63 ± 0.03 in AVO exposures, and 0.13 ± 0.07 in OCT exposures over the following 7 days, with similar results observed with mixture exposures (Figure 2.4 D). Chronic exposure to high doses of AVO and OXY resulted in complete extermination of daphnid populations in a similar 7-day time period (Figure 2.5) Perhaps most concerning of all, 7 µg/L OCT yielded identical mortality results at concentrations that have been previously observed in surface waters (Schaap and Slijkerman, 2018). The decreased survivorship of UVF-exposed daphnids resulted in a proportional decrease in the cumulative number of neonates produced by each treatment group, including 30% and 80% reductions respectively in acutely exposed AVO and OCT groups (data not shown). Despite surviving *Daphnia* demonstrating recovery following acute developmental exposures, toxicity to populations is evident, and large declines in number are possible in environmental conditions, which can potentially lead to trophic cascades of both predator and prey species of *Daphnia magna*, disrupting the ecosystem as a whole (C. Li et al., 2019; Miner et al., 2012).

The lack of observable physiological changes in OXY, and particularly OCT treatments in consideration with the overt chronic lethality at 7 µg/L, suggest that the endpoints traditionally observed over a 21-day OECD 211 reproduction test may not be an ideal toxicological assay for this particular class of compounds. Additional emphasis should be placed on biochemical assays focusing on oxidative stress and metabolic regulatory pathways, as these have been shown to be directly disrupted by UVF exposure (Ahn et al., 2019; Cocci et al., 2020; Rodríguez-Fuentes et al., 2015). Ecologically relevant behaviours should also be a prime candidate for experimentation, as our study demonstrates that acute AVO and OCT exposure results in decreased phototactic response.

2.4.5 Environmental implications

These results are promising in the context of environmental remediation. OCT and OXY half-lives are estimated to be on the scale of several days in surface waters up to several months, dependent on environmental parameters (Liu et al., 2013; Rodil et al., 2009; Semones et al., 2017; Vione et al., 2013). A reduction of contaminant inputs into the aquatic environment can ensure that UVF concentrations decrease below levels of concern without the need for active removal from the waters. This can be achieved through improved WWTP techniques, as current technologies suffer incomplete removal efficiency of many organic UVFs, and as such are a primary source of these compounds in the environment (Butkovskyi et al., 2016; Ma et al., 2019; O'Malley et al., 2019). The present results suggest that behavioural disruption of *Daphnia* migratory behaviours is temporary and can be recovered over several weeks. Compounds such as AVO that alter reproductive patterns under chronic conditions do not exhibit similar effects in acutely exposed neonates. While it is possible that complete recovery can occur within the same generation post-exposure, further testing is required to ensure that lingering effects do not occur in post-exposure generations, as UVFs are known bioaccumulators (Cocci et al., 2020; Gago-Ferrero et al., 2013a; He et al., 2019b).

The vast majority of UVF-containing plastics and commercially available sunscreens utilize a combination of UVF active ingredients in order to offer a more broad protection spectrum (Hanson et al., 2020; Muncke, 2011), therefore mixtures of several unique UV filters offer a more environmentally representative perspective of aquatic exposures. The data in the present study suggest that a 1:1:1 mixture of AVO, OCT and OXY does not alter the level of toxicity to daphnid neonates to any meaningful degree with respect to its individual components, as the mixture effects for each observed endpoint were highly comparable to the most toxic UVF (E.g., Figure 2.1 D-G, Figure 2.2 A, Figure 2.4 D). This is consistent with previous studies of various UVF mixtures in aquatic organisms and invertebrates, indicating that direct interactive effects between multiple UVFs are generally minimal,

and mixture toxicity is dictated by the concentration of the most toxic component (González and Martinez-Guitarte, 2018; Mao et al., 2018). It should be noted that UV stabilizers are another core component of commercial products due to their ability to promote UVF stability, therefore future research should consider these components in studies of complex mixture effects (Peng et al., 2020).

2.5 Conclusions

By far the most well-characterized compound present in this study, OXY proved to be the least toxic of the studied UV-filters, increasing metabolic rate in chronic 2 µg/L exposures. AVO also induced metabolic disruption, as well as significantly increased reproductive output of *Daphnia magna*. Phototactic ability was severely reduced in 48-hour 200 µg/L acute exposures to AVO and OCT, due to either reduced ability to detect light stimuli or lacking physical capability to efficiently respond. Perhaps the least studied of the tested compounds, OCT appeared to be the most toxic, causing a high degree of immobilization near environmental concentrations, in addition to proving highly lethal chronically at 7.5 µg/L. An overall absence of detectable responses in 21-day physiological assays following acute developmental exposures to high doses of UV-filters suggests that additional experimentation is required, as delayed mortality was observed post-exposure in all UVF treatments except OXY. Overall, the results suggest that UV-filters are capable of biologically and ecologically relevant disruptions to *Daphnia magna* at environmentally realistic concentrations.

2.6 Figures and tables

Table 2.1. Summary of stock solution parameters measured by ultra-performance liquid chromatography.

Stock	Nominal concentration (g/L)	Measured concentration (g/L)	Detection limits (µg/L)
AVO	10	10.00 ± 0.1	50
OCT	100	100.0 ± 0.5	100
OXY	10	10.00 ± 0.1	1.0
DMSO control	0	AVO: BDL OCT: BDL OXY: BDL	

BDL: below detection limit

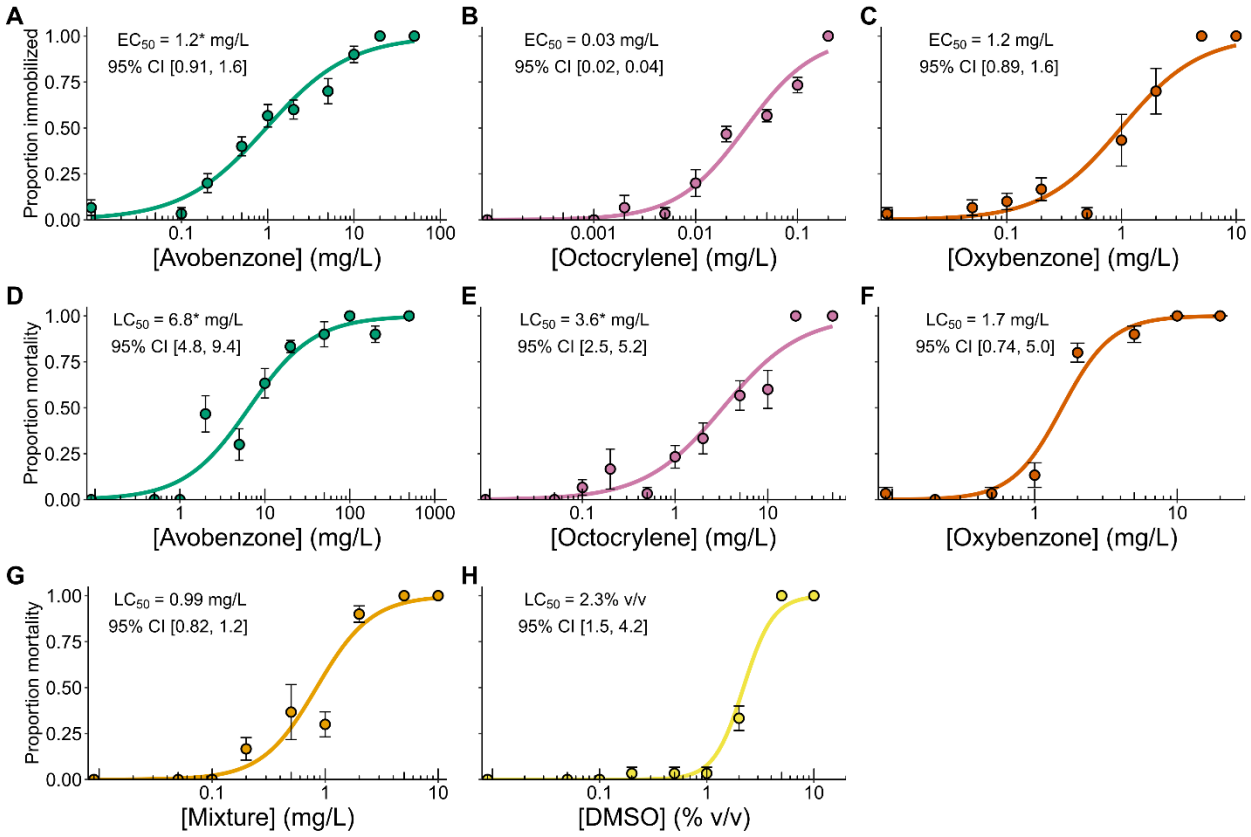

Figure 2.1. Median effect concentrations causing immobilization (EC50) (A-C) and median lethal concentrations (LC50) (D-G) of < 24-hour old *Daphnia magna* neonates exposed to individual UV filters and a 1:1:1 mixture for 48 hours. The LC50 of the vehicle control, dimethyl sulfoxide (DMSO) was determined to select the solvent concentration used for all UVF exposures (H). Each data point represents 6 replicates of n = 5 daphnids, bars represent ± SEM. * Indicates values that are above the solubility limit in water.

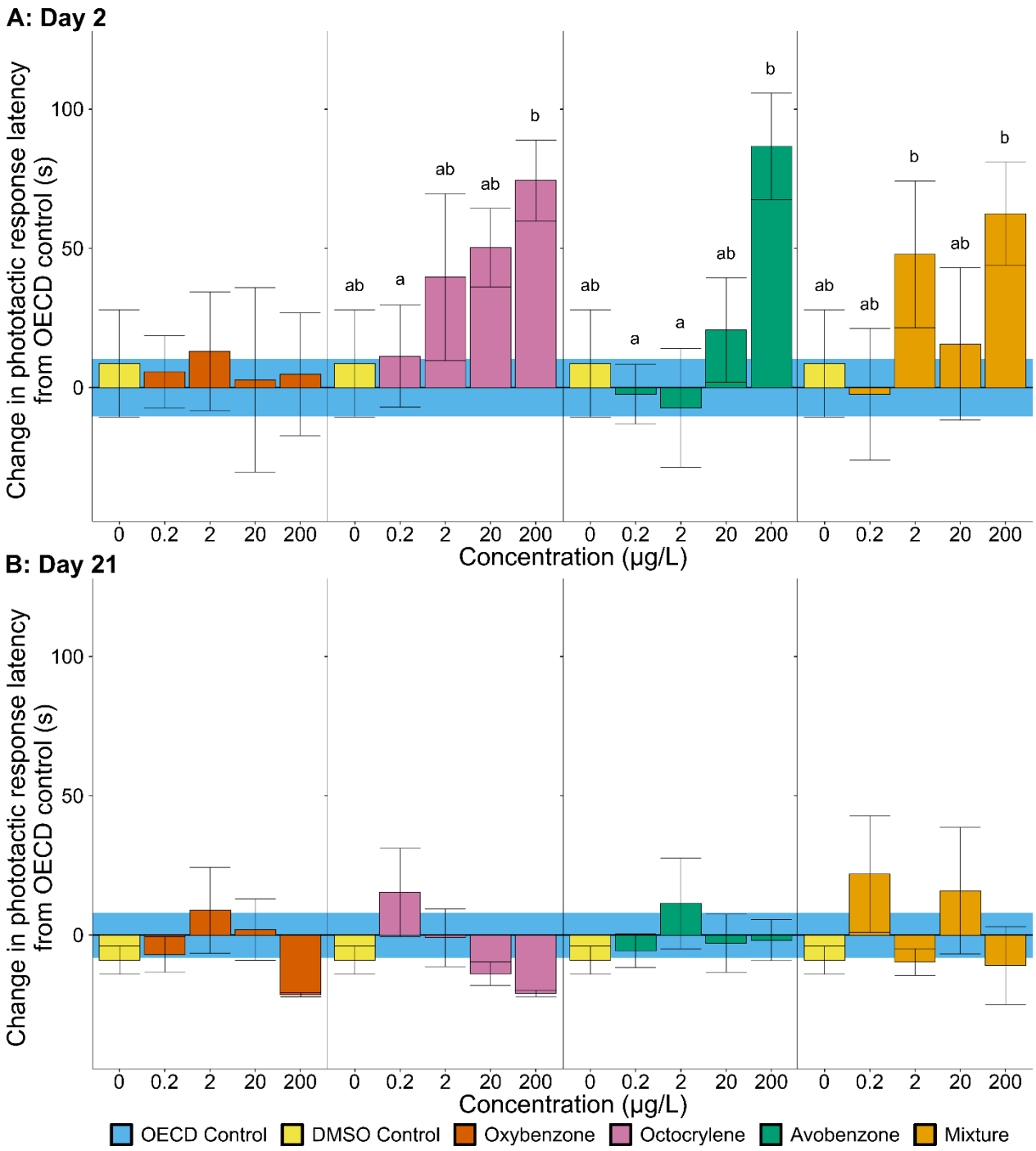

Figure 2.2. The mean change in phototactic response time in comparison to OECD water controls of < 24-hour old *Daphnia magna* neonates after a 48-hour exposure (A) and 19 days post exposure (B) to individual UV filters and a 1:1:1 mixture. The trial was considered as incomplete if daphnids did not finish in ≤ 5 minutes. Each treatment group was comprised of 30 individuals. Bars represent mean ± SEM. Different letters on the bars indicate a significant difference between concentrations within treatments, bars without letters are not considered to be significantly different (Kruskal-Wallis, $p < 0.05$).

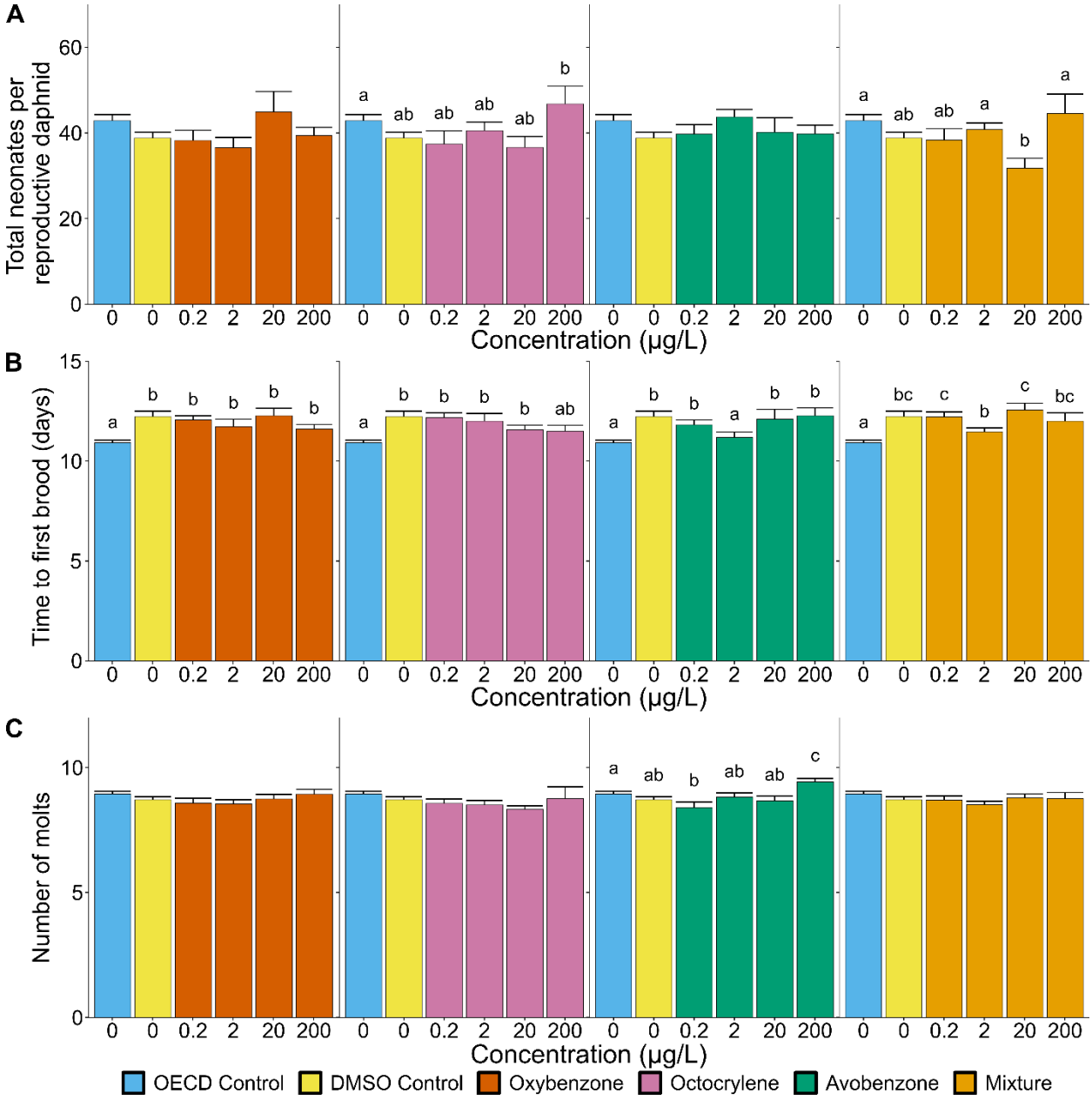

Figure 2.3. The number of neonates produced over 21 days per reproductive daphnid (A), the mean time to the release of the first brood (B) and the number of molts over 21 days (C) of < 24-hour old *Daphnia magna* neonates exposed to individual UV filters and a 1:1:1 mixture for 48 hours. Each treatment group was comprised of 30 individuals. Bars represent mean ± SEM. Different letters on the bars indicate a significant difference between concentrations within treatments, bars without letters are not considered to be significantly different (Kruskal-Wallis, $p < 0.05$).

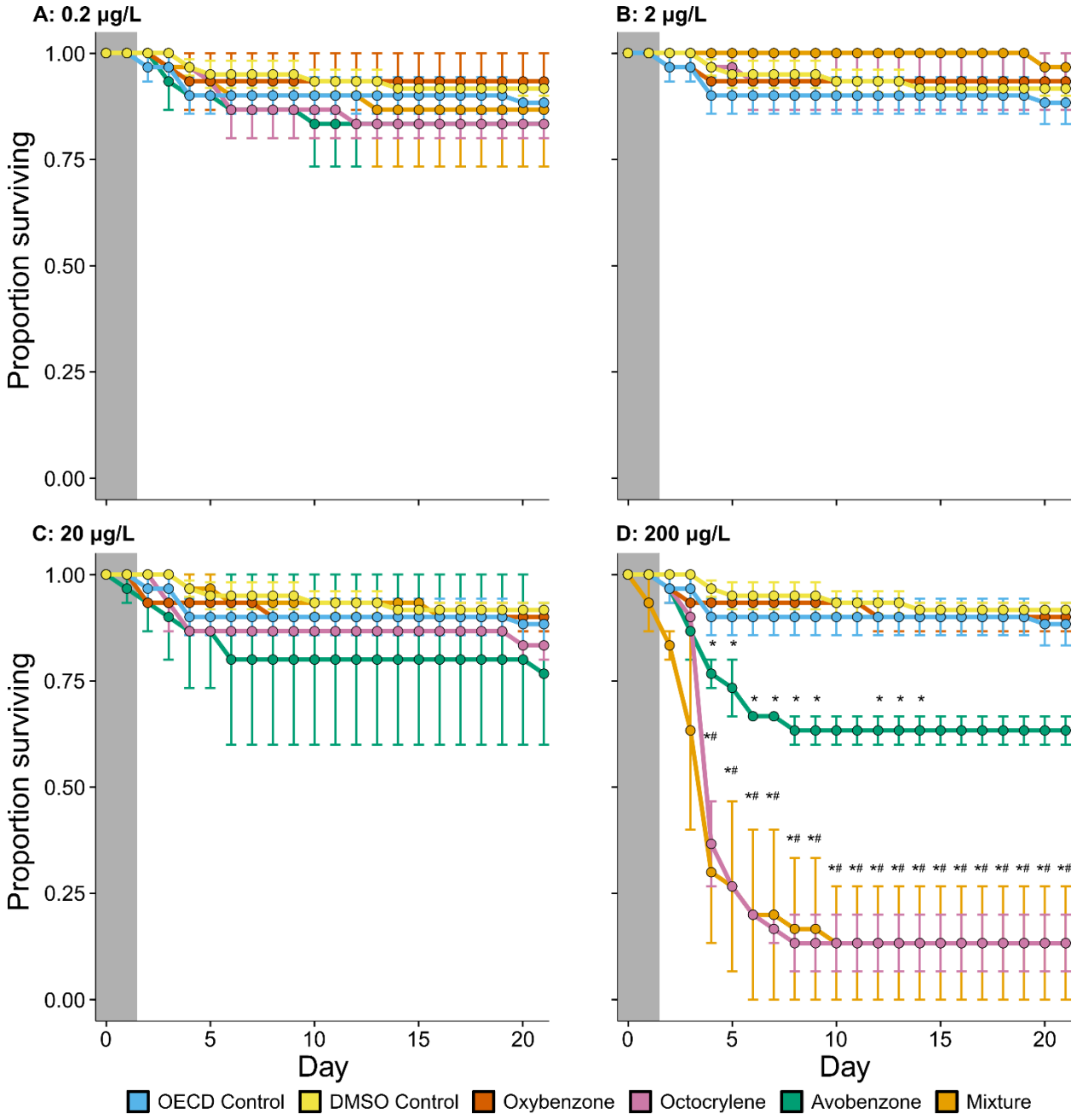

Figure 2.4. Survivorship curves of < 24-hour old *Daphnia magna* neonates exposed to individual UV filters and a 1:1:1 mixture for 48-hours at concentrations of 0.2 µg/L (A), 2 µg/L (B), 20 µg/L (C) or 200 µg/L (D). Grey regions represent exposure time. Daphnids were raised to 21 days in clean OECD water post-exposure. Each treatment group was comprised of 30 total individuals exposed in two batches of 15 individuals each. Points represent batch mean ± SEM. Groups differing from the DMSO control are indicated with *, groups differing from the OECD control are indicated with # (Kruskal-Wallis, $p < 0.05$).

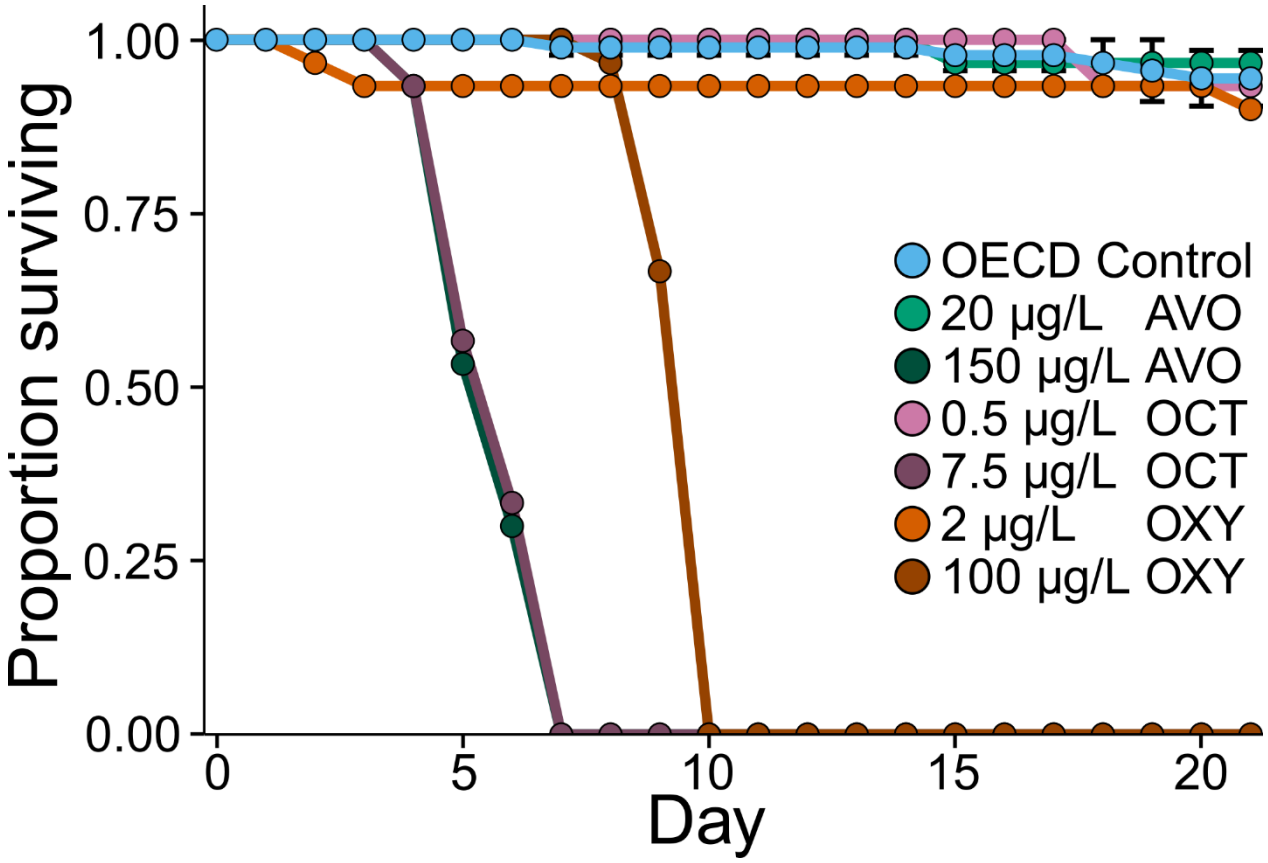

Figure 2.5. Survivorship curves of < 24-hour old *Daphnia magna* neonates exposed to individual UV filters or an OECD water control for 21 days. Each UV filter treatment group was comprised of 30 total daphnids exposed individually. OECD data are the means of 3 replicates of 30 daphnids. Bars represent mean ± SEM.

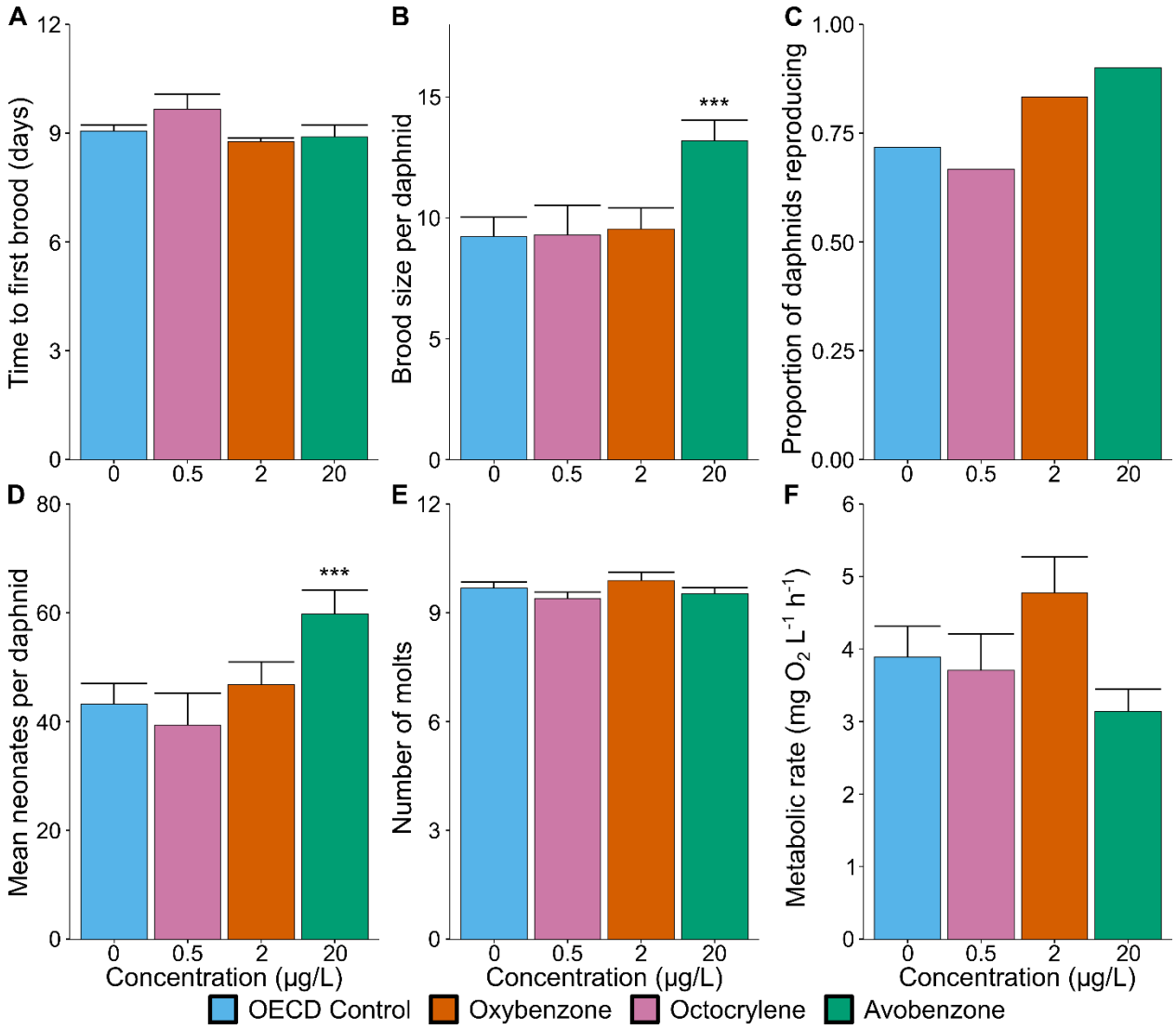

Figure 2.6. < 24-hour old *Daphnia magna* neonates were exposed to OECD water, or UV filter treatments for 21 days. Individual daphnids were scored daily for the time to first brood (A), average brood size per daphnid (B), the proportion of reproductive adults (C), the number of neonates released per individual (D) and the number of molts (E). Metabolic rate was measured at the end of the 21st exposure day (F). Stars indicate significance with respect to OECD control via Kruskal-Wallis test ($p < .05$).

Chapter 3: Can short-term data accurately model long-term environmental exposures? Investigating the multigenerational adaptation potential of *Daphnia magna* to environmental concentrations of organic ultraviolet filters

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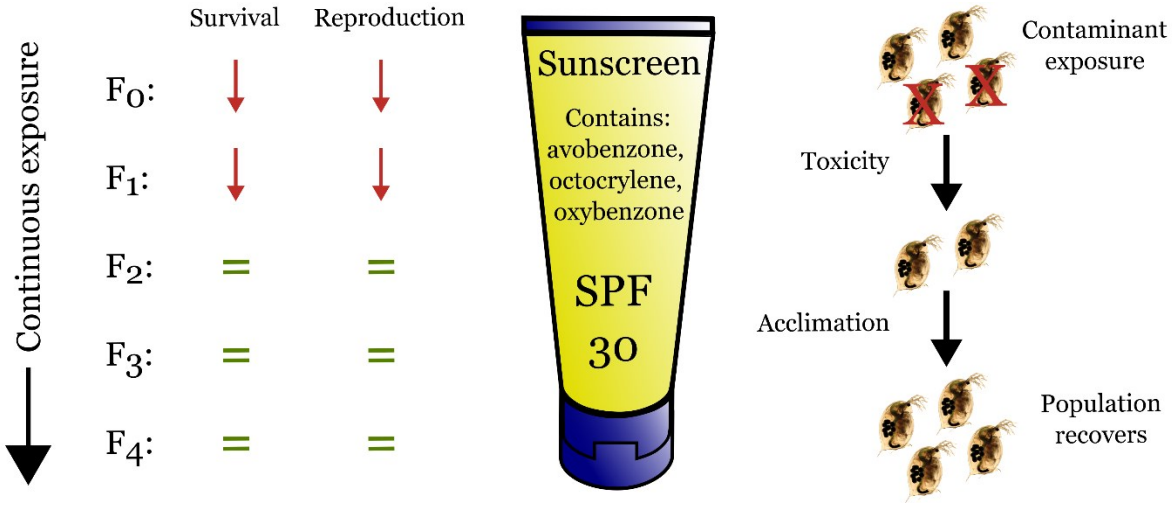


3.1 Introduction

Aquatic ecosystems across the globe are facing several challenges due to contaminants of concern, many of which are released directly into the environment through human activities. One class of these contaminants of concern are organic ultraviolet filters (UVFs) such as avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY), which are used to protect against harmful ultraviolet radiation (Berardesca et al., 2019). UVFs are present in a wide variety of pharmaceutical and personal care products including sunscreens, lip balm and lotions (Butkovskyi et al., 2016; Manová et al., 2013), in addition to their role as additives in outdoor-exposed products such as plastics, inks and product coatings (Cadena-Aizaga et al., 2020; Gago-Ferrero et al., 2013b; Muncke, 2011). UVFs from these sources will collect into wastewaters and ultimately enter into the environment through their incomplete removal in wastewater treatment plants (Emnet et al., 2015; Kasprzyk-Hordern et al., 2009; Wick et al., 2010). They can also directly enter the environment by leaching off of the skin when sunscreens or other UVF-containing products are used prior to entering the water for recreational purposes (Giokas et al., 2007; Labille et al., 2020; Peng et al., 2017b; Pintado-Herrera et al., 2017). Through these sources, contamination levels in natural waters are commonly reported in the range of 0.1 – 10 μg/L in many freshwater and marine environments (Bargar et al., 2015; Langford and Thomas, 2008; O'Malley et al., 2021; Sánchez Rodríguez et al., 2015; Tsui et al., 2014), although higher levels of 43 – 1,395 μg/L have been reported in some cases (Downs et al., 2016; Kasprzyk-Hordern et al., 2009; Vila et al., 2017).

These UVFs are believed to be endocrine disrupting compounds, and have been observed to bioaccumulate (Horricks et al., 2019; Mitchelmore et al., 2019), causing sublethal effects that include behavioural impairment (Boyd et al., 2021; Tao et al., 2020), chlorophyll bleaching (Danovaro et al., 2008; Downs et al., 2016), and reproductive impairments (Coronado et al., 2008; Yan et al., 2020). Previous research has indicated that UVF toxicity is of greatest concern for low trophic level organisms

such as invertebrates. Mortality has been reported at 24 h concentrations of 0.62 – 8 µg/L of OXY for a variety of coral species (Downs et al., 2016), and growth inhibition of sea urchins (*Paracentrotus lividus*) has been reported after 48 h exposure to 40 µg/L of OCT (Giraldo et al., 2017). In freshwater species such as *Daphnia magna*, complete mortality has been observed over 7-10 days of exposure to 150 µg/L AVO, 100 µg/L OXY and 7.5 µg/L OCT (Boyd et al., 2021). The range of concentrations found to cause impairment to aquatic biota overlaps with environmental concentrations, resulting in the implementation of UVF bans in countries including Aruba (Homan and Martinus, 2021), Palau (Republic of Palau, 2018), and several regions of the United States (Hawaii State Legislature, 2018; USVI Legislature, 2019).

Research interest has increased in recent years in an effort to better understand the risk UVF contamination poses to aquatic environments; however, the majority of studies have been limited to short term (< 21 d) exposures over single generations, leaving much unknown regarding the long-term ecotoxicological effects of contamination in the environment. Due to their widespread use, UVF contamination has been measured in the environment year-round (Astel et al., 2020; O'Malley et al., 2020; Tsui et al., 2015), suggesting that a long-term research approach is appropriate to better understand the threat posed. *Daphnia magna* are an ideal model species to help address this knowledge gap, as their rapid development allow for multigenerational studies to be conducted (Ebert, 2005). In addition, they are a key species in the food web of freshwater environments, serving as a common prey species for higher trophic levels (Hartnett, 2019), and have previously reported sensitivity to UVFs (Boyd et al., 2021). The present study sought to characterize the multigenerational effects of continuous exposure of *D. magna* to AVO, OCT and OXY at concentrations representative of a high-contamination scenario, and is the first to study > 2 generations of exposure in any species. This data will provide insight regarding the potential changes in toxicity as *Daphnia* populations may become more tolerant to UVF contaminants through adaptive phenotypic plasticity processes or selective

pressures against the most sensitive individuals of the population. Alternatively, daphnids may become more sensitive to contamination as toxicity induced damage accumulates across generations. Regardless of outcome, this data will be crucial to better understand the effects of long-term contamination in the environment, and to help determine if the alarming results of short-term studies carry forward to environmentally relevant concentrations in longer duration exposures.

3.2 Materials and methods

3.2.1 Daphnia culture maintenance

Adult *Daphnia magna* individuals were obtained from Aquatic Research Organisms (US, November 2021) to generate a culture housed in the Department of Biological Sciences at the University of Alberta. The culture consisted of < 2 month old cohorts of 30 daphnids each, housed in 1 L of dechlorinated City of Edmonton water (pH ≈ 7.6) prepared under Organization for Economic Co-operation and Development (OECD) guidelines (294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$, 5.80 mg/L KCl; OECD, 2008), referred to as OECD water in the following text. Complete water changes were performed every 2-3 days. For the complete water chemistry of the dechlorinated water, refer to Delompré et al. (2019b). Each cohort was fed a daily ration of 3 mL of yeast, cereal leaf and trout chow (YCT) mix, and 3 mL of 3 x 10^7 cells/mL freshwater green algae (*Raphidocelis subcapitata*) purchased from Aquatic Research Organisms and supplemented once per week with 100 µL of Roti-Rich invertebrate food (VWR, Canada). All *Daphnia* involved in this study were maintained at 21.4 ± 0.5 °C on a 14 h:10 h photoperiod.

3.2.2 Exposure solutions

All UVFs were dissolved in dimethyl sulfoxide (DMSO) to produce concentrated stock solutions for each of AVO, OCT and OXY (Sigma-Aldrich, US). These stocks were diluted to the desired UVF exposure concentrations in OECD water and dosed to contain 0.003% v/v DMSO. All

studies included two control groups: OECD water without additions, and a solvent control group of 0.003% v/v DMSO. For the multigenerational study, all stocks and exposure solutions were remade every 12 days on average to minimize UVF degradation (O'Malley et al., 2021) and were maintained in darkness prior to use for *D. magna* exposures. All glassware was cleaned before the start of experimentation by submersion in 5% HNO_3 for 24 h, rinsed with distilled water, then submerged in 10% EtOH for 24 h followed by a final rinse with distilled water. Between uses during the study, glassware was submerged overnight in 10% EtOH and rinsed with distilled water.

Water samples of the exposure solutions for the multigenerational study were taken from different batches of solutions and collected at the time of the first water change for the batch to allow for solutions to stabilize following expected sorption losses to the glass container that typically occur over the first 2 h (O'Malley et al., 2021). Samples were analyzed using an ultra-performance liquid chromatography – triple quadrupole mass spectrometry (LC-QQQMS, 6495C, Agilent, US) operated in positive electrospray ionization and data were acquired in multiple reaction mode. The transitions for the compounds are available in Table S3.1. Chromatographic separation was achieved using a ZORBAX Eclispse Plus C18 (2.1 mm × 50 mm × 1.8 µm, Agilent), at 40 °C with an injection volume of 1 µL. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), and the flow rate was set at 0.4 mL/min. The elution gradient was at 0–5 min, solvent B was increased from 30% to 50%, 5–8 min solvent B was increased to 95% and held at 95% for 1.5 min. Quantification of the three sunscreen compounds was accomplished using external calibration curves. Mixtures of the three sunscreen compounds were prepared at 7 concentration levels (0.98, 1.95, 3.91, 15.63, 31.25, 125.00, 250.00 µg/L) and injected into LC-QQQMS with each batch of samples under the same parameters listed in Table S3.1. The three calibration curves exhibited excellent linearity ($R^2 > 0.999$) and superior stability of response (relative standard deviation < 2% for three injections). The limit of detection and quantification of the three compounds was determined

according to United States Environmental Protection Agency guidelines (United States Environmental Protection Agency, 2016c).

3.2.3 Median lethal concentration study

A 21 d median lethal concentration (LC50) study was performed for each of the three UVFs following modified OECD test 202 and test 211 guidelines (OECD, 2012, 2004). Briefly, < 24 h old *Daphnia* neonates were exposed to UVF solutions of various concentrations (Table S3.2) for 21 d, with complete water changes occurring every 2 days. Each concentration contained 4 replicates of 5 daphnids in 20 mL of exposure solution. Each vial was fed 200 µL of YCT mix and green algae daily. Every 24 h, daphnids were scored under a dissecting microscope to determine mortality as defined by a lack of gill respiratory motion over a 15 s observation period. Daphnids in OECD water served as the control, and the average control mortality of 20% (acceptable under OECD test 211 guidelines (OECD, 2012)) was subtracted from each experimental group prior to lethal concentration estimations.

3.2.4 Multigenerational study

A series of 21 d exposures following OECD test 211 guidelines with modifications were performed on < 24 hour old *Daphnia* neonates collected from the culture (OECD, 2012). Neonates were individually exposed to one of five different treatments: OECD (water) controls, DMSO (solvent) controls, and UVFs (AVO, OXY, OCT) at approximately their 21 d LC20 concentration (Table 3.1). The 21 d LC20 concentration was chosen as 20% mortality has been established as an acceptable level in OECD guidelines during chronic exposures, allowing for the observation of sublethal effects (OECD, 2012). Each treatment contained 30 neonates individually exposed in 20 mL of the appropriate exposure solution in uncapped glass scintillation vials. Complete water changes were performed every 2 days. Each daphnid was fed 100 µL of YCT mix and green algae daily until day 19, after which they were fasted for 48 h in preparation for metabolic rate measurements conducted on the final day of

exposure for each generation (day 21). *Daphnia* were scored daily for mortality, molts, and reproductive effort, which was the sum of all reproduction categorized by living neonates, unhatched eggs released, and non-viable neonates that were deceased at the time of scoring. Neonates from parents producing their 3rd brood were collected on day 15 of each generation, the day at which the majority of daphnids released their 3rd brood, and pooled per treatment prior to randomly selecting 30 neonates to start the next generation. Each generation was exposed for a total of 21 d, with the following generation beginning on the 15th day of exposure of the previous generation. This process was repeated for a total of 5 generations of continuously exposed *Daphnia* (F0 – F4).

All daphnids surviving to exposure day 21 were randomly assigned to undergo either a phototaxis assay or metabolic rate determination, in a 1:1 ratio. For the phototaxis assay, daphnids were individually placed in the centre of clear polycarbonate tubes (34 cm length, 3 cm diameter) which contained approximately 175 mL of the appropriate exposure solution (Delompré et al., 2019a). These tubes were housed in darkness except for the final 5 cm on one end, which was illuminated from below with an approximately 170 lm lightbox. Responses were filmed top-down with a camera directly above the illuminated portion of the tubes. The assay was performed in a random order on assigned daphnids and videos were scored in a blind manner for the time taken for the daphnid to move into the illuminated region. A maximum of 5 minutes were allotted for each assay, after which trials were considered incomplete. Following the phototaxis assay, daphnids were gently blotted dry and weighed.

The metabolic rate of the remaining daphnids was measured with a 24-well glass optical fluorescence respirometry microplate (Loligo Systems, Denmark). All metabolic and behavioural assays were performed between 9 am and 4 pm. Individual *Daphnia* were randomly placed into each 500 µL well filled with the appropriate exposure solution with no headspace. A silicone gasket was placed on the microplate and weighted down to seal all wells, and the dissolved oxygen concentration was measured every 15 s over a 1 h period, followed by the weighing of each daphnid after

measurements were complete. The first 10 minutes of measurements were considered to be the acclimation period, and the overall metabolic rate was calculated by dividing the slope of the change in oxygen over the remaining 50 minutes of data by the mass of each daphnid using Formula 3.1 below. Estimated metabolic rates were corrected by subtracting the average change in oxygen concentration of control wells containing OECD water without daphnids from the same plate run.

Formula 3.1: $MR = \frac{\Delta O_{2_{exp}} - \Delta O_{2_{blank}}}{m}$

Where MR refers to the mass specific metabolic rate in mg O_2 L^{-1} h^{-1} mg^{-1}, $\Delta O_{2_{exp}}$ refers to the change in dissolved oxygen concentration of a well containing a daphnid in mg O_2 L^{-1} h^{-1}, $\Delta O_{2_{blank}}$ refers to the average change in dissolved oxygen concentration across all wells without daphnids on the same plate run in mg O_2 L^{-1} h^{-1} and m refers to the measured body mass of the daphnid in mg.

3.2.5 Statistics

R version 4.0.5 was used for all statistical analyses and figures (R Core Team, 2024). The intrinsic rate of population increase (r) was calculated via Lotka's equation (Lotka, 1913) by pooling all replicates within each treatment group and generation. Lethal concentration estimation was performed using the probit method from the "ecotox" package. Prior to testing, one-way ANOVA assumptions were assessed using Shapiro-Wilk and Levene's tests. Data that met all assumptions were tested via one-way ANOVA and Tukey tests, while data that did not meet the assumptions of normality and homoscedasticity and could not be transformed were tested via Kruskal-Wallis and *post hoc* Dunn's tests. Similar methods were used to determine if normality assumptions were met for comparisons of reproductive characteristics between individuals in the OXY F0 treatment group that did or did not survive the duration of the 21 d exposure, which were compared via Wilcoxon rank sum tests. All values are reported as mean ± standard error of the mean, and significance was determined at $\alpha = 0.05$.

3.3 Results and discussion

3.3.1 LC50 study and water chemistry

This study is the first to report long-term LC50s representative of chronic exposures in any organism for all three of the tested UVFs. Estimated 21 d LC50s were similar for AVO (42.0 µg/L; 95% CI 34.4 – 53.4) and OXY (53.5 µg/L; 95% CI 43.5-68.3), while OCT was approximately an order of magnitude more toxic with an LC50 of 5.35 µg/L (95% CI 4.64 – 6.17; Table 3.1). These values are considerably lower than the 48 h LC50s of 1,700 – 6,800 µg/L previously reported with this *D. magna* culture (Boyd et al., 2021); the increased toxicity is consistent with the longer exposure duration allowing for damage to the organism to accumulate over time (United States Environmental Protection Agency, 1992). This previous study also reported mortality data obtained from a 21 d chronic exposure that is consistent with the present study, as concentrations of 150 µg/L (AVO), 7.5 µg/L (OCT) and 100 µg/L (OXY) resulted in complete mortality within 10 days.

Exposure solutions for the multigenerational study were targeted at the estimated LC20 for each UVF; however, quantification of these solutions yielded concentrations 25-50% of the target nominal levels for AVO and OXY (Table 3.1). OCT was present in the exposure solutions at detectable but not quantifiable levels; therefore, it was considered to be present at the average of the limit of detection and the limit of quantification, a concentration of 0.6 µg/L. Several previous studies have reported lower than intended exposure concentrations of UVFs, often in a similar range of 10-50% of nominal concentrations (Blüthgen et al., 2012; Fel et al., 2019; Giraldo et al., 2017) due to adsorption of the lipophilic UVFs to particulate matter present in the water (Benedé et al., 2014) or the sides of glass containers (Cormier et al., 2019; O'Malley et al., 2021), and is likely to impact all studies using similar methods as the present manuscript.

3.3.2 Physiological responses to multigenerational exposures of UVFs

Mortality of both control treatments as well as AVO and OCT exposed daphnids remained below 20% in each of the 5 exposed generations (Figure 3.1). OXY exposures yielded high mortality in the F0 and F1 generations of 62% and 40% by day 21, respectively. Mortality remained consistent with all other treatments until day 17 of exposure in the F0 generation, after which a sharp decline in population occurred. This trend continued through to the F1 generation, where approximately 1/3 of OXY exposed daphnids experienced mortality on days 4-6, before the population stabilized for the remainder of the study. These results indicate an increase in toxicity beyond the expected 20% mortality at the targeted LC20 exposure concentration. This difference could be due to the differing densities of *Daphnia* between the LC50 and multigenerational studies, as the latter consisted of individual neonates exposed in the same volume of water (20 mL) as the 5 individuals in LC50 exposures, allowing for a proportionately greater quantity of UVF per organism to be present for potential uptake into the organism over the duration of the study.

Similar trends were observed in the reproductive characteristics of *Daphnia*, as the intrinsic rate of population increase was decreased by approximately 20% compared to control treatments (Table 3.2) due to early mortality and reduced neonate production in the F1 generation. By the F4 generation, the rate of increase of all treatments were similar and highly consistent with estimates from previous studies (Guilhermino et al., 2021; Yin et al., 2020), suggesting a recovery of OXY exposed daphnids across generations. OXY suppressed reproductive effort in the F0 (one-way ANOVA, $p < 0.001$) and F1 (one-way ANOVA, $p < 0.001$) generations (Figure 3.2 A). The average reproductive output of each OXY exposed daphnid decreased by approximately 30% (F0) and 40% (F1), followed by a minor overshoot of 20% greater reproductive effort in the F2 generation. No differences were apparent in AVO and OCT treatments in the F0 generation; however, small decreases of 15% and 23% respectively were noted in F1 daphnids. All treatments exhibited similarly high levels of reproduction by the F3 and

F4 generations, indicating a recovery of normal function despite the continued presence of UVFs. A previous study noted no effects on *Daphnia* reproduction after a 21 d exposure to 32 and 342 µg/L of OXY; however, the difference in results could be due to methodological differences, as this previous study utilized a different solvent at a higher concentration (0.05% EtOH) as well as a lower frequency in water changes and feeding (2x weekly; Sieratowicz et al., 2011), possibly reducing *Daphnia* metabolic rates and slowing the rate at which damage can occur (Meador et al., 1995). The diminished reproductive output of daphnids exposed to UVF contaminants indicates a burden on the organism requiring that energy is allocated away from reproductive processes in favour of survival by promoting xenobiotic defense mechanisms such as biotransformation, and cellular repair (Sokolova et al., 2012). In the context of these selected UVFs, resources may be diverted towards cytochrome P450 mediated demethylation and hydroxylation reactions in phase I and conjugation enzymes such as glucuronosyltransferases and sulfotransferases in phase II (Guesmi et al., 2020; Mao et al., 2020; Saunders et al., 2019); however, these were not quantified in the current study. A previous study noted an increase in catalase and glutathione-S-transferase activity in the F1 generation of *D. magna* exposed to 0.17 µg/L OXY, indicating that induction of antioxidant and biotransformation enzymes across generations are possible factors impacting daphnid responses to prolonged contamination (de Paula et al., 2022).

In addition to the impairments to overall reproductive output, the proportion of reproductive failure as measured through the presence of released but unhatched eggs and dead, non-viable neonates was increased in the F0 (Kruskal-Wallis, $p < 0.001$) and F1 (Kruskal-Wallis, $p = 0.012$) generations (Figure 3.2 B). This outcome primarily occurred in OXY exposed daphnids, as 13.3 ± 2.1% - 19.5 ± 5.5% of reproductive effort did not result in viable offspring, an approximately 4-fold increase in the failure rate in comparison to both controls. Overall, this had the effect of reducing the number of viable offspring by approximately 46% across the F0 (one-way ANOVA, $p < 0.001$) and F1 (one-way

ANOVA, $p < 0.001$) generations, a reduction of ~22 neonates per OXY exposed daphnid in comparison to the OECD and DMSO controls. The majority of this reduction can be attributed to the production of neonates that hatched from their eggs but did not survive to the day that they were released from their parent's brood chamber (Figure 3.2 C). It is possible that these non-viable neonates are indicative of a decrease in neonate quality due to maternal toxic stress (Dao et al., 2010), rendering neonates more sensitive to OXY exposure during their most vulnerable developmental stage (Enserink et al., 1990). Evidence for this is apparent in the mortality during OXY exposures, which is notable in that F0 mortality occurred after neonates were collected for the F1 generation (day 15), allowing for damage to the parent to accumulate prior to reaching lethal levels during this critical period in the study (Figure 3.1). This proposed weakening of the resulting neonates comprising the F1 generation could explain the onset of mortality 13 days earlier than the prior generation. Weakening of offspring through maternal toxic stress is further evident in the gradual increase in the proportion of reproductive effort resulting in the production of non-viable neonates over each progressive day in the F0 generation of OXY exposure (Figure 3.3 A). The overall quality of each successive brood was decreased in comparison to the previous until non-viable neonates surpassed the production of viable neonates as the dominant reproductive outcome by the start of the 4th brood (day 18). Minimal non-viable neonates were produced on days 19-21 of the exposure; however, reproduction overall greatly decreased on these days as well, contributing to the decrease in the average overall reproductive effort for OXY exposed daphnids in the F0 generation. The reverse trend can be observed in F1 OXY daphnids, as the overall proportion of non-viable neonates decreased over successive broods, reaching negligible levels by the 3rd brood (day 15). Previous studies have noted that OXY exposed *D. magna* individuals produced smaller offspring, indicative of a decrease in neonate quality (Im et al., 2022; Song et al., 2021). Additionally, it has been reported that adult *D. magna* OXY exposure can greatly inhibit the embryonic development of neonates (Im et al., 2022). Overall, these data suggest that there is a steady

increase in maternal stress over time in the F0 generation as a result of continuous OXY exposure, followed by gradual acclimation in the surviving F1 generation, which will be discussed in greater detail in section 3.3.3 below.

Minimal differences were observed in the time required to reach reproductive maturity as well as the number of broods released by each daphnid over the 21 d exposures in any treatment or generation (Table 3.2), providing further evidence that the reduction in overall reproduction is driven by changes to energy allocation within adult organisms over time rather than developmental delays shortening the period of reproduction during the study. This could indicate that the observed impairments are the result of an accumulation of damage to the organism through prolonged UVF exposure (Sokolova et al., 2012). If damage to daphnids were to occur early in the exposure, developmental delays would be expected, whereas damage accumulating later in the exposure often results in reproductive impairments instead (Ginjupalli and Baldwin, 2013). Adult *Daphnia* achieved similar wet masses at the end of each generation across all treatments (Figure 3.4 A), suggesting that the fraction of energy allocated to organism growth was similar regardless of UVF exposure, and that any increase in biotransformation and/or cellular repair energy usage perhaps came from the energy allocation for reproduction instead; however, this should be confirmed with future studies. Furthermore, minimal differences in the number of molts were observed, indicating that the rate of growth and frequency of reproduction were consistent across treatments (Table 3.2). Frequent molting occurs in *D. magna* as they develop to reproductive maturity as it is required for the organism to reach its adult body size, and required after maturity only when broods are released for reproduction (Dabrunz et al., 2011; Espie and Roff, 1995). Molting behaviours in *Daphnia* are largely controlled by the hormone 20-hydroxyecdysone (Baldwin et al., 1995) and its accompanying ecdysone receptor (Zhao, 2020). The UVFs utilized in this study are believed to be endocrine disrupting compounds that elicit disruptions primarily via estrogen and androgen receptor pathways (Klopčič and Dolenc, 2017;

Kunz and Fent, 2006b). Previous studies have noted increases in the expression of ecdysone receptor at 96 µg/L (Yang et al., 2021) and ultraspiracle protein at 166 µg/L OXY in *D. magna* (Lambert et al., 2021), indicating the potential for endocrine disruption of the ecdysone pathway; however, the latter study noted no effects of OXY on molting. Molting was similarly unaffected in a previous *D. magna* study utilizing concentrations of 20 µg/L AVO, 0.5 µg/L OCT and 2 µg/L OXY over 21 d, or following a 48 h exposure of 0.2 – 200 µg/L of each UVF (Boyd et al., 2021). No changes to the mass specific metabolic rate were detected across treatments for the duration of the study (Figure 3.4 B), and the measured metabolic rates in all treatments were similar to those reported in previous *D. magna* studies (Boyd et al., 2021; Lari et al., 2017). Considering that both mass and metabolic rate were similar across treatments, this indicates that the overall energy budget available to each daphnid was similar; therefore, differences in physiology and reproduction are a result of changes to energy allocation or metabolic efficiency rather than overall energy availability (Pörtner et al., 2005).

Finally, there were no apparent trends on the effects of UVF exposure on *Daphnia* phototactic responses (Figure S3.3). These results are consistent with those previously reported from a 48 hr exposure, as the concentrations used in this study are below those that were previously noted to cause impairment (Boyd et al., 2021). Additionally, the strongest effects were observed in 48 – 72 h old neonates, suggesting that any effects to *Daphnia* behaviour may be more prevalent earlier in development, consistent with the results of a previous study noting behavioural impairment of 7 d old *D. magna* individuals after 24 h exposure to 100 µg/L OXY (Yang et al., 2021). This data indicates that the risk of increased predation due to behavioural impairments is low in *Daphnia* populations exposed to environmental concentrations of UVFs (Brausch et al., 2011).

3.3.3 Implications

From the present study, it is clear that each successive generation of OXY exposed *Daphnia* was better acclimated than their parental generation as overall mortality decreased, and reproductive

characteristics demonstrated gradual improvement over time. This acclimation could arise as the result of selective pressures, as individual daphnids most sensitive to UVF contamination could have been removed from the population during the high mortality of the F0 and F1 generations, allowing for the surviving, less sensitive individuals to reproduce and carry the population forward. No differences in the quality of reproduction were observed between OXY exposed individuals that survived for the entirety of the F0 generation and those that did not (Wilcoxon rank sum, $p = 0.103$; Figure 3.3 B), indicating a lack of a dominant phenotype clearly favoured by selective pressures. Due to the procurement of laboratory *D. magna* strains from long lineages of clonal populations, the expected low genetic diversity suggests that minimal changes would occur across generations as a result of natural selection (Barata et al., 2002).

In the absence of selective pressures, it is likely that the observed gradual acclimation of *Daphnia* populations instead arose as a result of functions such as phenotypic plasticity, where epigenetic mechanisms alter the phenotype of an organism in an effort to better adapt to environmental changes (Chatterjee et al., 2019). A similar pattern of recovery was observed in a previous study following a single generation exposure to 111 µg/L OXY additive obtained from microplastic leachate, as the authors noted severely reduced reproduction in the exposed F0 generation of *D. magna* followed by incremental recovery in the unexposed F1 & F2 generations (Song et al., 2022). Interestingly, the authors noted that global DNA methylation was unchanged across the F0 – F2 generations, although they postulate that this may be due to a balance in hypo- and hypermethylation of different pathways. Although the observed recovery occurred after removal of OXY contamination, the results from Song et al. (2022) align with the present study, indicating the potential for rapid recovery of *D. magna* reproduction across generations despite chronic OXY exposure. Other studies have found that *Daphnia* can alter reproductive characteristics in order to optimize energy expenditure between ensuring offspring viability and maternal survivability in times of stress (Groeger et al., 1991; Koch et al., 2009;

Smith and Fretwell, 1974). Parents may also "predict" the environment of their offspring under the assumption that the environment is stable, modifying their offspring so that they are better acclimated to their environment, referred to as an "anticipatory parental effect" (Burgess and Marshall, 2014). Overall, these data demonstrate the possibility of gradual acclimation to OXY occurring over the first two generations of exposure. UVF concentrations were quantified from samples obtained across multiple batches of exposure solutions, which did not demonstrate high measured variability across samples, nor do the timing of new batches of exposure solutions correspond with changes in toxicity (Table 3.2); therefore, the observed acclimation of *Daphnia* populations across generations is not expected to be a result of corresponding differences in UVF exposure concentrations.

The characteristics of daphnids across all treatments were largely similar by the F2 generation, which is notable in that this is the first generation which would have been exposed to UVF contaminants as the F0 germline, representing the first daphnids to be continuously exposed through all stages of development (Jeremias et al., 2018). The F1 generation can be modified through anticipatory parental effects from the F0 generation (Burgess and Marshall, 2014). The F2 generation can be modified in the same manner from the F1 generation, and also can receive epigenetic modifications from the F0 generation as germline cells, allowing for a greater potential for acclimation to occur to a stressor as neonates benefit from the success of their parents and can be further modified (Walsh et al., 2014). It is also possible that the co-exposure of F2 germline cells during the F0 exposure may have offered a selective pressure favouring more resistance to UVFs, leading to the observed acclimation in later generations. Similarly, the co-exposure of the F1 embryos during the F0 generation would not offer a similar strength of selective pressure due to their more advanced developmental stage; therefore, leading to the increased toxicity in reproductive traits in the F1 generation (Poulsen et al., 2021). It is interesting to note that all treatments demonstrated a similar pattern of gradual improvements in reproductive output and metabolic rate over the first 2 – 3 generations. In this study, reproductive effort

increased by approximately 50% from the F0 – F4 generation in both control groups (Figure 3.2 A). Previous studies have noted similar increases to reproduction of 20 – 60% across successive generations of control groups (Jeremias et al., 2018; Maselli et al., 2017), suggesting the possibility of acclimation of *D. magna* individuals to the specific handling and methodology of an experiment over generations. It is also possible that these changes are indicative of recovery from initial suboptimal conditions in this study, potentially increasing the sensitivity of daphnids exposed to UVFs over early generations (Heugens et al., 2003). The timing and magnitude of changes observed in this study are highly consistent across all treatments, and the average reproductive effort of F0 control daphnids is similar to what has been reported in previous single generation studies utilizing identical methods (Boyd et al., 2022, 2021), and with differing methodology (Besseling et al., 2014; Rist et al., 2017). Additionally, the *Daphnia* culture used for this study is tested monthly with reference toxicant (copper) LC50s which consistently confirmed the overall health of the culture over the study period, suggesting that the observed changes are more likely the result of acclimation to experimental conditions rather than the result of stress from initial suboptimal conditions.

Minimal changes to *Daphnia* traits were noted over 5 exposure generations of OCT and AVO, with the exception of small decreases in reproductive output and quality in the F1 & F2 generations and a smaller adult body size in the F2 & F3 generations of AVO exposure. The concentrations of AVO and OCT utilized in this study were likely not high enough to induce the widespread toxic effects observed in OXY exposures; therefore, any adaptive pressures would be minimal in these populations. It is possible the later onset of these effects occurred due to the greater potential for bioaccumulation in F1 & F2 generations as a result of the longer exposure period through parental exposure (de Paula et al., 2022). This potentially greater accumulation of UVFs would allow for a proportionally greater toxic response; however, bioaccumulation was not quantified in the present study. Regardless, these data provide valuable insight into the long-term effects of UVF contamination to a key primary

consumer at environmental concentrations. The majority of reported OXY environmental concentrations are at or below the 16.5 µg/L exposure used in this study (Bargar et al., 2015; Downs et al., 2016; Kasprzyk-Hordern et al., 2009; Langford and Thomas, 2008; O'Malley et al., 2021; Sánchez Rodríguez et al., 2015; Tsui et al., 2014; Vila et al., 2017); therefore, these results are representative of a possible high contamination scenario, yet complete recovery of the population was observed despite continued exposure. It is assumed that populations in less contaminated environments would experience a smaller disruption to physiology over initial exposure generations, but they would acclimate and regain normal function over time, as observed in this study. Indeed, a two-generation exposure to 4.4 µg/L AVO, 4.4 µg/L OCT or 0.17 µg/L OXY produced a similar pattern of reduced reproduction in the F1 generation to all UVFs, yet minimal mortality occurred with OXY exposure, in accordance with the lower exposure concentration (de Paula et al., 2022). Although individual recovery did occur in later generations of the present study, there is still risk as the high mortality and supressed reproductive output would render *Daphnia* populations more susceptible to extirpation from other stressors (Robles and Ciudad, 2017). Future studies should consider variables that may further impact toxic outcomes in the environment such as co-contaminants and secondary stressors. A concurrent study was performed during the present study utilizing group exposures, aiming to shed light on the molecular level changes that occurred over each exposed generation (Boyd et al., 2025, in prep). This will provide additional insight regarding the potential mechanisms driving the acclimation of *Daphnia* to UVFs and allow for further assessment regarding the extent to which populations recovered during UVF exposure.

3.3.4 Conclusions

In summary, five generations of exposure of *D magna* to 6.59 ± 0.735 µg/L AVO or ~0.6 µg/L OCT proved to be minimally toxic, yielding only transient disruptions to reproductive characteristics and body size which were quickly recovered. Exposure to 16.5 ± 7.80 µg/L of OXY resulted in high

mortality, low reproductive output, and a high proportion of failed reproduction over the first two generations of exposure; however, populations demonstrated a remarkable capability to acclimate to the continued presence of contamination, perhaps as a result of adaptation through phenotypic plasticity. Overall, the results from this study indicate that evidence of toxicity obtained from short-term exposures may not accurately represent long-term outcomes in wild populations. For example, the single and trans-generational exposure results reveal potentially devastating effects on *Daphnia* populations exposed to OXY; however, these effects are entirely removed from the population within 3 generations of exposure at the tested concentrations. If a population is capable of surviving the initial exposure to UVFs, then it is possible that a full recovery through acclimation to the contamination can occur over several generations, allowing normal functions to be regained. As the concentration tested for each UVF is in the upper range of what has been measured in aquatic environments, these results can serve as a representation of potential long-term outcomes under a high contamination scenario. It is clear that additional complexity is required when modeling the effects of environmental contamination in an effort to ensure that any observed changes in organism function are representative of the wild populations that currently debated UVF bans are aiming to protect.

3.4 Figures and tables

Table 3.1. Estimated 21 d median lethal concentrations (LC50, LC20) and 95% confidence intervals (CI) of *Daphnia magna* exposed to one of three UVFs. LC50 and LC20 estimates are the means of 4 replicates of n = 5 daphnids, estimated using the probit method in the R "ecotox" package. Nominal and measured concentrations of UVF exposure solutions determined via ultra-performance liquid chromatography are indicated for the multigenerational experiment, as well as the limits of detection (LOD) and limits of quantification (LOQ). Measured concentrations are presented as the minimum – maximum measured concentration, and the means ± standard error. All values are expressed in µg/L.

	21-day exposure		Multigenerational exposure			
Treatment	LC50 (95% CI)	LC20 (95% CI)	Nominal concentration	Measured concentration Min - max (Mean ± SEM)	LOD	LOQ
AVO	42.0 (34.4, 53.4)	25.9 (17.6, 33.5)	25	6.07 - 7.11 (6.59 ± 0.735)	0.1	0.3
OCT	5.35 (4.64, 6.17)	3.47 (2.60, 4.18)	3	> 0.3 – < 0.9 (~ 0.6)	0.3	0.9
OXY	53.5 (43.5, 68.3)	30.1 (20.8, 39.7)	30	11.0 – 22.1 (16.5 ± 7.80)	0.1	0.3

Table 3.2. The number of days to release the first brood of offspring, the total number of broods produced, and the number of molts released of < 24-hour old *Daphnia magna* neonates exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids, which were pooled to estimate the intrinsic rate of population increase for each treatment group. Data are presented as mean ± SEM, different letters indicate a significant difference between treatments within generations, entries without letters are not considered to be significantly different at $p < 0.05$ (first brood & number of broods: Kruskal-Wallis, number of molts: one-way ANOVA).

		Generation				
	Treatment	F0	F1	F2	F3	F4
Days to first brood	OECD Control	9.07 ± 0.13	9.45 ± 0.20[a]	9.97 ± 0.11[a]	9.34 ± 0.09	9.17 ± 0.21
	DMSO Control	8.77 ± 0.92	9.30 ± 0.17[a]	9.89 ± 0.11[a]	9.97 ± 0.20	9.07 ± 0.07
	AVO	9.00 ± 0.12	9.72 ± 0.36[a]	10.2 ± 0.19[a]	10.0 ± 0.23	9.21 ± 0.12
	OCT	8.97 ± 0.20	9.59 ± 0.19[a]	9.97 ± 0.09[a]	9.97 ± 0.18	9.33 ± 0.27
	OXY	8.90 ± 0.13	10.7 ± 0.41[b]	9.48 ± 0.11[b]	9.93 ± 0.18	9.20 ± 0.10
Number of broods	OECD Control	4.75 ± 0.12	4.34 ± 0.12	4.20 ± 0.09	4.79 ± 0.08[a]	4.76 ± 0.13
	DMSO Control	4.88 ± 0.11	4.23 ± 0.09	4.15 ± 0.10	4.34 ± 0.10[b]	4.89 ± 0.06
	AVO	4.72 ± 0.08	4.14 ± 0.18	4.07 ± 0.10	4.59 ± 0.12[ab]	4.60 ± 0.19
	OCT	4.79 ± 0.11	3.96 ±0.21	4.17 ± 0.09	4.52 ± 0.09[b]	4.79 ± 0.08
	OXY	4.64 ± 0.20	4.11 ± 0.16	4.21 ± 0.08	4.52 ± 0.11[ab]	4.90 ± 0.06
Number of molts	OECD Control	9.75 ± 0.18	9.62 ± 0.14	9.43 ± 0.11[a]	9.79 ± 0.13	9.69 ± 0.11
	DMSO Control	9.56 ± 0.13	9.03 ± 0.17	9.63 ± 0.17[a]	9.59 ± 0.14	9.96 ± 0.11
	AVO	9.62 ± 0.14	9.25 ± 0.17	9.37 ± 0.15[a]	9.79 ± 0.12	9.67 ± 0.15
	OCT	9.89 ± 0.19	9.11 ± 0.15	8.55 ± 0.14[b]	9.38 ± 0.12	9.79 ± 0.09
	OXY	9.64 ± 0.28	9.44 ± 0.22	8.28 ± 0.16[b]	9.48 ± 0.14	9.97 ± 0.09
Intrinsic rate of population increase (d^{-1})	OECD Control	0.311	0.285	0.286	0.335	0.354
	DMSO Control	0.322	0.313	0.287	0.311	0.357
	AVO	0.313	0.271	0.270	0.307	0.347
	OCT	0.308	0.258	0.274	0.309	0.341
	OXY	0.294	0.237	0.330	0.311	0.351

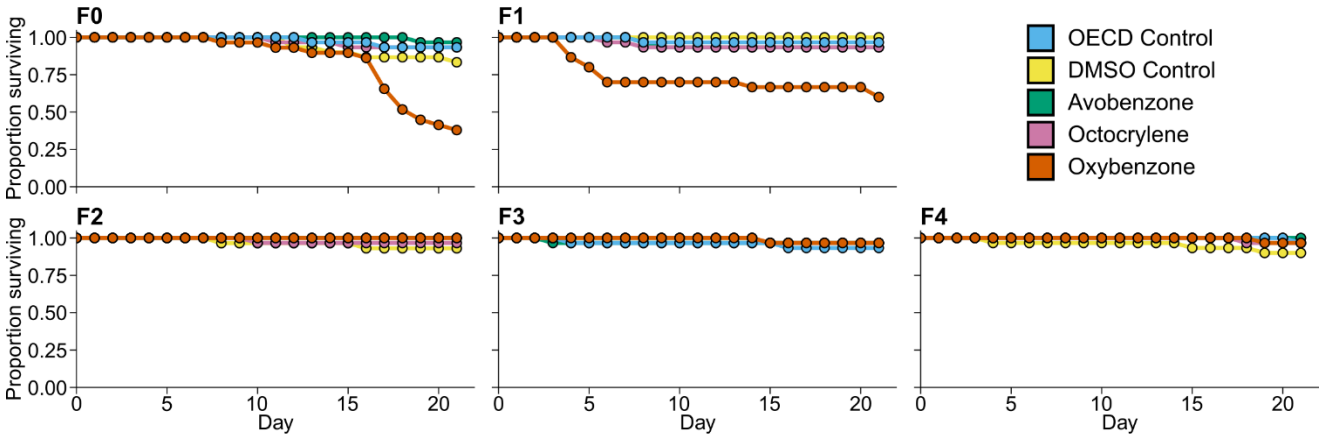


Figure 3.1. Survivorship curves of < 24-hour old *Daphnia magna* neonates exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids. Neonates from each treatment were collected on the 15th exposure day to begin the next generation of exposures.

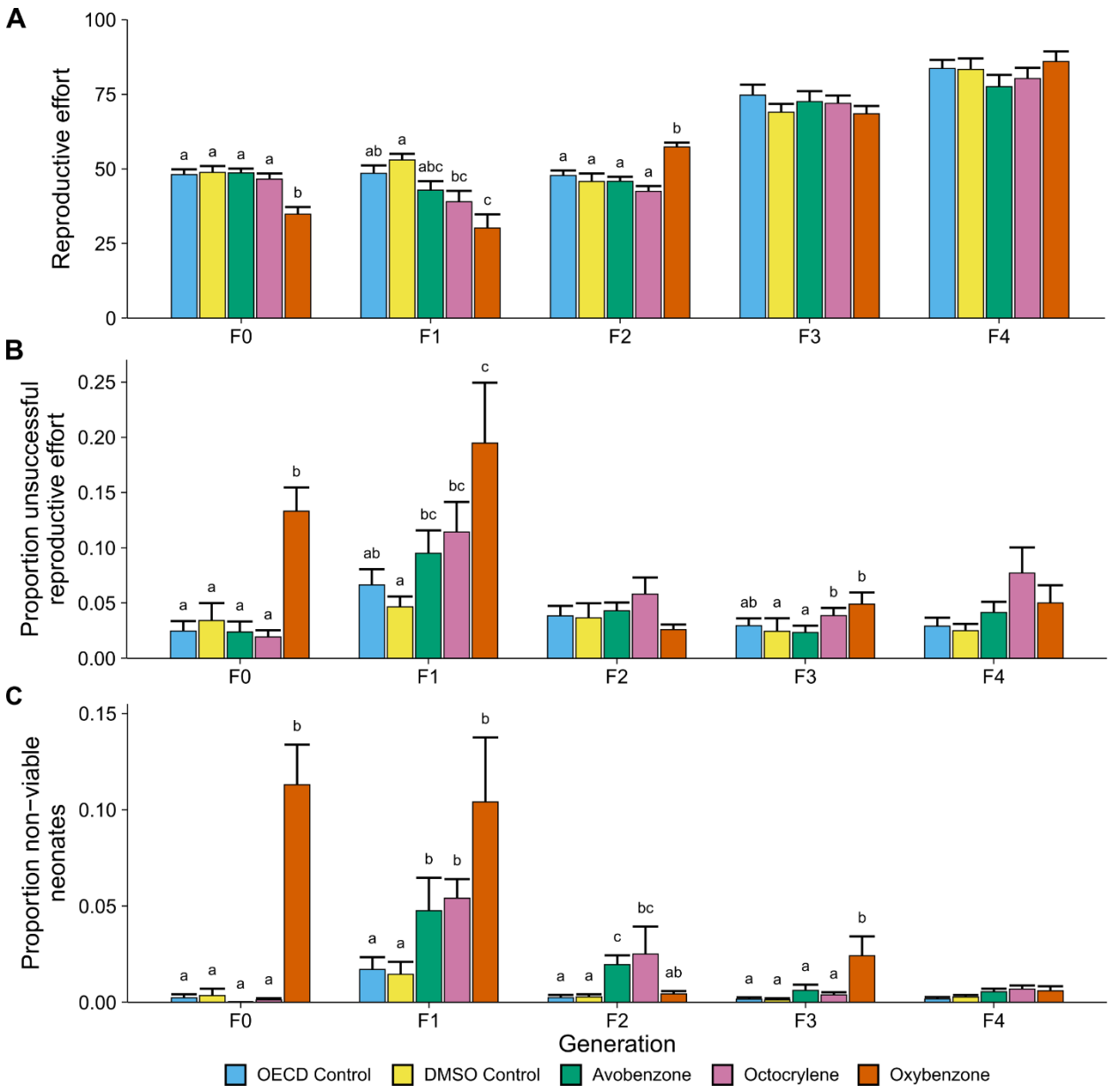


Figure 3.2. The average total reproductive effort (viable neonates + unhatched eggs + non-viable neonates) over 21 days (A), unsuccessful reproductive effort (unhatched eggs + non-viable neonates) produced per adult daphnid as a proportion of their overall reproductive effort (B) and non-viable neonates produced per adult daphnid as a proportion of their overall reproductive effort (C) of < 24-hour old *Daphnia magna* neonates exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids. Bars represent mean ± SEM. Different letters on the bars indicate a significant difference between treatments within generations, bars without letters are not considered to be significantly different at $p < 0.05$ (A: one-way ANOVA, B & C: Kruskal-Wallis).

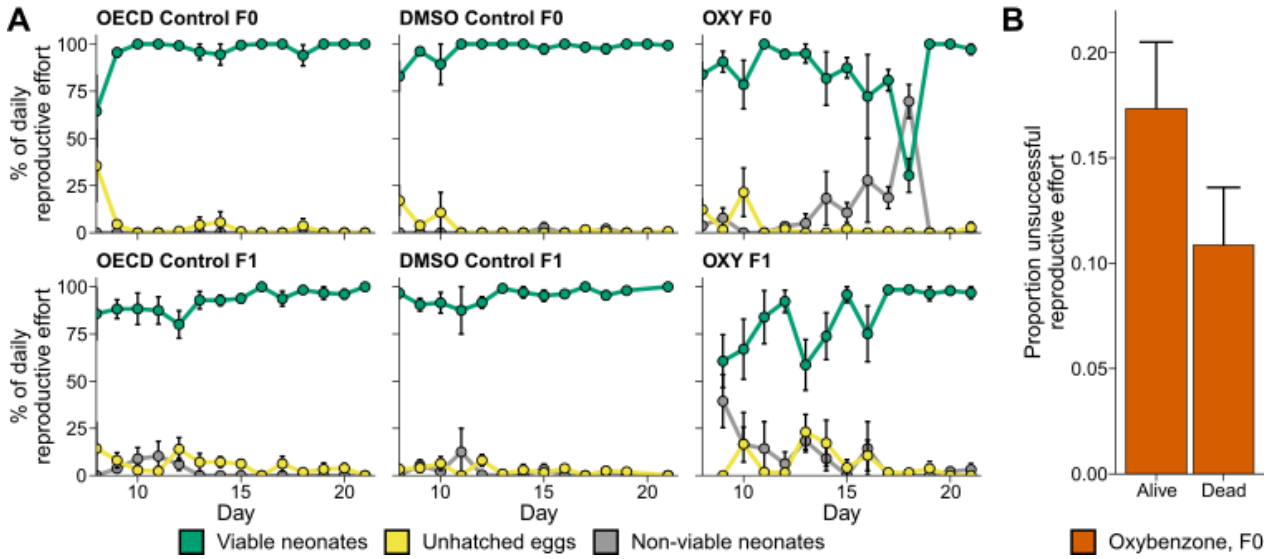


Figure 3.3. The percent of daily reproductive effort (A) due to viable neonates (green), unhatched eggs (yellow) or non-viable neonates (grey) of < 24-hour old *Daphnia magna* neonates exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). The proportion of unsuccessful reproductive effort (unhatched eggs + nonviable neonates) of OXY exposed daphnids in the F0 generation between individuals that did (alive) and did not (dead) survive the entire 21 d exposure (B) as a proportion of their overall reproductive effort (viable neonates + unhatched eggs + non-viable neonates). Each treatment was comprised 30 individually exposed daphnids. Data are presented as mean ± SEM. The proportion of unsuccessful reproductive effort did not significantly differ between alive and dead daphnids (Wilcoxon rank sum, $p > 0.05$). An expanded version of Figure 3A depicting all treatments and generations is available in Figure S3.2.

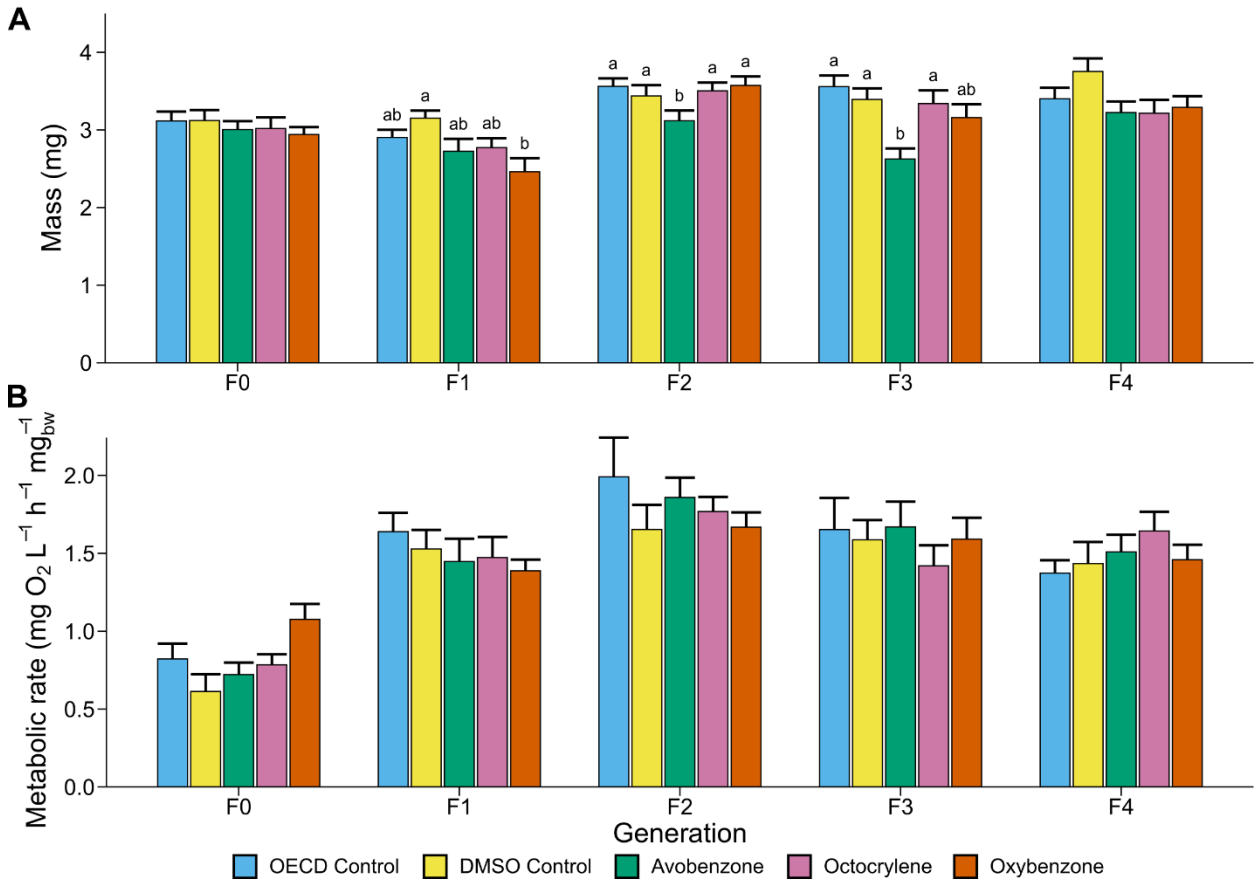


Figure 3.4. Whole body wet mass (A) and the mass specific metabolic rate (B) of 21-day old *Daphnia magna*. < 24-hour old neonates were exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids. Bars represent mean ± SEM. Different letters on the bars indicate a significant difference between treatments within generations, bars without letters are not considered to be significantly different (one-way ANOVA, $p < 0.05$).

3.5 Supplementary materials

Table S3.1. LC-QQQMS multiple reaction mode conditions and the external calibration curves for each UVF

Compounds	Retention Time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)	Calibration curve	R^2
Avobenzone	3.55	310	161	25	Y = 1098.42x + 172.12	1.000
Octocrylene	4.26	362	251	20	Y = 1403.89x + 1174.16	1.000
Oxybenzone	0.825	229	151	20	Y = 402.55x + 112.03	0.999

Table S3.2. Exposure solution concentrations utilized for 21 d median lethal concentration (LC50) determination for each UVF. Four replicates of n = 5 < 24 h old *Daphnia magna* neonates were exposed at each of the indicated concentrations for 21 days.

Treatment	Concentration (µg/L)
AVO	0, 2, 5, 10, 20, 40, 80, 120, 160, 200, 250, 300, 500, 1000
OCT	0, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10, 15, 20, 50
OXY	0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 150, 200, 300, 500

Table S3.3. Start and end dates for each generation of the multigeneration study.

Generation	Exposure begins (days since study start)	Exposure ends (days since study start)	Exposure duration (days)
F0	0	21	21
F1	15	36	21
F2	30	51	21
F3	45	66	21
F4	60	81	21

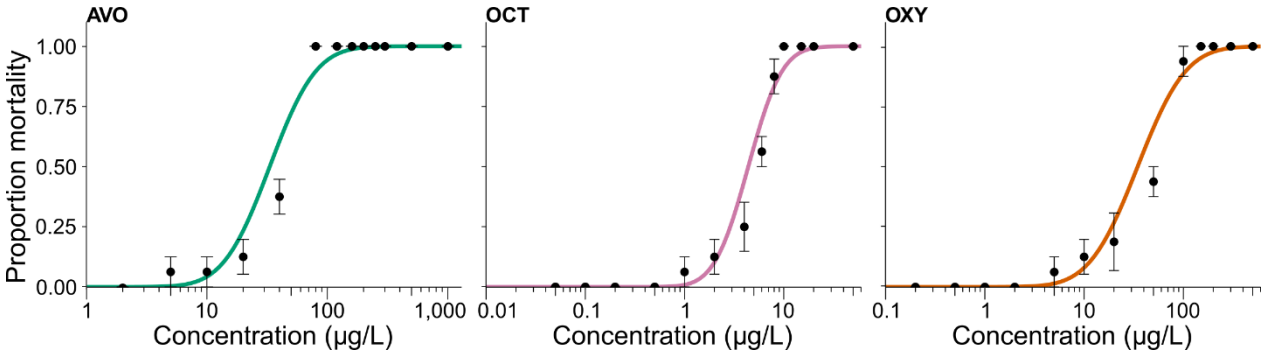


Figure S3.1. Median lethal concentration dose/response curves for *Daphnia magna* exposed to avobenzone, octocrylene or oxybenzone for 21 days. Four replicates of n = 5 < 24 h old *Daphnia* magna neonates were exposed at each UVF concentration for 21 days.

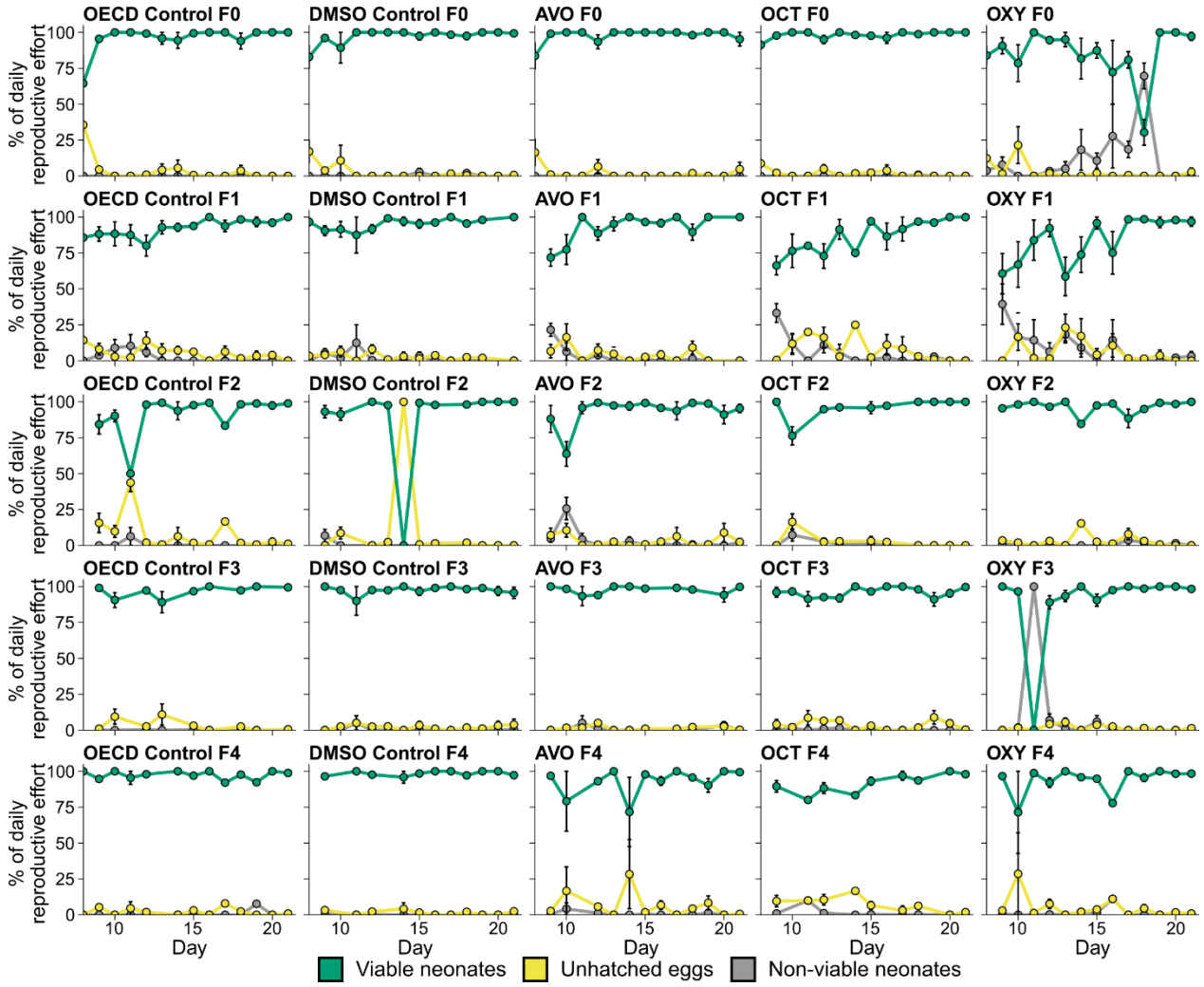


Figure S3.2. The percent of daily reproductive effort due to viable neonates (green), unhatched eggs (yellow) or non-viable neonates (grey) of < 24-hour old *Daphnia magna* neonates exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids. Data are presented as mean ± SEM.

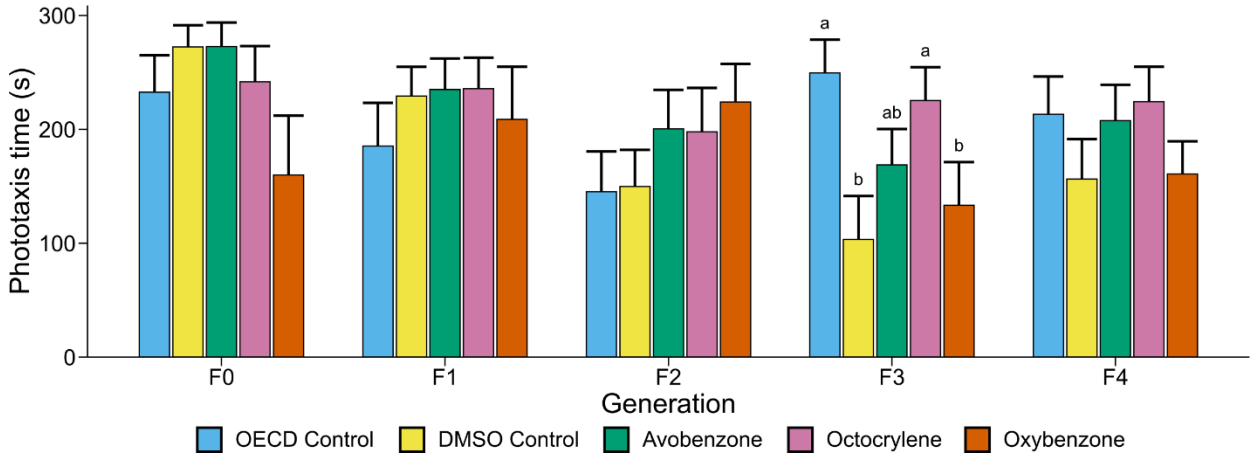


Figure S3.3. The weighted phototactic response time of 21-day old *Daphnia magna*. Trials were considered incomplete if daphnids did not finish in ≤ 5 minutes. Incomplete trials were assigned a maximum score of 300 s. < 24-hour old neonates were exposed to UV filter treatments for 21 days across 5 generations (F0 – F4). Each treatment was comprised 30 individually exposed daphnids. Bars represent mean ± SEM. Different letters on the bars indicate a significant difference between treatments within generations, bars without letters are not considered to be significantly different (Kruskal-Wallis, $p < 0.05$).

Chapter 4: Getting over it? A proteomic analysis of mechanisms driving multigenerational acclimation to organic ultraviolet filters in *Daphnia magna*

Boyd, A., Lummer, C., Mehta, D., Uhrig, R.G., Blewett, T.A., 2025. Getting over it? A proteomic analysis of mechanisms driving multigenerational acclimation to organic ultraviolet filters in *Daphnia magna*. In prep for Water Research.

4.1 Introduction

Organic ultraviolet filers (UVFs) such as avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY) are commonly used in many cosmetics and personal care products to protect against damage caused by harmful sunlight ultraviolet radiation (Berardesca et al., 2019). UVFs are widespread contaminants in a variety of aquatic environments due to sunscreen use during aquatic recreation (Giokas et al., 2007; Labille et al., 2020) and incomplete removal from wastewater treatment plant effluent (Emnet et al., 2015; Kasprzyk-Hordern et al., 2009; Wick et al., 2010), leading to concentrations of individual UVFs commonly reported in the range of 0.1 – 10 μg/L (Bargar et al., 2015; Langford and Thomas, 2008; O'Malley et al., 2021; Sánchez Rodríguez et al., 2015). As a result, many studies have been performed to investigate the toxicity of these compounds in a variety of species, which have suggested that the highest reported environmental concentrations of UVFs may pose a threat to the most sensitive aquatic invertebrate species. Reported effects include mortality of coral species such as *Stylophora pistillata* exposed to OXY (Downs et al., 2016), mortality of *D. magna* exposed to OCT (Boyd et al., 2021), and growth impairment of sea urchins (*Paracentrotus lividus*) exposed to OCT (Giraldo et al., 2017). The overlap in UVF concentrations found to be toxic to aquatic invertebrates and those reported in the most contaminated environments has prompted the countries of Aruba (Homan and Martinus, 2021), Palau (Republic of Palau, 2018), and several regions of the United States (Hawaii State Legislature, 2018; USVI Legislature, 2019) to institute bans of certain UVFs in order to protect fragile ecosystems.

Investigations of subcellular changes in response to UVF exposure have contributed towards the categorization of these contaminants as endocrine disrupting compounds due to their affinity for estrogen receptors (Molina-Molina et al., 2008; Suzuki et al., 2005). Large disruptions of metabolism have also been observed in vertebrates (Simons et al., 2022; Y.-K. Zhang et al., 2023; Ziarrusta et al., 2018b), with recent studies indicating that AVO, OCT and OXY are potential obesogens that can

interfere with lipid metabolism pathways controlled by peroxisome proliferator-activated receptors (Ahn et al., 2019; Ko et al., 2022; Shin et al., 2020). While subcellular research in invertebrates has been more limited, similar metabolic disruptions have been observed in corals (*Pocillopora damicornis* and *Sytlophora pistillata*; (Stien et al., 2019; Thorel et al., 2022); however, minimal disruptions were observed in aquatic fly larvae (*Chironomus riparius;* González and Martinez-Guitarte, 2018, *Sericostoma vittatum;* Campos, 2017). Investigations of the freshwater invertebrate, *Daphnia magna* have reported effects of OXY on hormonal regulation of molting (Lambert et al., 2021) as well as oxidative stress responses (de Paula et al., 2022; Yang et al., 2021); however, investigations beyond typical xenobiotic response mechanisms have been largely limited in this model species.

Most ecotoxicity data for UVFs and many other contaminants have been acquired from single generation exposures of organisms, with the results extrapolated to draw conclusions about the long-term threats facing at risk environments. While this approach is beneficial for the high throughput short-term experiments provide, the data produced may not always be an accurate representation of long-term toxicity to populations that have been exposed to contaminants across many generations, as mechanisms such as selective pressures or phenotypic plasticity may result in a population becoming more or less sensitive to any particular contaminant across subsequent generations of exposure. A previous study that was conducted in parallel to this one found that the toxicity experienced by the F0 generation of *Daphnia magna* populations exposed to AVO, OCT or OXY diminished until no evidence of impairment could be observed by the F3 generation of exposure in measurements of mortality, reproductive characteristics or metabolic rates, indicating that acclimation is indeed possible over long-term exposure to contaminants (Boyd et al., 2023a). The objective of this study was to identify the mechanisms driving this gradual acclimation by identifying protein level changes that occurred across generations of exposure. Quantifying protein level changes in response to novel UVF exposure will allow for mechanisms of toxicity to be identified. In addition, these results will help identify the

mechanisms through which daphnid populations are capable of acclimating to continued contamination, allowing for a determination of if the decreased UVF sensitivity observed across generations of *D. magna* exposure are the result of physiological adaptations through mechanisms such as phenotypic plasticity, or from selection of a more tolerant phenotype driven by the mortality that was observed in earlier generations of exposure (Boyd et al., 2023a).

4.2 Materials and methods

4.2.1 Daphnia culture maintenance

This study was performed concurrently to the research described in Boyd et al. (2023a) and used similar methodology. Briefly, a *Daphnia magna* culture was produced from gravid adults purchased from Aquatic Research Organisms (ARO; US) in November 2021 and maintained in the Department of Biological Sciences at the University of Alberta in Edmonton, Canada. Culture water was prepared from pH ≈ 7.6 dechlorinated City of Edmonton water according to Organization for Economic Co-operation and Development (OECD) guidelines to contain 294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$, and 5.80 mg/L KCl (OECD, 2012), referred to as OECD water in the following text. The chemical composition of the OECD water has been previously reported in Boyd et al. (2023b). Cohorts of 30 daphnids were each maintained in 1 L of OECD water to a maximum age of 2 months, with complete renewal of culture waters occurring every 2-3 days. Cohorts were provided a daily ration of 3 mL of 3 x 10^7 cells/mL freshwater green algae (*Raphidocelis subcapitata*) and 3 mL of yeast, cereal leaf and trout chow mix from ARO along a weekly supplement of 100 µL of Roti-Rich invertebrate food (VWR, Canada). All daphnids were maintained on a 14 h: 10 h photoperiod at 21.4 ± 0.5 °C.

4.2.2 Multigenerational exposures

D. magna neonates < 24 h old were exposed in groups of 25 to one of 5 treatments including independent control groups of untreated OECD water and a 0.003% dimethyl sulfoxide (DMSO) solvent control. Exposures to UVFs dissolved in 0.003% DMSO were performed using solutions with measured concentrations of 6.59 ± 0.735 µg/L AVO, ~0.6 µg/L OCT, or 16.5 ± 7.80 µg/L OXY for a period of 21 days following modified OECD test 211 guidelines (OECD, 2012), repeated across 5 generations of continuous exposure (F0 – F4) and 5 replicates per treatment. The UVF exposure concentrations were selected based on the estimated 21 d LC20 obtained during the concurrent study described in Boyd et al. (2023a), with both studies utilizing the same exposure stock solutions (Table 3.1). The UVF concentrations presented here are the average concentrations measured over the duration of both studies, described in detail in Boyd et al. (2023a), with additional information provided in the supporting information. Each group of 25 *Daphnia* were exposed in 20 mL of water per daphnid and provided a daily food ration of 100 µL each of YCT mix and green algae per daphnid, with food and water scaled to the number of surviving individuals on each day of the experiment (maximum 500 mL water, 2.5 mL of each food). Complete renewal of the exposure water occurred every 2 days, and each group of daphnids was scored daily for mortality, male daphnids and reproductive effort, which was defined as the sum of viable neonates, eggs released from the brood chamber that were unhatched, and non-viable neonates at the time of scoring. Subsequent generations began on the 17th day of exposure of the prior generation when the majority of daphnids released their 3rd brood by pooling all viable neonates released from all replicates of a single treatment and randomly selecting neonates to comprise the next generation of exposure.

4.2.3 Median lethal concentration experiments

48 h median lethal concentration (LC50) tests were performed on daphnids from unexposed parents (F0 offspring) and from neonates produced during the multigenerational experiment (F1 – F5

offspring) from parents on their 13th day of exposure when the majority of reproductive daphnids were releasing their 2nd brood. Neonates from the OECD and DMSO controls were tested against a common reference toxicant of copper to monitor the health of control daphnids, while neonates from UVF treatments were tested against their respective UVFs to determine if the overall sensitivity of each population changed across each generation. For each LC50, 4 replicates of 5 neonates < 24 h old were exposed in 20 mL of each concentration of test solution outlined in Table S4.1 for a total of 48 h, in accordance with OECD test 202 guidelines (OECD, 2004). Daphnids were fasted for the duration of the exposure, and mortality was determined by the absence of gill respiratory motion during a 15 s period of gentle agitation.

4.2.4 LC-MS/MS analysis and label-free quantitation

At the end of the 21-day exposure for each generation, surviving daphnids were vacuum filtered and quickly dissected to excise the brood chamber and developing eggs prior to flash freezing the remaining adult tissues in liquid N_2. 4 individuals per replicate and 3 total replicates per treatment from the F0, F1 and F3 generations were randomly selected for proteomic analysis. The F3 generation was selected for analysis rather than the F4 generation as it was the first one in which full recovery of the observed physiological functions occurred in Boyd et al. (2023a) for all UVF exposed daphnids.

Samples were processed following the method described in Mehta et al. (2022) without deviation. BoxCar data dependent acquisition (BoxCar DIA) liquid chromatography mass spectrometry (LC-MS) analysis was performed on a FAIMSpro mounted Fusion LUMOS hybrid Orbitrap mass spectrometer (ThermoFisher Scientific) as described in Rodriguez Gallo et al. (2023). Here, a 25 cm easy spray column (ES902; ThermoFisher Scientific) was run at a flow rate of 300 nL/min and the FAIMSpro was set to multi-CV setting -30, -50, -70. Data analysis was performed using a decoyed (reversed) version of the *D. magna* protein database obtained from UniProt (https://www.uniprot.org/) and Spectronaut v16 (Biognosys AG; Mehta et al., 2022).

4.2.5 Bioinformatics

The 26,600 proteins of the *D. magna* proteome were annotated with BLASTP (nr v5 database) and mapped to 68,399 gene ontology (GO) terms using Goa (2020,10) and InterProScan (version 5.51 – 83.0) as part of a previous analysis described in Boyd et al. (2023b). Of the 1,265 proteins quantified in this study, 93.7% were mapped to GO terms. REVIGO version 1.8.1 was used to remove obsolete and update outdated GO terms for the 1,350 proteins detected in the present study as well as to summarize related GO terms to provide a broad view of the biological processes related to these proteins, using all default settings with the exception of list size set to 0.5 (Supek et al., 2011).

4.2.6 Statistics

R version 4.3.2 was used to conduct all statistical analyses unless otherwise indicated (R Core Team, 2024). LC50s were estimated using the probit method of the "ecotox" package. Pairwise ratio test comparisons followed by a Benjamini & Hochberg p value correction were performed to compare resulting LC50 estimates. Reproduction and survivorship data were tested via one way ANOVA followed by post-hoc Tukey tests when normality and homoscedasticity assumptions were met as determined by Shapiro-Wilk and Levene's tests, respectively. Data that failed to meet these assumptions and could not be transformed were compared with Kruskal Wallis and post hoc Dunn's tests. Significance was determined at $\alpha = 0.05$ for all analyses, and all values are reported as the mean ± standard error of the mean unless indicated otherwise.

Perseus was used to perform one-way ANOVAs and post hoc Tukey's tests to identify significantly altered protein abundances. One-sided Fisher's exact tests using the Benjamini & Hochberg p value correction for false discovery rate was performed on proteins that differed from the DMSO control for each generation using the "clusterProfiler" package in R to identify significant positively enriched ($\log_2$ fold-change > 0.58) and negatively enriched (log2 fold-change < -0.58)

biological processes (BPs). Processes with fewer than 5 identified proteins were excluded from the analysis.

4.3 Results

4.3.1 Survivorship and reproduction changes

In the F0 generation, < 10% mortality occurred in all treatments over 21 d with the exception of OXY, where a steep increase in mortality was observed from 3% by day 16 to 55% on day 18, resulting in the early termination of that treatment (Figure 4.1). Similar patterns were observed in the F1 generation, with < 13% mortality occurring over 21 d in all treatments except OXY, which experienced 35% mortality within 5 d before the population stabilized for the remainder of the generation. Mortality in all treatments was approximately 20% or lower for the F2, F3 and F4 generations. OXY exposed daphnids had 30% and 21% reduced reproductive effort in the F0 generation compared to the OECD and DMSO controls, respectively (one-way ANOVA, $p = 0.001$), with no differences in reproductive effort observed over the F1 – F4 generations (one-way ANOVA, $p > 0.058$; Figure 4.2 A). 37% of reproductive effort from the OXY F0 generation resulted in non-viable neonates, compared to ≤ 0.1% for all other treatments (Kruskal-Wallis, $p = 0.0004$; Figure 4.2 B). Although statistical differences were detected (Kruskal-Wallis, $p < 0.004$), ≤ 2.1% of reproductive effort resulted in non-viable offspring for all treatments across the F1 – F4 generations.

4.3.2 Offspring LC50s

Copper 48 h LC50s for all generations of the OECD and DMSO control cultures ranged from 36.0 µg/L (95% confidence interval (CI): 29.6 – 43.9 µg/L) up to 66.4 µg/L (95% CI: 56.9 – 78.3 µg/L) with no trends apparent over the six tested generations (Figure 4.3). Statistical significance from the F0 neonates from unexposed parents was observed between the most extreme LC50s obtained for the DMSO culture (F1 & F3 generations; ratio test, $p = 0.0159$) but not for any generations of the OECD

culture ($p > 0.0531$). A similar lack of trends was observed for AVO LC50s, which ranged from 3.86 mg/L (95% CI: 2.93 – 5.39 mg/L) to 11.80 mg/L (95% CI: 9.52 – 15.1 mg/L) and OCT LC50s, ranging from 2.30 mg/L (95% CI: 1.63 - 3.28 mg/L) to 11.40 mg/L (95% CI: 7.86 – 19.6 mg/L). OXY LC50s also did not display a trend across generations, with LC50s ranging from 1.39 mg/L (95% CI: 1.18 – 1.68 mg/L) to 4.10 mg/L (95% CI: 3.41 – 5.12 mg/L).

4.3.3 Protein changes

Principal component analysis of all quantified proteins from the end of each generation revealed similar clustering of all treatments in the F0 generation with the exception of OXY exposed daphnids (Figure 4.4 A). OXY daphnids were clustered on the periphery of all other treatments in the F1 generation and overlapped entirely with all treatments in the F3 generation (Figure 4.4 B & C). 36.7% of significantly changing proteins in the F0 generation were found to be unique to OXY exposed daphnids (Figure 4.4 D), with each UVF treatment resulting in more distinct alterations in the F1 and F3 generations as the majority of significantly changing proteins (Fisher's exact test)were not shared between any two treatments (Figure 4.4 E & F). In the F0 generation, OXY resulted in the greatest number of significantly changing proteins with respect to the DMSO control (60 increased in abundance, 248 decreased in abundance), followed by OCT (25 increased, 170 decreased) then AVO (44 increased, 45 decreased). The overwhelmingly negative enrichment of BPs in the F0 generation was absent in the F1 generation, with 76 increased and 60 decreased proteins for OXY, 57 increased and 12 decreased for OCT, and 122 increased and 165 decreased for AVO. By the F3 generation, the majority of significantly changing proteins favoured increases in protein abundance, as OXY yielded 156 increased and 101 decreased proteins, OCT caused 133 increased and 50 decreased proteins, and AVO resulted in 76 increased and 57 decreased proteins compared to the DMSO control. Within UVF treatments, less than half of significantly altered proteins detected in the F0 generation persisted

through to the F3 generation, with 12.4%, 33.8% and 43.1% of proteins in common for AVO, OCT and OXY, respectively (Figure 4.4 G – I).

The majority of significantly changing proteins across all 3 generations were summarized into general functions related to metabolism (6 BPs) and immune response (4 BPs), with enrichment also detected for the cuticle chitin metabolic process BP, the glycosphingolipid biosynthetic process BP, the superoxide anion generation BP and the protein modification BP (Figure 4.5). For the F0 generation of OXY exposed daphnids, 4/5 of the BPs with the largest proportion of significantly decreased proteins were involved in metabolic processes, with 4/6 BPs related to metabolism demonstrating > 60% negative enrichment. Immune response related processes also demonstrated strong negative enrichment between 18.2% and 45.5% for related BPs, while cuticle chitin metabolism and glycosphingolipid biosynthetic processes had negative enrichment of 35.7% & 54.5%, respectively. Similar patterns were observed for OCT and AVO; however, minimal changes to immune response related BPs were noted for AVO in the F0 generation as all BPs had enrichment between -12.1% and 20.0% of proteins in their respective processes with the exception of the 34.4% negative enrichment of the superoxide anion generation BP observed for AVO. In the F3 generation, significant positive enrichment was observed for 2/6 metabolism-related BPs, 1/4 immune response BPs and the cuticle chitin metabolic process BP for OXY exposed daphnids. 5/6 BPs for metabolism had 28.0% - 57.1% of proteins significantly increased in abundance, while the glycosphingolipid biosynthetic process and the cuticle chitin metabolic process BPs had 31.8% & 35.7% positive enrichment, respectively. Only the leukotriene biosynthetic process BP had > 15% of proteins significantly increased in abundance (36.4%) for the immune response-related processes. A similar pattern of positive enrichment was observed for OCT, although the proportion of each BP that was enriched was slightly lower than observed for OXY, resulting in only significant positive enrichment of the carbohydrate metabolic process BP. All BPs had between -13.6% and 10.7% enrichment for the F3 generation of AVO exposed daphnids.

4.4 Discussion

The results from this study exposing *D. magna* in groups of 25 are consistent with the results obtained from the parallel study exposing individual daphnids described in Boyd et al. (2023a), which suggests that UVFs can be particularly toxic to previously unexposed *Daphnia* populations as novel toxicants. While large impairments were evident as a result of OXY exposure, minimal physiological changes occurred across the 5 generations of exposure to AVO or OCT, indicating that the concentrations used for the latter two UVFs were not sufficient to cause observable impairments to the daphnids. Although both studies used the same exposure solutions and the same *D. magna* population, the severity of OXY to the F0 generation was greater to the groups of daphnids used in this study. While the mortality caused by OXY exposure reached similar levels on day 18 (48% for individuals vs 55% for groups; Figure 4.1), a much larger proportion of reproductive effort resulted in non-viable offspring (11.3% for individuals vs 37.4% for groups; Figure 4.2). Despite the water volume and food ration being scaled to the number of surviving daphnids on each day, the differences in sensitivity between group and individual exposures may be caused by intraspecific competition for resources (Gust et al., 2016), as dominant individuals compete for a greater share of available food (Schoepf and Schradin, 2013; Ward and Webster, 2016). The differences between individual and group exposed daphnids will be discussed in further detail in Chapter 5).

4.4.1 Selective pressures vs physiological adaptation

Despite the toxicity of OXY observed in the F0 generation, populations appear to be capable of gradual acclimation to long-term sublethal concentrations, as mortality was reduced to 35% in the F1 generation before returning to background levels (< 20%) for the F2 - F4 generations. Similarly, the reproductive impairments observed in the F0 generation were largely absent by the F1 generation and beyond. The gradual decrease of organism sensitivity across generations could be the result of either selective pressures through the removal of more sensitive phenotypes in earlier generations (Leblanc,

1982), or the result of gradual acclimations obtained through epigenetic alterations that allow surviving organisms to better acclimate to their current environment over each subsequent generation (Chatterjee et al., 2019). The timing of the observed F0 mortality is notable in that it occurred largely after the collection of neonates to start the F1 generation (day 17); therefore, any sensitive phenotype in the population would have the opportunity to carry forward to the next generation, potentially contributing towards the mortality observed in the F1 generation. As the F1 mortality occurred earlier in development, only phenotypes allowing survival for at least 17 days of exposure to OXY would be able to continue, offering a potential explanation for the absence of mortality in the F2-F4 generations.

It is unclear whether a more sensitive phenotype was present within the exposed OXY population, as the majority of surviving daphnids on day 18 of the F0 generation demonstrated signs of stress including immobilization and body discolouration (personal observation), leading to the early termination of the generation so that proteomic analysis could be performed. It is likely that a complete loss of the population would have occurred within 1 – 2 days, reducing the likelihood of the presence of a "resistant" phenotype within the exposed population. The late onset of mortality in the F0 generation in combination with the exposure of individual daphnids in Boyd et al. (2023a) allowed for comparisons of reproductive endpoints between surviving daphnids and those that experienced mortality in the F0 generation, which did not reveal any appreciable differences in the measured endpoints, providing further indication that selection of a "resistant" phenotype may not be the cause of the gradual acclimation to UVFs observed across each generation of exposure. Additionally, the OECD test guideline requirement of maintaining *Daphnia* cultures through parthenogenic reproduction of clones in an effort to maintain low genetic diversity within test populations (OECD, 2012) further reduces the likelihood of multiple phenotypes within the population due to the expected low genetic diversity that would accompany a long lineage of a population of genetic clones (Barata et al., 2002).

Several previous studies have demonstrated that *D. magna* populations are capable of acclimating across several generations to exposures of contaminants such as copper, lead (Leblanc, 1982), zinc (Hochmuth et al., 2015; Muyssen and Janssen, 2001), cadmium (Ward and Robinson, 2005), wastewater effluent (Chatterjee et al., 2019), prochloraz (fungicide; Poulsen et al., 2021) and chlorpyrifos (insecticide; Maggio and Jenkins, 2022). In many of these studies, the authors reported low mortality, instead concluding that the observed acclimations were likely the result of internal mechanisms such as phenotypic plasticity rather than the result of selective pressures. A study by Leblanc (1982) concluded that toxicant resistance acquired by *D. magna* across generations of exposure was not hereditary in origin, but rather the result of physiological adaptations made over each exposed generation, as the toxicant resistance was lost during unexposed intermediate generations. Chatterjee et al. (2019) similarly concluded that physiological adaptations through the differential expression of a small number of specific proteins were sufficient to allow for an increase in *D. magna* toxicant resistance across 3 generations of exposure to wastewater effluent. Regardless of the nature of the observed acclimations in later generations of exposure, the resistance to UVFs acquired did not result in any meaningful changes in offspring LC50s, as similar patterns of random variability were present for the offspring of control daphnids exposed to a novel copper toxicant (Figure 4.3 A & B) as those from parents exposed to the same UVF toxicant for generations (Figure 4.3 C – E). The lack of observable trends in offspring LC50s indicate that the acclimations acquired by exposed populations offer lower levels of resistance that are more suitable for long-term, low concentration exposures rather than the extreme doses that are necessary for 48 h LC50 tests. A lack of appreciable changes in the 48 h EC50 of *D. magna* exposed to zinc has also been reported regardless of long term acclimation for up to 8 generations (Muyssen and Janssen, 2001). The magnitude of biological changes necessary in order to confer resistance to overwhelmingly large mg/L exposure doses would likely be extremely costly to daphnids, requiring diversion of energetic resources away from growth and development during a

critical early life stage (Ginjupalli and Baldwin, 2013). In order to better understand the nature of these population acclimations, quantification of more subtle sublethal endpoints is required.

4.4.2 Multigenerational proteomic alterations

Analysis of surviving daphnid proteomes at the end of the F0, F1 and F3 generations revealed a broad initial response to a novel contaminant exposure that became more refined over subsequent generations (Figure 4.4 A – C). Previous studies have observed similarly broad responses from *D. magna* after exposure to novel toxicants that become refined over generations of exposure through proteomic (Chatterjee et al., 2019) and metabolomic analysis (Poulsen et al., 2021). Considering that less than half of significantly changing proteins in the F0 generation persisted through to the F3 generation in any UVF treatment along with the decrease in toxic response in later generations (Figure 4.4 G – I), it would appear that daphnids were capable of optimizing the physiological responses necessary to confer resistance to prolonged contaminant exposure. It is interesting to note that while the specific proteins that were significantly altered in later generations tended to be unique to each UVF treatment (Figure 4.4 E & F), these proteins tended to belong to the same biological processes overall, particularly in the case of OCT and OXY (Figure 4.5).

Metabolic processes demonstrated the largest negative enrichments in the F0 generation across all UVFs followed by the largest positive enrichments in the F3 generation and can be summarized as functions primarily related to carbohydrate and lipid metabolism (Figure 4.5). Metabolomic and transcriptomic profiling of fish revealed disorders in both lipid storage and carbohydrate metabolic precursors due to OXY exposure in *Sparus aurata* (Ziarrusta et al., 2018b), disruption of liver lipid metabolism in *Amphiprion ocellaris* (Y.-K. Zhang et al., 2023), and dysregulation of triglyceride metabolism in *Danio rerio* larvae (Simons et al., 2022), while plants such as *Brassica rapa* L. spp. *chinensis* similarly experienced disruption of lipid and carbohydrate metabolic pathways, including a reorganization of the electron transport chain to maintain ATP synthesis despite potential damage to

mitochondrial processes (Li et al., 2023a). Downregulation of proteins governing iron and amino acid metabolism were reported in the marine bacterium *Epibacterium mobile* in response to OXY (Lozano et al., 2021), while OCT has been observed to cause similar disruptions to fatty acid metabolism and mitochondrial function in the coral *Pocillopora damicornis* (Stien et al., 2019). Recent studies in human keratinocytes and mesenchymal stem cells have found that AVO, OCT and OXY can bind to peroxisome proliferator-activated receptors that regulate adipose tissue development, suggesting that these UVFs are obesogens (Ahn et al., 2019; Ko et al., 2022; Shin et al., 2020).

It is interesting to note that no alterations in *D. magna* metabolic rates were observed between UVF treatments and the OECD or DMSO controls in any generation during the study of individually exposed daphnids in Boyd et al. (2023a), suggesting that the impairments to lipid and carbohydrate metabolic processes noted in the proteome of UVF exposed daphnids in the F0 generation are compensated for via alternate pathways. Li et al. (2023) proposed that *Brassica rapa* L. spp. *chinensis* resorted to protein catabolism to compensate for the disruption of normal metabolic pathways during OXY exposure. In the present study, all quantified proteins related to the protein catabolism BP were found to be significantly decreased in the F0 generation, and more than half significantly increased in the F3 generation of OXY exposure. This was inversed from the expected pattern if daphnids were indeed utilizing protein catabolism to compensate for impairment of carbohydrate and lipid metabolism. As only 7 proteins related to protein catabolism were detected in this study, it is possible that other unquantified proteins may explain how daphnids were able to maintain their overall metabolic rate despite the protein level disruptions observed to normal metabolic pathways; however, a more targeted approach may be necessary to identify the exact mechanism of compensation.

Biological processes related to immune response were the second largest category of enriched BPs identified, with processes related to opsonization and leukotriene biosynthesis demonstrating the largest negative enrichment observed in the F0 generation of OXY exposure (Figure 4.5). Opsonization

is important in tagging foreign objects such as pathogens for removal from the organism (Mucklow and Ebert, 2003), while leukotrienes play a role in the regulation of inflammatory responses to cell damage (Heckmann et al., 2008; Sangwoo Lee et al., 2023). It has been proposed that coral bleaching due to UVF or sunscreen exposure is caused by a large increase in viral infection (Danovaro et al., 2008), while immune-related biomarkers were observed to be strongly associated with OXY exposure in goldfish (*Carassius auratus*; Zhang et al., 2020). It would appear from the results of the present study that *D. magna* may also be susceptible to infection as a result of UVF exposure, contributing to the decreased viability of the F0 generation. A suppression of the immune system during the initial generation may leave daphnids particularly vulnerable to infection, but the trend towards positive enrichment of these processes in the F3 generation indicate that a strong immune response, particularly increased inflammatory processes, can be beneficial for daphnids to overcome the negative effects of OXY exposure. The laboratory water that these experiments were conducted in are not expected to be free of pathogens due to the lack of sterilizing procedures implemented; however, they are also not expected to contain an abundance of pathogens as the *D. magna* cultures used in this study have a history of demonstrated health, showing no clinical signs of infection. While the observed immune changes may still contribute towards the observed toxicity and subsequent recovery of the tested populations, other factors are likely to offer greater contributions towards the observed acclimation.

Also of note are the similar patterns of negative enrichment of chitin metabolism function in the F0 generation followed by positive enrichment in the F3 generation for OCT and OXY populations (Figure 4.5). Exoskeleton maintenance serves an important role in defending against environmental stressors as well as organism development and reproduction (Dabrunz et al., 2011; Espie and Roff, 1995). The molting process controlled by 20-hydroxyecdysone is required for developing daphnids to achieve a larger body size in addition to allowing adults to release broods as part of their reproductive cycle (Baldwin et al., 1995; Zhao, 2020); therefore, impairment of normal exoskeleton function can

interfere with many aspects of daphnid physiology. Impairment of chitin metabolism has been attributed as a mechanism of zinc toxicity in *D. magna* (Poynton et al., 2007), and increased chitin metabolism has been specifically linked to the natural resistance against the insecticide diflubenzuron found in the National Institute for Environmental Studies *D. magna* strain (Kato et al., 2022). In addition, upregulation of genes related to chitin metabolism has been observed in response to exposure of *D. magna* neonates to hydraulic fracturing wastewater (Blewett et al., 2017).

OXY has been demonstrated to upregulate ecdysone receptor (Yang et al., 2021) and ultraspiracle protein (Lambert et al., 2021), suggesting a potential for disruption of the exoskeleton or molting behaviours. Previous studies of *D. magna* have not observed impairments in the timing or number of molts during exposure to AVO, OCT or OXY (Boyd et al., 2021; Lambert et al., 2021), including no changes in molting observed in individually exposed daphnids performed concurrent to this study (Boyd et al., 2023a). The lack of observable effects on molting outcomes could indicate that the impacts of novel OXY exposure are related to the quality and structure of the exoskeleton, potentially serving to reduce the permeability of the integument to xenobiotics (Jeong et al., 2013) and pathogens (Locke, 2001). This may serve to benefit the organism through both reducing the rate at which xenobiotics can penetrate into the organism and also improve defenses against infection in concordance with the immune system alterations discussed above. A loss of chitinase activity has been associated with increased cuticle permeability in *Drosophilla melanogaster* (Dong et al., 2023) and impaired chitin organization similarly linked to increases sensitivity to multiple different xenobiotics in *Locusta migratoria* (Yu et al., 2022).

It is important to note that all treatments demonstrated an increase in the level of daily reproductive effort observed per surviving daphnid between the F2 and F3 generations (Figure 4.2). The magnitude and timing of this increase was consistent across all treatments in this study, and is similar to the pattern observed with the individually exposed daphnids discussed in Boyd et al. (2023a).

Previous *D. magna* studies have reported similar increases in control group reproduction ranging from 20 – 60 % across generations (Jeremias et al., 2018; Maselli et al., 2017), indicating that daphnids may be capable of acclimating to experimental handling and methodology over time. In this study, no significantly enriched biological processes were observed between the F0 and F3 generations of OECD control daphnids ($p > 0.332$), while negative enrichment of several biological processes related to metabolism were observed in the DMSO controls (Table S4.3). It is expected that prolonged exposure to any chemical, including solvents such as DMSO, will result in measurable protein level responses, even if larger effects on organism function are not observed. Alterations of metabolic and housekeeping functions were reported in response to an order of magnitude greater concentration of DMSO than used in the present study during an exposure of *Epibacterium mobile* (Lozano et al., 2021), highlighting the importance of maintaining the use of solvents at the lowest concentrations possible. It is assumed that the changes observed across generations of chemically exposed daphnids are in response to the chemical stressor rather than acclimation to experimental conditions; therefore, the mechanisms behind the observed increases in reproduction in later generations of *D. magna* experiments remain unclear.

4.4.3 Implications

It is interesting to note that none of the enriched BPs were related to detoxification processes (Figure 4.5). While mild perturbations to superoxide dismutase and the superoxide anion generation process were observed, none of these changes were significant with the exception of the decreased enrichment observed in response to AVO in the F1 generation. Increases in oxidative stress response factors in response to OXY have been reported in *Epibacterium mobile* (Lozano et al., 2021), *Brassica rapa* L. spp. *chinensis* (Li et al., 2023b), and *Carassius auratus*, with the latter also demonstrating increases in relevant UVF detoxification pathways (Zhang et al., 2020). In several other studies measuring broad changes in subcellular responses to UVF exposure, no appreciable changes in important detoxification pathways including enzymes such as cytochrome p450s, glutathione-S-

transferases, and carboxylesterases were observed (Lozano et al., 2021; Simons et al., 2022; Y.-K. Zhang et al., 2023; Ziarrusta et al., 2018b). Studies that directly targeted relevant detoxification enzymes have reported corresponding changes in enzyme activities in response to UVF exposure in *D. magna* (de Paula et al., 2022) and *Mytilus edulis* (Falfushynska et al., 2021), but not in *Chironomus riparius* (González and Martinez-Guitarte, 2018) or *Sericostoma vittatum* (Campos, 2017). Overall, this suggests that any alterations to detoxification pathways that may occur in response to UVF exposure may not be broad enough to be identified as a significant response pathway when using a more generalized approach such as proteomics or transcriptomics. Additionally, the development of larger detoxification capabilities may not be the energetically optimal solution in response to long-term contamination, as Chatterjee et al. (2019) reported a similar lack of enriched detoxification pathways responsible for the adaptation of *D. magna* to 3 generations of exposure to wastewater effluent, instead observing increased capacity for oxygen transport. Investment into mechanisms which prevent exposure or reduce the rate at which toxicants can enter into the organism may be a more favourable alternative, as organisms can invest their limited energetic resources into preventative mechanisms such as the potentially decreased integument permeability through increased chitin synthesis discussed above. If an organism's response is instead to increase detoxification capabilities, that would likely require energetic investments into biotransformation enzymes, excretion transporters for resulting metabolites, and cellular repair processes, as biotransformation of UVFs can generate metabolites of greater toxicity through bioactivation, which is known to occur in the case of OXY (Molina-Molina et al., 2008; Suzuki et al., 2005).

Overall, it would appear that the toxicity observed in the F0 generation of exposure is caused by a combination of metabolic disruptions that may interfere with how daphnids use their energetic resources. Although the results of this study suggest that UVFs, particularly OXY, can impart toxicity to *D. magna* as a novel toxicant, populations are capable of acclimating to long-term exposure through

gradual modifications of a small number of protein pathways. Long-term contamination offers a stable and predictable environment, which can allow for parents to modify the phenotype of their offspring as an anticipatory parental effect, altering the phenotype of their offspring to be better suited to their local environment in an effort to improve fitness (Burgess and Marshall, 2014). In this case, it would appear that subtle changes in metabolic and immune processes, as well as a decrease in integument permeability through increased chitin production are sufficient to allow daphnids to regain normal physiological functions after 3-4 generations of continuous exposure; however, it is important to note that while *D. magna* populations are clearly capable of overcoming long-term exposure to single stressors, the physiological changes required to do so may come at the cost of increased sensitivity to a secondary stressor. It is unclear which of the enriched pathways identified in this study are the leading causes of the observed acclimation due to the timing of sample collection for proteomic analysis occurring at the end of each generation, excluding all daphnids succumbing to mortality. A future study investigating the exact timing that each of the observed changes occurs in response to the exposure would be valuable in identifying the primary mechanism of physiological acclimation in *D. magna*.

4.4.4 Conclusions

The exposure doses used for each UVF in this study are above the level of contamination reported in the majority of aquatic environments (Cadena-Aizaga et al., 2020; Carve et al., 2021; Kwon and Choi, 2021; Mitchelmore et al., 2021). While the toxicity observed in the first generation of exposure is consistent with previous studies that suggested that these UVFs can pose a threat to invertebrate species (Boyd et al., 2021; Downs et al., 2016; Giraldo et al., 2017), the observed acclimations over subsequent exposure generations suggest that the long-term risk of UVF contamination is lower than originally believed. The lack of toxicity within an exposed population after 3-4 generations of exposure are more relevant to the outcomes expected in a natural environment due to the likelihood of prolonged exposure in most contamination scenarios. Considering the potential for *D.*

magna populations to physiologically adapt to prolonged exposure to a variety of contaminants across generations (Chatterjee et al., 2019; Hochmuth et al., 2015; Leblanc, 1982; Maggio and Jenkins, 2022; Muyssen and Janssen, 2001; Poulsen et al., 2021; Ward and Robinson, 2005), it is apparent that a long-term approach is required to adequately assess the long-term threats posed against wild populations of organisms that the current debate on UVF bans are seeking to protect.

4.5 Figures and tables

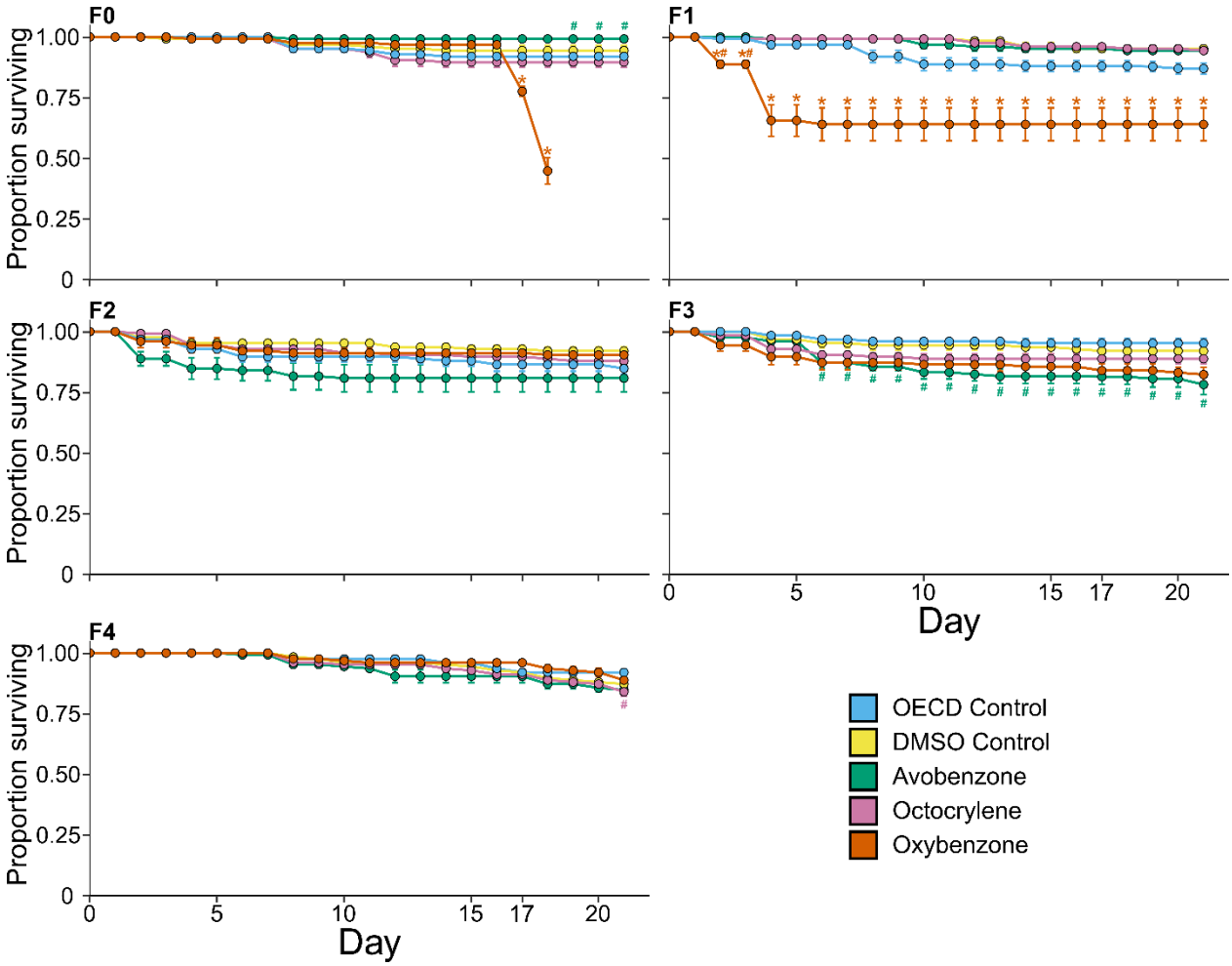


Figure 4.1. Survivorship curves of *D. magna* exposed to UV filter treatments over 21 days across 5 generations (F0 – F4). < 24 h old neonates were collected on the 17th day of exposure to begin each subsequent generation. Dots represent the mean ± SEM of 5 replicates of 25 exposed daphnids per treatment. Significant differences from the OECD and DMSO controls are represented by # and *, respectively (Kruskal-Wallis, $p < 0.05$).

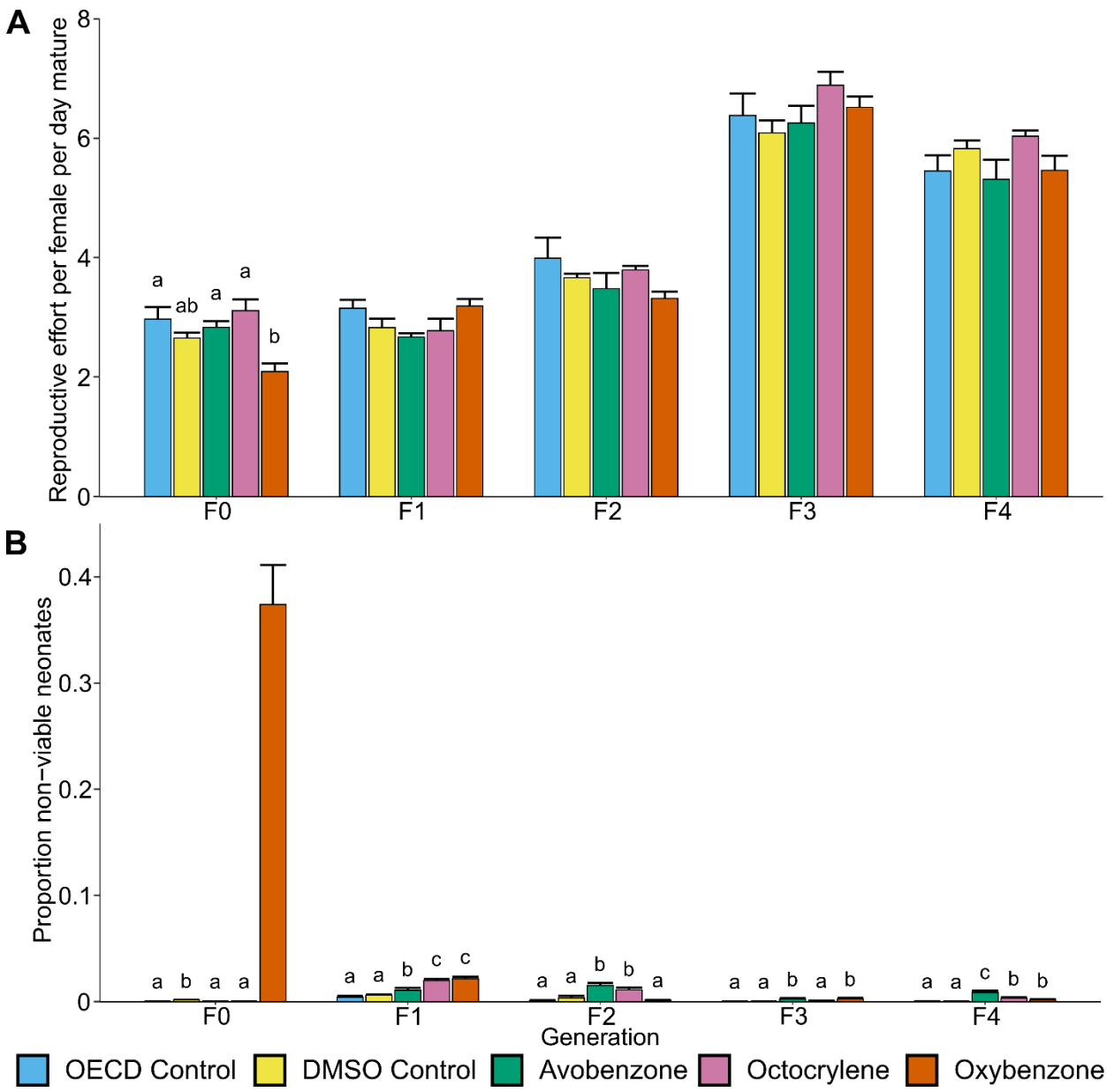


Figure 4.2. The average reproductive effort (viable neonates + non-viable neonates + unhatched eggs) of surviving female daphnids per day since the first daphnids reproduced starting on day 9 (A) and the proportion of reproductive effort resulting in non-viable neonates (B). < 24 h old *D. magna* neonates were exposed to UV filters for 21 d, with new exposure generations beginning on the 17th day of exposure across 5 generations (F0 – F4). Bars represent the mean ± SEM of 5 replicates of 25 daphnids per treatment. Different letters indicate significant differences between treatments within a generation at $p < 0.05$ (A: one-way ANOVA, B: Kruskal-Wallis).

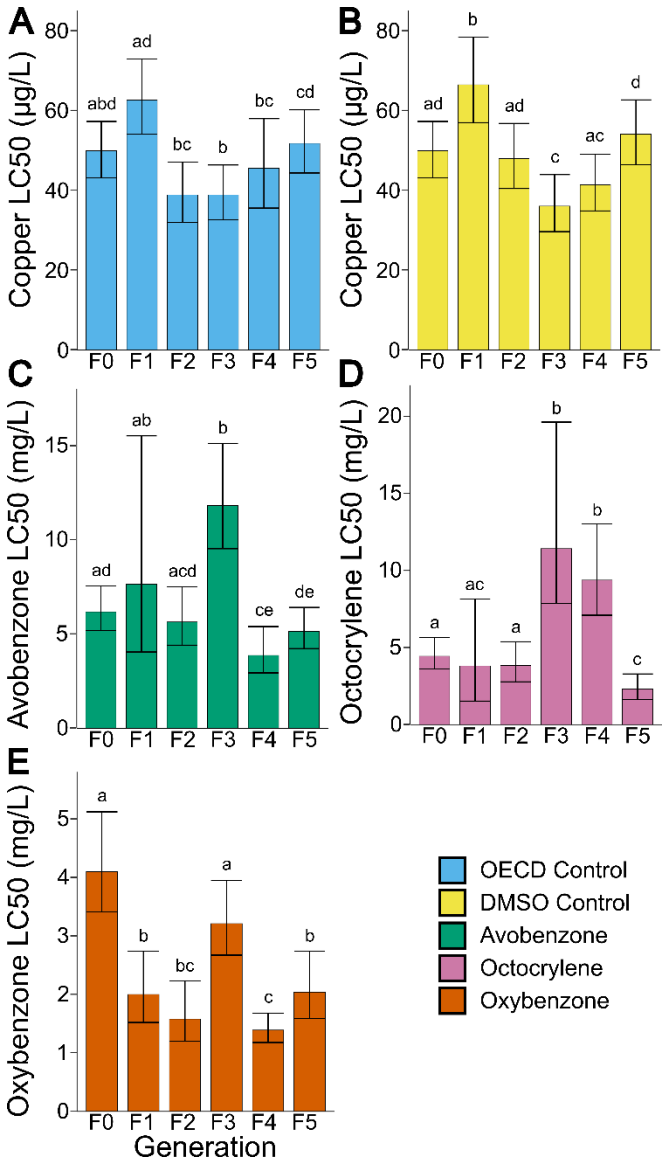


Figure 4.3. 48 h median lethal concentrations (LC50) of < 24 h old *D. magna* neonates exposed to copper or UV filters. < 24 h old neonates from the 2nd brood were obtained from 13 d old unexposed parents (F0), or from cultures exposed for up to 5 generations (F1 – F5) to an OECD water control (A), a DMSO solvent control (B), or UVFs (C-E). Treatment colouration indicates the exposure history of the parents that the neonates were obtained from. Bars represent the LC50 and the 95% confidence interval estimated using the probit method, representing 4 replicates of 5 exposed daphnids per test concentration (see Table S1). Different letters above each bar indicate significant differences between generations as determined by pairwise ratio tests with a Benjamini & Hochberg correction ($p < 0.05$).

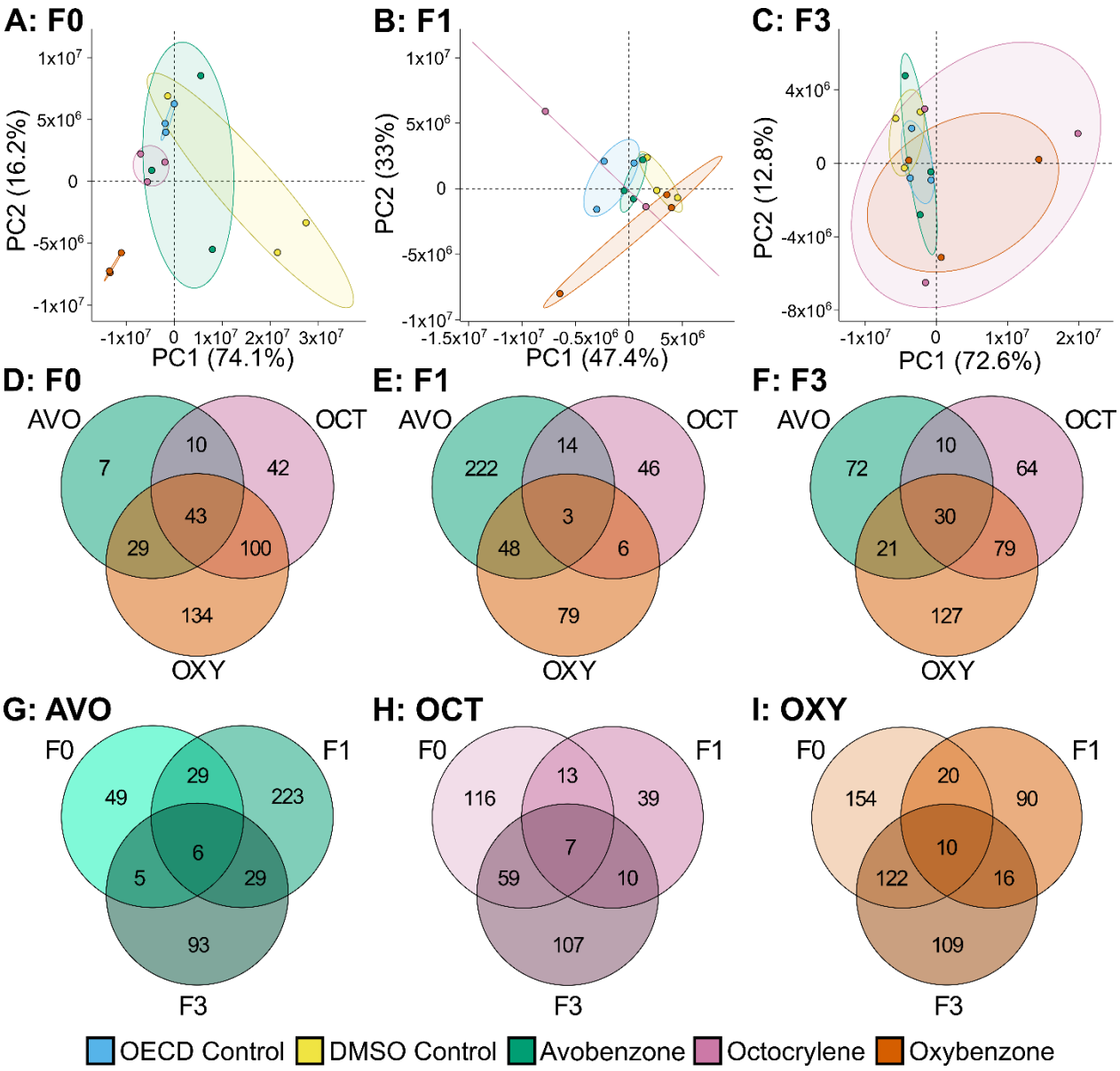


Figure 4.4. Principal component analysis of all quantified proteins from 21 d old daphnids (18 d for OXY F0) collected at the end of the F0 (A), F1 (B), and F3 (C) generations of exposure to UV filters. Each point represents 4 randomly selected females collected from the same exposure replicate that were pooled for protein quantification. Venn diagrams demonstrating the overlap between significantly changing proteins detected in each UVF with respect to the DMSO control are depicted within each generation (D – F), and across each generation within a single UVF treatment (G – I).

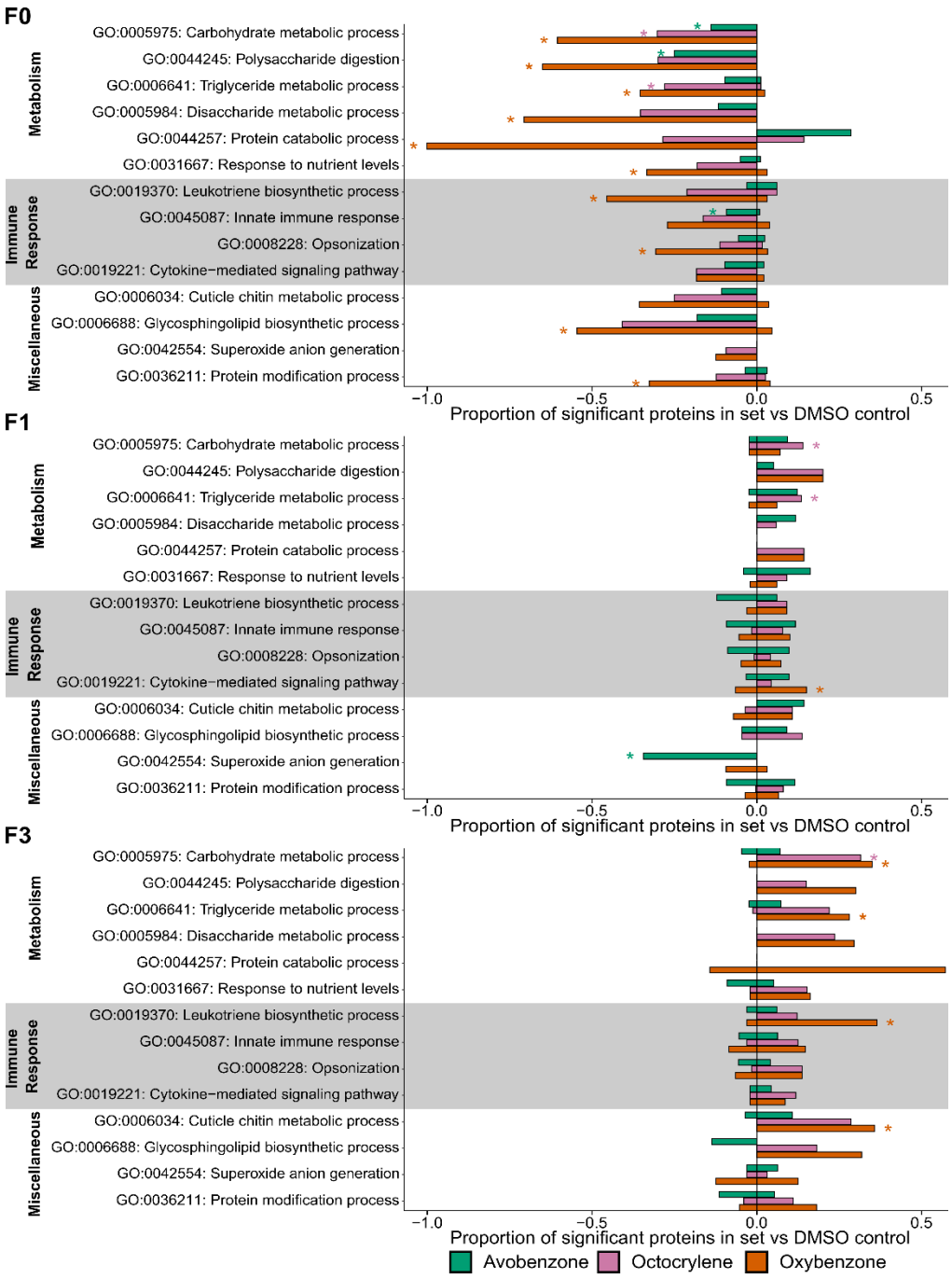


Figure 4.5. Summarized gene ontology (GO) for select biological processes for 21 d old daphnids (18 d for OXY F0) from the F0, F1 or F3 generation of UV filter exposure. Related GO terms were grouped under a single representative term using REVIGO version 1.8.1 (see Table S3) and assigned to a general function (bold labels on left, shaded regions). Bars represent the proportion of all quantified proteins belonging to each biological process that significantly differed in abundance from the DMSO control (Fisher's exact test FDR < 0.05). Asterisks indicate biological processes with increasing protein abundance (positive values) or decreasing protein abundance (negative values).

4.6 Supplementary materials

Table S4.1. Concentrations used during 48 h median lethal concentration (LC50) tests. For each concentration listed, 4 replicates of 5 < 24 h old *D. magna* neonates were exposed for 48 h. All values are expressed in units of mg/L.

Test chemical	Concentrations tested
Copper	0.005, 0.01, 0.02, 0.05, 0.075, 0.1, 0.2
Avobenzone	0.1, 0.2, 0.5, 1, 2, 5, 10, 20
Octocrylene	0.1, 0.2, 0.5, 1, 2, 5, 10, 20
Oxybenzone	0.1, 0.2, 0.5, 1, 2, 5, 10, 20

Table S4.2. Gene ontology (GO) terms for significantly altered proteins that were summarized under the representative biological processes shown in Figure 5. REVIGO version 1.8.1 was used with list size set to 0.5, with all other settings set to default to summarize GO terms under representative biological processes.

Representative biological process	Number of GO terms	Summarized GO terms
GO:0005975 – carbohydrate metabolic process	1	GO:0005975
GO:0044245 – polysaccharide digestion	6	GO:0044245, GO:0001951, GO:0035812, GO:0003096, GO:0044241, GO:0030421
GO:0006641 - triglyceride metabolic process	34	GO:0006641, GO:0006507, GO:0006656, GO:0006665, GO:0006672, GO:0006684, GO:0016255, GO:0036092, GO:0046513, GO:0006631, GO:0006635, GO:0006661, GO:0008610, GO:0008654, GO:0016042, GO:0033539, GO:0044242, GO:0044255, GO:0046854, GO:0006497, GO:0006506, GO:0006644, GO:0006650, GO:0008202, GO:0009395, GO:0030258, GO:0032049, GO:0036149, GO:0046474, GO:0046486, GO:0046503, GO:1901292, GO:0035965, GO:0006646
GO:0005984 - disaccharide metabolic process	9	GO:0005984, GO:0005993, GO:0009313, GO:0005991, GO:0005985, GO:0005989, GO:0009312, GO:0005992, GO:0005986
GO:0044257 - protein catabolic process	1	GO:0044257
GO:0031667 - response to nutrient levels	18	GO:0031667, GO:0007584, GO:0009267, GO:0032094, GO:0033280, GO:0042149, GO:0042262, GO:0042594, GO:0051593, GO:0033273, GO:0033189, GO:0033197, GO:0051780, GO:0071305, GO:0051365, GO:0061771, GO:0031669, GO:1990928
GO:0019370 - leukotriene biosynthetic process	6	GO:0019370, GO:0006691, GO:0001516, GO:0006693, GO:0019369, GO:0019371
GO:0045087 - innate immune response	16	GO:0045087, GO:0051673, GO:0009615, GO:0009617, GO:0009620, GO:0031640, GO:0032496, GO:0035821, GO:0042742, GO:0050829, GO:0050830, GO:0051707, GO:0071219, GO:0071222, GO:0098542, GO:0098586
GO:0008228 - opsonization	16	GO:0008228, GO:0002446, GO:0002252, GO:0002474, GO:0006955, GO:0019882, GO:0019886, GO:0035745, GO:0042267, GO:0043312, GO:0001867, GO:0001913, GO:0002443, GO:0002444, GO:0006956, GO:0035010
GO:0019221 - cytokine-mediated signaling pathway	19	GO:0019221, GO:0035455, GO:0070741, GO:0034612, GO:0035722, GO:0038111, GO:0070106, GO:0070555, GO:0071345, GO:0071346, GO:0071347, GO:0071352, GO:0071353, GO:0071356, GO:0098761, GO:1990830, GO:1990869, GO:0035456, GO:0070498
GO:0006034 - cuticle chitin metabolic process	13	GO:0006034, GO:0006044, GO:0006032, GO:0009253, GO:0006023, GO:0006027, GO:0006164, GO:0006796, GO:0009117, GO:0019637, GO:0019693, GO:0030203, GO:0030212
GO:0006688 - glycosphingolipid biosynthetic process	8	GO:0006688, GO:0006683, GO:0006680, GO:0006685, GO:0006689, GO:0019376, GO:0030148, GO:0046514
GO:0042554 - superoxide anion generation	2	GO:0042554, GO:0050665
GO:0036211 - protein modification process	6	GO:0036211, GO:0006306, GO:0018106, GO:0070647, GO:0080009, GO:1990481

Table S4.3. Significantly enriched biological processes detected between the F0 and F3 generation of DMSO exposed daphnids (Fisher's exact test FDR < 0.05). GO terms were grouped under a single representative term using REVIGO version 1.8.1.

Representative biological process	Proportion of proteins in set significantly changing
GO:0005975 - carbohydrate metabolic process	-0.419
GO:0005984 - disaccharide metabolic process	-0.529
GO:0006641 - triglyceride metabolic process	-0.293
GO:0006683 - galactosylceramide catabolic process	-0.455
GO:0007040 - lysosome organization	-0.500
GO:0019220 - regulation of phosphate metabolic process	-0.223
GO:0031667 - response to nutrient levels	-0.263
GO:0044245 - polysaccharide digestion	-0.450

Chapter 5: Differential development of individual and group exposed *Daphnia magna* persists across generations

Boyd, A., Blewett, T.A., 2025. Differential development of individual and group exposed *Daphnia magna* persists across generations. In prep for Proceedings of the Royal Society B.

5.1 Introduction

The goal of many toxicology studies is to understand complex environmental and ecosystem level outcomes. This is often achieved by using simple toxicity models, as resource constraints prevent experiments from achieving the level of complexity present in natural environments and the nature of many studies requires simplification of methods in order to account for confounding factors (Del Signore et al., 2016; van der Hoeven, 2004). In order to identify potential mechanisms of toxicity, detailed knowledge of specific biological pathways and functions are required, leading to the development of a standardized set of methodologies and model species (Carve et al., 2021; Persoone et al., 2009; Tkaczyk et al., 2021). This standardization has greatly reduced the differences between experiment methods and the potential for confounding variables to impact outcomes due to methodological differences (Baker, 2016), allowing for researchers to make comparisons between different scenarios, toxicants, and organisms.

Small modifications of these standardized methods are often made in order to better achieve the specific goals of a study. One common difference in toxicity studies is to use exposures of either multiple organisms in groups or isolated single organisms (Tincani et al., 2017). Group exposures allow for targeting population level endpoints, or the collection of tissues *en masse* for molecular level assays, while testing individual organisms allows researchers to link multiple traits together by accurately attributing them to the specific individual (Gust et al., 2016). Both methods provide benefits; however, despite researchers' best efforts to minimize the differences between these techniques, they may still subtly bias results. For example, social organisms may become stressed if exposed in isolation, potentially leading to altered behaviours or a stronger toxic response than if exposed in groups (Serra et al., 2007; Ward and Webster, 2016), while the reverse may be true for solitary organisms (Chelini et al., 2011; Schoepf and Schradin, 2013). Intraspecific competition may also lead to alterations in organism response that influence traits ranging from mortality to reproduction and

behaviour (Bezerra Da Silva et al., 2019; Faccoli, 2009; Gust et al., 2016; Noden et al., 2016; Tomas et al., 2005). Given the current replication crisis in academia, (Baker, 2016) it is important that these potential biases are understood so that data can be interpreted with the correct context, results from previous research can be replicated and accurate cross-study comparisons can be made.

Individual and group exposures have been widely used in many model species, including rodents (Chelini et al., 2011), fish (Tincani et al., 2017), and invertebrates such as *Daphnia magna* (Baldwin et al., 1995). A previous study by Gust et al. (2016) observed greater toxicity of metals in group exposed *Daphnia*, proposing that intraspecific competition led to an increase in organism sensitivity. Many studies reporting changes caused by the presence of conspecifics have largely attributed these effects to the costs associated with competing for access to a shared pool of resources (Bezerra Da Silva et al., 2019; Rinkevich and Loya, 1985). In contrast, Lowes et al. (2021) demonstrated that this effect may be driven by differences in exposure volumes and the relative evaporation rate rather than the number of organisms per container over a 48 h period. Regardless of the number of organisms exposed per container, studies tend to maintain a constant ratio of food and volume per individual by scaling up the guidelines outlined by various entities promoting methodological standardization, including the Organization for Economic Co-operation and Development, Environment and Climate Change Canada, and the United States Environmental Protection Agency; therefore, it is important to better understand what the effects of this study design may be, if any.

The objective of this study was to identify how exposure to toxicants individually or in groups alters *Daphnia magna* developmental characteristics and their sensitivity to toxicants. This was done by comparing data obtained from two concurrent experiments continuously exposing daphnids to ultraviolet filters (UVFs) avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY) over 5 generations utilizing individual organism exposures (Boyd et al., 2023a) or groups of *Daphnia* (Boyd et

al., 2025, in prep). As both of these studies were conducted using similar methods, densities, diet rations, and stressors, the existing data from these experiments can be leveraged to identify any effects that arose due to the number of organisms per container (1 vs 25). This data allows for comparisons to be made across multiple toxicants for a long experimental duration, allowing for potential method biases to be identified to provide additional context for future cross-study comparisons.

5.2 Materials and methods

5.2.1 Daphnia colony maintenance

Gravid *Daphnia magna* were purchased from Aquatic Research Organisms (US, November 2021) to create a colony maintained within the University of Alberta Biological Sciences Department comprised of groups of n = 30 < 2-month-old daphnids. *Daphnia* were held in dechlorinated water from the City of Edmonton at approximately pH 7.6, which was modified according to OECD guidelines to contain 294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$, and 5.80 mg/L KCl (OECD, 2012). This water is referred to as OECD water in the following text, and the composition has been previously characterized in Boyd et al. (2023b). All *Daphnia* were provided a diet of 3 mL of yeast, cereal leaf and trout chow (YCT) mix and 3 mL of 3 x 10^7 cells/mL of freshwater green algae (*Raphidocelis subcapitata*, Aquatic Research Organisms) to each group daily, supplemented once weekly with 100 µL of Roti-Rich invertebrate food (VWR, Canada). A 14 h:10 h photoperiod and a temperature of 21.4 ± 0.5 °C was maintained for the colony as well as all *Daphnia* used in experiments.

5.2.2 Exposure solutions

D. magna were exposed to solutions of individual organic ultraviolet filters (UVFs), including avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY). Stocks were produced by dissolving individual UVFs into dimethyl sulfoxide (DMSO), followed by diluting to the desired concentrations of UVFs in OECD water. All UVF exposure solutions were dosed to contain 0.003% v/v DMSO, and a

solvent control group was run for all experiments containing 0.003% v/v DMSO in OECD water. An additional control group of OECD water without additions was included for all experiments as well. To minimize the degradation of solutions, all stocks and exposure solutions were freshly remade every 12 days on average. The same exposure solutions were used for both the individual exposure experiment and the group exposure experiment, at concentrations of 6.59 ± 0.735 µg/L AVO, ~0.6 µg/L OCT and 16.5 ± 7.80 µg/L OXY as measured by ultra-performance liquid chromatography – triple quadrupole mass spectrometry and reported in Boyd et al. (2023a). OCT concentrations were present between the limit of detection (0.3 µg/L) and the limit of quantification (0.9 µg/L) and were considered to be present at the average of these two values. Prior to the start of experimentation, glassware was cleaned via 24 h submersion in 5% HNO_3 and 24 h submersion in 10% EtOH. Distilled water rinses occurred before and after each submersion. During experiments, overnight submersion in 10% EtOH followed by a distilled water rinse occurred between uses of glassware to remove residual organic UVFs.

5.2.3 Individual exposures

A series of 21 d reproductive tests following modified OECD test 211 guidelines were performed on *D. magna* neonates (OECD, 2012). Briefly, n = 30 < 24 h old neonates were individually exposed to one of five solutions of AVO, OCT, OXY, OECD control or DMSO control outlined in section 5.2.2 for 21 d, referred to as the chemical treatment in the following text. Daphnids were scored daily for mortality, molting, and reproductive effort, which was the sum of living neonates, unhatched eggs released and dead neonates at the time of scoring, with 100% water changes performed every 2 days. On day 15, when the majority of individually exposed *Daphnia* released their 3rd brood, all 3rd brood neonates were collected and pooled within each treatment, with 30 neonates randomly selected for the next generation of 21 d exposures, repeated for a total of 5 generations of continuous exposure (F0 – F4). All individually exposed daphnids were maintained in 20 mL of exposure solution in uncapped glass scintillation vials and fed a daily ration of 100 µL YCT mix and 100 µL green algae.

Daphnids were fasted beginning on day 19 for the final 48 h of exposure so that metabolic rate measurements could be performed. Refer to Boyd et al. (2023a) for more information regarding the methods and results of the individually exposed *Daphnia* experiment.

5.2.4 Group exposures

A similar experiment was performed concurrently to the individual exposure experiment outlined in section 5.2.3. Group exposures of < 24 h old *D. magna* neonates were performed with a total of 5 replicates of 25 individuals each all five treatment groups outlined above. Water and food were scaled to the population of each group, with rations of 20 mL of exposure solution and 100 µL YCT mix and 100 µL green algae provided for each living daphnid per day to match the conditions of the individual exposure experiment, to a maximum of 500 mL of water and 2.5 mL of each food type per replicate for populations that had not experienced any mortalities. Groups were housed in uncovered 600 mL glass beakers and scored daily for reproductive effort and mortality. Due to the consistent cyclical nature of *Daphnia* reproduction, broods were considered in 4 d windows as the number of broods released by each individual daphnid could not be tracked due to the nature of the group exposure (Figure 5.4). At the peak of the 3rd brood on day 17, all neonates were collected and pooled within treatments prior to the selection of 5 replicates of 25 individuals to start the next generation of 21 d exposures, repeated for 5 generations. Metabolic rate measurements were not performed on group exposed *Daphnia*; therefore, no fasting occurred on days 19-21. Refer to Chapter 4 above for more information regarding the methods and results of the group exposed *Daphnia* experiment.

5.2.5 Data analysis and statistics

Due to the nature of exposing groups of *Daphnia*, reproduction specific to each daphnid within a replicate could not be assessed; therefore, the amount of reproduction per each surviving female was

averaged across the number of days that the daphnid could be considered to be reproductively mature. The start of reproductive maturity was determined by the day at which daphnids of that exposure type began to reproduce, resulting in reproductive maturity being reached on day 9 for group exposed daphnids, and day 8 for individually exposed daphnids (Figure 5.4 B & C). To make comparisons of maturation time and brood frequency between individual and group exposed daphnids (referred to as exposure type in the following text), all daphnids within each group were assigned estimated brooding days. These were determined by summing the total reproduction of each 4-day brood and calculating the daily percentage of the brood released on each day of exposure per group replicate. This daily brood percentage was multiplied by the number of surviving female daphnids present for each day to determine the number of *Daphnia* estimated to have reproduced on each day, rounded to the nearest whole daphnid using the following equation:

$$\textbf{Formula 5.1: } X_n = \frac{R_n}{B_n} F_n$$

where X is the number of female daphnids reproducing on day n, R is the reproductive effort for day n, B is the total reproductive effort of the 4-day brood corresponding to day n, and F is the number of living females present on day n. To maintain a fair comparison, this method was applied to individually exposed *Daphnia* as well by considering all 30 individual replicates per treatment as one "group", with the corresponding 3-day brood windows outlined in Figure 5.4. The data produced for individually exposed daphnids by this model, referred to as "model data", was compared to the real observations recorded for this treatment type to validate and determine the biases of this model (refer to section 5.3.2 below; Figure 5.3), which found a small underestimation of both maturation time and brood frequency; therefore, group and individual exposures were compared using the model data when relevant to maintain a fair comparison between exposure types.

All statistical analyses were conducted using R version 4.3.2 (R Core Team, 2024). Lotka's equation was used to determine the intrinsic rate of population increase for each group exposed population (Lotka, 1913). To account for the differences in feeding that occurred between the individual and group exposed daphnids, statistical comparisons of mortality and reproduction excluded the 20th and 21st days of exposure, when the individually exposed daphnids were fasted. Assumptions of normality and homoscedasticity were tested via Shapiro Wilk and Levene's tests, respectively when necessary. Two-way repeated measure ANOVAs were performed to determine if the effects of each treatment group within each generation should be considered in the models comparing exposure types. Comparisons which indicated that only exposure type (i.e. individual vs group) yielded significant effects were pooled by chemical treatment and analyzed via one-way repeated measure ANOVAs with within generations, otherwise comparisons were made across exposure types within each chemical treatment and generation. When no chemical treatment effects were detected, figures are presented as the pooled data for all treatments; however, repeated analyses and figures separated by chemical treatment are available in the supplementary materials in these instances. Post-hoc pairwise t-test comparisons using the Benjamini-Hochberg correction were used to compare the exposure types within each generation and chemical treatment (when necessary). All values are reported as mean ± standard error of the mean, and significance was determined at $\alpha = 0.05$.

5.3 Results

5.3.1 Effects of chemical treatment and exposure type

Cumulative daphnid mortality over 19 days was similar across exposure types within chemical treatments and generations in most cases; however, mortality was slightly greater in group exposures when the per generation mortality was < 20% (Figure 5.1; two-way ANOVA, $p = 0.002$). Differences were observed between chemical treatments ($p = 0.012$), but no interactive effect of chemical treatment and exposure type was detected ($p = 0.404$). Group exposed *Daphnia* experienced approximately 5-fold

greater mortality to OXY in the F0 generation than individually exposed daphnids after 18 d; however, mortality in individually exposed daphnids increased to approximately the same level as grouped daphnids by the 21st day of exposure (data not shown).

The reproductive effort of surviving female daphnids per day of reproductive maturity was significantly decreased by 10.7 – 34.6% in group exposure types per starting population in all generations except for F3 (Figure 5.2 A; two-way ANOVA, $p < 0.0001$); however, there was no interaction between chemical treatment and exposure type ($p = 0.378$) nor any effect of chemical treatment ($p = 0.597$). Male daphnids were not observed in any treatment or generation of individual exposure; however, males were present in group exposures, increasing over each subsequent generation in all chemical treatments to a maximum of 10.6% of the population in the F4 generation (Figure 5.2 B; two-way ANOVA, $p < 0.001$). The intrinsic rate of population increase for each generation was lower in group exposures in all generations except for the F3 generation by an average of 0.0490 ± 0.0161 (Table 5.1; two-way ANOVA, $p < 0.001$). No effect was detected for chemical treatment ($p = 0.657$) or the interaction between chemical treatment and exposure type ($p = 0.689$).

5.3.2 Model validation

The time required for daphnids to release their first brood when comparing the data generated from the model to the experimentally derived data was similar, underestimating the maturation time by 0.218 ± 0.103 days on average across all generations; however, the data model assigned first brood days that were significantly earlier than the experimental data by 0.500 and 0.385 days in the F1 and F3 generations, respectively (Figure 5.3 A; two-way ANOVA, $p < 0.001$). The brood frequency determined by the model was 0.141 ± 0.0258 days fewer on average than the experimental data (Figure 5.3 B). This difference was statistically significant across all generations (two-way ANOVA, $p < 0.001$). These differences between the model data and the experimental data for the time to first brood and the brood

frequency were consistent across all chemical treatments, and no interaction between chemical treatment and data source was detected ($p > 0.241$).

Using the simulated data from the model to compare the individual and group exposure types revealed that *Daphnia* in group exposures reached reproductive maturity 1.14 ± 0.171 days later than daphnids in individual exposures (Figure 5.4 D; two-way ANOVA, $p < 0.001$), with no effect detected for chemical treatment ($p = 0.110$) or the interaction between treatment and exposure type ($p = 0.08$). A similar trend was observed with the brood frequency, as group exposures required 0.560 ± 0.0866 additional days between broods (Figure 5.4 E; two-way ANOVA, $p < 0.001$). No interaction was detected between chemical treatment and exposure type ($p = 0.068$), but an effect of chemical treatment was detected ($p < 0.001$). Repeating the analysis with each chemical treatment individually revealed a similar pattern of more days required between broods in group exposed daphnids across all chemical treatments and generations, ranging between 0.110 – 0.907 days (Figure S5.6; $p < 0.004$).

5.4 Discussion

For a detailed discussion of the toxicity of AVO, OCT, and OXY, refer to Boyd et al. (2023a) for physiological and behavioural outcomes in individually exposed daphnids, and Chapter 4: Getting over it? A proteomic analysis of mechanisms driving multigenerational acclimation to organic ultraviolet filters in *Daphnia magna* for physiological and proteomic alterations in group exposed daphnids.

From the data, it is apparent that exposure type did not influence the overall toxicity of UVFs to *Daphnia magna*, as both individual and group exposure types produced similar trends in various toxicity-indicative physiological responses such as mortality, reproduction, and maturation time across chemical treatments. The exception to this is the F0 generation of OXY exposure, which caused 5-fold greater mortality in group exposures after 18 d (Figure 5.1). While the mortality in the individually exposed daphnids reached a similar level by the 21st day of exposure, it is important to note that the

group F0 OXY exposure was terminated after 18 d to ensure the collection of sufficient tissues for proteomic analysis; therefore, it is unclear if the overall mortality would be greater in group exposures after 21 d, or if the mortality simply occurred a few days earlier than in individually exposed daphnids. It is interesting to note that when treatments and generations with > 20% mortality are excluded (F0 and F1 OXY), the mortality of all remaining treatments was more than double when daphnids were exposed in groups (9.98 ± 0.636%) than individually (4.07 ± 0.726%), indicating that exposure in groups may increase the "baseline" of mortality observed regardless of chemical treatment. A previous study by Gust et al. (2016) cited intraspecific competition as the cause of increased group mortality in both Cu and Pb exposures over 14 days when 20 *D. magna* were exposed at the same density and food ration as single daphnids, although the effect size (~3 – 5 fold increase) was greater than in the present study. Earlier life stages of *Daphnia magna* have also been observed to be more sensitive than adults to high organism densities, leading to higher overall mortality early in experiments (Viaene et al., 2015). While the overall 21 d mortality is low and well within the 20% threshold deemed acceptable for controls under OECD guidelines (OECD, 2012), the large relative difference between treatment types could be indicative of increased stress in group exposed daphnids.

The higher initial mortality of group exposed daphnids is reflected in the reproductive characteristics depicted in Figure 5.2 A and Table 5.1. The average reproduction per day that each surviving female daphnid was reproductively mature and intrinsic rate of population increase was consistently decreased in group exposures across all generations except for the F3 generation, suggesting that groups of daphnids may face increased stress that reduces the resources available for reproduction. Similarly, using the modeled data to compare the time to reach reproductive maturity and the frequency of reproduction revealed consistently large differences in *Daphnia* reproductive development due to the presence of conspecifics (Figure 5.4). The delay of reproductive maturity by more than 1 day accompanied with the decrease in brood frequency from approximately 3 days for

individuals to 3.5 days for groups resulted in consistent decreases in the intrinsic rate of population increase (Table 5.1). Cleuvers et al. (1997) noted similar delays in maturation and brood timing in group exposed *D. magna* as those reported in the present study; however, their results differ in that observed deviations only appeared in the offspring of parents raised in a group environment. Intraspecific competitive pressures have been observed to decrease growth and reproduction in corals (*Stylophora pistillata*), with negative effects disappearing if conspecifics experienced mortality early in the observation period (Rinkevich and Loya, 1985). Similar responses have been observed in sea urchins (*Paracentrotus lividus*; Tomas et al., 2005), beetles (*Tomicus destruens*; Faccoli, 2009) and mosquitos (*Aedes aegypti* and *Aedes albopictus*; Noden et al., 2016), indicating that intraspecific competition can have a large influence on the life history traits of a variety of invertebrates.

It is important to note that many of these studies investigating the effects of intraspecific competition utilize varying organism densities and food rations across treatment groups (Gust et al., 2016; Knillmann et al., 2012), while this study maintained a constant ratio of organisms:water:food specifically to minimize the effects of intraspecific competition, yet effects of conspecific presence were observed, indicating that some competitive pressures may still exist despite the scaling of resources to population size. While *D. magna* are not known to be a social species, social interactions can still occur between conspecifics regardless of a species' predisposition towards social interactions. Individually exposed organisms are guaranteed the same allotment of space and food, while an equivalent ratio of resources in group exposures can be disrupted by dominant individuals consuming more than their fair share of food (Ward and Webster, 2016). Group living can impart costs to organisms through social stress as individuals must expend additional energy interacting and competing with others (Schoepf and Schradin, 2013; Ward and Webster, 2016). This mild increase in stress within groups could lead to the shift in physiological baselines observed in this study. Negative interference due to conspecific cues have been shown to inhibit *D. magna* reproduction at densities lower than that

of the present study despite constant food concentrations (Goser and Ratte, 1994). DeLong et al. (2014) observed that density negatively influences metabolic rate of several species including *Daphnia ambigua* in the absence of food, proposing that the presence of conspecifics directly affects metabolic rate regardless of interference competition for food resources.

Conspecific presence similarly appeared to promote the development of male daphnids in group exposures, as virtually all daphnids were female in the F0 generation of both group and individual exposures, but the proportion of males within the population steadily increased across generations of groups exposures while zero individually exposed daphnids were male at any point of the study (Figure 5.2 B). *Daphnia magna* and other cyclic parthenogens rely on environmental cues for sex determination (Cornetti and Ebert, 2021) and are capable of reacting to waterborne cues indicating the presence of conspecifics (Booksmythe et al., 2018; Goser and Ratte, 1994; Hobaek and Larsson, 1990). Low densities of males can positively influence the production of males over females, signalling that a sexual reproduction strategy can increase maternal fitness; however, the absence of males can negatively influence the production of males due to a lack of sexually reproducing females to mate with (Booksmythe et al., 2018; Galimov et al., 2021; Li et al., 2022).

A previous study has proposed that the effects of intraspecific competition in group exposures render animals more sensitive to toxicants (Gust et al., 2016). A subsequent study by Lowes et al. (2021) suggested that this was due to concentration of the exposure solution caused by proportionately greater evaporation in smaller exposure volumes scaled to fewer individuals per exposure group. While concentration of the UVF solutions due to evaporation could be a factor influencing the mildly greater toxicity observed in specific endpoints, consistent differences between groups and individuals were noted in OECD controls in the present experiment. As there was a lack of contaminant to concentrate in these controls, this indicates that the effects of intraspecific competition should be considered in

conjunction with evaporation effects when comparing data obtained from individual and group experiments.

In the present study, instances of toxicity are proportional to alterations in the control response (e.g., increased mortality in group controls corresponds with increased mortality in group OXY exposures; Figure 5.1). We propose that the effects of exposure in groups changes an organism's baseline condition; what may appear to be an increase in toxicity is instead a proportional change with respect to a change in response in control organisms. As the presence of conspecifics resulted in notable changes in *D. magna* physiology despite equal access to resources and space, these differences may be of greater consequence in environmental exposures alterations to an organism's baseline condition due to intraspecific competition has the potential to amplify the toxicity of contaminants if access to resources is limited. It is possible that these alterations in organism response may mask subtle changes in organism physiology or mislead a researcher to conclude that mild toxicity may be present in certain instances with improperly designed experiments. These results also suggest that experiments that seek to combine the benefits of individual and group exposure types to maximize the breadth of data obtained must be designed carefully so that individually tracked physiological traits are not directly compared to molecular data obtained from groups of *Daphnia* without consideration for how the baseline of each exposure type may differ. These results highlight the importance of including independent controls within every experiment that match the condition of experimentally manipulated organisms so that confounding factors such as intraspecific competition can be appropriately accounted for when making comparisons to studies with differing methodologies. Considerations should be made for future research that investigate hormones and other indicators of stress in non-resource limited groups to better understand the mechanisms of the observed changes in *Daphnia* physiology and extend these comparisons to other species that are commonly used for both individual and group exposures.

5.4.1 Conclusions

This study found consistent changes in the physiology of *Daphnia magna* as a result of exposure individually or in groups, regardless of the presence of toxicants and despite a constant ratio of food and water per organism. These differences persist across generations and are often of consistent magnitude (e.g. delayed reproduction and lower brood frequency in groups), with the exception of an increasing proportion of males in group exposures across generations. These results suggest that the baseline response of *D. magna* is altered in a variety of physiological traits as a result of exposure in groups, perhaps due to competitive influences from conspecifics. This study highlights the importance of considering the context of data when making cross-study comparisons, as alterations in methodology seemingly as minor as the number of organisms exposed can influence the perception of relative toxicity of test substances.

5.5 Figures and tables

Table 5.1. The intrinsic rate of population increase (d^{-1}) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). Data are shown as mean ± SEM, p values are shown for pairwise t-test comparisons within generations with the Benjamini-Hochberg correction.

	Generation				
Type	F0	F1	F2	F3	F4
Group	0.228 ± 0.0236	0.226 ± 0.0157	0.242 ± 0.0126	0.305 ± 0.0145	0.285 ± 0.0182
Individual	0.309 ± 0.0102	0.271 ± 0.0294	0.288 ± 0.0204	0.311 ± 0.00725	0.349 ± 0.00653
p-value	2.14×10^{-7}	6.87×10^{-5}	2.39×10^{-6}	6.64×10^{-1}	2.13×10^{-7}

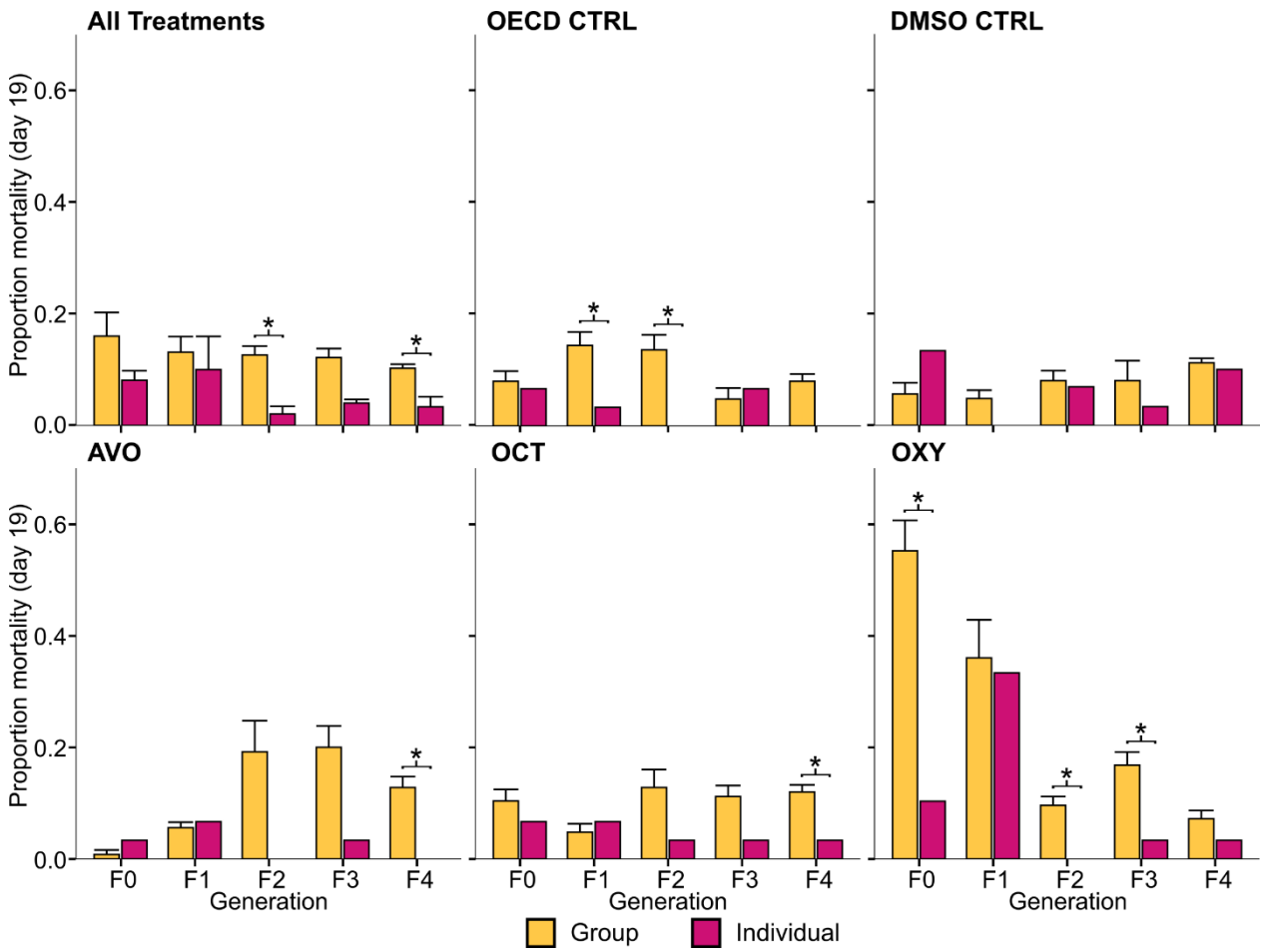


Figure 5.1. Mortality after 19 days of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). Individual exposures consisted of 30 replicates of single daphnids per treatment, while group type exposures consisted of 5 replicates of 25 daphnids per treatment. Bars represent mean ± SEM. Stars indicate a significant difference between the individual and group exposure mortality within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$). OXY F0 data depicts mortality on day 18 due to early termination of the group exposures for sample collection.

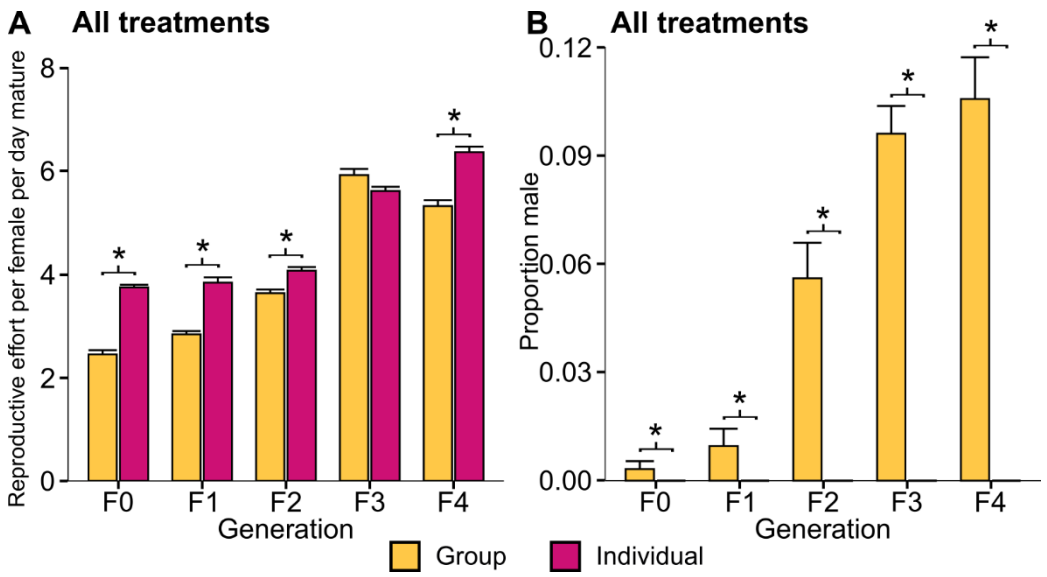


Figure 5.2. The average reproductive effort (viable neonates + unhatched eggs + non-viable neonates) of female *Daphnia magna* per day reproductively mature (A) and the proportion of male daphnids per starting population (B) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments across 5 generations (F0 – F4). Data are shown as mean ± SEM. Stars indicate a significant difference between the individual and group exposure treatment types within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$). All treatments were pooled for each comparison due to no significant effect of the chemical treatment, refer to Figure S5.1 & Figure S5.2 for figures depicting each chemical treatment separately.

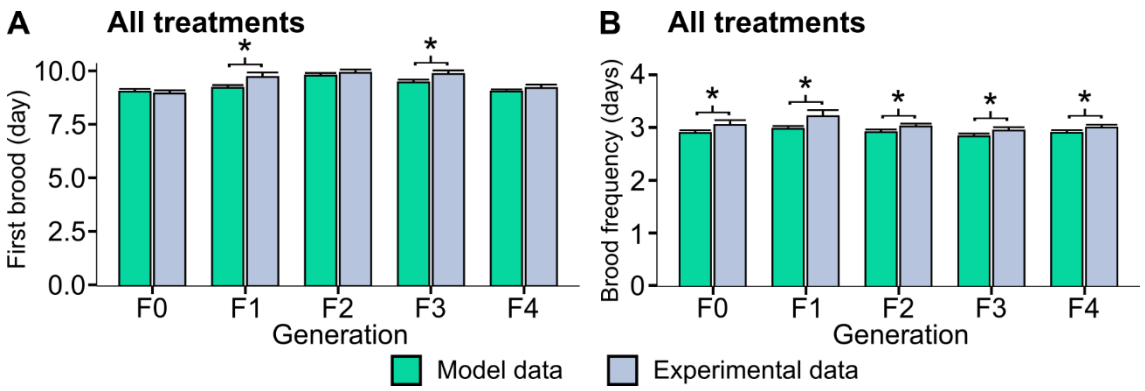


Figure 5.3. The number of days to reach reproductive maturity (A) and the number of days between broods (brood frequency) (B) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). "Experimental data" refers to the data obtained from 30 replicates of individually exposed *Daphnia* per treatment group and "Model data" refers to estimated data for individually exposed daphnids using the method described in section 5.2.5. Data are shown as mean ± SEM. Stars indicate a significant difference between the model and experimental data within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$). All treatments were pooled for each comparison due to no significant effect of the chemical treatment, refer to Figure S5.3 & Figure S5.4 for figures depicting each chemical treatment separately.

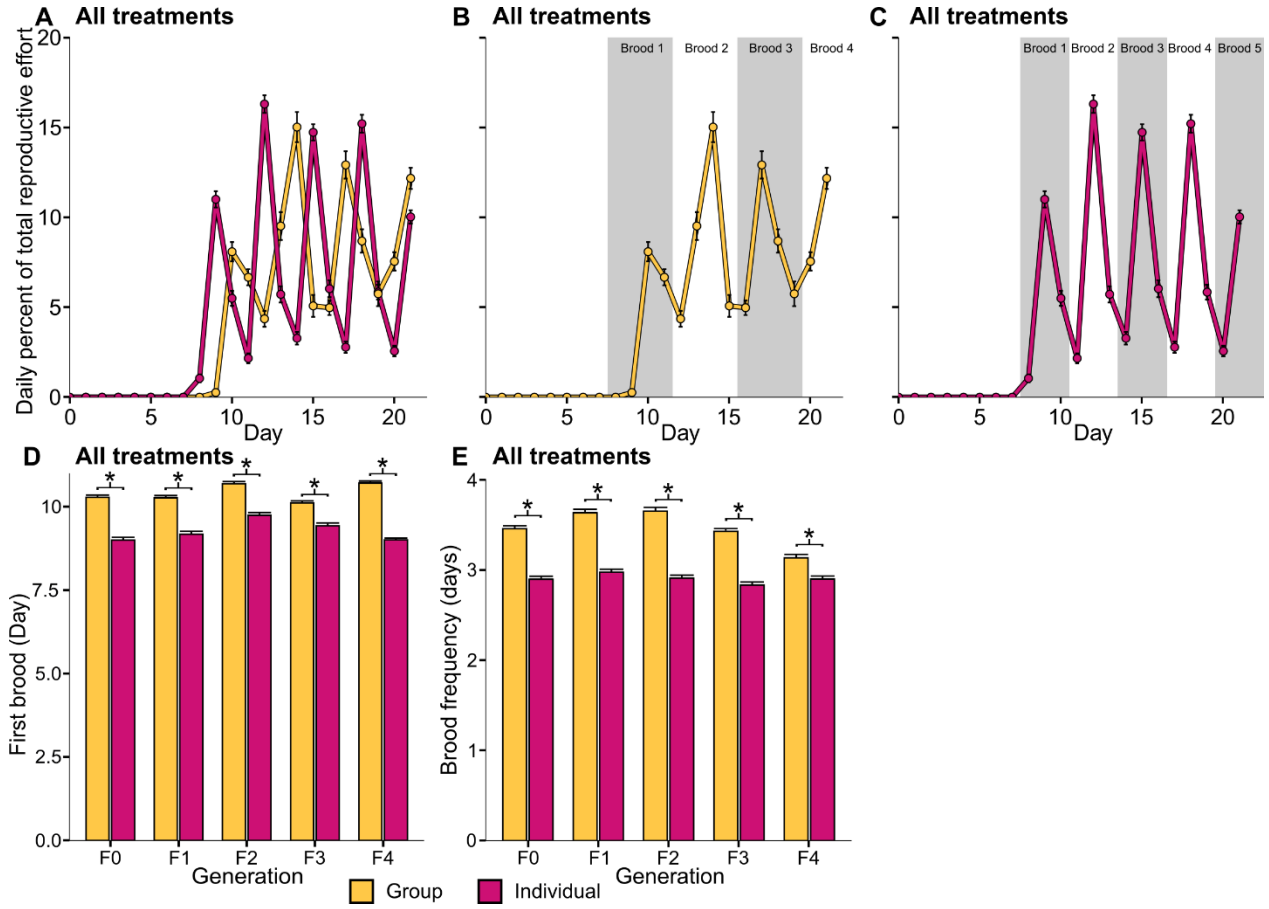


Figure 5.4. Daily reproductive effort (viable neonates + unhatched eggs + non-viable neonates) as a percentage of the total reproductive effort over 19 days of individually and group exposed *Daphnia magna* (A), with brood designations highlighted (B – C) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). The number of days to reach reproductive maturity (D) and the number of days between broods (brood frequency; E) are shown using the data estimation method described in section 5.2.5. Data are shown as mean ± SEM. Stars indicate a significant difference between the individual and group exposure treatment types within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$). All treatments were pooled for each comparison due to no significant effect of the chemical treatment, refer to Figure S5.5 & Figure S5.6 for figures depicting each chemical treatment separately.

5.6 Supplementary materials

Table S5.1. The intrinsic rate of population increase (d^{-1}) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). Data are shown as mean ± SEM, p values are shown for pairwise t-test comparisons within generation and chemical treatments with the Benjamini-Hochberg correction.

		Generation				
Chemical treatment	Type	F0	F1	F2	F3	F4
OECD control	Group	0.238 ± 0.0103	0.233 ± 0.0105	0.253 ± 0.0123	0.312 ± 0.00550	0.297 ± 0.00753
	Individual	0.310	0.284	0.285	0.324	0.354
	p-value	1.22×10^{-2}	1.22×10^{-2}	1.56×10^{-2}	1.88×10^{-2}	1.21×10^{-2}
DMSO control	Group	0.229 ± 0.0104	0.231 ± 0.0106	0.240 ± 0.00386	0.262 ± 0.00651	0.287 ± 0.00722
	Individual	0.322	0.313	0.286	0.310	0.356
	p-value	6.05×10^{-3}	6.05×10^{-3}	6.05×10^{-3}	1.45×10^{-2}	6.05×10^{-3}
AVO	Group	0.237 ± 0.0101	0.221 ± 0.0164	0.229 ± 0.0117	0.294 ± 0.0205	0.256 ± 0.0148
	Individual	0.312	0.269	0.269	0.305	0.347
	p-value	1.28×10^{-2}	1.28×10^{-2}	1.28×10^{-2}	3.13×10^{-1}	1.28×10^{-2}
OCT	Group	0.242 ± 0.0123	0.240 ± 0.00713	0.243 ± 0.00820	0.319 ± 0.00847	0.301 ± 0.00958
	Individual	0.307	0.256	0.273	0.308	0.340
	p-value	1.28×10^{-2}	1.86×10^{-2}	1.28×10^{-2}	5.69×10^{-2}	1.28×10^{-2}
OXY	Group	0.193 ± 0.0293	0.207 ± 0.0101	0.247 ± 0.0136	0.303 ± 0.0105	0.284 ± 0.00357
	Individual	0.293	0.234	0.329	0.310	0.350
	p-value	1.40×10^{-2}	1.48×10^{-2}	1.33×10^{-2}	2.34×10^{-1}	1.33×10^{-2}

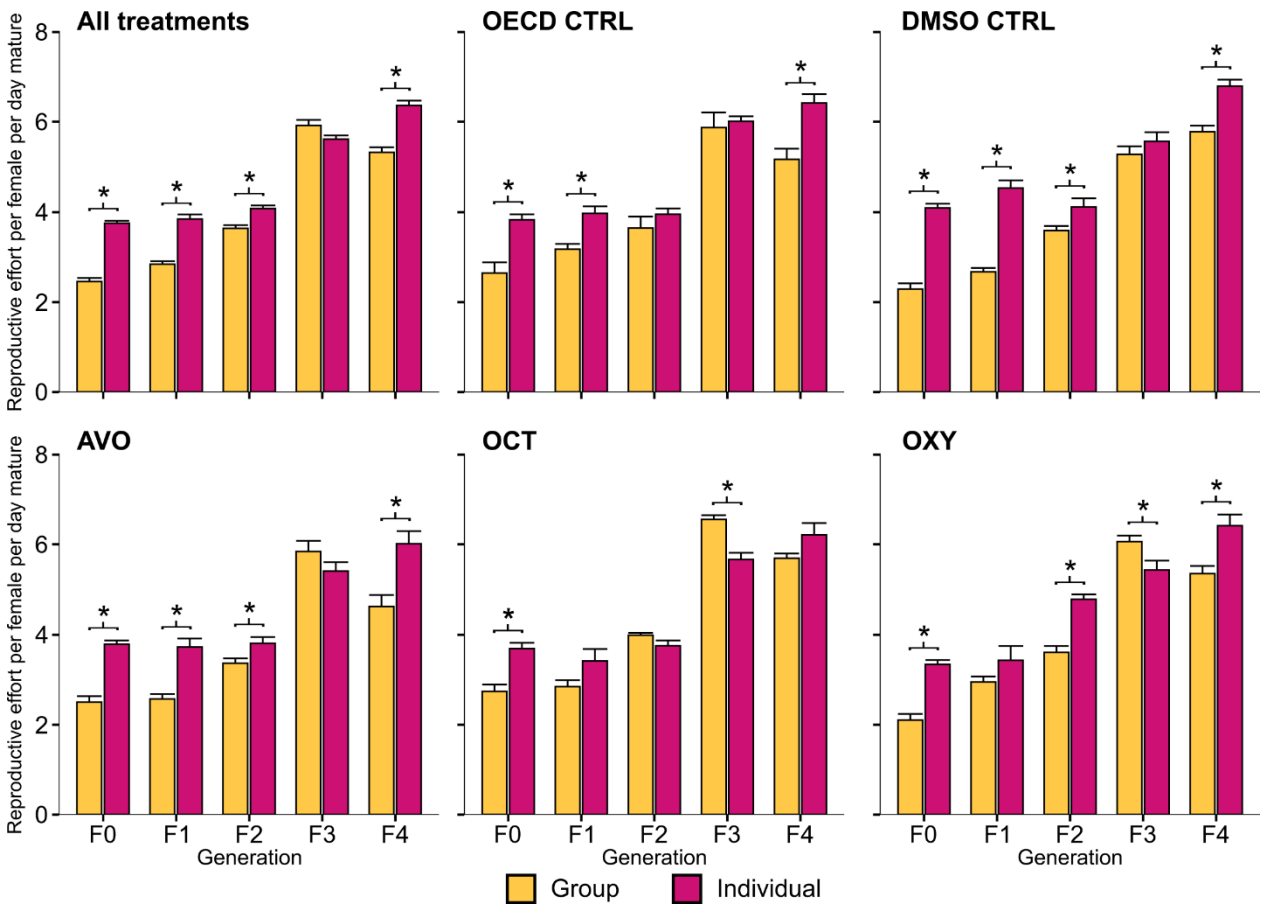


Figure S5.1. The average reproductive effort (viable neonates + unhatched eggs + non-viable neonates) of female *Daphnia magna* per day reproductively mature. Individual exposures consisted of 30 replicates of single daphnids per treatment, while group type exposures consisted of 5 replicates of 25 daphnids per treatment. Bars represent mean ± SEM. Stars indicate a significant difference between the individual and group exposure mortality within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

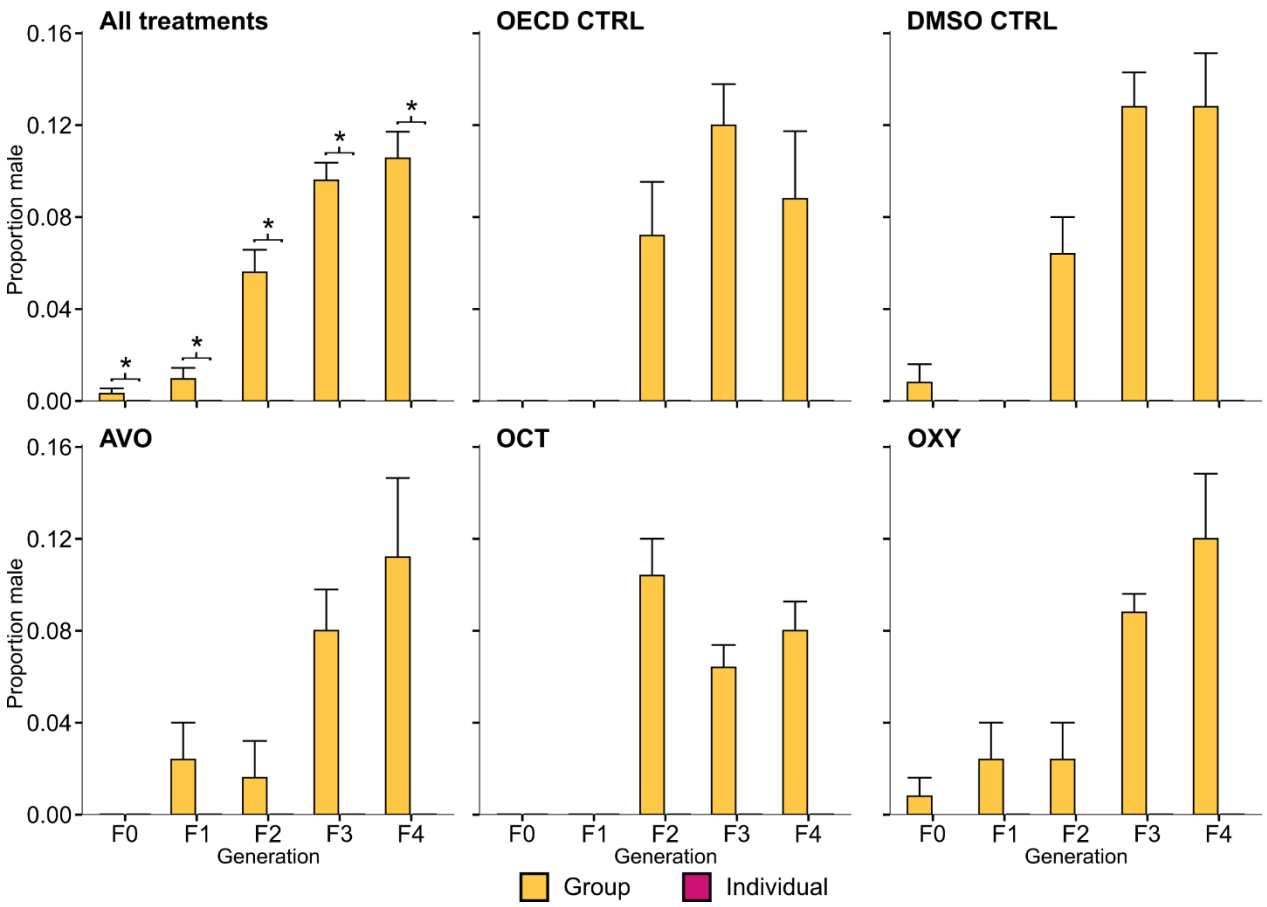


Figure S5.2. The proportion of male daphnids per starting population of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments across 5 generations (F0 – F4). Individual exposures consisted of 30 replicates of single daphnids per treatment, while group type exposures consisted of 5 replicates of 25 daphnids per treatment. Bars represent mean ± SEM. Stars indicate a significant difference between the individual and group exposure mortality within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

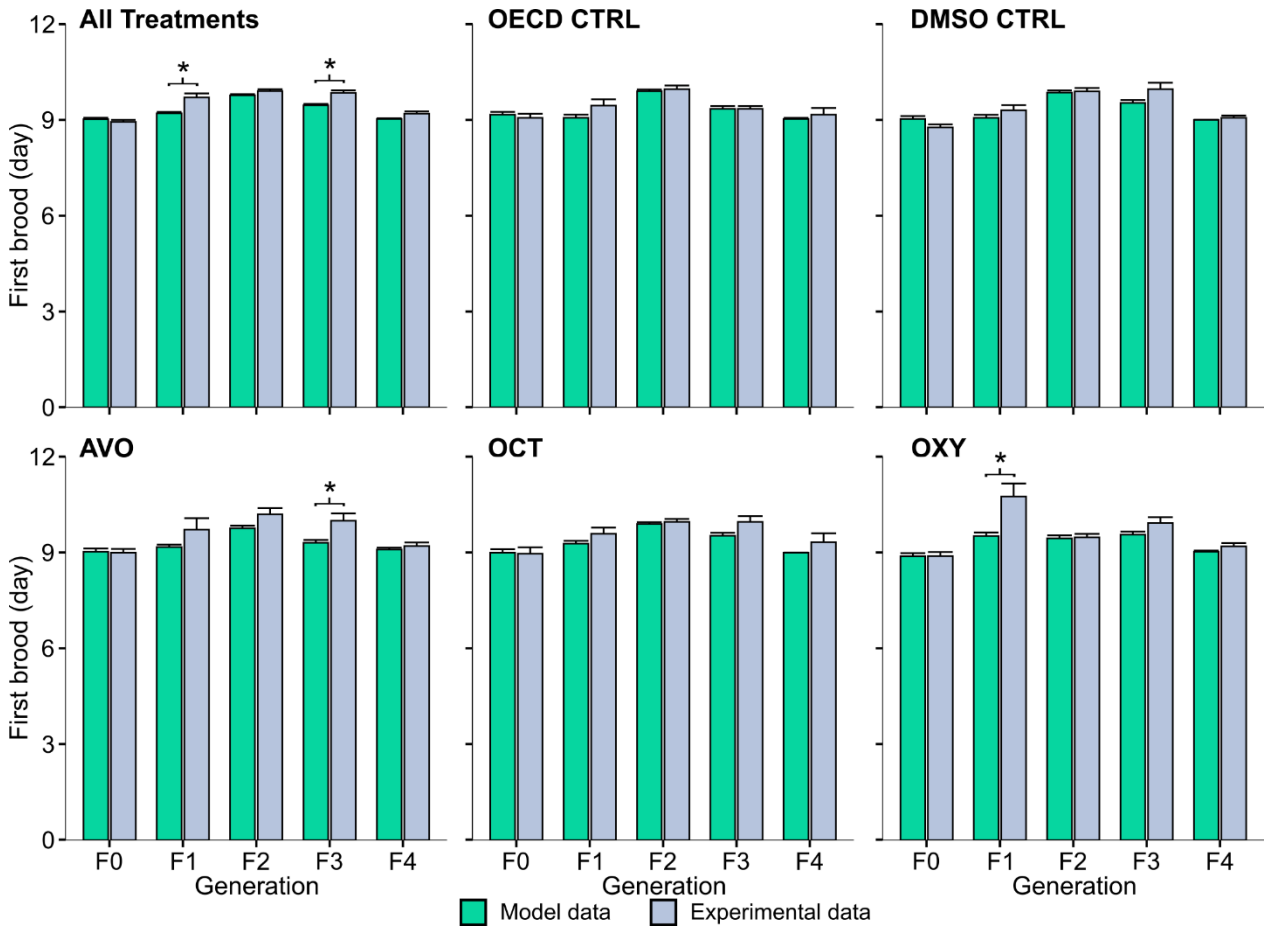


Figure S5.3. The number of days to reach reproductive maturity of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). "Experimental data" refers to the data obtained from 30 replicates of individually exposed *Daphnia* per treatment group and "Model data" refers to estimated data for individually exposed daphnids using the method described in section 5.2.5. Data are shown as mean ± SEM. Stars indicate a significant difference between the model and experimental data within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

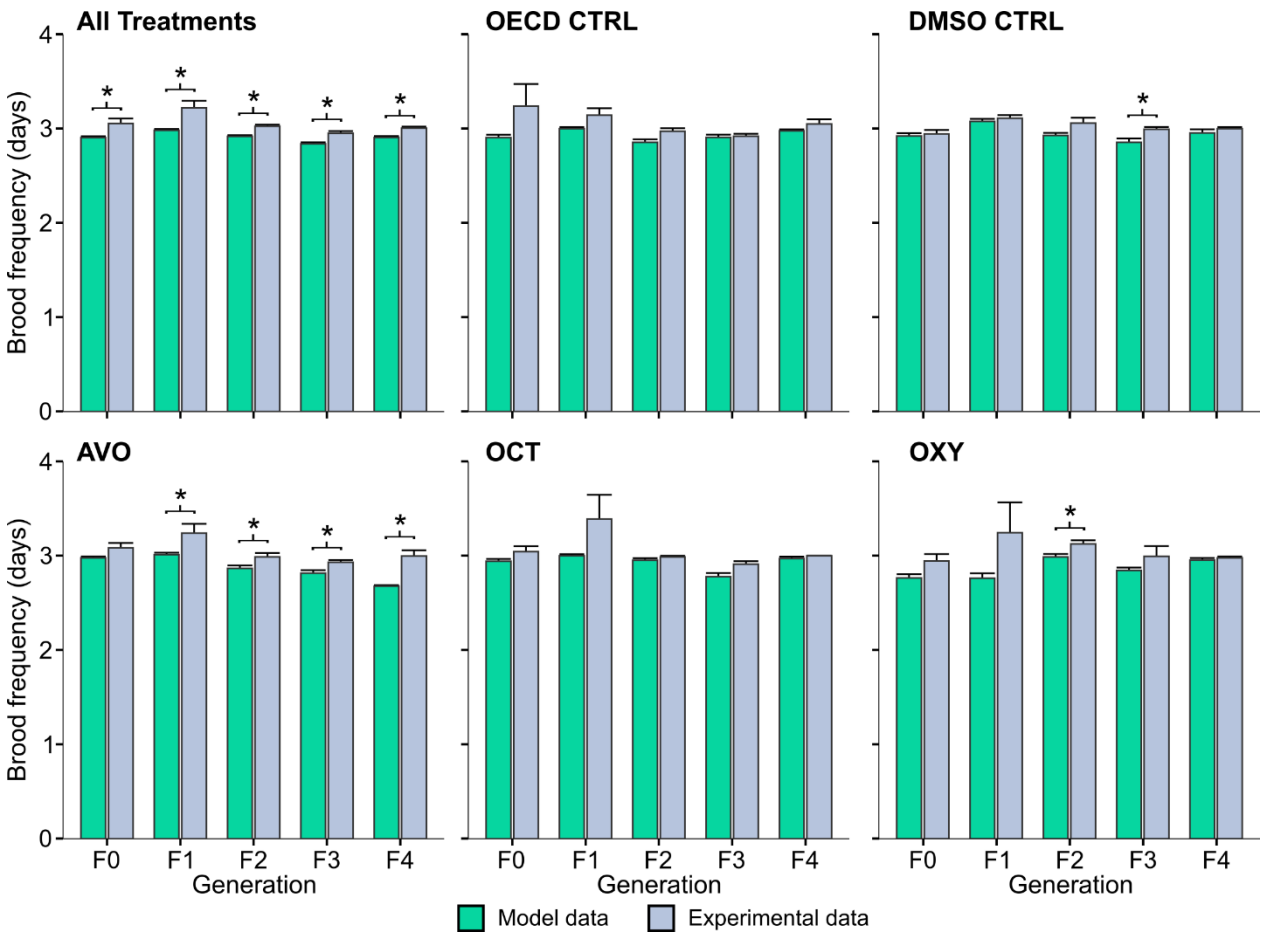


Figure S5.4. The number of days between broods (brood frequency) of < 24-hour old *Daphnia* magna neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4). "Experimental data" refers to the data obtained from 30 replicates of individually exposed *Daphnia* per treatment group and "Model data" refers to estimated data for individually exposed daphnids using the method described in section 5.2.5. Data are shown as mean ± SEM. Stars indicate a significant difference between the model and experimental data within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

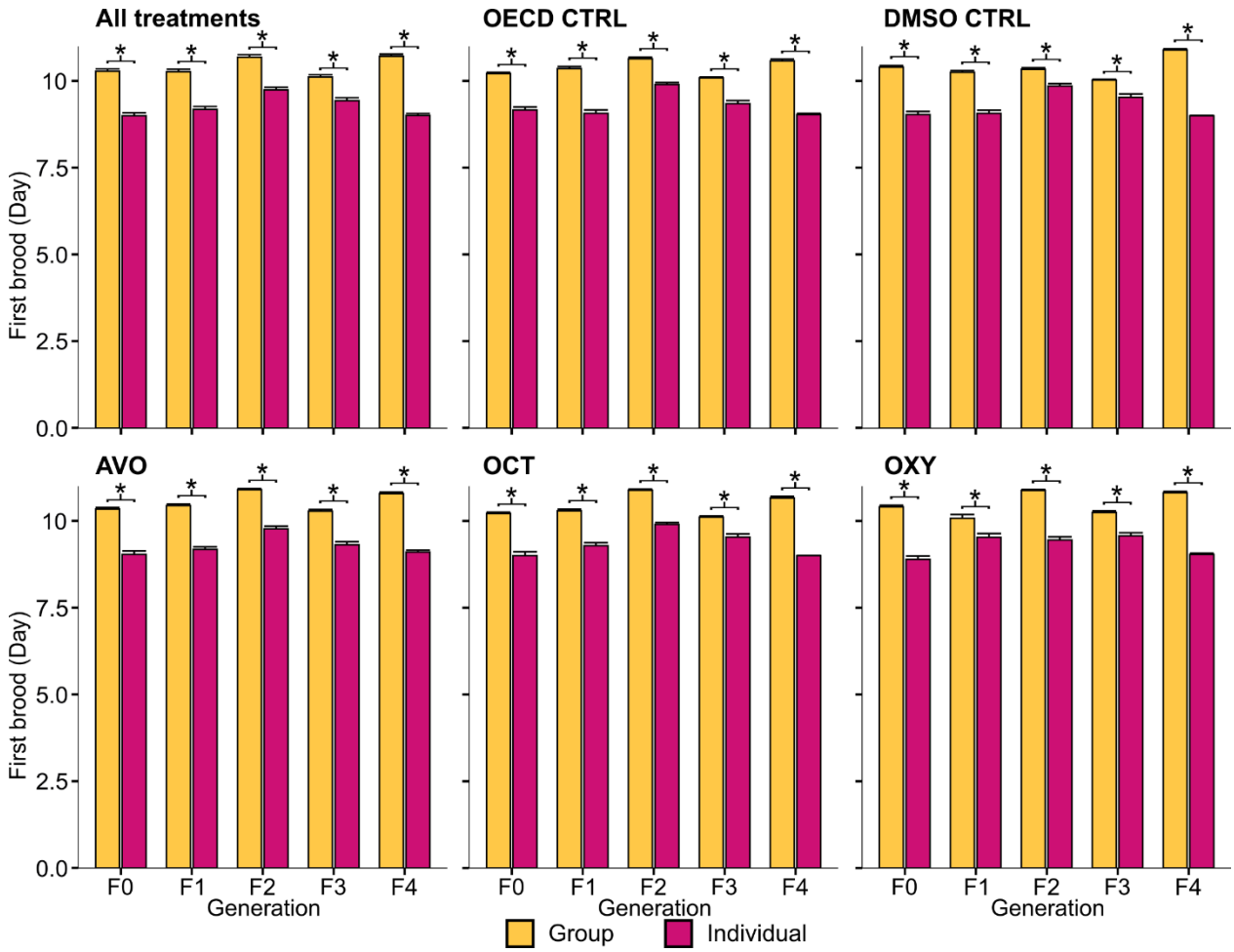


Figure S5.5. The number of days to reach reproductive maturity of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments across 5 generations (F0 – F4) are shown using the data estimation method described in section 5.2.5. Individual exposures consisted of 30 replicates of single daphnids per treatment, while group type exposures consisted of 5 replicates of 25 daphnids per treatment. Bars represent mean ± SEM. Stars indicate a significant difference between the individual and group exposure mortality within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

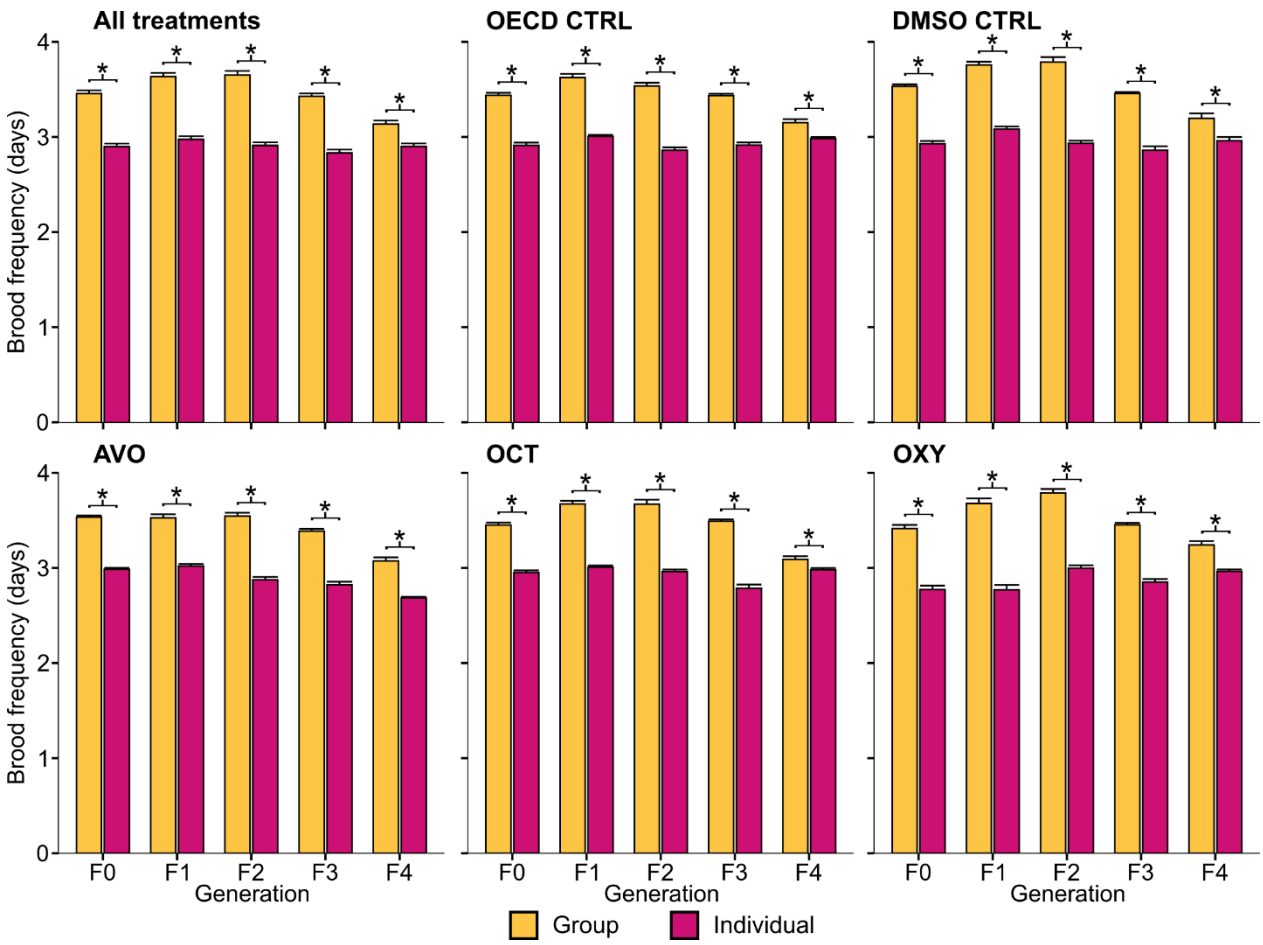
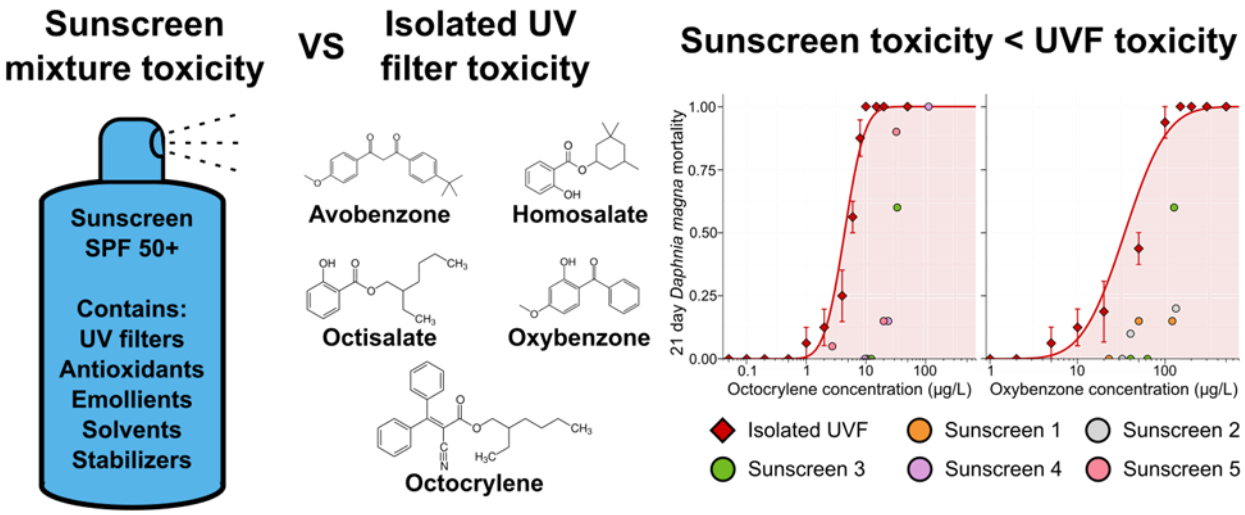


Figure S5.6. The number of days between broods (brood frequency) of < 24-hour old *Daphnia magna* neonates exposed to UVF treatments for 19 days across 5 generations (F0 – F4) are shown using the data estimation method described in section 5.2.5. Individual exposures consisted of 30 replicates of single daphnids per treatment, while group type exposures consisted of 5 replicates of 25 daphnids per treatment. Bars represent mean ± SEM. Stars indicate a significant difference between the individual and group exposure mortality within a generation (two-way repeated measure ANOVA, $\alpha = 0.05$).

Chapter 6: Are sunscreens better together? A comparison of the toxicity of individual ultraviolet filters and off-the-shelf sunscreens to *Daphnia magna*

Boyd, A., Martin, S., Legge, A., Blewett, T.A., 2024. Are sunscreens better together? A comparison of the toxicity of individual ultraviolet filters and off-the-shelf sunscreens to *Daphnia magna*. Environmental Pollution (accepted with revisions, July 28, 2024).



6.1 Introduction

Understanding the threats posed to aquatic ecosystems due to anthropogenic contamination has become a research priority, with contaminants of concern such as organic ultraviolet filters (UVFs) facing increasing scrutiny in recent years (Downs et al., 2016). UVFs such as avobenzone (AVO), homosalate (HMS), octisalate (EHS), octocrylene (OCT), and oxybenzone (OXY) are commonly used in a variety of personal care products such as lip balms, lotions and sunscreens to provide protection against harmful ultraviolet radiation from sunlight (Berardesca et al., 2019; Manová et al., 2013). Through their use, UVFs are known to contaminate many aquatic environments by leaching into the water when UVF-containing products are worn during recreational activities (Giokas et al., 2007; Labille et al., 2020; Langford and Thomas, 2008), resulting in concentrations of 0.1 – 10 µg/L commonly detected in freshwater and marine environments across the globe (Bargar et al., 2015; Downs et al., 2016; Kasprzyk-Hordern et al., 2009; O'Malley et al., 2021; Sánchez Rodríguez et al., 2015; Tsui et al., 2014; Vila et al., 2017).

Due to their widespread presence as environmental contaminants, there has been increased focus investigating the impacts of UVFs on aquatic biota, revealing that the most sensitive species can experience toxicity occurring at the upper range of environmental concentrations. Overall, the highest threat of UVF contamination has been reported in lower trophic level organisms, including mortality and bleaching of several coral species (Downs et al., 2016), inhibition of sea urchin growth (Giraldo et al., 2017), and mortality and reproductive impairment of *Daphnia magna* (Boyd et al., 2023a, 2021). These findings have contributed towards the banned use of certain UVFs in several regions such as Aruba (Homan and Martinus, 2021), Palau (Republic of Palau, 2018) and regions of the United States (Hawaii State Legislature, 2018; USVI Legislature, 2019) in an effort to protect the most at risk species from further contamination.

For these reasons, UVFs have received increased research focus in recent years with the majority of toxicity studies focusing on the impacts of individual UVFs, as these compounds serve as useful and distinct indicators of environments impacted by anthropogenic influences. Sunscreens are among the most commonly used products containing UVFs; however, UVFs account for less than half of the overall product volume, as antioxidants, emollients, solvents, and stabilizing compounds can all be present within the product formulation (Table 6.1; Manová et al., 2013; Mitchelmore et al., 2021; Sánchez-Quiles and Tovar-Sánchez, 2015; Varrella et al., 2022). As one of the major routes of entry into the environment for UVFs is sunscreen leaching off of skin during activities such as swimming (Labille et al., 2020), it stands to reason that other chemical components from sunscreen products would enter the environment through similar means; however, measures of environmental contamination to date have focused on the active ingredients within these skin care products (Kasprzyk-Hordern et al., 2008; Mitchelmore et al., 2021), leaving little information regarding the possible environmental contamination of other ingredients within sunscreen mixtures.

The complexity of sunscreen mixtures raises the possibility that other chemicals within the product may alter the toxicity of these well-studied UVFs in the environment as examples of additive (Salomão et al., 2014), synergistic (Drzymała and Kalka, 2020) or antagonistic toxicity (Godoy and Kummrow, 2017) have been reported through research of other complex mixtures of pharmaceutical and personal care products. Any modification of the toxicity of UVFs in a complex mixture has the potential to either increase the number of species that are considered to be threatened by UVF contamination or reduce the perception of risk for vulnerable species. The sensitivity of the freshwater invertebrate, *Daphnia magna*, to these UVFs has been previously characterized (Boyd et al., 2023a, 2021); therefore, this knowledge can be leveraged to determine if assessing the toxicity of individual UVFs is representative of sunscreen mixture toxicity in a common model organism. To address these knowledge gaps, the objectives of this study were:

1) Determine the toxicity of five commercially available sunscreen products.
2) Assess if overall sunscreen toxicity was similar to the toxicity of the individual UVFs within the product.
3) Assess if the toxicity of sunscreens was proportional to the total quantity of UVFs within the product.

Through understanding how sunscreen mixture toxicity is influenced by UVF content, we can determine if continued studies assessing the toxicity of UVFs in isolation are the most pragmatic approach in understanding the threats that sunscreen and UVF contamination pose to the environment.

6.2 Materials and methods

6.2.1 Daphnia magna culture maintenance

A culture of female *Daphnia magna* was maintained at the University of Alberta, produced from gravid individuals purchased from Aquatic Research Organisms (ARO; US) in November 2021. Cohorts of 30 individuals were cultured in 1 L of water prepared from dechlorinated City of Edmonton water (pH ≈ 7.6) according to Organization for Economic Co-operation and Development (OECD) guidelines to contain 294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$, and 5.80 mg/L KCl (OECD, 2012), referred to as OECD water in the following text. The composition of the OECD water has been previously quantified in Boyd et al. (2023b). Cohorts were fed a daily ration of 3 mL of 3 x 10^7 cells/mL freshwater green algae (*Raphidocelis subcapitata*; ARO) and 3 mL of yeast, cereal leaf and trout chow (YCT) mix (ARO). Full renewal of the culture water occurred every 2-3 days, and cohorts were produced from < 24 h old neonates from the second brood or later, maintained to a maximum age of 2 months. Daphnids were maintained on a 16:8 photoperiod at 20.9 ± 0.7 °C for the duration of this study.

6.2.2 Sunscreen solution preparation and quantification

Five sunscreen spray products were purchased locally in Edmonton, Canada for this study in October 2022, referred to as SS1 – SS5 in this manuscript. These sunscreens were selected to cover a range of sun protection factors (SPF) across three different product brands (Table 6.1). In addition, they are available at a variety of different national retail chains within Edmonton and are therefore easily acquired by consumers for normal use. Each of the five sunscreens contained at least 4 of 5 commonly used UVFs of AVO, HMS, EHS, OCT and OXY. Exposure solutions of each sunscreen were produced by diluting collected spray (considered to be 100% v/v sunscreen) from each product into OECD water. Fresh exposure solutions were produced every 48 h during all experiments in this study. Additional stocks of HMS and EHS were dissolved in dimethyl sulfoxide (DMSO) and further diluted in OECD water to produce exposure solutions to each UVF in isolation. All exposure solutions were produced to contain 0.01% DMSO for all LC50 and 21 d exposures of HMS and EHS.

Three replicates of water samples were collected from separate exposure solution stocks for each treatment in the 21 d experiment to quantify UVF content. All UVFs were analyzed with HPLC-tMS^2, performed using a Vanquish UHPLC System (Thermo Fisher Scientific, Germany) with a Kinetex 1.7 µm C8 Biphenyl analytical column. (2.1 x 50 mm) with a pore size of 100 Å (Phenomenex, US), thermostated at 45°C (HMS, EHS) or 50°C (AVO, OCT, OXY) followed by mass spectrometric detection using an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, Germany).

For the quantification of AVO, OCT and OXY, the buffer gradient system was composed of 0.5 mM ammonium fluoride in water as mobile phase A and methanol (Optima LC-MS) as mobile phase B. For the separation of analytes, a 2 µL aliquot was loaded onto the column at a flow rate of 0.45 mL/min and an initial buffer composition of 30 % mobile phase A and 70% mobile phase B. Elution of the analytes was done by using a linear gradient from 70% to 90% mobile phase B for 2 minutes, 90%

to 98% mobile phase B for 0.1 minutes, stationary at 98% mobile phase B for 0.9 minutes, then returned to 70% mobile phase B in 0.5 minutes. Mass spectra were acquired using a heated electrospray ionization (HESI) source in positive mode. Mass spectrometric conditions were: sheath gas 50, auxiliary gas 10, sweep gas 1, ion transfer tube at 325°C, vaporizer at 350°C, spray voltage static at 3400 V, and RF lens 70. Mass spectra were acquired using a targeted MS^2 scan with an isolation window of 1.8 Da. 1 mg/mL standards for each compound of interest were prepared in methanol, as well as subsequent stock dilutions. A stock for the HESI method was prepared containing 100 µg/mL AVO, 500 µg/mL OCT, and 100 µg/mL OXY. Calibration solutions were diluted with a final solvent concentration of 80% methanol and 20% water. An internal standard of benzophenone-d_{10} was prepared in methanol at a concentration of 1000 ng/mL.

For the analysis of HMS and EHS, the buffer gradient system was composed of water as mobile phase A and methanol as mobile phase B. For the separation of analytes, a 2 µL aliquot was loaded onto the column at a flow rate of 0.5 mL/min with an initial buffer composition of 5% mobile phase A and 95% mobile phase B. Elution of the analytes was done by using a linear gradient from 5% to 88% mobile phase B over a period of 0.2 minutes, held at 88% mobile phase B for 3 minutes, 88% to 98% mobile phase B over a period of 0.1 minutes, held at 98% mobile phase B for 0.4 minutes, then returned to 5% mobile phase B in 0.1 minutes. After a 0.4-minute equilibration at 5% mobile phase B, a linear gradient was used to wash the column from 5% to 98% mobile phase B in 0.5 minutes, held at 98% for 0.3 minutes and back to 5% over 0.2 minutes. This was repeated twice before equilibration of the column and injection of the next sample. Mass spectra were acquired using an APCI (atmospheric pressure chemical ionization) source in negative mode. Mass spectrometric conditions were: sheath gas 45, auxiliary gas 10, sweep gas 2, ion transfer tube at 325°C, vaporizer at 400°C, current at 10 µA, and RF lens 70. Mass spectra were acquired using a targeted MS^2 scan with an isolation window of 1.6 Da. A stock for the APCI method was prepared containing 100 µg/mL HMS and 100 µg/mL EHS, diluted

to a final solvent concentration of 50% methanol and 50% water. An internal standard of methyl salicylate was prepared in 50% methanol at a concentration of 2000 ng/mL.

For all UVFs, data acquisition and analysis was performed using Trace Finder™ 5.1 (Build 203) software (Thermo Fisher Scientific), with mass tolerance set to 5 ppm. Final calibration solutions and unknown samples were prepared by mixing 900 µL of sample with 100 µL of internal standard. Two peaks were obtained for AVO, which were summed to determine the overall AVO concentration in each sample.

6.2.3 48 h median lethal concentration study

48 h median lethal concentration (LC50) and median effect concentration (EC50) tests were conducted for each sunscreen sample (SS1-SS5) as well as homosalate and octisalate individually. 48 h (Boyd et al., 2021) and 21 d LC50s (Boyd et al., 2023a) have been previously determined for AVO, OCT and OXY using the same *D. magna* colony; therefore, these three UVFs were not retested for this manuscript. Each test was conducted according to OECD test 202 guidelines (OECD, 2004), with a total of 6 replicates performed containing 5 exposed < 24 h old daphnids at each test concentration. Daphnids were scored at the end of the 48 h exposure for mortality defined by an absence of gill respiratory motion for the LC50 estimation, or immobilization defined by the presence of gill respiratory motion paired with an inability of the organism to move to a new location over a 15 s observation period for the EC50 estimation. OECD water controls were performed alongside all exposures, and an additional control of 0.01% DMSO was performed with EHS and HMS exposures.

6.2.4 21 d exposure study

21 d exposures were performed for all sunscreens starting at approximately 1/20 of the estimated 48 h LC50 for each sunscreen obtained from the LC50 experiment (0.0005% v/v; referred to as the high dose). Subsequent exposures of 0.0001% v/v (medium dose) and 0.00005% v/v (low dose)

were performed based on the results of the previous exposures. Additional 21 d exposures to EHS and HMS at nominal concentrations of 10, 50 and 100 µg/L were performed to assess the toxicity of the individual UVFs. All 21 d exposures were performed alongside an OECD (water) control without additions, while the EHS and HMS exposures contained an additional 0.01% DMSO (solvent) control exposure. Exposures to AVO, OCT and OXY were not performed directly in this study as these three UVFs have been extensively tested with the same *D. magna* colony over 1 (Boyd et al., 2021) and 5 generations of continuous exposure (Boyd et al., 2023a); therefore, only exposures of EHS and HMS were required in this study to complete the dataset.

All 21 d exposures were performed according to modified OECD test 211 guidelines (OECD, 2012). Individual *D. magna* neonates < 24 h old were exposed in 50 mL of their respective treatment solutions, replicated 20 times per treatment for a total of 21 d. Complete water changes occurred every 2 days, and daphnids were provided a daily ration of 100 µL each of freshwater green algae and YCT mix for 19 days, after which daphnids were fasted for 48 h in preparation for metabolic rate measurements. All daphnids were scored daily for mortality, and the production of viable neonates, non-viable neonates and released but unhatched eggs at the time of scoring. The three reproductive measurements were summed to determine total reproductive effort.

At the end of the 21 d exposure, surviving daphnids were assessed for metabolic rate in a random order using a 24-well optical fluorescence respirometry glass microplate (Loligo Systems, Denmark). Daphnids were placed into a well containing 500 µL of their respective exposure solutions with no headspace, which were then sealed with a silicone gasket underneath a weighted block. The concentration of dissolved oxygen in each well was then recorded every 15 s over a 1 h period. Upon completion of the metabolic rate assay, daphnids were imaged under a microscope, after which the daphnids were then placed into an incubator to dry at 55°C overnight before weighing the following

morning to obtain the organism's dry mass. Daphnid body length was determined with ImageJ by measuring from the edge of the carapace above the eye spot to the tip of the tail (Figure S6.2).

The first 10 minutes of oxygen data from the metabolic rate assessment were treated as the acclimation period and excluded from the analysis, while the mass specific metabolic rate was calculated using Formula 6.1 below.

Formula 6.1: $MR = \frac{\Delta O_{2exp} - \Delta O_{2blank}}{m}$

The mass specific metabolic rate (MR) was determined by calculating the difference between the change in oxygen of the well containing the daphnid (ΔO_{2exp}) and the average change in oxygen of all blank wells containing OECD water without any daphnids on the same microplate (ΔO_{2blank}) to obtain a corrected change in oxygen over the 50-minute observation period. This was then divided by the dry mass of the daphnid to determine the final mass specific metabolic rate of the daphnid in mg O_2 L^{-1} h^{-1} mg^{-1}. All mass specific metabolic rates with a correlation coefficient < 0.9 were excluded from the analysis.

6.2.5 Statistics

Data analysis was performed using R version 4.3.2 (R Core Team, 2024). The probit method from the "ecotox" package was used for LC50 and EC50 estimates. Data obtained from the 21 d exposures were assessed for normality and homoscedasticity via Shapiro-Wilk and Levene's tests, respectively and parametric data was tested with a one-way ANOVA and Tukey test, while non-parametric data that could not be successfully transformed were tested via Kruskal-Wallis and Dunn's tests. Significance was determined at $\alpha = 0.05$, and all values are reported as a mean ± standard error of the mean unless indicated otherwise. 48 h LC50 and EC50 data for AVO, OCT and OXY were originally analyzed using the logit method when published in Boyd et al. (2021); therefore, the original

raw data was re-analyzed using the probit method to maintain consistency across all data included in this manuscript.

6.3 Results

6.3.1 Sunscreen solution water chemistry

EHS (< 2.31 – 55.6 µg/L) and AVO (1.15 – 45.4 µg/L) were measured at the lowest concentrations across the sunscreen samples while OCT (2.73 – 113 µg/L) was detected at moderate levels and HMS (4.65– 129 µg/L) and OXY (22.9 – 133 µg/L) were the most abundant UVFs in the sunscreens in which they were present. The total concentration of UVFs measured in the highest dose of each sunscreen did not correlate with the SPF indicated on the product label, as SS4 (SPF 30) contained 343 ± 22.1 µg/L of UVFs, while SS3 and SS5 (SPF 50+) contained 213.4 ± 19.2 µg/L and 124 ± 5.15 µg/L, respectively (Table 6.2). Of the three sunscreens tested from the same brand, higher SPF formulations typically contained greater total UVF concentrations in the low and medium doses; however, SS1 (SPF 15) contained more UVFs (308 ± 61.9 µg/L) than SS2 (276 ± 22.7 µg/L; SPF 30) and SS3 (213.4 ± 19.2 µg/L; SPF 50+) at the high exposure dose.

6.3.2 48 h LC50 and EC50 experiments

EHS was moderately more toxic to *D. magna* with an estimated 48 h LC50 of 3.41 mg/L (95 % CI: 2.86, 4.18) compared to HMS, with an LC50 of 9.86 mg/L (95 % CI 8.42, 11.8; Table S6.2). SS1 was the most toxic sunscreen tested with a 48 h LC50 of 0.00872 % v/v (95 % CI 0.00734, 0.0106), while all other sunscreens yielded similar LC50s of 0.0101 – 0.0273 % v/v. Both UVFs and all sunscreens tested in this study induced immobilization of daphnids at moderately lower EC50 concentrations than their corresponding LC50 concentrations for each test solution.

6.3.3 21 d exposure experiments

All low and medium sunscreen doses as well as OECD controls resulted in ≤ 15% mortality over the 21 d exposure (Figure 6.1 A & B). In the high exposure dose, SS1 and SS2 resulted in ≤ 20% mortality, while SS3 caused 60% mortality, with SS4 and SS5 causing 100% and 90% mortality, respectively (Figure 6.1 C). Similar trends were apparent in the physiological endpoints observed, with no effects on total reproductive effort observed for the low dose of each sunscreen, while medium doses of SS3 and SS4 resulted in 30% and 44% lower total reproduction compared to the OECD control (Kruskal-Wallis, $p = 0.0174$; Figure 6.2 A). Reproductive effort was reduced by 75% - 93% for SS1, SS2 and SS3 at the high exposure dose, while the two surviving SS5 daphnids failed to produce any viable or non-viable form of reproduction (Kruskal-Wallis, $p < 0.0001$). The proportion of reproductive effort resulting in failure (unhatched eggs or non-viable neonates) was 40 – 59% for the high doses of SS1, SS2 and SS3, compared to 1.7% in control daphnids (Kruskal Wallis, $p < 0.0001$; Figure 6.2 B). Daphnids released their first brood 2.6 – 3.1 days later during exposure to high doses of SS1, SS2 and SS3 than control daphnids (Kruskal Wallis, $p < 0.0001$); Figure 6.2 C). The mass specific metabolic rate was unaffected by any sunscreen treatment (one-way ANOVA, $p \geq 0.093$; Figure 6.2 D); however, daphnids achieved a smaller body size by the 21st day of exposure to high doses of SS1, SS2, SS3 and SS5, with a 40 – 62% lighter dry mass (one-way ANOVA, $p < 0.0001$; Figure 6.2 E) and a 20 – 30% smaller body length (Kruskal Wallis, $p < 0.0001$; Figure 6.2 F).

Daphnids exposed to EHS or HMS at nominal concentrations up to 100 µg/L experienced ≤ 15% mortality. Minimal effects of EHS or HMS on daphnid physiology were observed in any endpoint, but significance was detected in these treatments for the time to release the first brood and daphnid body length; however, all treatments were found to be within 10% of both OECD and DMSO controls for both endpoints (Figure 6.3).

6.3.4 Sunscreen toxicity vs UVF content

Daphnids exposed to sunscreens containing AVO experienced mortality at concentrations up to 1.3-fold lower than those causing an equivalent level of mortality when exposed to AVO alone over 21 d (Figure 6.4 A). The opposite trend was observed for sunscreens containing OCT, as daphnids survived concentrations up to 5.6-fold greater in sunscreens than OCT in isolation (Figure 6.4 B). Daphnids exposed to any dose of SS3, low and medium doses of SS4, or the medium and high doses of SS5 survived OCT concentrations at or above the 10 µg/L that resulted in 100% mortality to OCT in isolation, including ≤ 20% mortality for daphnids exposed to the low and medium doses of SS3 and SS4, as well as the medium dose of SS5. Similarly, sunscreens containing OXY caused an equivalent level of mortality as OXY in isolation at up to 9-fold greater concentrations (Figure 6.4 C). The high doses of SS1 and SS2 contained OXY concentrations greater than those causing > 94% mortality over 21 d to daphnids exposed to OXY alone; however, mortality was ≤ 20% in both cases.

The severity of impairment of each endpoint observed correlated poorly with the total UVF concentration of each sunscreen. Although a significant relationship was detected in all cases presented in Figure 6.5, the adjusted coefficients of determination were low, ranging from 0.3120 – 0.4675 for comparisons of mortality, body length, dry mass, number of broods and the proportion of reproductive effort resulting in viable offspring. Only the total reproductive effort of daphnids over 21 d demonstrated a moderately high correlation with sunscreen total UVF content, with an R^2 of 0.6641.

6.4 Discussion

6.4.1 Toxicity of sunscreen mixtures

All 5 tested sunscreen mixtures yielded similar levels of acute toxicity with all 48 h LC50 and EC50 values within 0.5 orders of magnitude regardless of brand or UVF content (Table S6.2, Figure S6.1). In comparison to the least toxic LC50 (i.e., SS5), SS1, SS2 and SS3 contained 1.7 – 2.5-fold

higher total UVF concentrations and 2.7 – 3.1-fold lower LC50s (Table S6.3). In contrast, SS4 had a 2.8-fold higher total UVF concentration but yielded an LC50 nearly identical to SS5, suggesting that while the individual UVFs contribute towards toxicity, other aspects of the sunscreen mixtures may need to be considered to fully assess mixture toxicity. The relationship between UVF concentrations and sunscreen toxicity is similarly unclear over chronic 21 d exposures, as the sunscreens containing the most (SS4) and least (SS5) total UVFs were equally the most toxic in the highest tested dose, resulting in ≥ 90% mortality and inhibiting all reproduction from any surviving daphnids (Figure 6.1 & Figure 6.2). Sunscreens that contained OCT rather than OXY (SS4 & SS5) were more toxic than those containing OXY instead of OCT (SS1 & SS2), which is consistent with previous data demonstrating a higher toxicity of OCT to *D. magna* (Boyd et al., 2023a, 2021). The increased toxicity of OCT may be explained by a greater potential to cause oxidative stress at several orders of magnitude lower concentrations than OXY, as observed in studies of *Danio rerio* (Gayathri et al., 2023; Zhang et al., 2021).

The differences in the relationship between sunscreen toxicity and total UVF content across acute and chronic exposures may be fundamental to the exposure duration, as compounds that are not dependent upon the organism's metabolism to exert toxicity typically manifest toxic effects earlier than those requiring bioactivation (Buhler and Williams, 1988). Indeed, several OXY metabolites have been reported to have a higher estrogenic activity than the parent molecule (Molina-Molina et al., 2008; Suzuki et al., 2005). In addition, the feeding of organisms during the 21-d exposure allows for dietary routes of exposure to contribute towards toxicity in addition to the normal external exposure route. Lipophilic compounds in each sunscreen mixture such as the UVFs, α-tocopherol acetate, and retinyl palmitate may adsorb to the ingested food, with previous studies noting that dietary uptake of UVFs such as OCT in several species of fish is a relevant uptake pathway contributing towards whole body burdens (Pawlowski et al., 2019; Peng et al., 2017a). Dietary exposure of *D. magna* via the same

species of green algae used in this study has been observed to induce decreased reproduction after exposure to similarly lipophilic compounds such as pesticides (Bessa Da Silva et al., 2016), and brominated flame retardants (Evandri et al., 2003). Several of the listed ingredients that were unique to the most toxic sunscreens (SS4 & SS5) are lipophilic compounds (e.g. butyloctyl salicylate, C12-15 alkyl benzoate, dicaprylyl carbonate, and stearoxytrimethylsilane); therefore, the additional dietary exposure of daphnids to these sunscreen ingredients may have contributed towards the differences in toxicity observed between each tested product.

Previous studies of sunscreen mixtures have observed reduced toxicity for products that do not contain preservatives such as benzyl benzoate (Corinaldesi et al., 2017), or acrylate copolymers (Varrella et al., 2022). Known toxic preservatives are absent from the sunscreen products tested in this study; however, all five sunscreens contained octylacrylamide copolymers which may have contributed to the toxicity observed in the high doses of all samples (Figure 6.2 A). The higher toxicity of SS4 and SS5 can likely be attributed towards the unique ingredients within each product. Although no single ingredient was in common between only SS4 and SS5, notable ingredients include the surfactant/emulsifier polypropylene glycol-5-ceteth-20 which was only present in SS4, and the emollient caprylic triglyceride present in SS3 and SS5. These ingredients are capable of reducing the surface tension of water (Fiume et al., 2016; Sohn, 2020), which can be indirectly toxic to *Daphnia* by causing individuals to become trapped on the surface, inhibiting their filter feeding behaviours (Blewett et al., 2018). Araújo et al. (2020) observed that shrimp (*Palaemon varians*) tend to avoid sunscreen contaminated waters, but that some products could diminish this avoidance behaviour through an induced immobilization of the exposed organisms; however, it is unclear whether this immobilization was caused by the inclusion of specific ingredients in the sunscreen or due to the differences in testing lotions, gels, and sprays. Overall, the sunscreens in this study were > 1 order of magnitude more toxic to *D. magna* than those tested previously through exposures to flatworms (*Convolutriloba macropyga;*

McCoshum et al., 2016), corals, (*Xenia elongate;* Danovaro et al., 2008; McCoshum et al., 2016) or sea urchins (*Paracentrotus lividus*; Varrella et al., 2022); however, it cannot be determined from the available data if this is due to differences in the products tested, organism habitat (freshwater vs marine) or species sensitivity.

6.4.2 Comparative toxicity of sunscreens to individual UVFs

The toxicity of HMS and EHS was assessed over acute and chronic exposures to complement the existing data available on AVO, OCT and OXY toxicity from this *D. magna* colony. The 48 h EC50 for HMS was similar to the value reported for *D. magna* by Marcin and Aleksander (2023), but EHS was approximately an order of magnitude more toxic. The 48 h LC50s determined for HMS and EHS were similar to those previously determined for AVO, OCT and OXY from this culture (Table S6.2; Boyd et al., 2021) and from independent research groups (Jang et al., 2016; Molins-Delgado et al., 2016; Park et al., 2017; Sieratowicz et al., 2011). The lack of 21 d toxicity of HMS or EHS in any measured endpoint at nominal concentrations up to 100 µg/L (Figure 6.3) is in stark contrast to the known toxicity of AVO, OCT and OXY, which includes 60% mortality and 30% decreased reproductive effort caused by 16.5 µg/L OXY, the 21 d LC50 of 42 µg/L AVO (Boyd et al., 2023a), or the 100 % mortality observed after a 7-d exposure to 7.5 µg/L of OCT (Boyd et al., 2021). A study of various coral species observed bleaching as a result of exposure to sunscreen mixtures as well as OXY alone, but not HMS (Danovaro et al., 2008). While UVFs are overall considered to be endocrine disrupting compounds which can elicit effects through estrogen receptor α pathways, EHS and HMS have been demonstrated to have minimal estrogenic activity in comparison to OXY (Kunz and Fent, 2006b), and HMS exhibits several orders of magnitude lower anti-androgen activity in comparison to AVO (Jiménez-Díaz et al., 2013; Klopčič and Dolenc, 2017). Although HMS and EHS have not received as much toxicity research focus as other UVFs, the data presented above suggest that these

particular UVFs pose a minimal threat to *D. magna* compared to AVO, OCT or OXY; therefore, HMS and EHS are unlikely to be the primary determinants of toxicity in these sunscreen mixtures.

The chronic lethality of sunscreen mixtures to *D. magna* did not align well with the 21 d LC50 results presented in Boyd et al. (2023a) for AVO, OCT and OXY (Figure 6.4). Daphnids experienced mortality at slightly higher rates when exposed to sunscreens than when exposed to AVO alone; however, AVO was the least abundant UVF along with EHS detected in exposure solutions. Only the high dose of SS4 contained AVO levels near the 21d LC50 concentration, rendering it a minor component of sunscreen mixtures in comparison to HMS, OCT or OXY. Interestingly, daphnids were capable of surviving long-term exposure to SS3, SS4 and SS5 doses containing OCT concentrations in excess of the 100% mortality threshold for the UVF in isolation (10 µg/L). Similar results were observed for SS1 and SS2 with respect to OXY content, as ≤ 20% mortality occurred despite the presence of OXY in each sunscreen at levels exceeding the 90% mortality threshold for the UVF alone, while a smaller but still notable decrease in sunscreen lethality was observed in SS3 with respect to OXY content. The toxicity of the sunscreen mixtures poorly correlated with the total UVF content, as only reproductive effort ($R^2 = 0.6641$) and body length ($R^2 = 0.4675$) yielded coefficients of determination > 0.4 (Figure 6.5). These results are consistent with a previous study that similarly concluded that sunscreen lotion toxicity to sea urchins (*Paracentrotus lividus*) appears to be moderately independent of the UVF content (Varrella et al., 2022), suggesting that the overall severity of UVFs may be moderated by interactions with other components of the sunscreen mixtures.

Overall, it would appear that the toxicity of UVFs is decreased due to antagonistic interactions within the sunscreen mixtures, leaving the possibility that other components of the mixture are serving as the main drivers of toxicity. Alternatively, UVFs may still be the key determinants of toxicity but are instead functioning at a reduced potency. Antagonistic interactions can arise from the formation of complexes between different mixture components, preventing the compounds involved from entering

into biota or interacting with the molecular targets that normally result in toxicity. Some studies of UVF mixtures did not report antagonistic toxicity between mixtures of AVO, OCT and OXY (Boyd et al., 2021), or AVO and OCT (Park et al., 2017); however, others have observed antagonism within mixtures of OXY, OCT and octinoxate (ethylhexyl methoxycinnamate; Li et al., 2018) and AVO, OCT and octinoxate (Park et al., 2017). Altogether, these previous findings suggest that specific combinations of UVFs may be required for antagonistic interactions between UVFs to occur, as it has been proposed that certain combinations of UVFs are more likely to compete for interactions with their target biological structures when they possess high structural similarity (Marcin and Aleksander, 2023). EHS, HMS, and OXY all possess an aromatic hydroxyl group, EHS, HMS and OCT contain an ester group, while AVO and OXY contain a ketone group, offering several opportunities for competitive antagonistic interactions to occur within biota. A study by Molins-Delgado et al. (2016) noted that interactions between UVFs and other mixture components such as Ag nanoparticles can also lead to reduced toxicity through the potential aggregation of toxicants, reducing the surface area of toxicants available for biological interactions. From the currently available evidence, it appears likely that antagonistic interactions between UVFs is the primary cause of the lower than anticipated toxicity of sunscreen mixtures; however, additional research is required in order to understand any potential interactions occurring with other components in these complex mixtures and how they may impact their overall toxicity.

6.4.3 Implications

Due to the lack of studies directly quantifying the release of all sunscreen mixture components into the environment, it is difficult to assess the level of threat posed by these sunscreen products as it is unclear to what extent that sunscreen components leach into the water during regular use. Varrella et al. (2022) estimated an annual release of 43 – 679 µL/L (0.0043% - 0.0679% v/v) of sunscreens into coastal waters of the Mediterranean Sea based on the assumption that 25% of dermally applied

sunscreens would be leached into the water. The highest tested chronic sunscreen dose is similar to the conservative end of this estimate, suggesting that these sunscreens would pose a large threat to aquatic environments. Alternatively, if all non-UVF sunscreen ingredients are assumed to enter the environment in proportion to the UVFs released, then the current practice of quantifying UVF concentrations in environmental samples would allow for the overall release of sunscreen mixtures to be extrapolated. Using this method, the concentrations of UVFs quantified in the lowest exposure dose for each sunscreen (Table 6.2) would be representative of the top 5 most contaminated environmental samples reported for each individual UVF (Downs et al., 2016; Kasprzyk-Hordern et al., 2009; Langford and Thomas, 2008; Sánchez Rodríguez et al., 2015; Tsui et al., 2014; Vila et al., 2017), suggesting that the threat posed is quite low.

Due to the chemical complexity of sunscreen mixtures, it is difficult to determine the relationship between environmental concentrations of UVFs and inactive sunscreen ingredients, rendering it difficult to assess the risk posed by these sunscreens with any degree of certainty. The measured concentrations of UVFs within each mixture did not consistently align with the sunscreen dosage, as the medium doses of SS1-SS4 contained only 1.5 - 2.6-fold more UVFs than their respective low doses in contrast to the expected 5-fold increase. Similarly, the high dose of SS2 and SS3 contained 64% and 35% respectively of the anticipated 10-fold increase in UVFs over the low dose of each mixture (Table 6.2), suggesting that the measured concentration of UVFs in receiving waters may not necessarily scale linearly with sunscreen dose. This could be due to the expected UVF concentrations in high test doses approaching each chemical's respective solubility limits in water, with some samples exceeding the solubility of AVO (27 µg/L) and OCT (40 µg/L) in pure water (Mitchelmore et al., 2021). The inclusion of solvents within each mixture would improve the solubility of the UVFs; however, it should be expected that a greater quantity of UVFs are lost to precipitation (Benedé et al., 2014) or adsorption to the container walls (Cormier et al., 2019; O'Malley et al., 2021)

at higher concentrations, which has been reported in many previous studies that have reported UVFs at 10 – 50% of their nominal concentrations (Blüthgen et al., 2012; Boyd et al., 2023a; Fel et al., 2019; Giraldo et al., 2017). Future research should be targeted towards quantifying the release of sunscreen inactive ingredients with respect to UVFs, which would allow for researchers to assess if measuring UVF contamination provides a reasonable representation of sunscreen pollution. Additionally, surveys of UVF environmental contamination should seek to include common inactive ingredients such as α-tocopherol acetate, octylacrylamide copolymers or glycerin so that the environmental input of UVFs from sunscreen use can be differentiated from other sources. This study tested only sunscreen spray products; however, considerations should be made for other product types such as lotions and creams which are known to utilize different product formulations (Geoffrey et al., 2019) that may influence UVF toxicity in a different manner than the spray products tested in this study.

6.4.4 Conclusions

The results from this study suggest that the chemical complexity of sunscreen mixtures prevents researchers from adequately modelling their environmental impacts through tests of UVFs alone. The toxicity observed from each sunscreen spray was not consistent with the overall quantity of UVFs measured within each solution and could not be attributed to high quantities of a single UVF. The possibility of antagonistic effects between the combined UVFs and/or the inactive ingredients in the product formulations reduced the toxicity of each tested product with respect to predictions based on the toxicity of each individual UVF. It can be concluded that complex mixture interactions have the potential to greatly impact the toxicity of UVFs; therefore, studies which seek to model threats posed to the environment by sunscreen contamination should include considerations for the influences of sunscreen ingredients beyond UVFs, including the environmental effects and fates of each ingredient within the product mixture. Due to the myriad of chemical interactions that can occur in environments with complex contamination profiles, continuing to use tests of individual UVFs to model sunscreen

contamination may not be representative of environmental outcomes. Further research is needed in order to understand the interactions between active and inactive sunscreen ingredients if the environmental impacts of sunscreen contamination are desired to be understood through single chemical tests.

6.5 Figures and tables

Table 6.1. The composition of each tested sunscreen as indicated on the manufacturer's product label. Active ingredients are listed as the percent v/v of the total formulation, while inactive ingredients are listed as present (+) or absent (-) in the formulation.

Sunscreen	SS1	SS2	SS3	SS4	SS5
Brand	A	A	A	B	C
SPF	15	30	50+	30	50+
Active ingredients					
Avobenzone	2%	2%	3%	3%	3%
Homosalate	10%	15%	15%	10%	10%
Octisalate	5%	5%	5%	5%	5%
Octocrylene	-	-	10%	3.75%	4%
Oxybenzone	3%	4%	6%	-	-
Inactive ingredients					
DL- α-tocopherol acetate	+	+	+	+	+
Denatured alcohol	+	+	+	+	+
Fragrance	+	+	+	+	+
Octylacrylamide copolymers	+	+	+	+	+
Glycerin	+	+	+	-	+
Aloe barbadensis leaf juice	+	+	+	-	-
Ascorbic acid	+	+	+	-	-
Retinyl palmitate	+	+	+	-	-
Caprylic triglyceride	-	-	+	-	+
Butyloctyl salicylate	-	-	-	-	+
C12-15 alkyl benzoate	-	-	-	+	-
Dicaprylyl carbonate	-	-	-	+	-
DL-panthenol	-	-	-	-	+
Epilobium angustifolium extract	-	-	-	-	+
Helianthus annuus seed oil	-	-	-	-	+
Polypropylene glycol-5-ceteth-20	-	-	-	+	-
Stearoxytrimethylsilane	-	-	-	-	+

Table 6.2. Measured UVF composition of each sunscreen tested at three different exposure doses: low (0.00005% v/v), medium (0.0001% v/v) and high (0.0005% v/v). All values are presented as the mean of 3 replicates ± SEM, expressed in units of µg/L. BDL indicates concentrations below detection limits.

Sunscreen	Exposure dose	Avobenzone	Homosalate	Octisalate	Octocrylene	Oxybenzone	Total UVFs
SS1	Low	2.81 ± 0.191	9.19 ± 0.948	BDL	BDL	22.9 ± 2.85	34.9 ± 3.38
	Medium	8.31 ± 1.67	26.1 ± 3.77	7.06 ± 1.21	BDL	50.1 ± 12.6	91.6 ± 19.0
	High	24.1 ± 6.11	114 ± 22.9	48.9 ± 10.4	BDL	121 ± 23.0	308 ± 61.9
SS2	Low	0.996 ± 0.569	9.38 ± 2.87	BDL	BDL	32.4 ± 8.16	42.8 ± 11.3
	Medium	1.95 ± 0.586	17.5 ± 3.84	3.10 ± 1.55	BDL	40.3 ± 4.68	62.9 ± 10.4
	High	16.5 ± 2.53	98.5 ± 15.3	27.3 ± 5.27	BDL	133 ± 3.07	276 ± 22.7
SS3	Low	2.36 ± 0.689	7.52 ± 2.02	BDL	10.6 ± 1.58	40.5 ± 6.16	60.9 ± 9.79
	Medium	4.24 ± 0.755	13.7 ± 0.986	2.33 ± 2.33	12.4 ± 2.10	62.9 ± 3.12	94.7 ± 4.88
	High	8.57 ± 1.15	35.6 ± 3.32	8.71 ± 1.13	33.6 ± 5.19	127 ± 8.74	213.4 ± 19.2
SS4	Low	7.42 ± 0.706	10.3 ± 1.87	BDL	9.62 ± 2.25	BDL	27.4 ± 4.63
	Medium	12.6 ± 0.697	26.7 ± 1.96	6.24 ± 1.59	23.7 ± 1.27	BDL	69.3 ± 5.36
	High	45.4 ± 4.17	129 ± 6.30	55.6 ± 3.40	113 ± 8.24	BDL	343 ± 22.1
SS5	Low	3.57 ± 0.123	4.65 ± 0.784	BDL	2.73 ± 0.345	BDL	11.0 ± 0.320
	Medium	12.1 ± 1.33	22.7 ± 3.97	5.92 ± 1.26	19.9 ± 3.24	BDL	60.6 ± 9.45
	High	18.5 ± 1.53	52.7 ± 5.58	20.8 ± 2.45	32.5 ± 2.32	BDL	124 ± 5.15

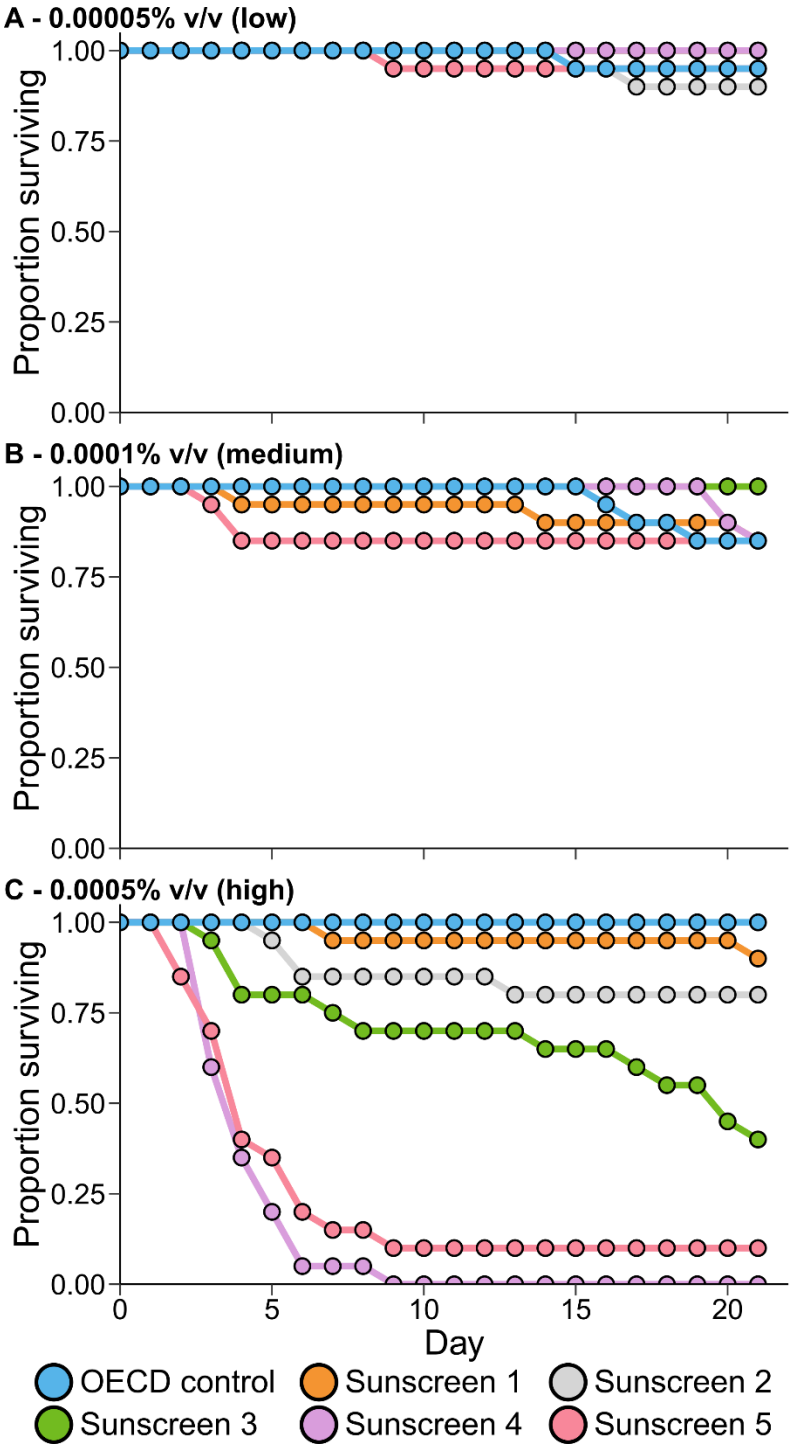


Figure 6.1. Survivorship curves of < 24 h old *D. magna* neonates exposed to low (0.00005% v/v; A), medium (0.0001% v/v; B) and high (0.0005% v/v; C) doses of sunscreens for 21 d. Each point represents the proportion of surviving daphnids on each day out of 20 total replicates per treatment.

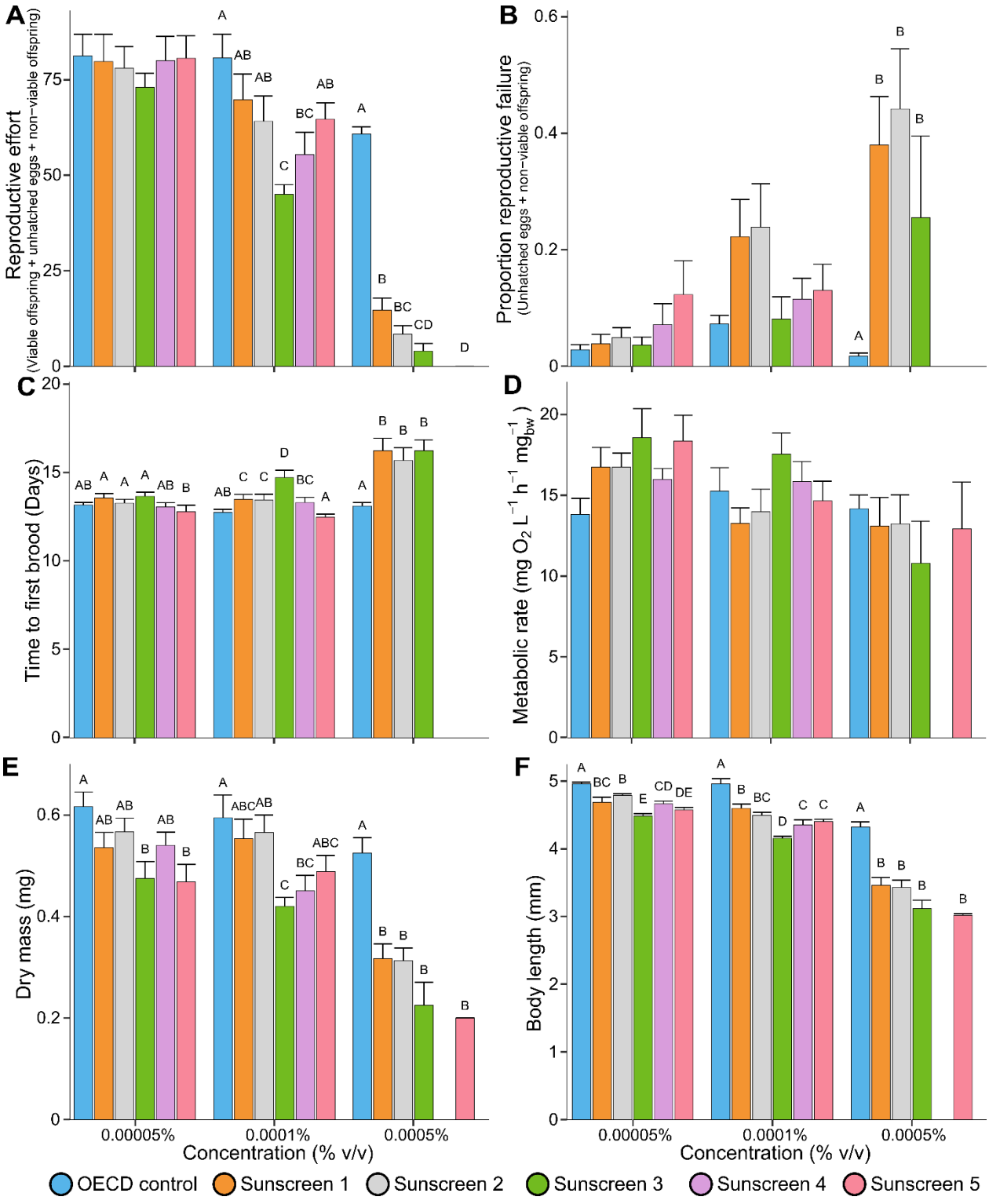

A
Reproductive effort
(Viable offspring + unhatched eggs + non−viable offspring)
B
Proportion reproductive failure
(Unhatched eggs + non−viable offspring)
C
Time to first brood (Days)
D
Metabolic rate (mg O_2 L^{-1} h^{-1} mg_{bw}^{-1})
E
Dry mass (mg)
F
Body length (mm)
0.00005%
0.0001%
0.0005%
Concentration (% v/v)
OECD control
Sunscreen 1
Sunscreen 2
Sunscreen 3
Sunscreen 4
Sunscreen 5

Figure 6.2. Physiological parameters of < 24 h old *D. magna* neonates exposed to three concentrations of sunscreens over 21 d. Average reproductive effort (A), failed reproduction as a proportion of reproductive effort, (B) and maturation time (C) summarize all reproduction observed over 21 d from daphnids surviving the entire exposure period. The mass specific metabolic rate (D), organism dry mass (E), and body length (F) were measured on the 21st day of exposure. All bars represent the mean ± SEM of 20 starting replicates per treatment. Different letters above the bars indicate a significant difference between treatments within an exposure dose via one-way ANOVA (D, E) or Kruskal Wallis test (A, B, C, F). Bars without letters are not considered to be significantly different at $\alpha = 0.05$.

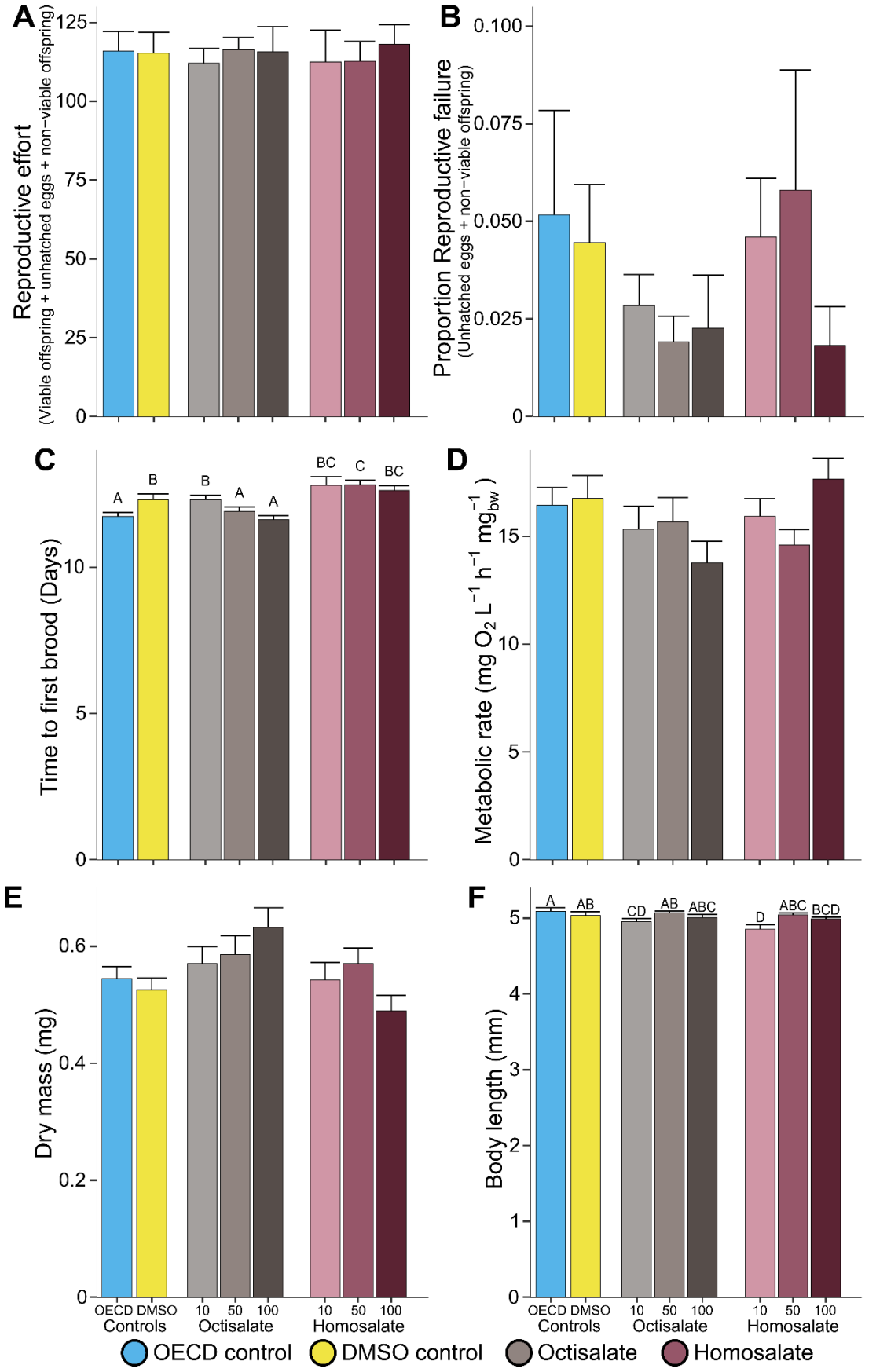

A
Reproductive effort
(Viable offspring + unhatched eggs + non−viable offspring)
125
100
75
50
25
0
B
Proportion Reproductive failure
(Unhatched eggs + non−viable offspring)
0.100
0.075
0.050
0.025
0
C
Time to first brood (Days)
A
B
B
A
A
BC
C
BC
10
5
0
D
Metabolic rate (mg O_2 L^{-1} h^{-1} mg_{bw}^{-1})
15
10
5
0
E
Dry mass (mg)
0.6
0.4
0.2
0
F
Body length (mm)
A
AB
CD
AB
ABC
D
ABC
BCD
5
4
3
2
1
0
OECD DMSO
Controls
10 50 100
Octisalate
10 50 100
Homosalate
OECD control
DMSO control
Octisalate
Homosalate

Figure 6.3. Physiological parameters of < 24 h old *D. magna* neonates exposed to three concentrations of homosalate or octisalate, or water (OECD) or solvent (DMSO) controls over 21 d. Average reproductive effort (A), failed reproduction as a proportion of reproductive effort, (B) and maturation time (C) summarize all reproduction observed over 21 d from daphnids surviving the entire exposure period. The mass specific metabolic rate (D), organism dry mass (E), and body length (F) were measured on the 21st day of exposure. All bars represent the mean ± SEM of 20 starting replicates per treatment. Different letters above the bars indicate a significant difference between treatments via one-way ANOVA (A, D) or Kruskal Wallis test (B, C, E, F). Bars without letters are not considered to be significantly different at $\alpha = 0.05$.

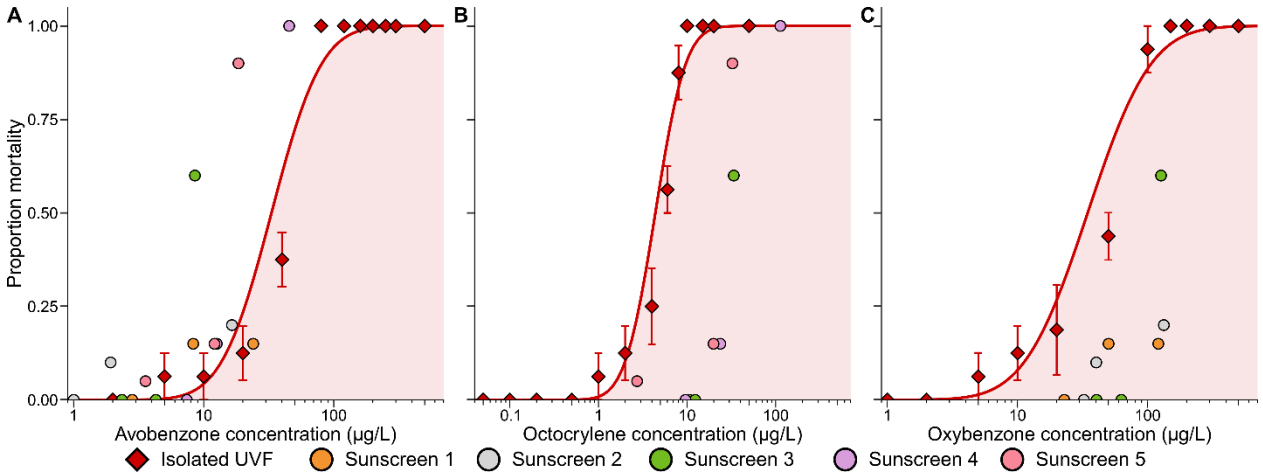


Figure 6.4. The proportion of mortality observed over 21 d exposure to sunscreens vs the measured concentration of avobenzone (A), octocrylene (B) or oxybenzone (C) within the sunscreen mixtures. Red diamonds represent the mean ± SEM of *D. magna* surviving a 21 d exposure to each UVF in isolation and the red curve represents the resulting estimated LC50 curve as determined by 4 replicates of 5 daphnids exposed at each tested concentration, using data previously published in Boyd et al. (2023a). Coloured circles within the red shaded region to the right of the LC50 curves indicate instances where *D. magna* experienced higher mortality when exposed to the UVF in isolation (21 d LC50; Boyd et al., 2023a) than when exposed to sunscreens containing that UVF (present study).

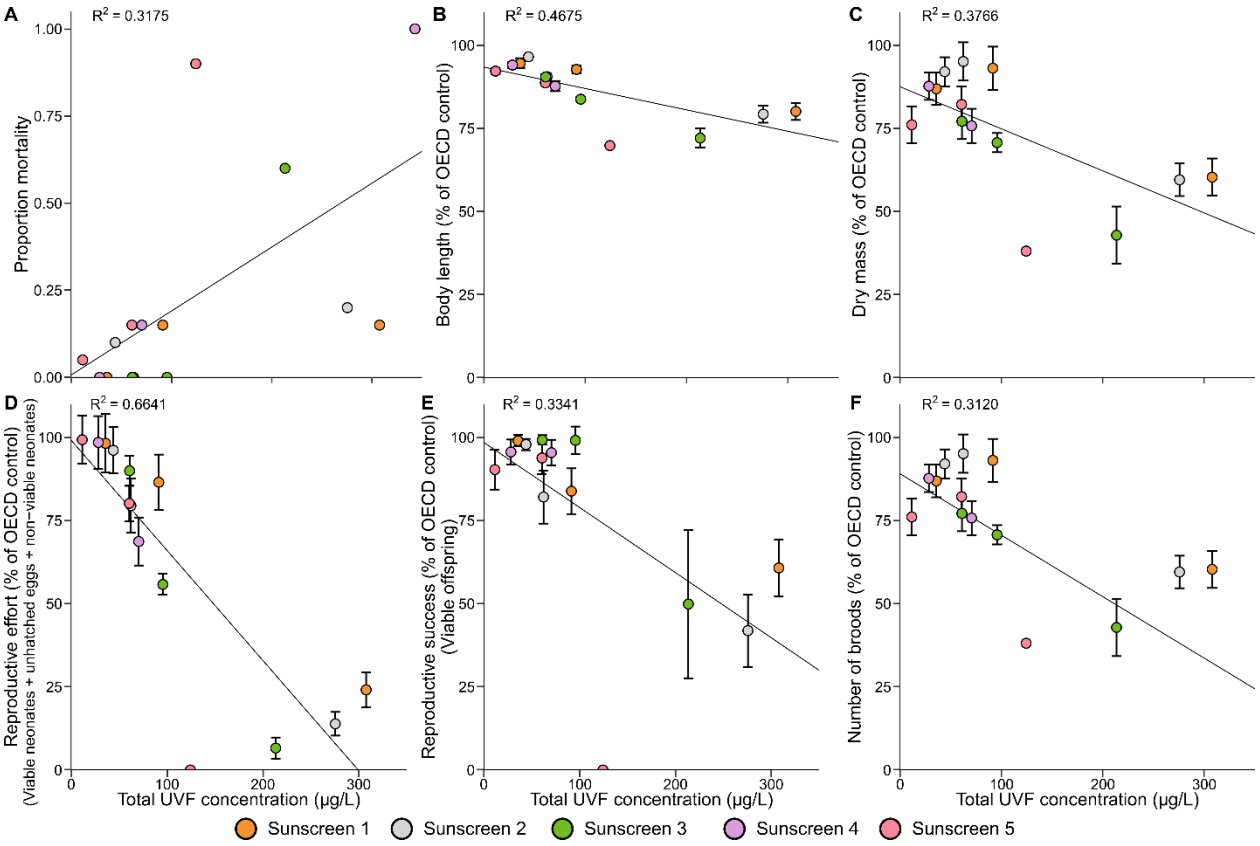


Figure 6.5. The proportion of *D. magna* experiencing mortality by the 21st day of exposure to sunscreens vs the total concentration of all UVFs within the respective treatment (A). Daphnid body length (B) and dry mass (C) were quantified on the 21st day of exposure in surviving replicates while reproductive effort (D), successful reproduction (viable neonates) as a proportion of reproductive effort (E) the number of broods (F) are derived from all reproduction observed in surviving replicates over 21 d. Data presented in panels B-F are scaled as a percentage of the mean value observed in the OECD (water) control treatment. Each point represents the mean ± SEM of 20 starting replicates, while the lines represent the fitted linear regression model of each measured characteristic vs the total UVF concentration of each treatment. The adjusted coefficient of determination (R2) of each regression is indicated above each panel.

6.6 Supplementary materials

Table S6.1. Exposure solution concentrations tested for 48 h median lethal concentration (LC50) and median effect concentration (EC50) experiments. 6 replicates of 5 < 24 h old *D. magna* neonates were exposed at each of the indicated concentrations, which are indicated in % v/v for sunscreen samples and mg/L for EHS and HMS.

Treatment	Concentrations tested
SS1	0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05
SS2	0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05
SS3	0.0001, 0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05
SS4	0.00002, 0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2
SS5	0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2
Homosalate	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100
Octisalate	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100

Table S6.2. Estimated 48 h median lethal concentrations (LC50) and median effect concentrations (EC50) of *D. magna* exposed to sunscreens or individual UVFs. Values are expressed as the means and 95% confidence interval from 6 replicates of 5 < 24 h old neonates per test concentration. Values are expressed as % v/v for sunscreen samples and mg/L for individual UVFs. Confidence intervals listed as NA could not be estimated by the probit model. Data from Boyd et al. (2021) were re-analyzed from the original manuscript to match the methods used for all other data from the present study (see manuscript section 6.2.5).

UVF	48 h LC50 (95% CI)	48 h EC50 (95% CI)	Data source
Avobenzone	22.4 (6.64, 53.5)	2.61 (1.74, 3.62)	Boyd et al., 2021
Homosalate	9.86 (8.42, 11.8)	2.97 (2.52, 3.56)	This study
Octisalate	3.41 (2.86, 4.18)	1.58 (NA, NA)	This study
Octocrylene	6.53 (5.36 8.17)	0.0557 (0.0457, 0.0697)	Boyd et al., 2021
Oxybenzone	2.21 (1.77, 2.89)	1.43 (1.18, 1.80)	Boyd et al., 2021
SS1	0.00872 (0.00734, 0.0106)	0.00197 (0.00157, 0.00265)	This study
SS2	0.0101 (0.00865, 0.0121)	0.00223 (0.00190, 0.00287)	This study
SS3	0.0103 (0.00882, 0.0123)	0.00303 (0.00231, 0.00405)	This study
SS4	0.0254 (0.0214, 0.0312)	0.00506 (0.00385, 0.00670)	This study
SS5	0.0273 (0.0221, 0.0348)	0.00455 (0.00288, 0.00664)	This study

Table S6.3. The ratios of acute toxicity (48 h LC50) and the total UVF content of each sunscreen product were calculated with respect to the sunscreen yielding both the least toxic (highest) 48 h LC50 and the lowest total UVF concentration (SS5). Larger values in the LC50 ratio indicate higher toxicity with respect to SS5, and larger values in the total UVFs ratio indicates a higher UVF concentration than observed in SS5.

Sunscreen	LC50 vs SS5	Total UVFs vs SS5
SS1	3.13	2.48
SS2	2.70	2.23
SS3	2.65	1.72
SS4	1.08	2.77
SS5	1.00	1.00

Table S6.4. Limits of detection and limits of quantification for each UVF during sunscreen sample analysis. All values are expressed in µg/L.

Analyte	Limit of detection	Limit of quantification
Avobenzone (small)	0.388	1.18
Avobenzone (large)	0.504	1.53
Homosalate	1.92	5.82
Octisalate	2.31	7.00
Octocrylene	0.306	0.929
Oxybenzone	0.485	1.47

Table S6.5. Summary of analysis characteristics for the heated electrospray ionization method (HESI). NCE refers to normalized collision energy. Avobenzone (small) refers to the first peak obtained during elution, while avobenzone (large) refers to the second peak obtained.

Compound	Formula	Adduct	Retention time (min)	Resolution	m/z	Target peak	Confirming peak	NCE (%)
Benzophenone-d_{10}	$C_{13}D_{10}O$	+H	0.76	60000	193.1432	110.0648	N/A	45
Avobenzone (small)	$C_{20}H_{22}O_3$	+H	1.71	45000	311.1642	161.0961	135.0441	41
Avobenzone (large)	$C_{20}H_{22}O_3$	+H	2.30	45000	311.1642	161.0961	135.0441	41
Octocrylene	$C_{24}H_{27}NO_2$	+H	2.50	45000	362.2115	250.0863	232.0757	15
Oxybenzone	$C_{14}H_{12}O_3$	+H	1.09	60000	229.0859	151.0389	105.0335	43

Table S6.6. Summary of analysis characteristics for the atmospheric pressure chemical ionization method (APCI). NCE refers to normalized collision energy.

Compound	Formula	Adduct	Retention time (min)	Resolution	m/z	Target peak	Confirming peak	NCE (%)
Methyl salicylate	$C_8H_8O_3$	-H	1.4	60000	151.0401	91.0189	N/A	50
Homosalate	$C_{16}H_{22}O_3$	-H	1.9	60000	261.1496	93.0346	137.0244	48
Octisalate	$C_{15}H_{22}O_3$	-H	1.9	60000	249.1496	137.0244	93.0346	42

Table S6.7. Dilution concentrations for calibration solutions (Cal) used for UVF quantification. All concentrations are provided as ng/mL. Avobenzone (small) refers to the first peak obtained during elution, while avobenzone (large) refers to the second peak obtained.

Compound	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
Benzophenone-d_{10}	100	100	100	100	100	100	100	100
Avobenzone (small)	1	2.5	5	10	25	50	100	200
Avobenzone (large)	1	2.5	5	10	25	50	100	200
Octocrylene	5	12.5	25	50	125	250	500	1000
Oxybenzone	1	2.5	5	10	25	50	100	200
Methyl salicylate	200	200	200	200	200	200	200	200
Homosalate	5	25	50	75	125	250	500	750
Octisalate	5	25	50	75	125	250	500	750

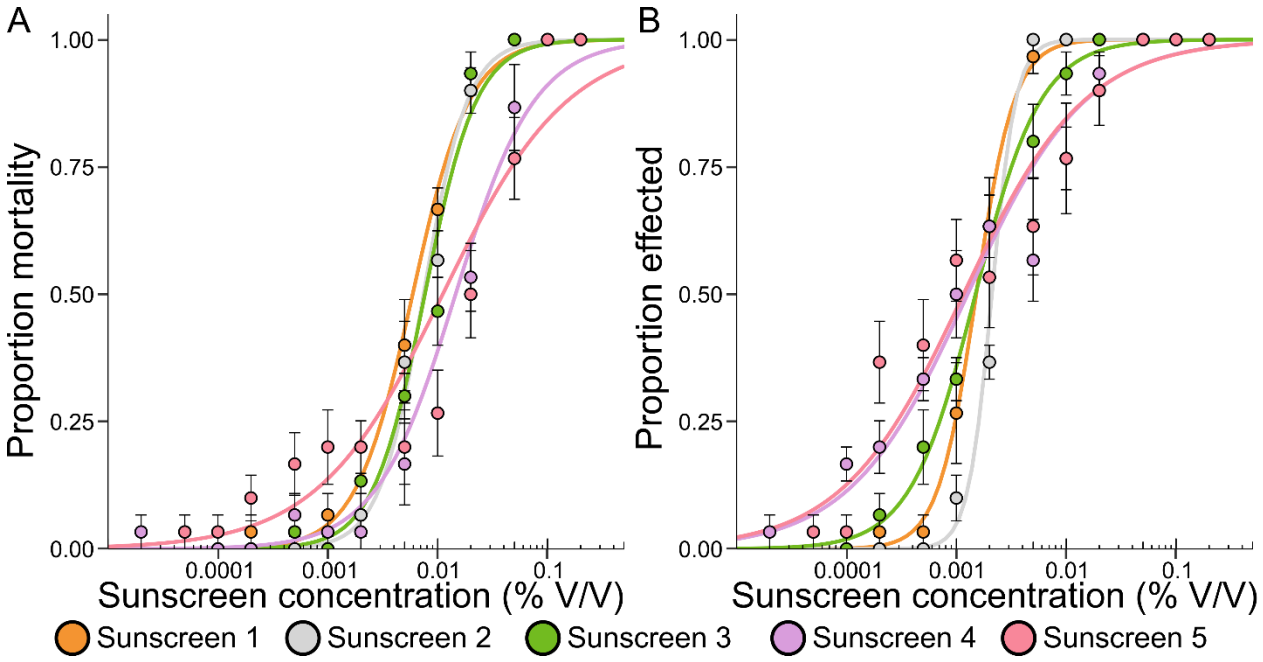


Figure S6.1. Dose/response curves of *D. magna* exposed to sunscreens to determine the median lethal concentration (LC50; A), and the median effect concentration (EC50; B). Each point represents the mean ± SEM of 6 replicates of 5 < 24 h old neonates exposed to the test concentration for 48 h.

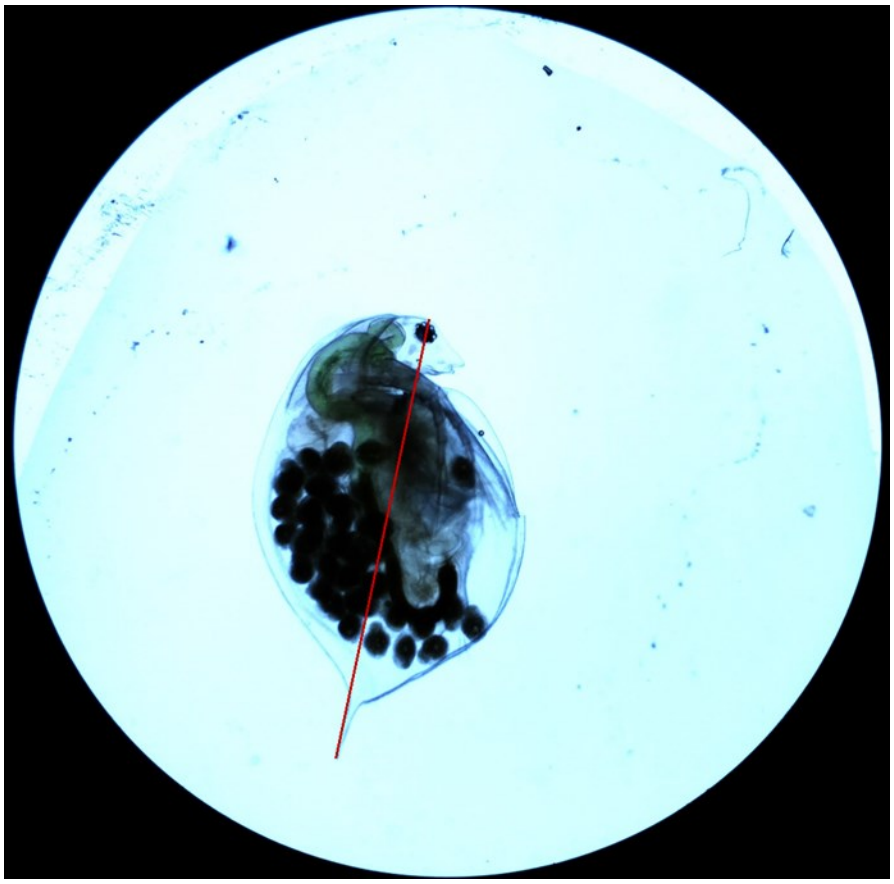


Figure S6.2. The method used to measure *D. magna* body length is depicted by the red line. Daphnids were measured from the edge of the carapace above the centre of the eye spot to the tip of the tail.

Chapter 7: Are standard test species still relevant? A comprehensive assessment of *Daphnia pulex* reared in laboratory and wild environments and their responses to organic ultraviolet filters

Boyd, A., Stewart C.B., McCuaig, J.D., Martin, S., Legge, A., Doty, D., Mitchelmore, C.L., Blewett, T.A., 2025. Are standard test species still relevant? A comprehensive assessment of *Daphnia pulex* reared in laboratory and wild environments and their responses to organic ultraviolet filters. Submitted to Environment International.

7.1 Introduction

The increasing quantity and variety of anthropogenic contaminants released into natural environments since the Industrial Revolution is a growing cause for concern (aus der Beek et al., 2016; Nunes et al., 2023; Stehle and Schulz, 2015). To address the environmental risk of contaminants, standardized toxicity test guidelines have been developed that simulate study systems with simplified models of their real-world counterparts, allowing critical factors (i.e., the contaminant under study) driving these observed outcomes to be identified. These standard test methods are used extensively in regulatory studies to provide defensible, reliable and reproducible data; however, these surrogate organisms are limited in species diversity and the resulting data may have decreased environmental relevance for real-world situations (Laskowski et al., 2010; Van de Perre et al., 2018). Creating standardized test practices to accommodate lab-based research has increased the accessibility and output of research through reduced costs and complexity in comparison to performing field-based research. While this has the benefit of increasing the rate at which data can be generated and the reproducibility of research due to the commonality of methods between research groups that follow the same test guidelines (Romero-Blanco and Alonso, 2022; Van de Perre et al., 2018), all lab-based research requires assumptions to be made of the methodology used in their testing to accommodate the simplification of real-world environments (McCarty, 2012). It is critical that these assumptions are tested to confirm their validity, and to ensure that the data generated from any study are interpreted appropriately in the context of the specific methodology used.

Lab-based research uses model species that have been cultured in the laboratory to reduce environmental variability associated with an organism's life history traits (e.g., age, timing of reproduction), prior exposure to chemical or biological stressors (e.g., pathogens), and other traits such as circadian rhythm (e.g., light cycles, temperature) and resource availability (Romero-Blanco and Alonso, 2022; Shaw et al., 2008). Rearing common test species under stable conditions has allowed for

the creation of laboratory subtypes; lineages that have acclimated to the specific conditions of the lab environment that exhibit genetic and phenotypic stability through selected inbreeding within a small population (Brown et al., 2012; Igawa et al., 2015; Lagisz et al., 2011), which could result in a lowered environmental plasticity (Swillen et al., 2015). Through the use of these lab subtypes, researchers are able to control the effects of biological variability in their studies (Shaw et al., 2008), which can be further leveraged in the case of cyclical parthenogens such as *Daphnia magna* and *Daphnia pulex*, where test guidelines require that organisms reproduce strictly through parthenogenic rather than sexual reproduction (Environment and Climate Change Canada, 2016; OECD, 2012; United States Environmental Protection Agency, 2016b), further reducing the variability of lab populations as the resulting offspring are genetic clones.

The use of these laboratory lineages of model organisms presents a critical assumption that these populations are accurate representations of their wild counterparts; however, this may not be the case as the stable conditions in which lab populations are cultured in are antithetical to the variable and stochastic nature of real-world ecosystems (Laskowski et al., 2010; Romero-Blanco and Alonso, 2022; Van de Perre et al., 2018). Previous studies have demonstrated many differences between lab and wild populations in a variety of model species, observing that wild populations can exhibit greater variability in response to stressors in *Daphnia magna* (Barata et al., 2000), *Daphnia dentifera* (Stewart Merrill et al., 2019) and *Gasterosteus aculeatus* (Robertson et al., 2016). In several species of teleost fish, wild populations have been demonstrated to be more resilient to stressors (Carline and Machung, 2001; Hirakawa and Salinas, 2020), perhaps developing more robust compensation strategies due to living in variable natural environments (Vincent, 1960). In the case of *Caenorhabditis elegans*, copper sensitivity was observed to decrease with longer lab domestication periods, suggesting that lab populations become highly specialized for their specific culture conditions (Heaton et al., 2022). Others have found that relative stressor sensitivity is determined on a case-by-case basis, dependent upon the

specific stressor, species and population tested (Romero-Blanco and Alonso, 2022). Many of these studies captured wild organisms and reared them under lab conditions so that genetic factors could be isolated from environmental influences (Barata et al., 2000; Hirakawa and Salinas, 2020; Morgan et al., 2019; Stewart Merrill et al., 2019); however, by doing so these studies have assumed that transferring wild organisms into synthetic laboratory water that is typically much less chemically complex will not have a confounding influence on the organisms' performance. While many environmental factors such as seasonality, predation, and competition can be mitigated by bringing wild organisms into the lab, other factors such as disease, water quality and composition can be specific to the water that organisms are cultured in; therefore, replacing a wild organism's natural water with laboratory water has the potential to influence how that organism responds to stressors during experimentation (Laskowski et al., 2010), which has not been adequately assessed to date.

This study sought to determine how responses to chemical stressors can deviate between lab-based research and natural environments by comparing lab and wild populations of *D. pulex* when in their ancestral waters, and when reared in the water of their counterparts (i.e., ancestral vs lab-made waters). These objectives were tested with organic ultraviolet filters (UVFs) avobenzone (AVO), octocrylene (OCT) and oxybenzone (OXY). UVFs are active ingredients in sunscreens and personal care products to protect against ultraviolet radiation (Berardesca et al., 2019) and can ubiquitously contaminate aquatic environments by leaching off skin when wearing sunscreen in the water (Giokas et al., 2007; Labille et al., 2020; Peng et al., 2017b; Pintado-Herrera et al., 2017), or through incomplete removal from wastewater treatment plant effluent (Emnet et al., 2015; Kasprzyk-Hordern et al., 2009; Wick et al., 2010), resulting in a typical range of coastal and freshwater environmental concentrations from 10 ng/L up to 10 µg/L (Mitchelmore et al., 2021; National Academies of Sciences, Engineering, and Medicine, 2022; Tovar-Sánchez et al., 2020). These UVFs were selected as the chemical stressors for this study as previous lab and wild toxicology comparisons have focused on standard, inorganic

toxicants such as copper and cadmium (Barata et al., 2000; Heaton et al., 2022); therefore, data on a new class of emerging contaminants can prove valuable in furthering our understanding and validation of current risk assessment practices. In addition, the toxicity of these UVFs has been previously assessed in *D. magna*, with reported effects including 100% mortality to 7.5 µg/L OCT after 7 d (Boyd et al., 2021) and 60% mortality to 16.5 µg/L OXY over 21 d (Boyd et al., 2023a). Assessment of the toxicity of these contaminants in the closely related species of *D. pulex* can additionally further our understanding of the risks posed to freshwater environments. Recent analyses of risk have determined risk quotients < 1 for most available data, indicating that the risk of UVFs to many environments is low; however, these studies often cite concerns regarding the quality and environmental relevance of toxicity data as a challenge towards conducting reliable risk assessments (Carve et al., 2021; Duis et al., 2022; Mitchelmore et al., 2021; National Academies of Sciences, Engineering, and Medicine, 2022). Much of our current knowledge on UVF toxicity in freshwater environments stems from research using lab organisms; therefore, these chemicals present an opportunity to assess the applicability of current toxicity data towards risk assessment outcomes, as moderate deviations in sensitivity could either solidify UVFs as a threat to the environment or categorize them as low risk contaminants that require only periodic monitoring to ensure contamination remains at safe levels in most environments.

7.2 Materials and methods

7.2.1 Sample site description and wild Daphnia collection

Wild *Daphnia* were collected from Driedmeat Lake, Alberta, Canada (52.876204, -112.755848) on June 1st, 2023. Driedmeat Lake is a 10 km long ribbon lake, approximately 15 km south of Camrose, Alberta (population 19,000; Statistics Canada, 2023) and is primarily surrounded by agricultural uses. Water and *Daphnia* were collected within 2 m of the shoreline at Tillicum Beach (unincorporated area, population 130; Statistics Canada, 2023). Low levels of recreational activity were present during the sampling times (< 10 people), including fishing and canoeing. Specimens were collected via bucket

catch and returned to the University of Alberta in Edmonton and candidate *Daphnia pulex* individuals were identified by the morphology of the postabdominal claw (Figure 7.1). Further genetic confirmation of the *D. pulex* species identity was obtained by sequencing the cytochrome c oxidase subunit I gene (COI) in the F2 generation of cultured wild daphnids, which is described in further detail in the supplementary materials below. Water was collected by submersible pump held approximately 20 cm below the surface and 20 cm above the lake bottom to minimize collection of surface algae and disturbance to the lakebed. Collection occurred monthly to account for changes to the lake water composition over time while rearing test cultures within the lab. Additional information regarding the sampling times and locations is available in the supporting information.

7.2.2 Daphnia culture maintenance

Water from Driedmeat Lake was filtered by pumping all collected water through double layered filter floss 3x to remove invertebrates and fibrous algae while minimizing the removal of other components in the water. The resulting water is referred to as lake water in the following text. Stocks of each water type were held in closed 55-gallon plastic drums under constant aeration. Dechlorinated City of Edmonton water was prepared with 294 mg/L $CaCl_2$, 123 mg/L $MgSO_4$, 64.8 mg/L $NaHCO_3$ and 5.80 mg/L KCl to produce culture water, prepared according to Organization for Economic Co-operation and Development (OECD) guidelines (OECD, 2012), referred to as OECD water in the following text. The chemical composition of both OECD (lab) and lake water is detailed in Table 7.1.

A laboratory *Daphnia pulex* culture was produced from individuals acquired from Aquatic Research Organisms (ARO; US) in August 2022 and continuously cultured in the University of Alberta Biological Sciences Department at approximately 22°C, pH 7.8 and a 14:10 h photoperiod. This culture originates from the US Environmental Protection Agency (EPA) Newtown, Ohio lineage, and has been cultured continuously by ARO since July 1985 (S. Sinitski, ARO, personal communication, June 14, 2023). New cultures were produced from this laboratory lineage on June 14th, 2023, and maintained on

a 16:8 photoperiod under fluorescent lighting to match the housing conditions of the collected wild *Daphnia* for this study. The pH of the laboratory cultures used in this study was adjusted to 8.6 to match the pH of the collected lake water. All *Daphnia* cultures and experiments were maintained at 22.5 ± 0.7°C on a 16:8 photoperiod for the duration of this study, and the spectral composition of the light used for all culturing and experiments performed in the laboratory during this study is depicted in Figure S7.1.

Cultures of lab *D. pulex* in OECD water and wild *D. pulex* in lake water (referred to as ancestral waters in the following text) were used to produce cultures of lab *D. pulex* in lake water, and wild *D. pulex* in OECD water (referred to as non-ancestral waters). The terminology used to describe each of the four *Daphnia* cultures is outlined in Table S7.5. Cultures in non-ancestral waters were created by raising < 24 h old neonates from the ancestral water culture in a 50:50 mixture of OECD and lake water for 1 week before becoming permanently maintained in 100% non-ancestral water for the remainder of the study. The four resulting cultures were maintained by housing 30 individuals in 1 L of their respective water changed every 2-3 days and fed a daily ration of 3 mL of 3 x 10^7 cells/mL freshwater green algae (*Raphidocelis subcapitata*) and 3 mL of yeast, cereal leaf and trout chow (YCT) mix, each purchased from ARO. Neonates from the 3rd brood and later were used to create the following generation of each culture. All experiments were performed using < 24 h old neonates comprising the F2 generation of each culture living in their specific rearing conditions for the study.

7.2.3 Exposure solutions and water chemistry analysis

pH was monitored approximately once per week in OECD and filtered lake water stocks using an Orion Star A211 pH probe (Thermo Scientific, US; Table S7.4). Ammonia, nitrite, and nitrate nitrogenous waste products were monitored using colourimetric freshwater test kits following the instructions provided (API, US). Major ions as well as dissolved and total organic carbon and inorganic elements were quantified from lake water samples from each collection date, as well as each OECD

water stock used during the 21-d experiment. Samples were filtered and analyzed at the Biogeochemical Analytical Service Laboratory, an ISO/IEC 17025 accredited facility at the University of Alberta. Organic carbon was quantified using a Shimadzu TOC-L Total Organic Carbon Analyzer following US EPA method 415.1 (United States Environmental Protection Agency, 1979), chloride and sulphate were quantified using a Dionex DX600 Ion Chromatography System following US EPA method 300.1 (Hautman et al., 1997), and inorganic elements were quantified using an Agilent 7900 Inductively Coupled Plasma-Mass Spectrometer following American Public Health Association method 3125B (American Public Health Association, 2012).

The UVFs AVO, OCT and OXY were dissolved in methanol (MeOH) to produce individual stock solutions (MilliporeSigma, US). Separate dilutions were prepared in OECD and lake water, with each exposure containing 0.5% v/v MeOH for the 48 h median lethal concentration study, and 0.001% v/v MeOH for the 21 day chronic exposure study. Fresh stocks and exposure solutions were made at the start of both 48 h and 21 d experiments, with a complete refresh of all stocks and exposure solutions occurring approximately halfway through the 21 d experiment to minimize UVF degradation (Table S7.10). Exposure solutions for the 21 d experiment were held in darkness under constant aeration to ensure mixing until aliquoted into test vessels for *Daphnia* exposures. All glassware was rinsed in hexane and submerged in 5% HNO_3 for 24 h, washed with distilled water, then submerged in 10% EtOH for 24 h followed by a final distilled water rinse prior to experimentation. During experiments, used glassware was submerged in 10% EtOH for 24 h and then washed with distilled water prior to reuse.

20 mL aliquots of each chemical treatment in OECD and lake water were collected in 40 mL volatile organic analysis amber glass vials once per week on days 0, 7, 14 and 21 of the chronic 21 d study from the exposure solutions, which were shipped overnight (0 – 4°C) to the Chesapeake Biological Laboratory at the University of Maryland Center for Environmental Science for extraction

and UVF quantification. Upon receipt of the samples at the Chesapeake Biological Laboratory, a liquid:liquid extraction procedure was used (refer to Conway et al., 2021), which has been shown to provide reproducible recoveries of all UVFs tested > 70% (and so no QAQC adjustments were made in this study). 5 mL of hexane was added to the 20 mL of sample water, and the samples shaken for 2 hours at room temperature. The solutions were allowed to separate and 100 µL of the hexane was added to a 1.5 mL amber glass vial containing a methanol:internal standard mix (i.e., 900 µL MeOH and 25 µL internal standard (200 µg/L in MeOH)). Samples were run on an Agilent 6420 LC QQQ MS/MS. Samples were run in triplicate at a flow rate of 0.2 mL/min and isocratically at 97% methanol/3% of 0.1% formic acid water. The injection volume was 10 µL and a severe wash-step of 40 sec flushing with isopropyl alcohol was implemented, due to the extreme adsorption of OCT. An ACE Excel C18-PfP, 150mm, 2.1 mm column was used to retain the UVFs. The instrument limit of detection (LOD) and limit of quantification (LOQ) for all UVFs were 0.01 µg/L and 0.02 µg/L, respectively.

7.2.4 Median lethal concentration study

Acute 48 h median lethal concentration (LC50) and median effect concentration (EC50) tests were performed for each UVF following OECD test 202 guidelines (OECD, 2004). Each test concentration (refer to Table S7.6) contained n = 5 < 24 h old neonates in 20 mL of exposure solution that were fasted for the duration of the test, replicated 4 times. After 48 h, daphnids were scored under a dissecting microscope to determine mortality, defined as a lack of gill respiratory movement over a 15 s period. Immobilization was determined by the presence of gill respiratory movement accompanied by a lack of whole-body locomotion. Paired controls of OECD water without additions and 0.5% MeOH were performed alongside each replicate, with replicates exhibiting < 20% stress in controls (1/5 daphnids) considered acceptable, while ensuring that < 10% (2/20) of control daphnids exhibited stress across all replicates (OECD, 2012). Solvent concentrations above the OECD recommendation of 0.01% v/v were required to obtain complete dose/response curves (OECD, 2019); therefore, LC50 and

EC50 tests of MeOH were also performed to ensure that the solvent concentration used for the acute tests was below the no-observed effect concentration for each daphnid culture (Figure S7.2; OECD, 2019). These tests were used to establish a range of toxicity to determine the concentrations used for the 21 d experiment described in section 7.2.5 below, but primarily used concentrations above the solubility limit of each UVF (AVO = 27 µg/L, OC = 40 µg/L, OXY = 6,000 µg/L; National Academies of Sciences, Engineering, and Medicine, 2022). Additional reference toxicant LC50s were performed with Cu to validate the condition of each culture during experimentation.

7.2.5 21 d chronic exposure study

A 21 day chronic exposure study was performed following OECD test 211 guidelines (OECD, 2012), exposing daphnids to one of three concentrations (10, 50 or 100 µg/L, nominal) of AVO, OCT or OXY (Table 7.3), a water control without additions, or a 0.001% v/v MeOH solvent control (OECD, 2019). The solvent concentration used for 21 d exposures was an order of magnitude below the OECD recommendation of 0.01% v/v (OECD, 2012). These 11 treatments were repeated across all 4 *Daphnia* cultures described above for a total of 44 unique treatments. All treatments within a single culture group were started on the same day, with each of the four culture group exposures starting on consecutive days to minimize temporal differences between various treatments (Table S7.9). These exposures were performed as simultaneously as possible to minimize the duration of time that wild daphnids were held in the laboratory environment while still ensuring that experiments were performed on F2 generation neonates, in order to minimize the changes that may occur over time by nature of removing wild *Daphnia* from their natural environment. For each treatment, 20 replicates of < 24 h old neonates were individually exposed in 50 mL of their respective treatment solutions. Exposures were performed through static renewal, with a 100% refresh occurring every 2 days. Daphnids were provided with a daily ration of 200 µL each of YCT and freshwater green algae until the 19th day of exposure, after which fasting occurred in preparation for metabolic rate measurements. Complete water

changes were performed every 2 days, and daphnids were scored daily for mortality and reproductive effort, which was the sum of viable neonates and non-viable neonates (those that were dead at the time of scoring each day). Unhatched eggs that were released from the parent daphnid were scored but not included in the data analysis due to the difficulty in obtaining accurate egg counts due to the presence of small debris in lake water treatments. Each daphnid was scored on day 10 and 21 for the presence visible epibionts (*Brachionus rubens)* using a 0-3 rating system (Table S7.11 & Table S7.12).

Assessments of metabolic rate followed by imaging to obtain body length were performed on all surviving daphnids on the 21st day of exposure in a random order. A 24-well glass optical fluorescence respirometry microplate (Loligo Systems, Denmark) was used to assess metabolic rate by placing individual daphnids into 200 µL wells filled with the appropriate exposure solution. All headspace was removed from the well, which was then sealed with a silicone gasket and weighed down. Dissolved oxygen was then recorded every 15 s for a total of 1 h per plate. Upon completion of the metabolic rate assay, daphnids were imaged under an inverted optical microscope to obtain core body length (edge of the carapace above the eye spot to the base of the tail) and tail length measurements following the method described in Figure S7.9. Daphnids were then placed into an incubator at 45°C overnight and dry body weights were obtained using an Orion Cahn C-35 microbalance (Thermo Fisher, US).

The first 10 minutes of data for the metabolic rate assay were considered the acclimation period and excluded from the analysis. The rate of change in oxygen over the remaining 50 minutes of data was divided by the dry mass of each daphnid to determine the mass specific metabolic rate using Formula 7.1 described below. Blank wells containing OECD or lake water with no *Daphnia* were performed on each microplate run as appropriate, with the average change in dissolved oxygen of all blanks of a plate subtracted from the daphnid metabolic rates obtained from that same plate to correct for oxygen consumed by other biological activity in the water.

Formula 7.1: $MR = \frac{\Delta O_{2_{exp}} - \Delta O_{2_{blank}}}{m}$

Where MR refers to the mass specific metabolic rate of a daphnid (mg O_2 L^{-1} h^{-1} mg^{-1}), $\Delta O_{2_{exp}}$ and $\Delta O_{2_{blank}}$ refer to the change in dissolved oxygen over time measured in a well containing a daphnid or the average of all blank wells on a plate, respectively (mg O_2 L^{-1} h^{-1}) and m refers to the dry mass of the daphnid (mg). All calculated mass specific metabolic rates with a correlation coefficient < 0.9 were excluded from the analysis. Additionally, one microplate run containing individuals from the lab *Daphnia* in OECD water treatments was excluded due to unexplained high oxygen consumption in the blank wells > 2 SD above the mean for all other blank wells for OECD water-based treatments.

7.2.6 Statistics

All statistical analyses and figures were generated using R version 4.3.1 (R Core Team, 2024). Lotka's equation (Lotka, 1913) was used to estimate the intrinsic rate of population increase (r) by pooling all replicates within each treatment group. Nominal LC50 and EC50 concentrations were estimated using the probit method provided by the package "ecotox". Differences between LC50s and EC50s within a *Daphnia* culture were assessed by pairwise ratio test using the Benjamini & Hochberg p-value correction for false discovery rate. For all other statistical tests, normality was assessed via Shapiro-Wilk test, and homoscedasticity was assessed using Levene's test. Data that met all assumptions were tested with a one-way ANOVA and Tukey test, while data that did not meet all assumptions and could not be transformed were tested with a Kruskal-Wallis and Dunn's test with significance assessed at $\alpha = 0.05$. All values are reported as a mean ± standard error of the mean unless otherwise indicated.

7.3 Results

7.3.1 Base water and exposure solution chemistry

Driedmeat Lake waters typically exhibited a greater diversity and greater concentrations of dissolved and total analytes than OECD water (Table 7.1). Of note is an approximately 9-fold greater concentration of organic carbon in lake water, 4.6-fold greater sulfate, and the presence of dissolved elements such as As, Cu, Fe, and Ni at concentrations of 1.1 – 9.3 µg/L in lake water that were below detectable limits in OECD water, with the exception of 0.930 ± 0.0250 µg/L Cu detected in OECD water. OECD waters contained a 3-fold greater concentration of Ca and a 6.8-fold greater concentration of dissolved Al; however, the latter is driven by an exceptionally high Al concentration detected in the August 6 sample of OECD water (Table S7.3). All analytes in both water types were typically temporally consistent, except for high Cu measured on June 1 and high Fe on July 21 in the lake water samples.

Low control levels of all tested UVFs were detected in both water types, with AVO and OXY concentrations < 0.02 µg/L, and OCT concentrations reaching 0.0950 ± 0.0691 µg/L in OECD water and 0.0325 ± 0.0177 µg/L in lake water (Table 7.2). UVF concentrations in the 21-d experiment in freshly made OECD water solutions were 33.2 ± 6.29% and 47.8 ± 5.15% for AVO and OCT, decreasing to 15.5 ± 8.39% and 10.9 ± 5.41% after 1 week (Table S7.8). Similar patterns were observed in the lake water solutions, with AVO and OCT quantified at 46.6 ± 8.48% and 47.8 ± 5.39% in fresh solutions while concentrations after 1 week were 28.3 ± 10.4% and 8.94 ± 3.74%, respectively. In both water types, OXY was quantified at 67.7± 2.86% in the first set of freshly made exposure solutions but was not detectable in the second set of fresh exposure solutions. Despite this, OXY was detected at low levels in both sets of exposure solutions after 1 week at 8.69 ± 6.99% in OECD water and 0.184 ± 0.115% of nominal concentrations in lake water. Average AVO concentrations were approximately 1.6-fold greater in the medium and high lake water exposure solutions than the OECD solutions; however,

these differences were not statistically significant (t-test, $p > 0.220$), while OCT and OXY concentrations were similar in both water types across all tested concentrations (Table 7.3).

7.3.2 Median lethal and effect concentrations

48-h acute toxicity was similar between lab and wild *Daphnia* in ancestral waters for all UVFs (Figure 7.2), with nominal LC50s ranging from 2.39 mg/L OXY (95% CI 1.92 – 3.13) to 5.65 mg/L AVO (95% CI 3.92 – 8.42), although a statistical difference was detected with OCT despite the similar estimated LC50s (ratio test, $p = 0.023$). Wild *Daphnia* in OECD were more sensitive to UVFs (ratio test, $p < 0.001$), with nominal LC50 concentrations ranging from 0.392 mg/L OCT (95% CI 0.314 – 0.510) to 0.710 mg/L AVO (95% CI 0.521 – 0.960). LC50s were greater for lab *Daphnia* in lake water than all other colonies (ratio test, $p < 0.05$), with LC50s ranging from 3.82 mg/L OXY (95% CI 2.65 – 6.20) to 27.0 mg/L AVO (95% CI 21.5 – 35.7). The EC50 trends for all UVFs were similar to the LC50 trends; however, nominal EC50 values were moderately lower than the corresponding LC50 in all cases. An LC50 of approximately 3% v/v MeOH was observed for all *Daphnia* colonies except for wild *Daphnia* in OECD water, which had an LC50 of 1.44% v/v (95% CI 1.21 – 1.76; ratio test, $p < 0.001$). All LC50 and EC50 results can be found in Table S7.7.

7.3.3 21 d chronic exposure study

Daphnid mortality over 21 d was similar for both the water and solvent controls within each culture, with populations in ancestral water experiencing 10-20% mortality compared to 25-30% mortality for cultures in non-ancestral water (Figure 7.3). Mortality was observed in all *Daphnia* cultures in a dose-dependent manner due to UVF exposure; however, populations in non-ancestral water experienced much higher mortality in all treatments. A large increase in mortality was observed in the lab *D. pulex* in lake water culture for all UVF treatments beginning on the 11^{th} day of exposure, resulting in 50 – 65% mortality in low exposure doses, 75 – 85% mortality in medium doses and 90 –

95% mortality in high exposure doses. Greater mortality was typically observed in the other non-ancestral water population of wild *D. pulex* in OECD water than in either population in ancestral water; however, mortality ≤ 30% was observed in low and medium UVF exposure doses, compared to 55% mortality in the AVO and OXY high doses, while the OCT high dose resulted in 80% mortality after 4 days, and 100% mortality after 12 days. Both lab and wild *Daphnia* populations in ancestral waters experienced ≤ 25% mortality in all UVF treatments, except for lab *Daphnia* in OECD water, which experienced 40% and 55% mortality when exposed to high doses of AVO and OXY, respectively, and wild *Daphnia* in lake water experiencing 45% mortality to the OCT high dose.

Differences in the number of viable neonates produced by daphnids surviving the entire 21 d exposure were found between chemical treatments within the lab *Daphnia* in OECD culture (Figure 7.4), as approximately 30% fewer neonates were produced when exposed to medium and high doses of AVO (125 ± 35.5 & 130 ± 32.8 neonates, respectively) compared to the water (177 ± 22.5) and MeOH controls (187 ± 30.7; one-way ANOVA, $p = 0.0123 – < 0.001$). Wild *D. pulex* exposed to lake water produced 55% fewer neonates when exposed to the high dose of OCT (82.6 ± 33.3) than either water (186 ± 33.0) or MeOH controls (182 ± 23.4; one-way ANOVA, $p < 0.001$). *Daphnia* raised in non-ancestral water produced approximately 25% fewer neonates than their counterparts in ancestral waters in both control treatments, and 15% fewer neonates across all UVF treatments on average; however, these differences varied across all chemical treatments and were more consistent between lab *Daphnia* populations. No differences were observed between either lab or wild *Daphnia* population in ancestral waters in any chemical treatment, apart from 28% fewer neonates produced by lab daphnids exposed to the medium dose of AVO (one-way ANOVA, $p = 0.002$) and the high dose of OCT previously noted in the wild *D. pulex* in lake water population (one-way ANOVA, $p < 0.001$).

No differences in the mass specific metabolic rate were observed within any daphnid culture across chemical treatments (Figure 7.5; Kruskal Wallis, $p \geq 0.198$). The metabolic rate of wild *Daphnia*

in lake water was approximately 20% lower than lab *Daphnia* in OECD water on average across all treatments; however, statistical differences were only detected in the water control, and the medium doses of AVO and OXY. Minimal differences were observed across chemical treatments or colonies in the time required to reach reproductive maturity, with the majority of daphnids releasing their first brood on the 7^{th} or 8^{th} day of the experiment (Figure S7.5). Similarly, most daphnids regardless of treatment or culture produced 6-7 broods over the duration of the experiment (Figure S7.6). Minimal trends were observed in both dry mass and core body length measurements for all daphnid populations and UVF treatments, apart from a 35% decrease in dry mass (Figure S7.8; $p < 0.001$) and a 17% decrease in core body length of wild *Daphnia* in lake water exposed to high doses of OCT (Figure S7.10; $p < 0.001$).

7.4 Discussion

This study represents the first comparison of lab and wild *D. pulex* populations and is the first to directly investigate the impact of culture water on the performance of lab and wild organisms, which revealed several major differences between each test population, driven primarily by whether organisms were cultured in ancestral or non-ancestral waters. All nominal LC50 and EC50 values for lab and wild *D. pulex* populations in ancestral waters were similar to those previously reported in *D. magna* for all chemicals when tested using International Organization for Standardization (ISO) water (Jang et al., 2016; Molins-Delgado et al., 2016), filtered tap water (Park et al., 2017), or OECD water (Boyd et al., 2021). Within this study, both populations in ancestral waters differed greatly in comparison to their counterparts in non-ancestral waters during acute (Figure 7.2) and chronic tests (Figure 7.3 & Figure 7.4).

UVF concentrations for the 21 d experiment were typically < 60% of nominal concentrations in freshly made exposure solutions, and < 40% of nominal in 1 week aged solutions (Table S7.8), indicating the loss of large quantities of each UVF from the aqueous phase of each solution. Previous

studies have reported similar decreases for these UVFs (Blüthgen et al., 2012; Conway et al., 2021; Fel et al., 2019; Giraldo et al., 2017), which has been attributed to the adsorption of chemical to vessel walls (Cormier et al., 2019; O'Malley et al., 2021) and to particulate matter within the water (Benedé et al., 2014). Photodegradation can further reduce UVF concentrations during experiments (Jentzsch et al., 2019; Semones et al., 2017), which would also impact the exposure dose to each daphnid during the present study. The low concentrations (< 0.1 μg/L) of all UVFs detected within both OECD and lake control are also notable. Several studies have reported low levels of UVFs in drinking water (Díaz-Cruz et al., 2012; N. Li et al., 2019; Pai et al., 2020); therefore, the UVFs in the OECD control water could be from pre-existing contamination of the dechlorinated tap water used to produce the water for this study, or from contamination that was introduced during the study. Similarly, the UVFs in the lake water could be from recreational activity (Labille et al., 2020) on Driedmeat Lake or introduced contamination during the study. Regardless of the origin, the concentrations in each water control are several orders of magnitude below the doses used in this study and are expected to have minimal impact on the results discussed below. Also of note is the continued decrease in measured UVF concentrations 1 week after dosing (Table S7.8). Despite the lower exposure doses in aged solutions followed by an increase in UVF concentration upon solution renewal, toxicity appeared to occur independently of solution renewal as mortality increased several days prior to renewal. This suggests that exposure to these UVFs during earlier developmental stages can result in lasting damage that manifests after organism maturation.

7.4.1 Effects of culture water

Wild *Daphnia* in OECD water demonstrated a consistently higher sensitivity to all tested chemicals over 48 h, suggesting that these organisms may be weakened due to the different water type despite having been cultured in it for several generations, which is discussed in greater detail below. High mortality was also observed during 21 d exposures to high UVF concentrations compared to both

populations in ancestral waters (Figure 7.3). The sensitivity of this population is greater than suggested by these results, as very high background mortality (> 40%) was present in untreated controls during initial experimentation; therefore, only neonates that appeared to be in good health via high swimming activity were selected for all 48 h and 21 d experiments described in this manuscript so that background mortality could be maintained at a lower level comparable to the other test populations, while neonates for all other populations were randomly selected from all available neonates at the start of each experiment. Despite this, control mortality for this population reached 25% by the end of the 21 d exposure, rendering the test of the wild *Daphnia* in OECD water population as invalid according to chronic test guidelines, highlighting the extent to which these daphnids were impaired due to the change in culture water used.

Similar trends were observed during the initial culturing of the *Daphnia* populations prior to experimentation; however, it is interesting to note that the initial wild *Daphnia* in OECD generation experienced approximately 20% mortality within 14 days compared to the approximately 40% mortality observed in the following cultured generation prior to the experiments described in this manuscript (data not shown). It is possible that the continued weakening of these daphnids is due to a lack of certain nutrients in OECD water, as essential trace nutrients such as Cu and Mn were present at 70 – 80% lower concentrations while Fe was not detectable in OECD water (Table 7.1). Other important nutrients such as iodine and zinc may also be lacking in the OECD water used; however, these were not quantified as part of this study. Indeed, previous studies have noted a reduction in *D. magna* performance in media containing insufficient Se (Elendt and Bias, 1990) or Cu (Bossuyt and Janssen, 2003), or reduced *D. pulex* growth rates with reduced Fe (Lind and Jeyasingh, 2018). Lam and Wang (2008) reported that the detrimental effects caused by Se deficiency in *D. magna* did not manifest until the F1 generation, as Se is required for glutathione peroxidase function, potentially allowing for cell membrane damage to accumulate over time in deficient organisms. While earlier

generations can experience decreased performance due to trace nutrient deficiencies, recovery has also been reported to occur in populations that have been acclimated to low nutrient levels over longer periods of time (Bossuyt and Janssen, 2003) as populations of many aquatic species are known to adapt to the specific inorganic profile of their local environment (Chapman and Wang, 2000), which may explain the consistently higher performance of lab *Daphnia* in OECD water despite the lower trace nutrient levels. It should be noted that many of these nutrients can likely also be obtained through dietary means; therefore, dissolved analyte concentrations cannot be expected to fully account for the differences observed between each *Daphnia* culture in this study.

The effects of each culture water are likely also driven by the presence of ligands such as particulate organic matter (POM) and dissolved organic carbon (DOC), the latter of which was present in high concentrations in the lake water (Table 7.1). These ligands could offer a protective effect by reducing chemical bioavailability through adsorbing the lipophilic UVFs and preventing them from entering the organism through external exposure routes due to the large size of the chemical-DOC complex (Haitzer et al., 1998), which could contribute towards the apparent decreased sensitivity of lab *Daphnia* in lake water during 48 h acute tests to all UVFs. Previous studies have observed reductions in the uptake and toxicity of other lipophilic chemicals such as pyrethroids (Day, 1991) and polycyclic aromatic hydrocarbons (Ruotsalainen et al., 2010) in the presence of similar concentrations of DOC as those reported in this study. This proposed reduction in acute toxicity due to lake water DOC would be similarly relevant for the wild *D. pulex* in lake water population. This would imply that wild daphnids in lake water were more sensitive to these UVFs than their lab counterparts, but this increased sensitivity was counterbalanced by the reduced availability of chemicals in the exposure solution due to DOC adsorption, resulting in nominal LC50 and EC50 values that are similar to those estimated for lab *Daphnia* in OECD water. The minimal differences in the nominal 48 h LC50s of the less lipophilic MeOH (National Academies of Sciences, Engineering, and Medicine, 2022; National Center for

Biotechnology Information, 2024) in each population cultured in ancestral water is not consistent with this hypothesis, implying that either wild daphnids are more sensitive to only UVFs but not MeOH, or that factors in the lake water other than high DOC are the primary driver of the observed differences in acute toxicity between each population. Further investigation is required to fully assess these differences.

Although lab *Daphnia* in lake water proved to be more resilient than wild *Daphnia* in OECD water during acute chemical exposures, chronic 21 d exposure to UVFs caused high levels of mortality in all tested UVF concentrations (Figure 7.3). For all chemical treatments, mortality greatly increased after the 10^{th} day of exposure, indicating that other factors, in addition to the UVFs, are major contributors towards the observed population decline. While a clear increase in mortality can be observed with increasing UVF doses, the 30% mortality that occurred in both water and solvent controls for this population indicate that culturing lab *D. pulex* in lake water impaired daphnid performance to the extent that which standard toxicity tests would be deemed inviable. Surviving daphnids also exhibited reduced reproduction compared to their counterparts in ancestral water in the absence of chemical stressors (Figure 7.4). Unlike the reduced viability of the wild *Daphnia* in OECD population discussed above, lab daphnids cultured in lake water had greater access to waterborne trace nutrients than those cultured in OECD water. Additionally, the lake water used during experimentation contained moderate levels of nitrogenous waste products (Table S7.4), including approximately 0.75 ± 0.25 mg/L nitrite. Zhang et al. (2023) reported that nitrite concentrations of 1.0 and 3.0 mg/L induced 20% and 80% *D. pulex* mortality after 12 d, respectively. Although the nitrite concentrations in the present study were below these concentrations, it is possible that they were high enough to provide an increased level of stress to the daphnids, reducing their capacity to cope with the additional stress of the UVFs, as evidenced by the lower mortality observed in the water and MeOH control groups (Figure 7.3). It would appear that while the lab *D. pulex* in lake water culture could sufficiently acclimate to the

novel culture water to mitigate the very high mortality reported in the wild *D. pulex* in OECD culture, the surviving organisms are left in a weakened state that rendered them highly susceptible to toxicity from any tested UVF dose.

The microbial environment of the lake water is another important consideration for each population. The lake water likely contains a greater diversity of species including bacteria, protozoa and fungi which could be both beneficial and harmful, depending on each species present (Akbar et al., 2022). Wild *D. pulex* may be partially dependent upon beneficial interactions with certain microorganisms in the lake water that are not sufficiently compensated for when in OECD water. Conversely, culturing lab *D. pulex* in lake water could expose them to harmful pathogens that are novel to this population. In addition, the degradation of UVFs is influenced by the microbial environment (S. H. Lee et al., 2020; Liu et al., 2012), potentially altering the degradation products present within each water type due to differences in the relative contribution of major degradation pathways including photolysis and biodegradation (metabolism). It is clear that many factors could contribute towards the lower viability of both lab and wild *D. pulex* when cultured outside of their ancestral waters, indicating that an organism's response to stressors during experimentation is a product of many factors beyond the specific organism itself.

7.4.2 Dissolved organic carbon influences on UVF toxicity

The measured UVF concentrations in OECD and lake water were similar in all treatments except for the 1.6-fold higher concentrations of AVO detected in lake water for the medium and high exposure doses (Table 7.3). Due to the high concentrations of DOC present in the lake water (Table 7.1), it is likely that an appreciable quantity of UVFs were adsorbed to the DOC present in lake water, transferring UVFs from the aqueous phase to the particulate phase of the exposure solutions, which has been demonstrated to reduce the bioavailability of lipophilic molecules by shifting the major routes of exposure from external routes to dietary routes (Akkanen et al., 2012). While this may reduce the

toxicity of UVFs during acute exposures, food consumption becomes a greater factor for the larger, mature daphnids present during the longer 21-d exposures (Kankaala and Eloranta, 1987). *Daphnia* are capable of consuming DOC and POM in the water as an alternative food source to phytoplankton (Cole et al., 2006; McMeans et al., 2015; Wenzel et al., 2021), providing another dietary exposure route for both lake water populations in addition to the normal dietary exposure that can occur through the uptake of UVFs into algae (Mao et al., 2017). The likely reduced external uptake due to reduced concentration of free UVFs in the lake water may be counterbalanced with an increase in dietary uptake via DOC; however, further research is necessary to determine whether this results in a net increase or decrease in toxicity. The high concentration of DOC as well as algal cells in the lake water should also be noted due to the potential greater access to food for both lake water populations compared to their OECD water counterparts. This was counteracted by providing all daphnids an excess of food during daily feedings to ensure satiation regardless of culture water to minimize the effects of differences in total food availability, as daphnids would instead be limited by the rate at which they can ingest food instead.

7.4.3 Differences between lab and wild populations

As noted previously, toxicity of all tested chemicals was most similar during acute exposures between both lab and wild *D. pulex* populations in ancestral water (Figure 7.2); however, large deviations in sensitivity to UVFs were observed over the 21 d exposures (Figure 7.3 & Figure 7.4). These results indicate that standardized test methods can produce results that are relevant to wild populations despite the differences in test waters and population sources for acute exposures in the case of UVFs, but the differences between lab and wild test populations diminishes the accuracy of the standard model over longer duration exposures. Previous research has demonstrated that incorporating environmentally relevant conditions during experimentation frequently produces results that differ from standard toxicity test results within a single *Daphnia* test population, including temperature

(Guilhermino et al., 2021), exposure duration (Boyd et al., 2023a), resource availability (Stevenson et al., 2017), water composition (Yue Li et al., 2024), intraspecific interactions (Gust et al., 2016), predation and parasitism (Coors and De Meester, 2008). It is unsurprising that different test populations within a species will also produce deviations in toxicity response. Indeed, previous studies have observed large differences in toxicity between separate populations of *D. magna* (Guan and Wang, 2006; Pereira et al., 2017) and *D. pulex* (Jiang et al., 2013), with the largest changes observed between wild and lab-reared clones (Barata et al., 2000).

A previous comparison of lab and wild *D. magna* by Barata et al. (2000) found that the mean tolerance of each population to chemical stressors was similar, but wild populations demonstrated greater variability in tolerance due to high phenotypic plasticity and genetic variation that was largely absent in both lab populations tested. In contrast, the present study observed differences in the tolerance of lab and wild daphnids in ancestral waters across each chemical treatment; namely that lab *D. pulex* in OECD water were more sensitive to AVO and OXY, while wild *D. pulex* in lake water were more sensitive to OCT (Figure 7.3). A meta analysis of the ECOTOX database by Romero-Blanco and Alonso (2022) concluded that wild aquatic organisms, particularly crustaceans such as *Daphnia*, are typically less sensitive to organic toxicants than their lab counterparts, although the authors noted that organism sensitivity was specific to each chemical and species tested, in line with the results of the present study. This variation in sensitivity is evident through the differing sublethal responses observed between each population, as both exhibited reduced reproduction in response to the UVFs they were respectively most sensitive to (Figure 7.4); however, wild *D. pulex* in lake water also presented a largely reduced body mass and moderately reduced core body length (Figure S7.8 & Figure S7.10), indicating that the strategies used by each population to cope with chemical stress are varied. Within each population, the metabolic rate did not vary greatly in response to any chemical treatment, suggesting that each population allocates their metabolic budget in different ways (Figure 7.5). The

30% lower metabolic rate of wild daphnids in the absence of a chemical stressor indicates that their total energy budget is smaller, requiring more trade-offs to be made between growth, survival, and reproduction. Differing responses to stressors as a result of restricted metabolic budget have been previously reported, with *D. magna* somatic growth sacrificed in favour of reproduction under a restricted diet (Enserink et al., 1995; Im et al., 2020b). Evidence of differing resource allocation strategies between lab and wild daphnids is also evident in their morphology, as wild *D. pulex* developed a large tail spine that was 1/3 the size of their core body length (Figure 7.1, Figure S7.12), indicating an investment into anti-predator defences that was largely absent in lab-reared daphnids with tail spines of 1/8 of their core body length. Previous research has demonstrated that tail spine development comes at the cost of delayed maturation, reduced reproduction, and a smaller core body size (Walls and Ketola, 1989), the latter of which was also observed in the wild *Daphnia* populations in this study (Figure S7.10).

Despite these metabolic limitations observed in wild daphnids, they demonstrated a higher tolerance for AVO and OXY. Differences in the physical and chemical properties of each UVF could influence their uptake and potential metabolism (Kwon and Choi, 2021), contributing towards the observed deviations in toxicity within each *Daphnia* population. Although the exact biotransformation enzymes involved in the detoxification of each UVF is unknown and has not been studied in crustaceans such as *Daphnia*, it is believed that OCT undergoes a Phase I carboxylesterase reaction (Falfushynska et al., 2021; Saunders et al., 2019) while AVO and OXY typically undergo oxidation or hydrolysis reactions with CYP450 enzymes instead (Guesmi et al., 2020; Sujin Lee et al., 2023; Watanabe et al., 2015). Glutathione conjugates have been observed for all three UVFs, indicating common Phase II processing (Guesmi et al., 2020). As a result, it is possible that the population of wild daphnids used in this study possess specific enhanced metabolic pathways through higher enzyme expression or differing enzyme isoforms that are beneficial in the detoxification of molecules such as

AVO and OXY, but not OCT. The lake that these daphnids were collected from is surrounded by agricultural uses, primarily barley and canola farms (personal observation), and encompasses a watershed area of 7,220 km^2 (Mitchell and Prepas, 1990). Low levels of pre-existing UVFs were measured in collected lake waters (Table 7.2); however, Driedmeat Lake receives the wastewater effluent from several upstream municipalities that provide services to > 30,000 people (Mitchell and Prepas, 1990; Statistics Canada, 2023). These waters are not expected to be free of the pharmaceutical and personal care products that are commonly detected downstream of wastewater treatment plants (Kasprzyk-Hordern et al., 2008; McCance et al., 2018); however, these were not quantified in the present study. Exposures of groups of daphnids were performed concurrently to this study that will target protein level changes in a future manuscript, providing insight into the validity of this hypothesis as well as the mechanisms driving the differences between each test population.

Additional biotic influences were present in this study, as two species of epibionts were inadvertently collected alongside the wild daphnids and colonized the test populations for the duration of the experiment (Figure S7.13). The rotifer *Brachionus rubens* (identified genetically) was present only in the wild *D. pulex* in OECD culture (Table S7.12), as only a few individuals were present on the collected wild daphnids, all of which were randomly assigned to the same culture conditions before the presence of the rotifer was detected, isolating it to a single test population. The ciliate *Vorticella sp.* (identified morphologically) was ubiquitously present on collected daphnids and quickly colonized all four test populations equally due to the difficulty in detecting these microscopic organisms on living daphnids. The effect of each epibiont species on the results generated in this study are expected to be minimal, as these organisms attach themselves to the carapace of *Daphnia* as a means of avoiding ingestion (Diéguez and Gilbert, 2011) and otherwise sustain themselves via filter feeding from the water rather than any parasitic interaction with the daphnid. A study by Kankaala and Eloranta (1987) determined that *D. longispina* of a similar size as the *D. pulex* in the present study would consume

approximately 90% of available food in the presence of the < 100 *Vorticella*/daphnid observed in the present study, with similar observations made between *B. rubens* and *D. pulex* by Gilbert (1985); therefore, these epibionts are expected to exert a minimal competitive pressure on test organisms. Reference toxicant LC50 tests were performed with Cu as part of this study, revealing no differences between lab *D. pulex* in OECD water with epibionts in this study (47.4 µg/L, 95% CI 40.4 – 56.8) or *D. pulex* tested by Mount and Norberg (53 µg/L, 95% CI 46 - 61; 1984). The reference LC50 was also similar to those obtained from testing *D. pulex* from the same supplier (ARO) in water with a similar inorganic profile as the OECD water used in the present study (Villavicencio et al., 2005).

7.4.4 Implications

Both populations of *D. pulex* in this study were less sensitive to all three UVFs than *D. magna* previously tested in the same laboratory (Boyd et al., 2023a, 2021). For lab and wild populations in ancestral waters, evidence of toxicity was mostly constrained to the highest exposure doses for each UVF, which exceeded the maximum solubility of AVO (27 µg/L) and OCT (40 µg/L) in pure water in some measurements (Mitchelmore et al., 2021; Table S7.8), making it difficult to delineate the observed impairments as a consequence of chemical toxicity caused by UVF interactions with biotic targets, or physical toxicity through mechanisms such as UVF precipitates accumulating around the gills or within the digestive tract, blocking normal functions (Duis et al., 2022; National Academies of Sciences, Engineering, and Medicine, 2022). In the case of OCT, only fresh exposure solutions for the highest exposure dose exceeded the solubility limit, with measured concentrations much lower than maximum solubility after 1 week. As discussed previously, UVF concentrations typically decrease rapidly in new solutions; therefore, it is likely that OCT concentrations exceeded water solubility during the chronic study for only a small duration of the experiment. The concentrations causing toxicity also exceed all measured freshwater environmental concentrations except one each for OCT (Vila et al., 2017) and OXY (Kasprzyk-Hordern et al., 2009); resulting in low risk posed to wild *D.*

pulex populations based on the results of this study. Additionally, *D. magna* have been demonstrated to be capable of acclimating to sublethal concentrations of these UVFs over multiple generations (Boyd et al., 2023a), which would further reduce any risk to wild *Daphnia* populations if *D. pulex* are similarly capable of acclimation. When applying the toxicity of these UVFs to the environment, it is important to acknowledge that the chronic study does not represent a definitive toxicity test, which would require 5 concentrations of each UVF to be tested.

Although the specific mechanism cannot be directly identified in this manuscript, it is evident that these *D. pulex* populations acclimate to their specific environmental conditions over periods of time beyond what a typical study utilizing wild caught organisms requires, facing increased stress when cultured in a novel water source. Both lab and wild *D. pulex* cultured in non-ancestral water were impaired to the extent that which toxicity tests would not pass standard validity criteria due to the impairments observed in control organisms, indicating the extent to which changing an organism's culture water can impact their performance during experimentation. These results are problematic in the context of previous lab and wild population comparisons that reared wild organisms in laboratory water, as the wild organisms tested in those studies may have had reduced performance due to the novelty of their culture water, potentially leading researchers to incorrect conclusions. It is important that researchers are cognizant of how the methods used for any study influence the results generated, so that conclusions can be drawn in the appropriate context. It is also important to note that this study used a single wild population, as differences could exist between different wild population sources (Jiang et al., 2013); therefore, further investigations of a variety of different wild populations in a single species are needed.

Additionally, these results indicate that lab and wild populations of D. *pulex* differ in response to chemical stressors; however, the nature of these differences are specific to each individual chemical tested. The lack of a consistent difference in responses between lab and wild *D. pulex* highlights the

challenges associated with generating toxicology data that is an accurate representation of environmental outcomes. Test guidelines that require organisms to be raised in fundamentally unnatural conditions could lead to "unnatural" results that are less relevant to environmental outcomes (Calisi and Bentley, 2009). The issue of applying laboratory data to natural environments can be overcome when conducting risk assessments using data from a variety of species and species sensitivity distributions, as these approaches have been demonstrated to be sufficiently conservative to protect against the uncertainty of laboratory data (Hose and Van Den Brink, 2004; Selck et al., 2002; Versteeg et al., 1999). The applicability of laboratory studies is more challenging in cases where data is limited, such as when the stressor of interest is of local/regional interest, or when novel stressors are initially identified as concerns warranting further study.

7.4.5 Conclusions

While utilizing lab populations provides a consistent baseline for a wide range of research, more research is required to understand how these models can or cannot be applied to wild populations and natural environments. Recommending that all studies utilize wild-caught organisms housed in conditions that are as "natural" as possible would greatly increase the cost and time invested into each individual study and would likely prove to be a net negative for environmental toxicology research by reducing the number of studies performed. Instead, studies that directly target wild-caught populations of organisms should take additional care to ensure that the performance of their research organisms is not negatively impacted by their housing and test conditions by incorporating a greater variety of baseline testing prior to, or during primary experimentation. In addition, studies that directly compare lab organisms to several different wild populations are needed at regular intervals so that the deviations of model species from their wild counterparts can be better monitored and understood. It is to be expected that organisms reared in fundamentally different environments will respond differently to stressors; however, if the nature and magnitude of these differences can be understood, then risk

assessment models can be revised to better account for the uncertainty that is inherent to biological research.

7.5 Figures and tables

Table 7.1. Inorganic profile of OECD (2 replicates) and Driedmeat Lake waters (4 replicates) over the study duration. Values are presented as mean ± SEM. BDL indicates analytes that were below the method detection limits for all replicates. Means for samples with both BDL and quantifiable replicates were calculated using LOD/2 for BDL measurements.

	OECD water		Driedmeat Lake water		LOD	
Analyte	Dissolved	Total	Dissolved	Total	Dissolved	Total
Cl (mg/L)	24.3 ± 1.23		36.3 ± 1.34		0.03	
SO4 (mg/L)	18.3 ± 1.77		85.4 ± 7.09		0.04	
Organic carbon (mg/L)	1.95 ± 0.250	1.82 ± 0.225	17.4 ± 0.381	16.8 ± 0.286	0.1	0.1
Ca (mg/L)	127 ± 2.35	121 ± 3.81	42.2 ± 1.69	40.1 ± 1.94	0.01	0.08
K (mg/L)	3.64 ± 0.403	3.50 ± 0.128	12.3 ± 0.373	11.5 ± 0.283	0.05	0.01
Mg (mg/L)	26.7 ± 0.963	25.0 ± 0.400	23.6 ± 0.295	22.6 ± 0.343	0.001	0.01
Na (mg/L)	33.3 ± 2.44	30.5 ± 2.66	73.4 ± 2.68	69.6 ± 2.48	0.005	0.08
Ag (µg/L)	BDL	BDL	BDL	BDL	0.005	0.05
Al (µg/L)	31.7 ± 27.6	204 ± 173	4.63 ± 0.887	11.5 ± 8.53	0.49	6
As (µg/L)	BDL	BDL	2.16 ± 0.123	2.01 ± 0.125	0.04	0.04
Ba (µg/L)	89.9 ± 11.3	65.5 ± 1.33	109 ± 10.8	74.1 ± 3.04	0.05	0.2
Be (µg/L)	BDL	BDL	0.0300 ± 0.0238	BDL	0.01	0.3
Cd (µg/L)	BDL	BDL	BDL	BDL	0.02	0.07
Co (µg/L)	0.0800 ± 0.0250	BDL	0.260 ± 0.0125	BDL	0.01	0.1
Cr (µg/L)	0.130 ± 0.0250	BDL	0.0500 ± 0.0350	0.600 ± 0.320	0.02	0.1
Cu (µg/L)	0.930 ± 0.0250	BDL	3.25 ± 2.22	1.84 ± 1.34	0.01	1
Fe (µg/L)	BDL	BDL	9.31 ± 1.30	71.3 ± 66.3	0.8	10
Mn (µg/L)	0.200 ± 0	BDL	1.03 ± 0.236	12.7 ± 7.39	0.04	0.04
Mo (µg/L)	0.900 ± 0.0500	0.780 ± 0.0250	1.33 ± 0.0829	1.25 ± 0.0791	0.01	0.01
Ni (µg/L)	BDL	BDL	1.10 ± 0.251	BDL	0.02	2
Pb (µg/L)	BDL	BDL	0.110 ± 0.0315	BDL	0.01	0.08
Sb (µg/L)	BDL	BDL	0.240 ± 0.0239	BDL	0.02	0.1
Se (µg/L)	BDL	BDL	BDL	BDL	0.01	2
Sn (µg/L)	BDL	BDL	0.270 ± 0.227	0.350 ± 0.301	0.09	0.09
Sr (µg/L)	386 ± 40.4	363 ± 40.2	346 ± 11.0	335 ± 8.14	0.04	0.2
Ti (µg/L)	BDL	BDL	BDL	BDL	0.01	0.05
V (µg/L)	0.230 ± 0.0250	BDL	1.03 ± 0.0946	1.01 ± 0.0898	0.01	0.1

Table 7.2. Measured UVF concentrations in untreated OECD and Driedmeat Lake water (water control) and MeOH control solutions over the duration of the 21-d experiment. Values are presented as the mean ± SEM of 4 replicates analyzed in triplicate. All values are expressed in µg/L.

	OECD water		Driedmeat Lake water	
Analyte	Water control	MeOH control	Water control	MeOH control
AVO	BDL	0.00925 ± 0.0930	0.00575 ± 0.00580	0.00475 ± 0.00480
OCT	0.0770 ± 0.0670	0.0950 ± 0.0691	0.0198 ± 0.0178	0.0325 ± 0.0177
OXY	0.0190 ± 0.0190	0.0150 ± 0.0111	0.00800 ± 0.00460	0.0173 ± 0.0169

Table 7.3. Measured UVF concentrations in exposure solutions made from OECD or Driedmeat Lake water for the 21-d experiment. Values are presented as the mean ± SEM of 4 replicates analyzed in triplicate. BDL indicates analytes that were below the method detection limits. All values are expressed in μg/L.

Treatment	Nominal concentration	OECD water measured concentration	Driedmeat Lake water measured concentration	LOD	LOQ
AVO low	10	1.23 ± 0.743	1.34 ± 0.684	0.01	0.02
AVO medium	50	14.5 ± 7.40	24.4 ± 4.35	0.01	0.02
AVO high	100	30.7 ± 9.03	50.2 ± 11.0	0.01	0.02
OCT low	10	2.53 ± 1.05	1.71 ± 0.934	0.01	0.02
OCT medium	50	16.7 ± 5.52	18.0 ± 5.13	0.01	0.02
OCT high	100	29.5 ± 15.6	25.6 ± 12.2	0.01	0.02
OXY low	10	2.84 ± 1.65	1.40 ± 1.39	0.01	0.02
OXY medium	50	9.33 ± 9.05	9.45 ± 9.33	0.01	0.02
OXY high	100	18.8 ± 17.3	15.9 ± 15.9	0.01	0.02

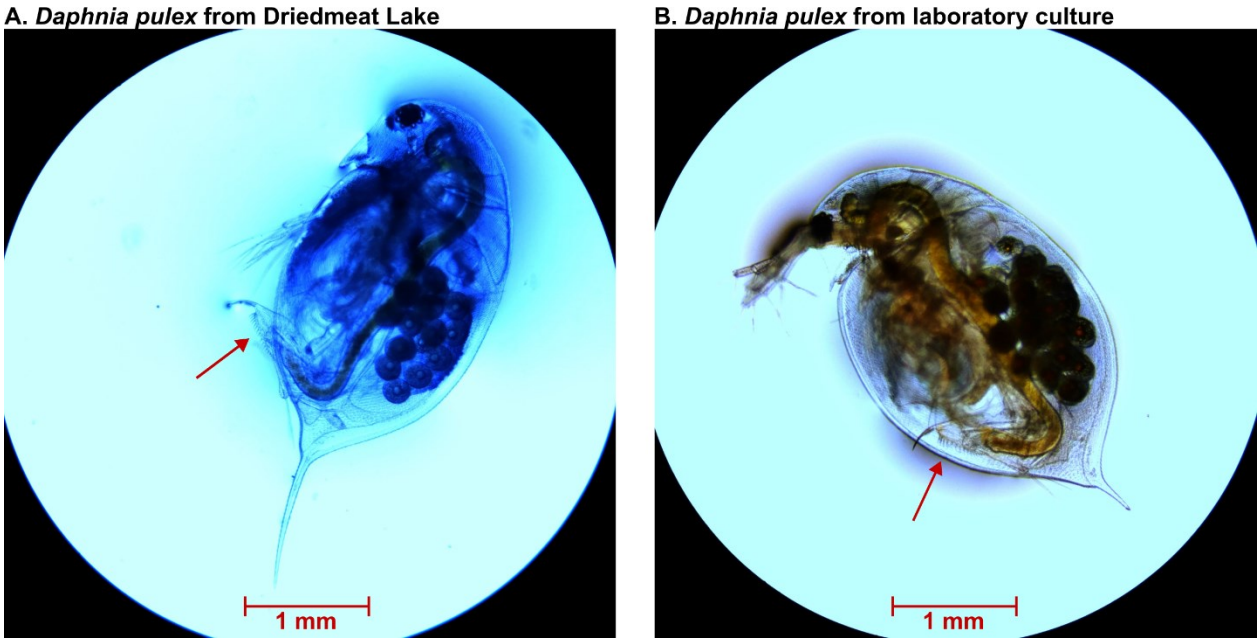


Figure 7.1. Representative images of 21-d old wild *D. pulex* collected from Driedmeat Lake (A), and lab *D. pulex* reared at the University of Alberta (B). Scale bars indicate a 1 mm distance on each panel. The postabdominal claw used for morphological identification is indicated by an arrow.

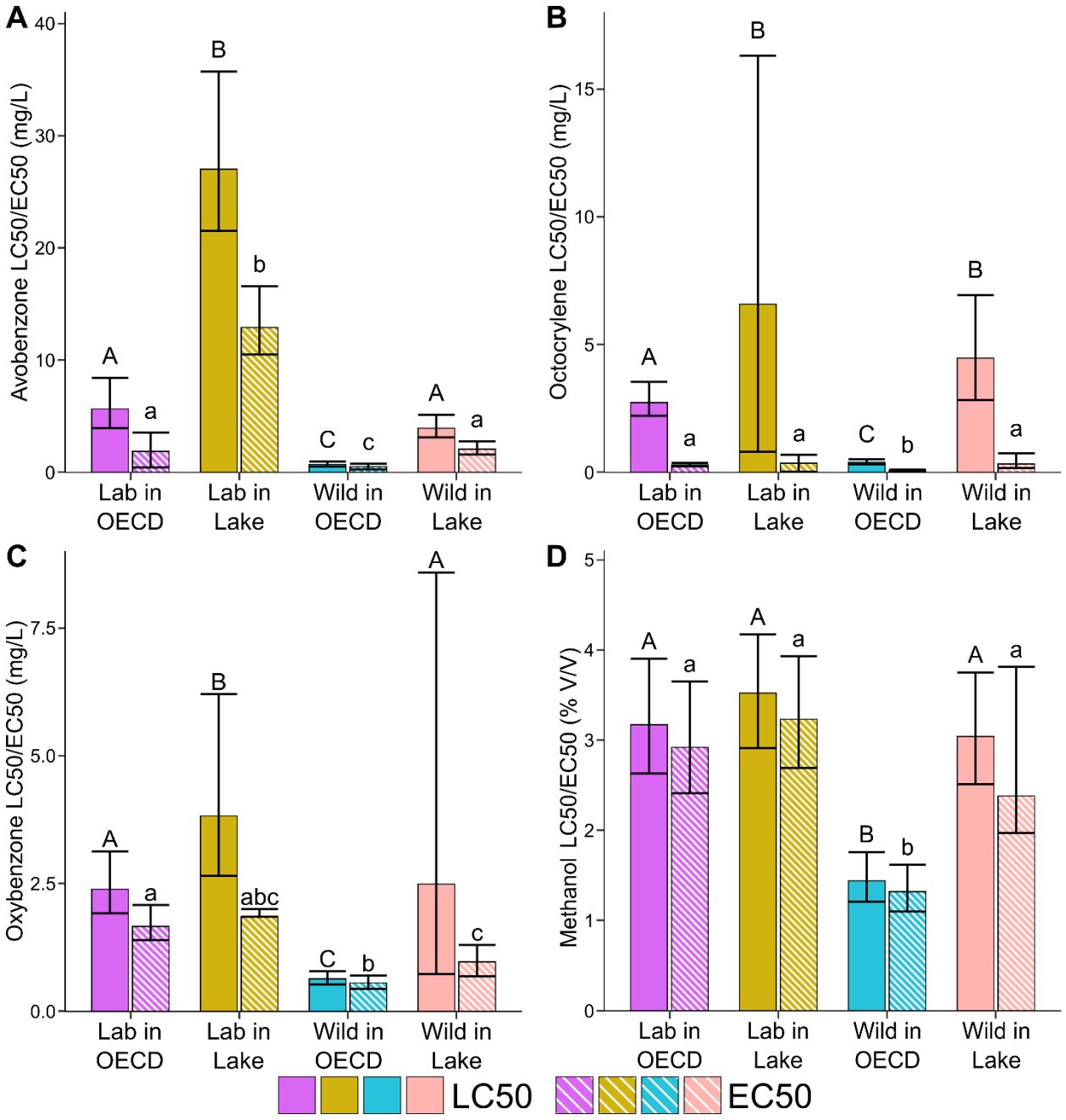


Figure 7.2. Nominal 48-hour median lethal concentrations (LC50; solid bars) and median effect concentrations causing immobilization (EC50; striped bars) of < 24 h old *D. pulex* neonates exposed to individual UVFs (A-C) or a methanol solvent control (D). Bars represent the mean ± 95% confidence interval determined from 4 replicates of 5 exposed daphnids per test concentration. Different letters on the bars indicate a significant difference between LC50s (uppercase letters) and EC50s (lowercase letters) for each *Daphnia* colony as determined by pairwise ratio tests with a Benjamini & Hochberg correction ($p < 0.05$). Tested concentrations are available in Table S7.6.

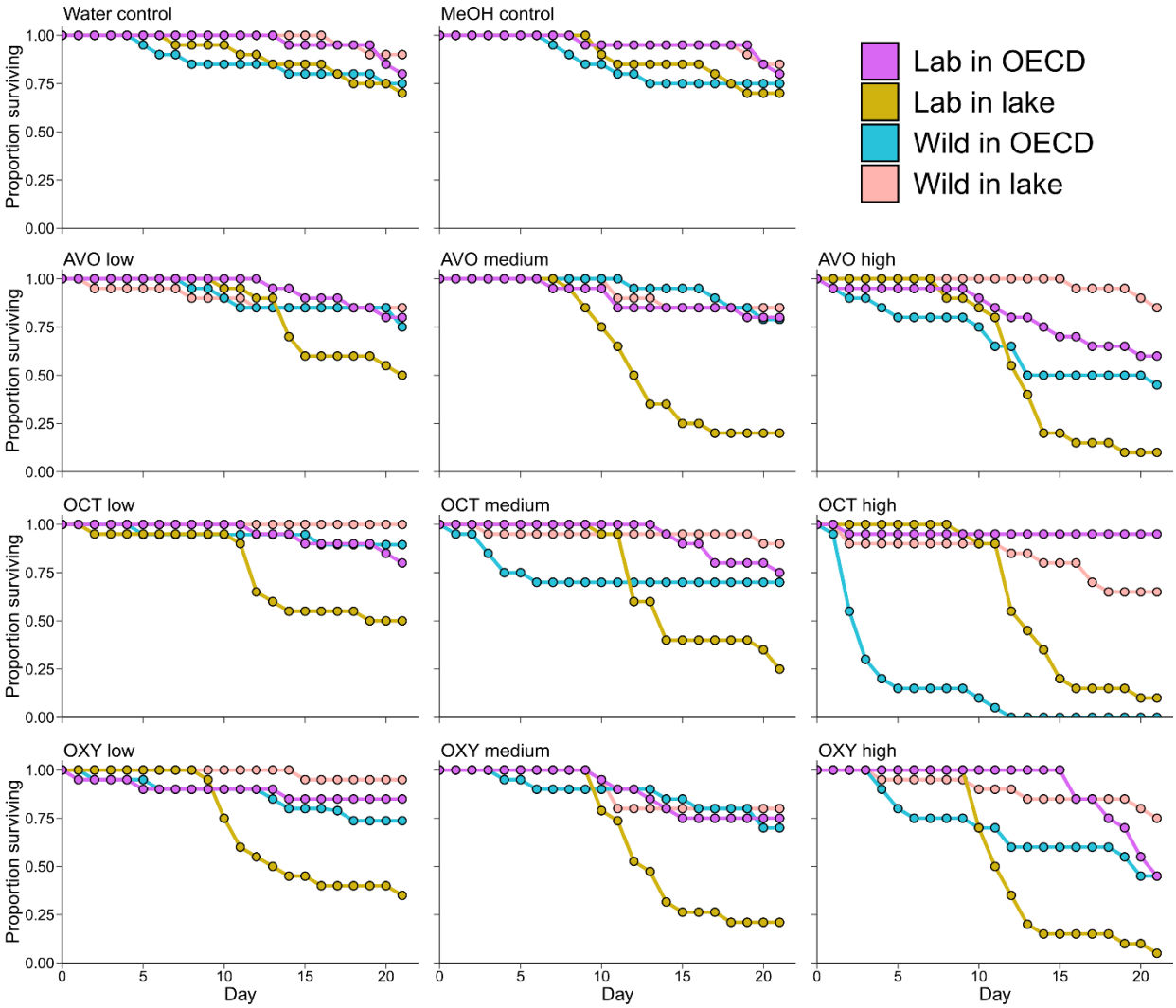


Figure 7.3. Survivorship curves of < 24 h old *D. pulex* neonates from each of the four test cultures exposed to one of three concentrations of UVFs, a MeOH solvent control, or a water control without additions for 21-d. Each point represents the proportion of surviving daphnids on each day of the experiment from 20 replicates of individually exposed daphnids per treatment.

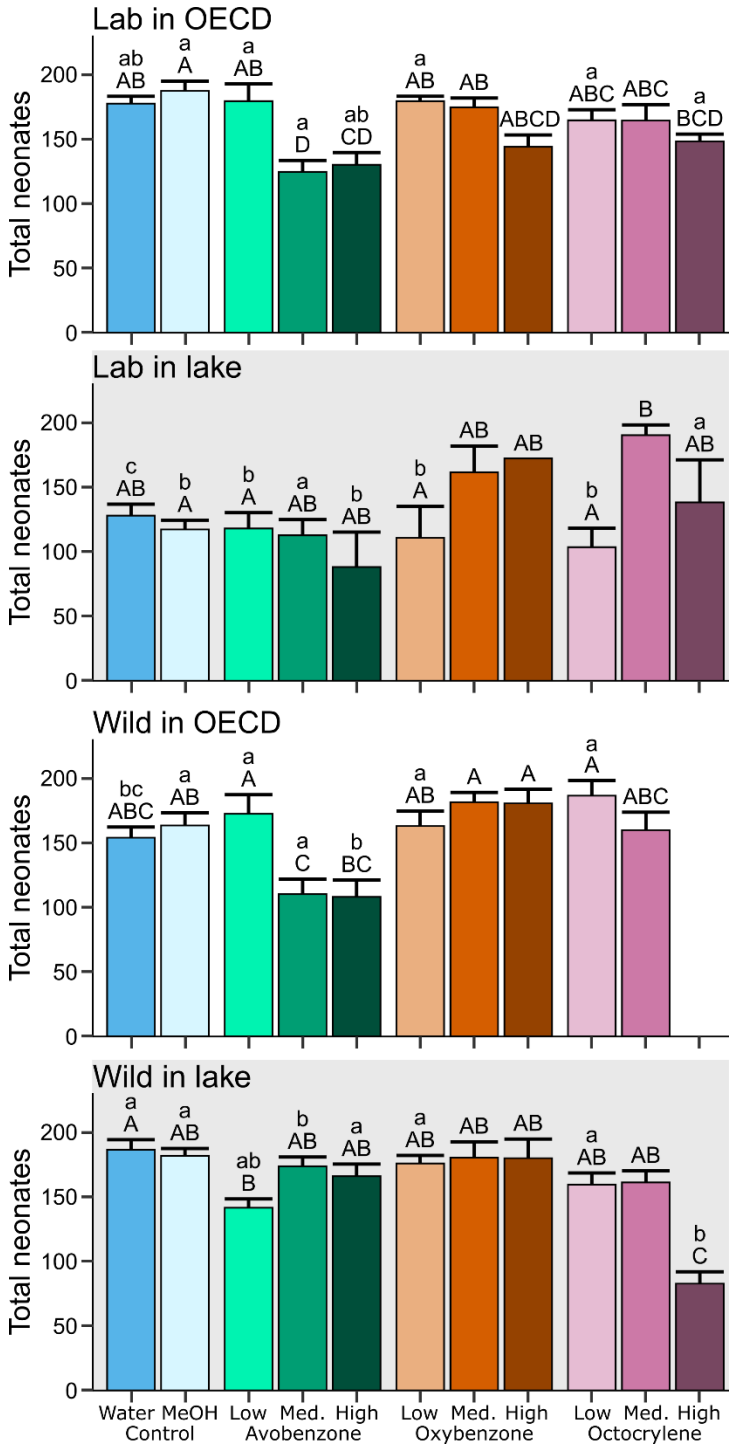


Figure 7.4. The average number of viable neonates produced by *D. pulex* surviving a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

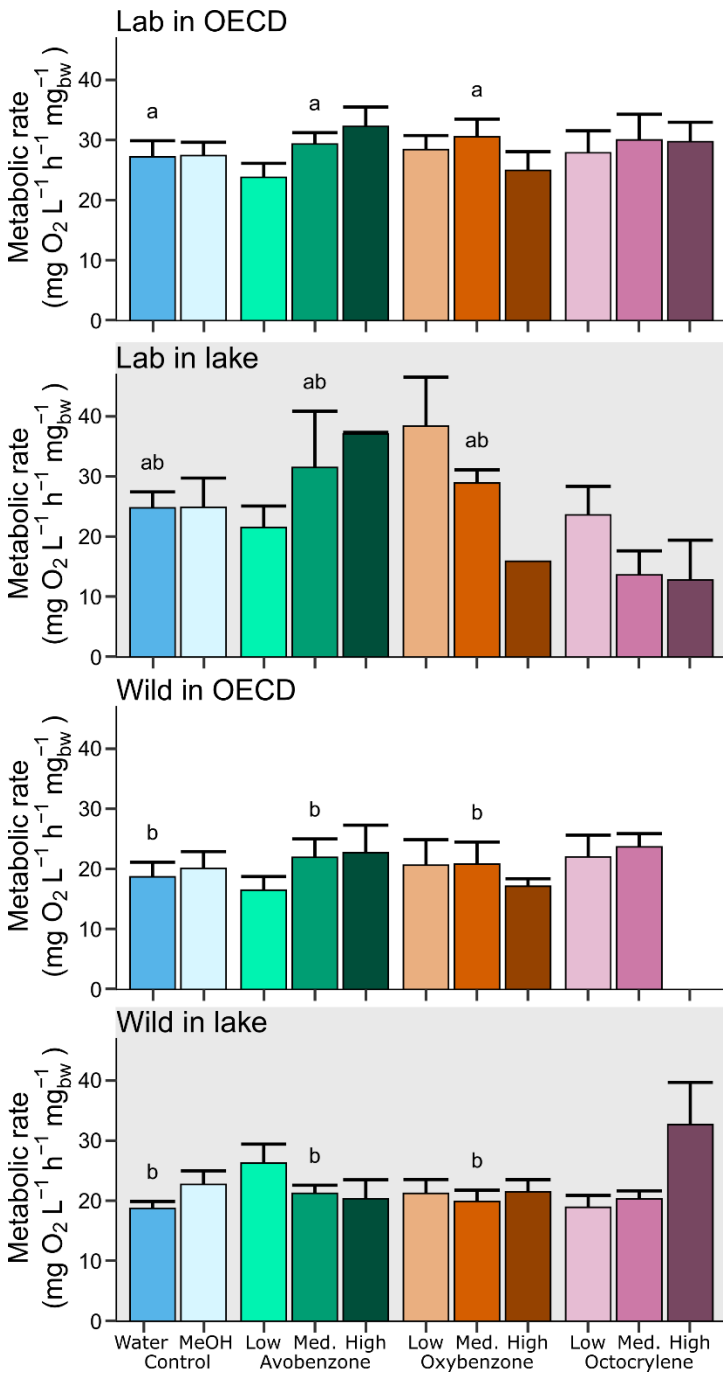


Figure 7.5. The mass specific metabolic rate of surviving *D. pulex* after a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment at the start of the experiment. Different letters on the bars indicate a significant difference between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (Kruskal Wallis, $p < 0.05$). No statistical differences were detected between chemical treatments within a test culture (horizontal comparison within a panel).

7.6 Supplementary materials

7.6.1 Genotyping methodology

Daphnids from the F2 generation of the wild population in lake water and the lab population in OECD water were collected to confirm the species identity genetically. Samples of 5 randomly selected daphnids were collected from a culture beaker, with 6 total replicates for each *Daphnia* population collected from different culture beakers. Each daphnid was cut in half prior to DNA extraction with a DNeasy Blood & Tissue kit (Qiagen, Netherlands). Samples were incubated overnight prior to collecting the final elution. Polymerase chain reaction (PCR) was performed targeting the cytochrome c oxidase subunit I gene (COI) by adding 5 µL of DNA to 20 µL of PCR reaction solution, consisting of 10X buffer (Fisher Scientific, US), 25 mM $MgCl_2$ (Fisher Scientific), 2 mM deoxynucleotide triphosphates (Fisher Scientific), 5 µM of each primer (LCO1490 and HCO2198; Folmer et al., 1994), 5 U/µL Taq polymerase (Fisher Scientific) and 20 mg/mL bovine serum albumin (New England Biolabs, US). PCR reactions were performed on 1:200 dilution samples with the following thermoprotocol: 95°C for 3 minutes, followed by 5 cycles of 95°C for 30 s, 45°C for 40 s, 72°C for 1 min, then 25 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 1 min, followed by a final extension of 72°C for 10 min. The resulting products were visualized via gel electrophoresis on a 1% agarose gel to confirm PCR reaction success and were Sanger sequenced on an ABI 3730 DNA Analyzer. The resulting sequences were then compared to known COI homologues with a NCBI BLAST search of GenBank sequences to obtain species identifications.

Table S7.1. Sampling dates and locations for wild daphnid and lake water collection.

Sample	Date	Location
Wild *Daphnia*	June 1, 2023	52.876065, -112.755026
Lake water	June 1, 2023	52.876297, -112.756672
Lake water	June 27, 2023	52.876297, -112.756672
Lake water	July 21, 2023	52.876297, -112.756672

Table S7.2. COI DNA primers used for *D. pulex* species identification.

Primer label	Sequence (5’ – 3’)
LCO1490	GGTCAACAAATCATAAAGATATTGG
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA

Table S7.3. Inorganic profile of OECD and Driedmeat Lake waters at each sampling date. July 21 Driedmeat Lake water was sampled after collection from the lake (fresh) and when producing the second set of exposure solutions on Aug 6th for the 21-d experiment (aged). OECD water was sampled when producing the first (July 23rd) and second (August 6th) sets of exposure solutions. BDL indicates analytes that were below the method detection limits.

	OECD water				Driedmeat Lake water							
	July 23		August 6		June 1		June 27		July 21 (fresh)		July 21 (aged)	
Analyte	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total
Cl (mg/L)	25.5	N/A	23.1	N/A	35.4	N/A	32.9	N/A	38.6	N/A	38.3	N/A
SO4 (mg/L)	20.1	N/A	16.6	N/A	66.4	N/A	82.6	N/A	96.3	N/A	96.2	N/A
Organic carbon (mg/L)	2.20	2.04	1.70	1.59	18.4	16.2	17.1	16.5	17.2	17.5	16.6	16.9
Ca (mg/L)	124	125	129	117	47.1	45.2	40.4	35.9	39.6	40.3	41.8	39.1
K (mg/L)	4.04	3.63	3.24	3.37	11.2	10.7	12.7	12.0	12.6	11.8	12.7	11.4
Mg (mg/L)	25.8	24.6	27.7	25.4	22.8	21.7	23.8	23.1	23.6	23.2	24.2	22.5
Na (mg/L)	35.7	33.2	30.8	27.9	67.2	63.7	70.6	67.4	77.6	74.3	78.1	73.1
Ag (µg/L)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Al (µg/L)	4.10	31.2	59.2	376	3.30	BDL	5.50	BDL	6.70	37.1	3.10	BDL
As (µg/L)	BDL	BDL	BDL	BDL	2.35	2.15	1.80	1.65	2.25	2.20	2.25	2.05
Ba (µg/L)	78.7	66.9	101	64.2	86.0	76.6	106	65.1	107	77.7	138	77.3
Be (µg/L)	BDL	BDL	BDL	BDL	0.100	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Cd (µg/L)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Co (µg/L)	0.0500	BDL	0.100	BDL	0.300	BDL	0.250	BDL	0.250	BDL	0.25	BDL
Cr (µg/L)	0.10	BDL	0.150	BDL	0.150	1.25	BDL	1.05	BDL	BDL	BDL	BDL
Cu (µg/L)	0.900	BDL	0.950	BDL	9.90	5.85	1.10	BDL	0.900	BDL	1.10	BDL
Fe (µg/L)	BDL	BDL	BDL	BDL	8.75	BDL	8.10	BDL	13.1	270	7.30	BDL
Mn (µg/L)	0.200	BDL	0.200	BDL	1.00	8.30	0.700	1.80	1.70	34.5	0.700	6.25
Mo (µg/L)	0.950	0.80	0.850	0.75	1.10	1.05	1.30	1.20	1.45	1.40	1.45	1.35
Ni (µg/L)	BDL	BDL	BDL	BDL	1.85	BDL	0.900	BDL	0.800	BDL	0.850	BDL
Pb (µg/L)	BDL	BDL	BDL	BDL	0.200	BDL	0.0200	BDL	0.100	BDL	0.100	BDL
Sb (µg/L)	BDL	BDL	BDL	BDL	0.250	BDL	0.200	BDL	0.200	BDL	0.300	BDL
Se (µg/L)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Sn (µg/L)	BDL	BDL	BDL	BDL	0.950	1.25	BDL	BDL	BDL	BDL	BDL	BDL
Sr (µg/L)	346	323	426	403	365	346	336	311	320	343	363	BDL
Ti (µg/L)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
V (µg/L)	0.200	BDL	0.250	BDL	1.15	1.10	0.750	0.750	1.15	1.15	1.05	BDL

Table S7.4. pH and nitrogenous waste products in OECD and lake stock waters from each collection date. Values are presented as mean ± SEM. Water characteristics were tested approximately weekly for the duration of use for each particular collection.

Parameter	OECD water	June 1 lake water	June 27 lake water	July 21 lake water (fresh)	July 21 lake water (aged)
pH	8.60 ± 0.0700	8.81	8.92 ± 0.078	8.85 ± 0.160	8.71 ± 0
Ammonia (mg/L)	0 ± 0	0.25	0.13 ± 0.07	0.38 ± 0.16	0.25 ± 0
Nitrite (mg/L)	0 ± 0	0	0 ± 0	0.50 ± 0.50	1.0 ± 0
Nitrate (mg/L)	0 ± 0	2.5	1.7 ± 1.7	6.3 ± 3.8	10 ± 0

Table S7.5. Terminology used to describe the four *D. pulex* cultures used in this study.

Culture name	*D. pulex* source	Culture water	Culture condition
Lab in OECD	Aquatic Research Organisms	OECD	Ancestral water
Lab in lake	Aquatic Research Organisms	Lake	Non-ancestral water
Wild in OECD	Driedmeat Lake	OECD	Non-ancestral water
Wild in lake	Driedmeat Lake	Lake	Ancestral water

Table S7.6. Nominal exposure solution concentrations utilized for 48 h median lethal concentration (LC50) and median effect concentration (EC50) determination for each UVF and the methanol solvent in each of the four *Daphnia* cultures. Four replicates of n = 5 < 24 h old *Daphnia pulex* neonates were exposed at each of the indicated concentrations for 48 h. UVF concentrations are indicated in mg/L, while MeOH concentrations are indicated in % v/v. Values in bold for UVFs are below the water solubility limit.

Culture	Treatment	Concentration (mg/L or *% v/v)
Lab in OECD	AVO	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OCT	**0.01, 0.02**, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20
	OXY	**0.2, 0.5, 1, 2, 5**, 10, 20, 50
	MeOH*	0.5, 1, 2, 5, 10, 20
Lab in lake	AVO	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OCT	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OXY	**0.2, 0.5, 1, 2, 5**, 10, 20, 50
	MeOH*	0.5, 1, 2, 5, 10, 20
Wild in OECD	AVO	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OCT	**0.01, 0.02**, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OXY	**0.1, 0.2, 0.5, 1, 2, 5**, 10, 20
	MeOH*	0.2, 0.5, 1, 2, 5, 10, 20
Wild in Lake	AVO	0.1, 0.2, 0.5, 1, 2, 5, 10, 20
	OCT	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50
	OXY	**0.2, 0.5, 1, 2, 5**, 10, 20, 50
	MeOH*	0.5, 1, 2, 5, 10, 20

Table S7.7. Nominal 48-hour median lethal concentrations (LC50) and median effect concentrations causing immobilization (EC50) of < 24 h old *D. pulex* neonates exposed to individual UVFs or a methanol solvent control. Values are expressed as the mean and 95% confidence interval determined from 4 replicates of 5 exposed daphnids per test concentration. UVF concentrations are indicated in mg/L, while MeOH concentrations are indicated in % v/v. Confidence intervals listed as NA could not be estimated by the probit model.

Culture	Endpoint	Avobenzone	Octocrylene	Oxybenzone	Methanol
Lab in OECD	LC50	5.65 (3.92, 8.42)	2.73 (2.21, 3.54)	2.39 (1.92, 3.13)	3.17 (2.63, 3.90)
	EC50	1.87 (0.434, 3.54)	0.291 (0.238, 0.369)	1.66 (1.39, 2.08)	2.92 (2.41, 3.65)
Lab in lake	LC50	27.0 (21.5, 35.7)	6.58 (0.804, 16.3)	3.82 (2.65, 6.20)	3.52 (2.91, 4.17)
	EC50	12.9 (10.5, 16.6)	0.359 (NA, NA)	1.85 (1.85, 2.00)	3.23 (2.69, 3.93)
Wild in OECD	LC50	0.710 (0.521, 0.960)	0.392 (0.314, 0.510)	0.636 (0.523, 0.782)	1.44 (1.21, 1.76)
	EC50	0.514 (0.276, 0.764)	0.0776 (0.0597, 0.106)	0.556 (0.444, 0.700)	1.32 (1.10, 1.62)
Wild in lake	LC50	3.930 (3.12, 5.13)	4.47 (2.83, 6.94)	2.49 (0.732, 8.58)	3.04 (2.51, 3.75)
	EC50	2.07 (1.60, 2.78)	0.329 (0.177, 0.744)	0.967 (0.682, 1.30)	2.38 (1.97, 3.81)

Table S7.8. Measured UVF concentrations in exposure solutions at each of four sampling dates during the 21-d experiment. Values are presented as the mean ± SD of triplicate analysis of each sample. BDL indicates analytes that were below the method detection limits. All values are expressed in µg/L.

OCT				OXY			
Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
0.277 ± 0.027	0.029 ± 0.012	BDL	0.002 ± 0.014	0.076 ± 0.006	BDL	BDL	BDL
0.073 ± 0.006	BDL	0.005 ± 0.004	0.001 ± 0.005	0.017 ± 0.004	BDL	0.015 ± 0.010	BDL
0.301 ± 0.034	0.044 ± 0.016	0.029 ± 0.020	0.006 ± 0.016	0.047 ± 0.007	BDL	0.013 ± 0.003	BDL
0.068 ± 0.007	0.058 ± 0.003	0.004 ± 0.004	BDL	0.068 ± 0.004	BDL	0.001 ± 0.007	BDL
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3.98 ± 0.073	0.449 ± 0.024	4.67 ± 0.034	1.01 ± 0.026	N/A	N/A	N/A	N/A
2.23 ± 0.012	0.310 ± 0.015	4.13 ± 0.120	0.168 ± 0.021	N/A	N/A	N/A	N/A
16.2 ± 0.202	2.49 ± 0.244	29.3 ± 0.11	18.8 ± 0.209	N/A	N/A	N/A	N/A
22.8 ± 0.425	7.53 ± 0.198	30.1 ± 0.497	11.8 ± 0.258	N/A	N/A	N/A	N/A
68.1 ± 1.27	4.16 ± 0.062	41.4 ± 1.06	4.35 ± 0.085	N/A	N/A	N/A	N/A
41.4 ± 0.827	10.2 ± 0.287	50.9 ± 0.720	0.020 ± 0.016	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	6.78 ± 0.166	4.35 ± 0.085	BDL	0.242 ± 0.003
N/A	N/A	N/A	N/A	5.57 ± 0.047	0.020 ± 0.016	BDL	0.015 ± 0.011
N/A	N/A	N/A	N/A	36.5 ± 0.264	BDL	BDL	0.853 ± 0.028
N/A	N/A	N/A	N/A	37.4 ± 0.228	BDL	BDL	0.367 ± 0.023
N/A	N/A	N/A	N/A	70.8 ± 1.28	0.187 ± 0.035	BDL	4.35 ± 0.085
N/A	N/A	N/A	N/A	63.7 ± 0.773	BDL	BDL	0.020 ± 0.016

		AVO			
Treatment	Water base	Week 1	Week 2	Week 3	Week 4
Water control	OECD	BDL	BDL	BDL	BDL
	Lake	BDL	BDL	0.023 ± 0.005	BDL
MeOH control	OECD	BDL	BDL	0.037 ± 0.012	BDL
	Lake	BDL	BDL	0.019 ± 0.015	BDL
AVO low	OECD	0.981 ± 0.033	0.075 ± 0.003	3.39 ± 0.313	0.479 ± 0.018
	Lake	1.86 ± 0.033	0.069 ± 0.005	3.03 ± 0.022	0.402 ± 0.010
AVO medium	OECD	17.0 ± 0.065	0.643 ± 0.042	N/A	25.9 ± 0.179
	Lake	31.3 ± 0.223	12.1 ± 1.15	29.8 ± 2.45	24.4 ± 1.39
AVO high	OECD	42.3 ± 1.90	6.07 ± 0.188	45.9 ± 1.09	28.6 ± 0.093
	Lake	71.1 ± 1.82	25.8 ± 1.07	37.4 ± 0.370	66.4 ± 2.67
OCT low	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A
OCT medium	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A
OCT high	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A
OXY low	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A
OXY medium	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A
OXY high	OECD	N/A	N/A	N/A	N/A
	Lake	N/A	N/A	N/A	N/A

Table S7.9. Start and end dates for each *Daphnia* culture for the 21-d exposure.

Culture	Exposure state date	Exposure end date	Exposure duration (days)
Wild in OECD	July 25, 2023	Aug 15, 2023	21
Lab in lake	July 26, 2023	Aug 16, 2023	21
Wild in lake	July 27, 2023	Aug 17, 2023	21
Lab in OECD	July 28, 2023	Aug 18, 2023	21

Table S7.10. Start and end dates for each exposure stock used for the 21-d exposure.

Date stock produced	Date stock used	Date stock ended	Usage duration (days)
July 23, 2023	July 25, 2023	Aug 6, 2023	13
Aug 6, 2023	Aug 7, 2023	Aug 18, 2023	12

Table S7.11. Epibiont scoring scale description for day 10 and day 21 of the 21-d exposure experiment.

Score	Description
0	No visible epibionts
1	< 25 % daphnid body covered by epibionts
2	25 – 50 % daphnid body covered by epibionts
3	> 50 % daphnid body covered by epibionts

Table S7.12. Percent of surviving daphnids with a visible epibiont presence on day 10 and day 21 of the 21-d exposure experiment.

	Lab in OECD		Lab in lake		Wild in OECD		Wild in lake	
Treatment	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21
OECD control	0	0	0	0	23.5	26.7	0	0
MeOH control	0	0	0	0	17.6	33.3	0	0
AVO low	0	0	0	0	5.3	11.8	0	0
AVO medium	0	0	0	0	10	26.7	0	0
AVO high	0	0	0	0	0	0	0	0
OCT low	0	0	0	0	11.1	11.8	0	0
OCT medium	0	0	0	0	7.1	7.1	0	0
OCT high	0	0	0	0	0	0	0	0
OXY low	0	0	0	0	5.6	0	0	0
OXY medium	0	0	0	0	11.1	0	0	0
OXY high	0	0	0	0	0	0	0	0

Table S7.13. The intrinsic rate of population increase estimated using the Lotka equation.

Treatment	Lab in OECD	Lab in lake	Wild in OECD	Wild in lake
Water control	0.390	0.421	0.363	0.433
MeOH control	0.440	0.424	0.400	0.430
AVO low	0.442	0.454	0.400	0.409
AVO medium	0.400	0.416	0.384	0.433
AVO high	0.360	0.416	0.324	0.439
OCT low	0.414	0.418	0.419	0.423
OCT medium	0.418	0.439	0.366	0.395
OCT high	0.426	0.381	-0.0304	0.362
OXY low	0.424	0.426	0.394	0.454
OXY medium	0.429	0.437	0.409	0.438
OXY high	0.410	0.429	0.359	0.431

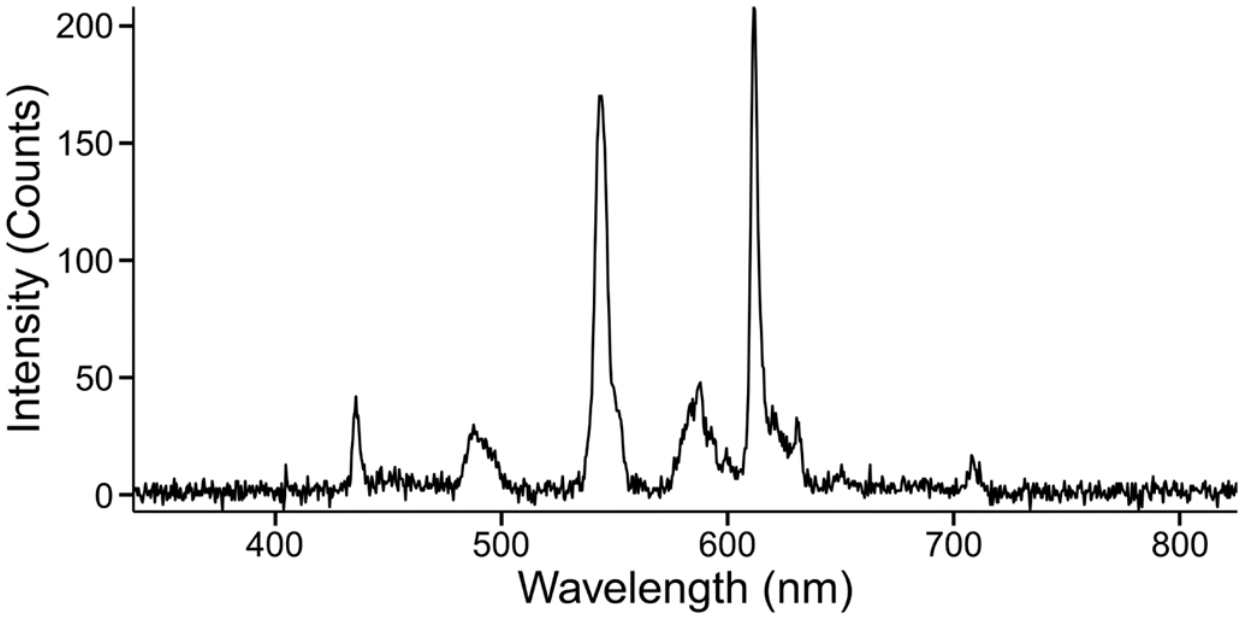


Figure S7.1. The spectral composition of the lighting conditions used to maintain each *Daphnia pulex* culture and to perform all experiments in this study.

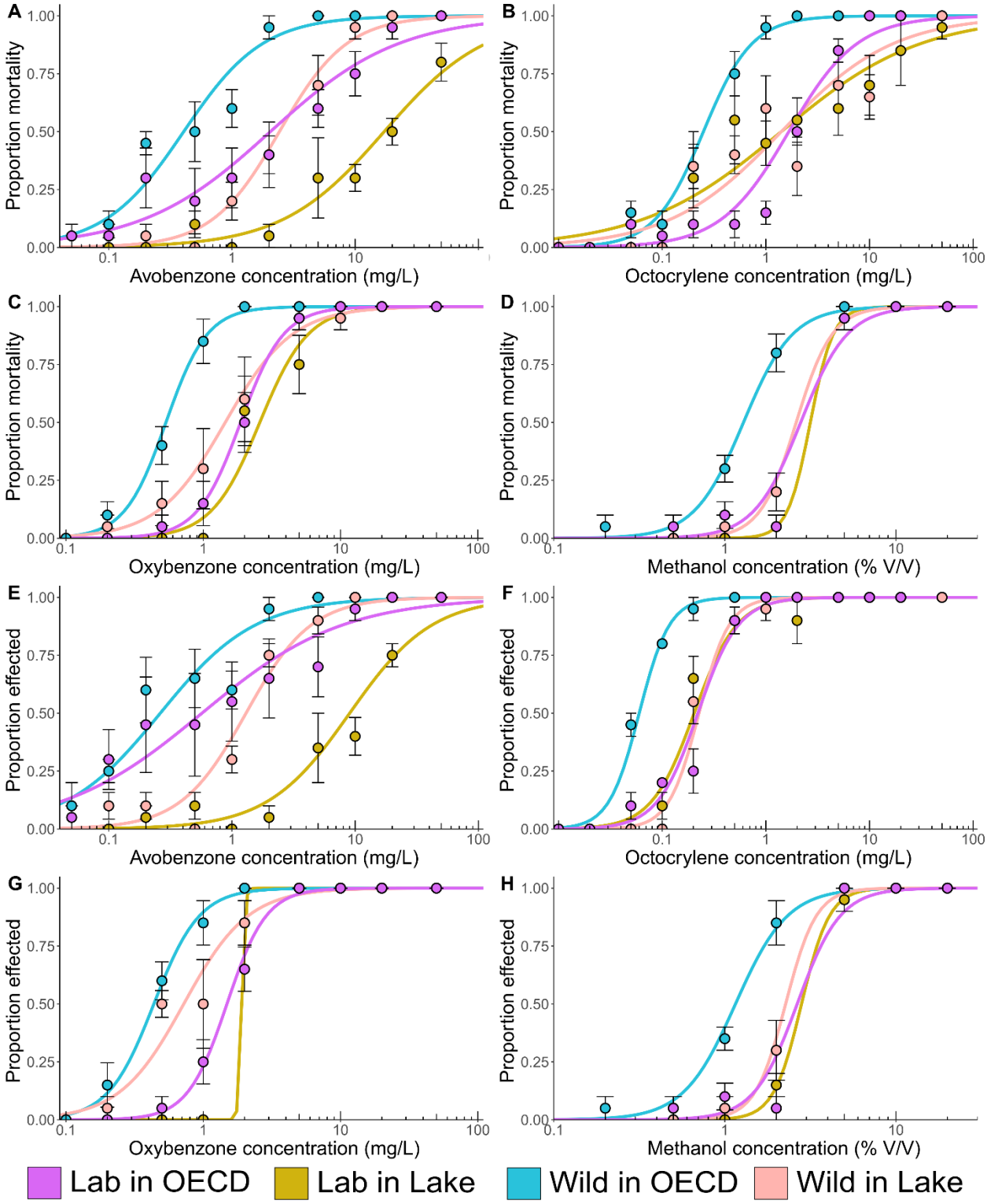


Figure S7.2. Nominal 48-hour median lethal concentration (A-D) and median effect concentration (E-H) dose/response curves for *D. pulex* populations. Each data point represents the mean ± SEM of 4 replicates containing 5 < 24 h old daphnids each.

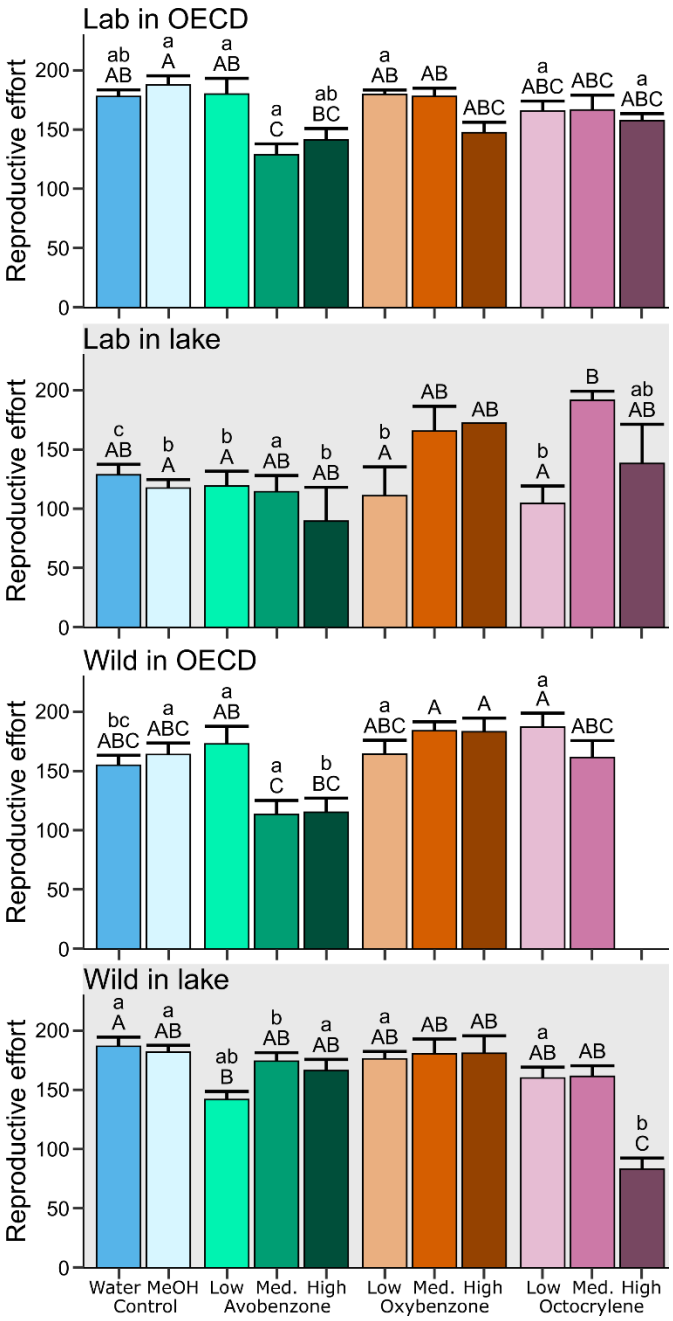


Figure S7.3. The average reproductive effort (viable neonates + non-viable neonates) produced by *D. pulex* surviving a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

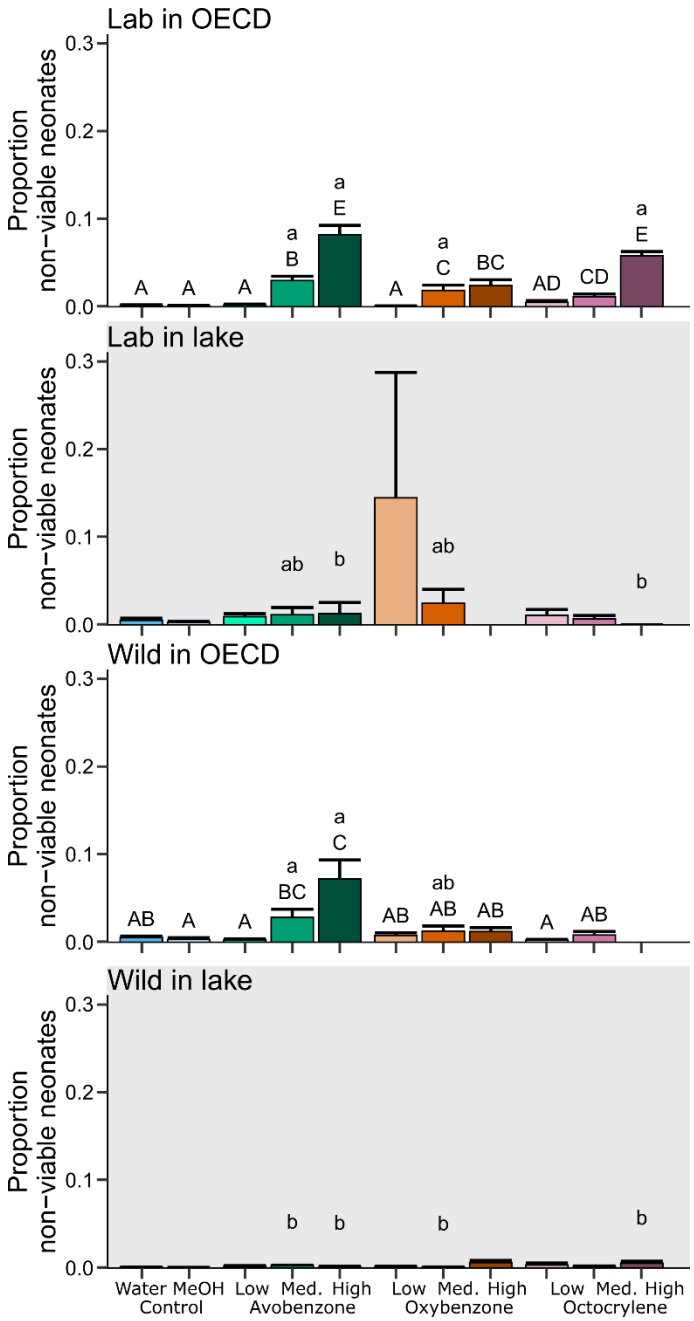


Figure S7.4. Non-viable neonates produced as a proportion of reproductive effort (viable neonates + non-viable neonates) of *D. pulex* surviving a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

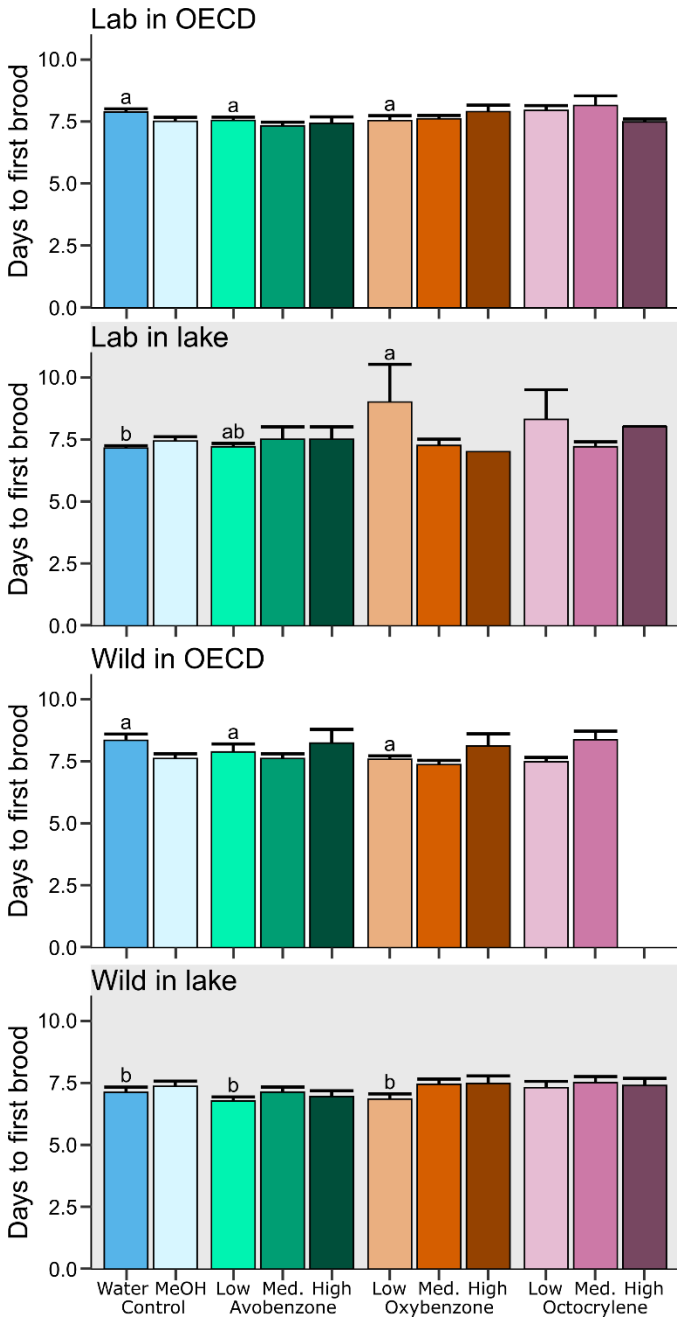


Figure S7.5. The number of days required for *D. pulex* to release their first brood of offspring during a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (Kruskal Wallis, $p < 0.05$). No statistical differences were detected between chemical treatments within a test culture (horizontal comparison within a panel).

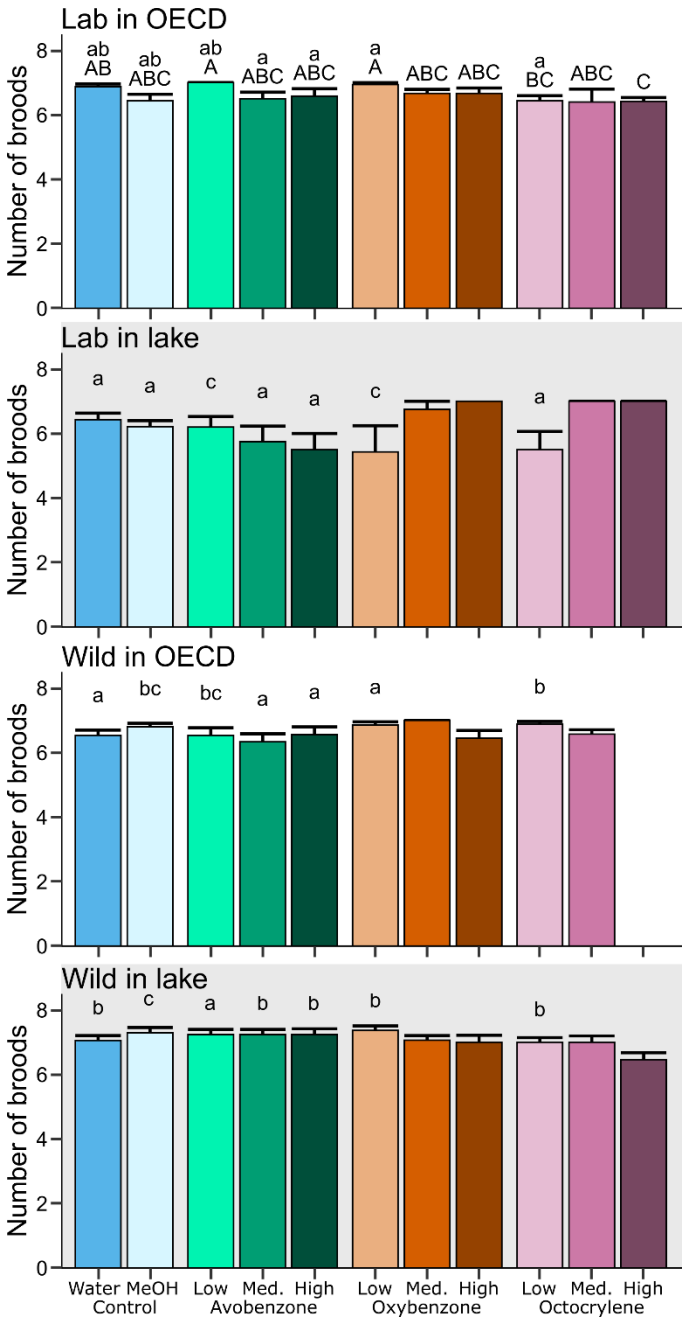


Figure S7.6. The number of broods released by each surviving *D. pulex* over a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (Kruskal Wallis, $p < 0.05$).

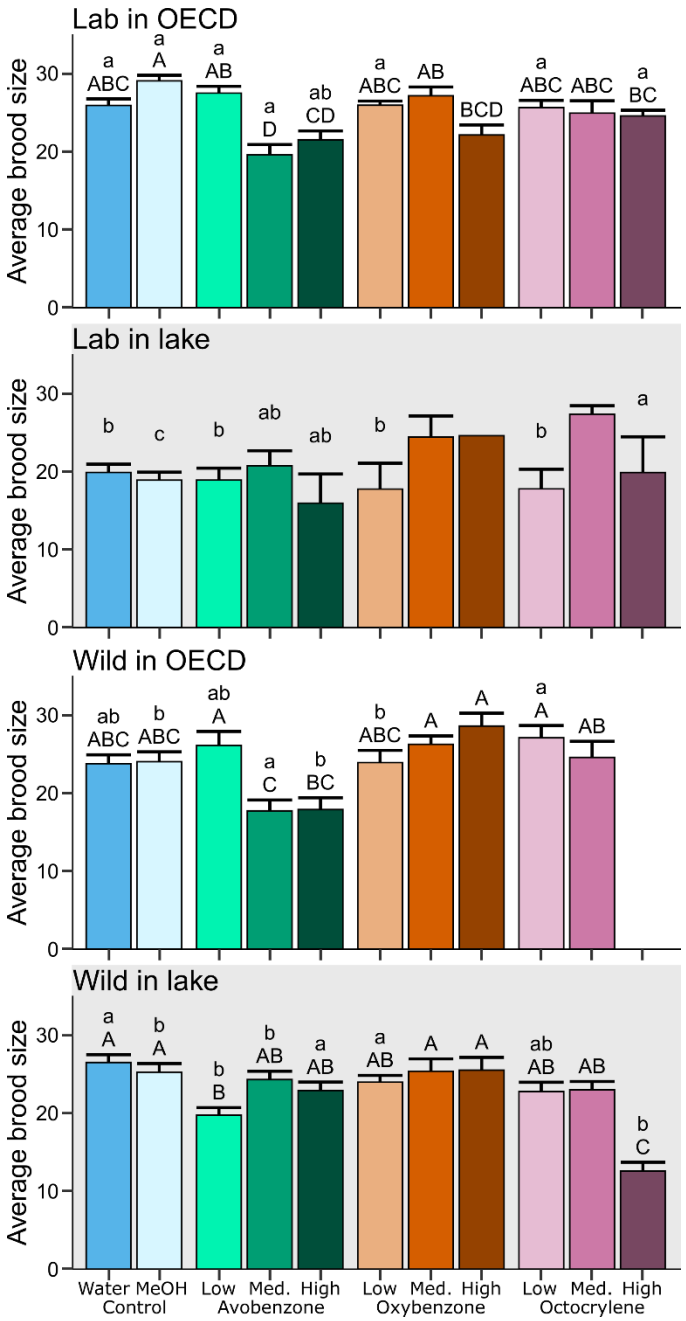


Figure S7.7. The average reproductive effort (viable neonates + non-viable neonates) per brood released by *D. pulex* surviving a 21-d exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

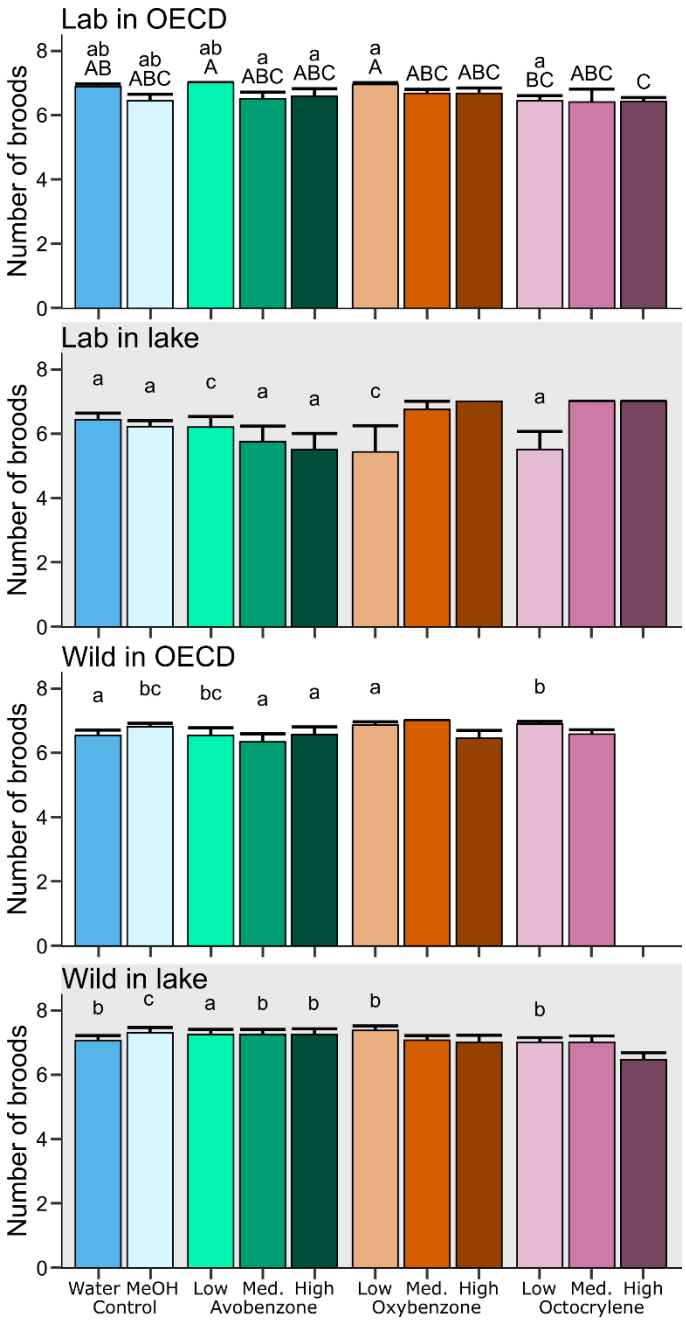


Figure S7.8. The average dry mass of 21-d old *D. pulex* surviving exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

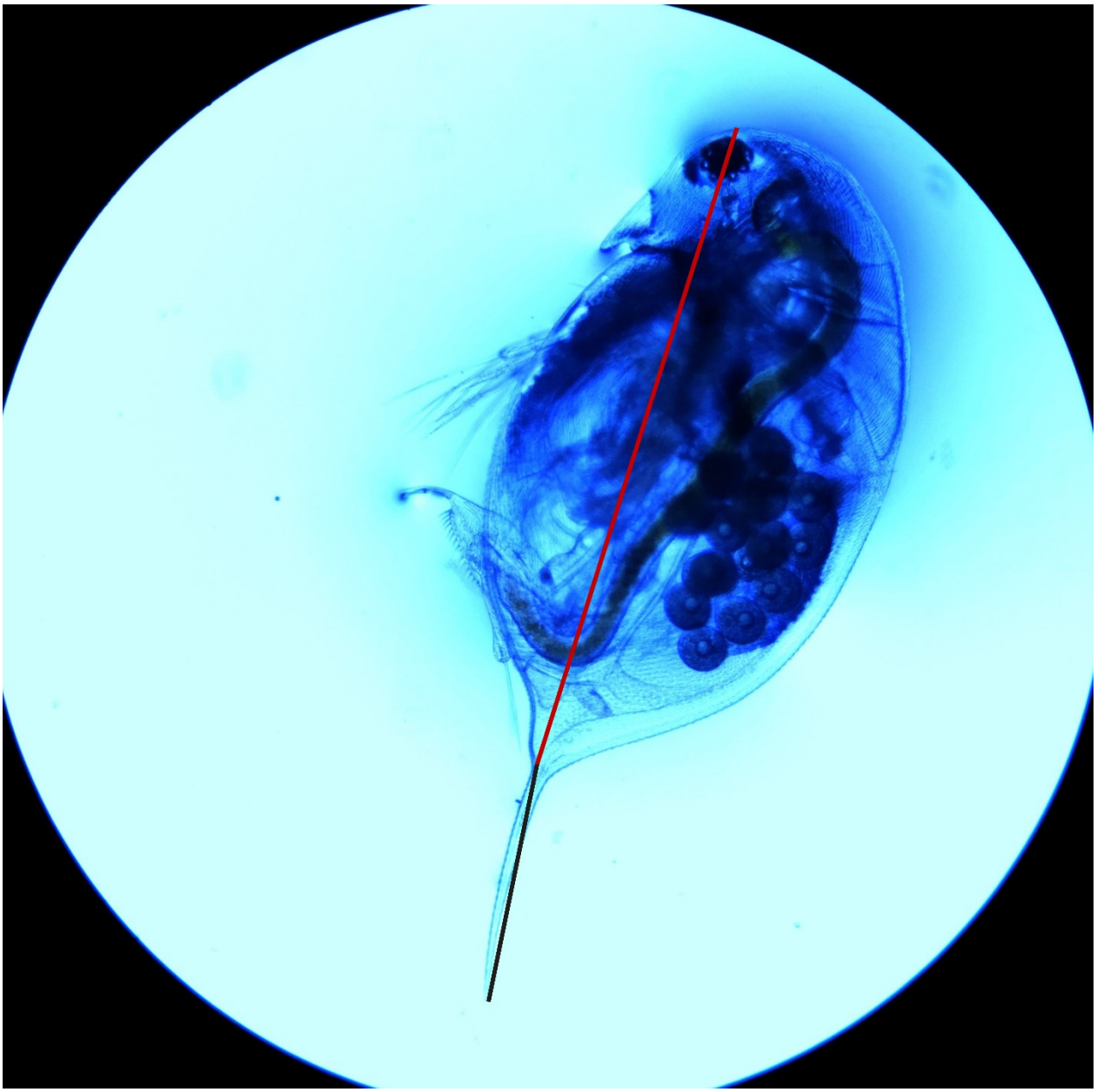


Figure S7.9. Measurements taken to obtain daphnid core body length (red) and tail length (black). Core body length was measured from the edge of the carapace above the eye spot to the base of the tail. Tail length was measured from where the core body measurement ended at the tail base to the tip of the tail spike.

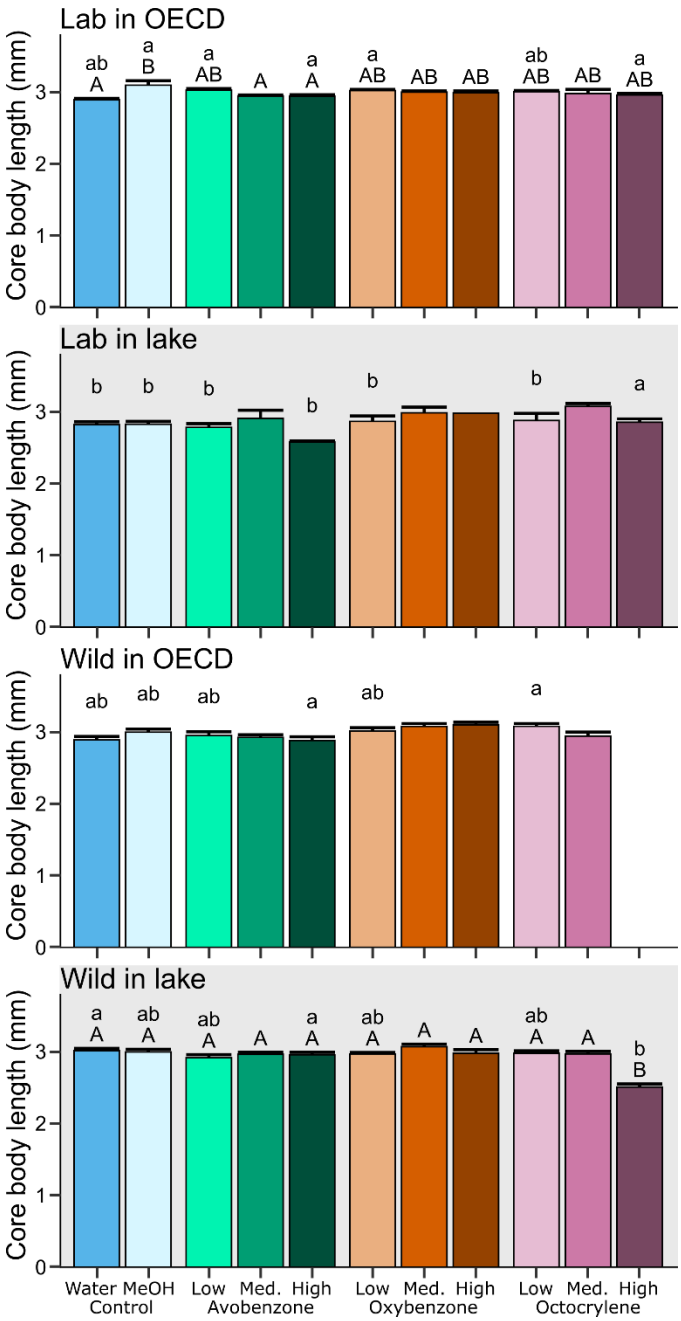


Figure S7.10. The average body length of 21-d old *D. pulex* surviving exposure to UVFs, excluding the tail region. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

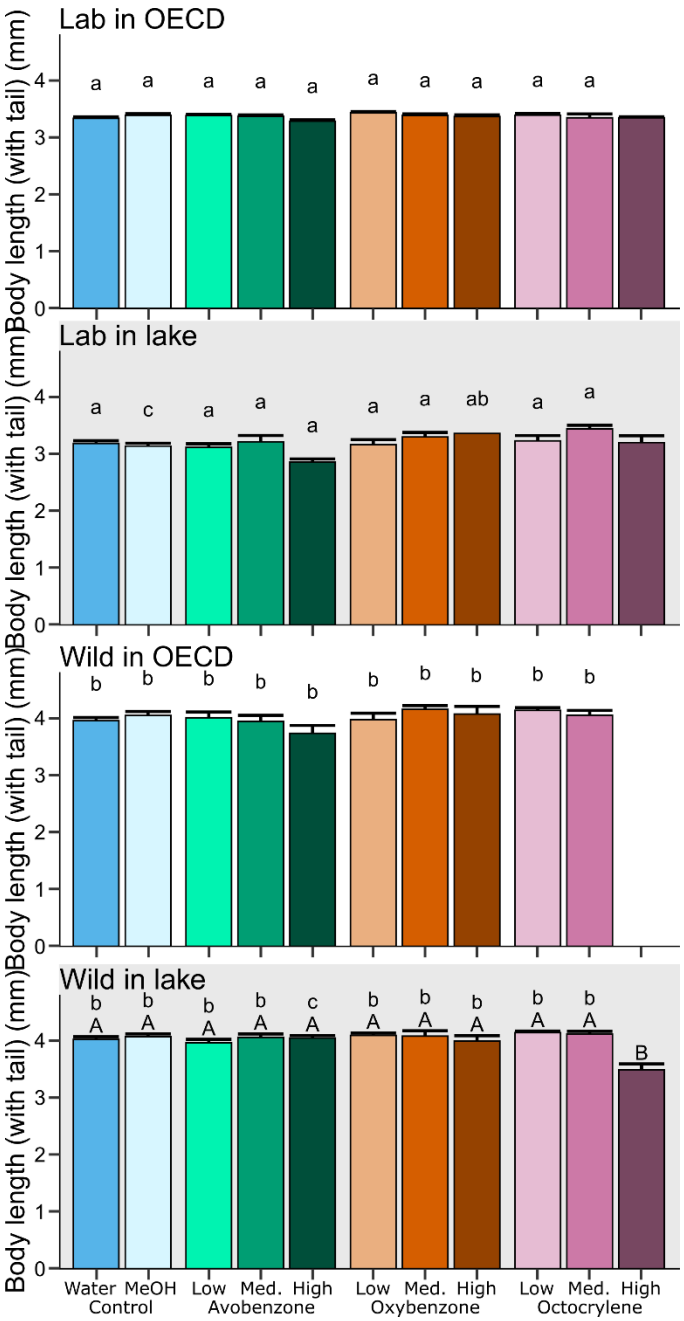


Figure S7.11. The average tail length of 21-d old *D. pulex* surviving exposure to UVFs. Bars represent mean ± SEM of 20 starting replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (Kruskal Wallis, $p < 0.05$).

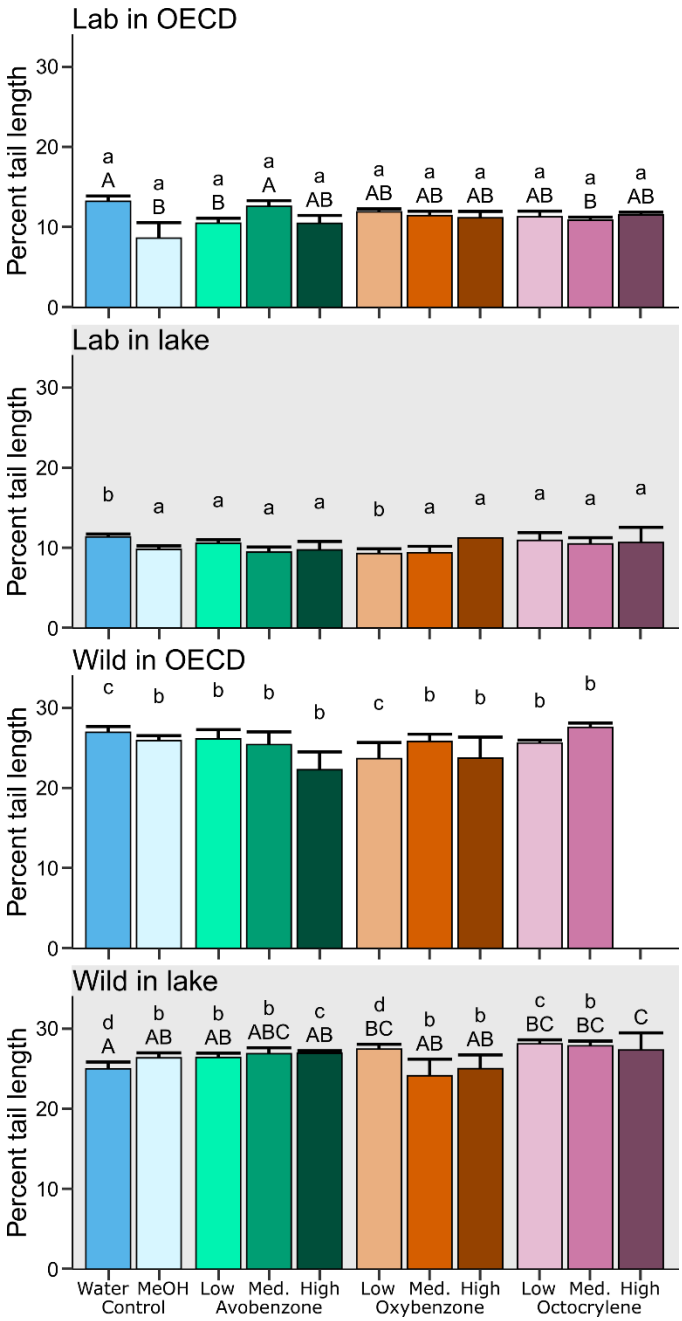


Figure S7.12. The average tail length of 21-d old *D. pulex* surviving exposure to UVFs as a percentage of their total body length. Bars represent mean ± SEM of 20 replicates per treatment. Different letters on the bars indicate a significant difference between chemical treatments within a test culture (uppercase letters; compared horizontally within a panel) or between test cultures within a chemical treatment (lowercase letters; compared vertically across panels). Bars without letters are not considered to be significantly different for that comparison (one-way ANOVA, $p < 0.05$).

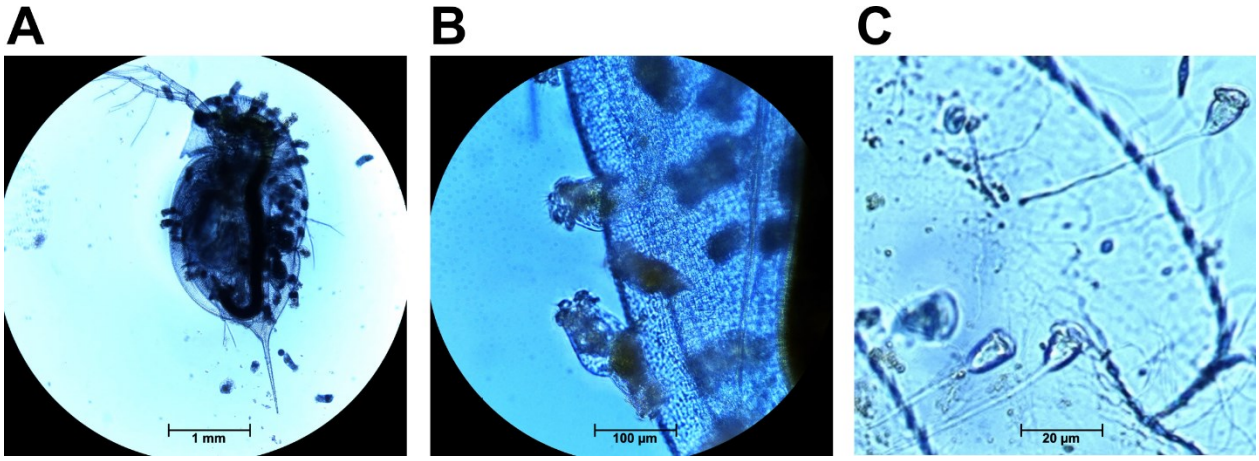


Figure S7.13. Epibionts observed on *D. pulex*, including *Brachionus rubens* on an adult daphnid (A & B), and *Vorticella sp*. (C) on a shed molt.

Chapter 8: Conclusions

From the results of the studies outlined in this thesis, it is clear that different outcomes can be obtained depending on which environmental factors are considered, or what methods are used. Data obtained in the absence of additional environmental factors (Chapters 2 & 3) indicate that the tested UVFs are particularly toxic to *D. magna* and may pose a threat to natural environments. When additional environmentally relevant factors are considered, the toxicity is reduced (Chapters 3 & 6), which instead suggests that the risk UVFs pose to freshwater environments is lower than expected from standardized test results. Investigating protein level changes revealed that UVF toxicity may be driven by disruptions to daphnids' metabolism and immune responses (Chapter 4). In addition, the validity of several common toxicology assumptions was not supported by these investigations (Chapters 3, 5, 7), demonstrating the extent to which the methods used can influence the results obtained.

8.1 Ultraviolet filter toxicity

Each tested UVF was toxic to *Daphnia* in varying degrees, with OCT typically exerting effects at the lowest concentrations and/or of the greatest severity, followed by OXY (Chapters 2-7). AVO was the least toxic of the three primary UVFs tested, infrequently causing severe impairments to daphnids, while HMS and EHS were equally the least toxic overall to *D. magna*, causing no observable impairments to daphnids beyond the determination of a 48 h LC50 for each chemical (Chapter 6). The relative toxicity of UVFs in this thesis is largely consistent with the current literature consensus (National Academies of Sciences, Engineering, and Medicine, 2022), with the exception that OXY has had greater previously reported toxicity to lower trophic level organisms such as corals (Danovaro et al., 2008), and algae (Mao et al., 2017). The relative toxicity of AVO and OXY to *D. magna* has been inconclusive in published literature, with single generation toxicity favouring OCT as more toxic (Park et al., 2017; Sieratowicz et al., 2011),

while two generation toxicity favours OXY (de Paula et al., 2022). It is possible that the greater number of studies indicating that OXY is the most toxic of these UVFs may be an artifact of the greater research effort invested into investigating OXY vs OCT, or the result of species-specific differences in sensitivity.

8.1.1 Avobenzone

AVO was capable of impairing the phototactic response of neonates and causing latent mortality after a 48 h exposure at nominal concentrations of 200 µg/L, and 100% mortality after 7 d of exposure to 150 µg/L (Chapter 2). Small increases to the proportion of reproduction resulting in non-viable neonates was observed in the F1 and F2 generations of 21 d exposure to 6.59 µg/L (Chapter 3), and reproduction was decreased after 21 d exposure to 14.5 µg/L for laboratory *D. pulex* (Chapter 7). The majority of impairments observed in this thesis occurred at concentrations above the maximum water solubility of AVO (27 µg/L), and all occurred at concentrations > 5 fold greater than the maximum contamination reported in freshwater environments (1.13 µg/L; O'Malley et al., 2021). The lowest observed effect concentration (LOEC) of 6.59 µg/L is consistent with a similar report of reproductive impairment in the F1 generation of *D. magna* exposed to 4.4 µg/L (de Paula et al., 2022), providing further evidence that *Daphnia magna* are the most sensitive species to AVO currently known. It is important to note that this LOEC was followed by a complete recovery in all tested endpoints in later generations of the same population (Chapters 3 & 4); therefore, the long-term impacts of AVO contamination can be expected to be lower than this LOEC suggests. As AVO is currently the only organic UVF approved for use by the US FDA that offers protection in the UV-A spectrum (National Academies of Sciences, Engineering, and Medicine, 2022), these results do not support any restrictions in the use of AVO in skin care products as the consequences to human health through

increased skin inflammation and melanomas are expected to impose a larger societal cost (Adler and DeLeo, 2020; Green et al., 2011).

8.1.2 Octocrylene

OCT caused more impairments across a wider range of endpoints, with some impairments occurring at concentrations an order of magnitude lower than those causing an equivalent effect for AVO or OXY. These effects include the impairment of neonate phototactic response at 200 µg/L and a nominal 48 h immobilization EC50 of 30 µg/L in *D. magna* (Chapter 2) and immobilization EC50s of 291 – 329 µg/L in lab and wild *D. pulex* (Chapter 7), which suggests that OCT is capable of disrupting daphnid locomotion. Impairments to reproduction in terms of quantity and quality were observed in the F1 and F2 generations of *D. magna* exposed to ~0.6 µg/L (Chapter 3), and in wild *D. pulex* exposed to 25.6 µg/L for 21 d (Chapter 7). These reproductive impairments in addition to the nominal 21 d LC50 of 5.35 µg/L indicate that OCT exposure can have high impacts to daphnid fitness, with a LOEC that has been reported in 36 different locations (10 freshwater, 24 marine) to date (Apel et al., 2018; Bratkovics et al., 2015; Bratkovics and Sapozhnikova, 2011; Cadena-Aizaga et al., 2022; Chisvert et al., 2017; Downs et al., 2024, 2022a, 2022b; Gago-Ferrero et al., 2011; García-Pimentel et al., 2023; Haunschmidt et al., 2010; Kameda, 2011; Langford and Thomas, 2008; Mandaric et al., 2017; Milinkovitch et al., 2024; Moeder et al., 2010; Negreira et al., 2010; O'Malley et al., 2021; Sánchez Rodríguez et al., 2015; Schaap and Slijkerman, 2018; Tsui et al., 2014; Vila et al., 2018a, 2018b, 2017, 2016). The recovery of *D. magna* populations across generations in Chapter 3 paired with the reduced toxicity of OCT in sunscreen mixtures in Chapter 6 suggests that the LOEC in the environment may be higher than single chemical studies suggest, and that populations are capable of acclimating to sublethal levels of contamination. Regardless, it would be beneficial to consider

transitioning away from the use of OCT, as there are a variety of UV-B spectrum UVFs currently approved for use that current data suggest are less toxic, such as HMS or EHS (National Academies of Sciences, Engineering, and Medicine, 2022).

It is interesting to note that this thesis has the highest reports of OCT toxicity to date, as LOECs of 40 µg/L for *Mytilus galloprovincialis* larvae development and *Paracentrotus lividus* growth were the most severe ecologically relevant impairments previously identified (Giraldo et al., 2017). This may be partially accounted for by the longer exposure duration used for the most severe effects reported in this thesis (21 d, 2 – 3 generations) compared to the values reported by Giraldo et al. (2017) after 48 h. The nominal immobilization EC50 of 30 µg/L (Chapter 2) is much lower than a previously reported nominal EC50 of 3,180 µg/L in *D. magna* (Park et al., 2017), and the nominal 21 d LC50 of 5.35 µg/L along with the reproductive impairments at ~0.6 µg/L in the F1 and F2 generations of continuous exposure (Chapter 3) are in contrast with the lack of reported effects after 2 generations of exposure to 4.4 µg/L reported by de Paula et al. (2022). These differences may be indicative of differing sensitivities of the *D. magna* cultures used by each research group, as Park et al. (2017) tested daphnids produced from ephippia purchased from Daphtoxkit F™ (Belgium), while de Paula et al. (2022) tested daphnids from a Brazilian culture of unreported origin. The studies in this thesis tested *D. magna* from Aquatic Research Organisms (ARO), US, which have common ancestry with the US EPA Newtown, Ohio lineage (S. Sinitski, ARO, personal communication, June 14, 2023); however, the toxicity of OCT was consistent across studies that tested daphnids procured from ARO in 2019 (Chapter 2) and 2021 (Chapters 3-6). In addition, several toxicity values presented in this thesis were obtained by independent researchers, as the 48 h EC50 and LC50 from Chapter 2 were obtained by C. Stewart, while similar 48 h EC50 and LC50s were obtained by A. Boyd (Chapter 4). The

repeatability of OCT toxicity within this thesis suggests that the differences with previous literature may be indicative of genetic variation across research subcultures.

8.1.3 Oxybenzone

OXY did not cause behavioural impairments to daphnids, and produced a much smaller concentration range that could cause immobilization to *D. magna* than AVO or OCT (48 h EC50: 1,200 µg/L, 48 h LC50: 1,700 µg/L; Chapter 2). Chronic exposures were capable of causing > 40% mortality, > 40% decreased reproduction and > 12% reproductive failure in the F0 and F1 generations at 16.5 µg/L (Chapters 3 & 4), as well as 55% mortality to lab *D. pulex* at 18.8 µg/L over 21 d (Chapter 7). The technical LOEC of 10 µg/L (nominal) was obtained via the 21 d LC50 in Chapter 3; however, daphnids were capable of acclimating to 16.5 µg/L if they survived the initial exposure, indicating that greater concentrations than this would be required to exert long-term impairments on an exposed population. These impairments occurred at much lower concentrations than the no observed effects reported by Sieratowicz et al. (2011) after a 21 d exposure of an unknown lineage of *D. magna* to 342 µg/L, and at higher concentrations than the reproductive impairments reported in the second generation of a Brazilian culture exposed to 0.17 µg/L by de Paula et al. (2022). Similar to OCT, these differences may be indicative of varying sensitivities of each daphnid culture used, but the level of OXY toxicity reported in this thesis was consistent and repeatable across studies, including data obtained from different researchers.

Twenty locations (4 freshwater, 16 marine) have been reported to have concentrations > 16.5 µg/L to date (Downs et al., 2024, 2022a, 2022b, 2016; Fenni et al., 2022; Kasprzyk-Hordern et al., 2009; Nejumal et al., 2021), but the majority of these are constrained to two regions (Hawaii and the southwest coast of India). Considering that OXY is the most well studied UVF by a large margin, confidence is greater that encountering an environment with such high levels

of contamination is rare. In addition, the reduced toxicity of OXY in sunscreen mixtures reported in Chapter 6 further reduces the threat posed to freshwater environments. The actions taken by various governments to restrict the use of OXY near coral reefs (Bonaire Pros, 2018; Hawaii State Legislature, 2018; Homan and Martinus, 2021; Republic of Palau, 2018; USVI Legislature, 2019) appears to be an adequate protective measure for marine environments, and an expansion of these restrictions to freshwater environments is not necessary at this time. If future research identifies a second low toxicity UV-B spectrum UVF while investigating OCT alternatives, then proactive measures to reduce or replace OXY in skin care products could be taken; however, this would be a low priority action in the context of freshwater environments.

8.1.4 Homosalate and Octisalate

Although the investigation of HMS and EHS toxicity was limited to a single study, the lack of observable effects on *D. magna* mortality and physiological characteristics after a 21 d exposure to nominal concentration of 200 µg/L suggest that these UVFs may be less toxic than AVO, OCT or OXY. Literature regarding HMS and EHS toxicity is currently limited, but current data suggests a similar lack of toxicity to corals (Danovaro et al., 2008), fish (Kunz et al., 2006), or various cell culture assays (Kunz and Fent, 2006b; Rehfeld et al., 2018). A lack of data should not be interpreted as a lack of threat; however, initial results suggest that these UVFs are promising candidates to serve as "environmentally friendly" alternatives to OCT and OXY to offer protection in the UV-B spectrum. Considering that HMS and EHS are among the most commonly used UV-B protecting UVFs (National Academies of Sciences, Engineering, and Medicine, 2022), further research may be warranted to better understand their environmental safety. The highest environmental concentrations for both UVFs reported to date are typically < 3 µg/L (Bargar et al., 2015; Benedé et al., 2016; Mitchelmore et al., 2019; Tsui et al., 2014; Vila et

al., 2017); therefore, future investigations of current effects are a low priority due to the large differences between the currently known levels of toxicity and the extent of environmental contamination, but would be a higher priority if either UVF is desired to be used as an alternative to OCT or OXY.

8.1.5 Mechanisms of action

AVO and OCT were capable of both impairing phototactic behaviour and immobilizing *D. magna* neonates (Chapter 2), suggesting the potential for neurotoxicity. While AVO has not had reported neurotoxic effects to date (Ruszkiewicz et al., 2017), OCT has been demonstrated to impair acetylcholine esterase activity in zebrafish embryos at nominal concentrations of 50 µg/L (Gayathri et al., 2023), which has the potential to cause muscle paralysis with other toxicants (Colovic et al., 2013). The mixed results previously published regarding OXY neurotoxicity (Campos, 2017; Campos et al., 2017; Huo et al., 2016; Ma et al., 2017; Wang et al., 2021) in addition to the smaller concentration range causing immobilization and a lack of phototactic impairment in Chapter 2 indicate that OXY may have less neurotoxic potential than AVO or OCT.

Analysis of *D. magna* proteomes in Chapter 4 revealed that the toxicity of AVO, OCT and OXY may be attributed to widespread impairments of carbohydrate, saccharide and protein metabolism processes, which is consistent with recent studies indicating that these UVFs are obesogens (Ahn et al., 2019; Ko et al., 2022; Shin et al., 2020). Additional impairments of immune response processes including leukotriene biosynthesis, opsonization and cytokine signalling suggest that the mechanism proposed by Danovaro et al. (2008) of UVFs causing toxicity to corals by increasing their susceptibility to viral infections is also a mechanism of toxicity for AVO, OCT and OXY to *D. magna*. The recovery of later generations of exposed

populations appears to be driven by a decrease in exoskeleton permeability via increased chitin synthesis along with a boosted immune response to counteract the consequences of any UVFs that enter into the organism.

D. pulex growth was only impacted by OCT (Chapter 7), which is consistent with previously reported growth inhibition of *Paracentrotus lividus* (Giraldo et al., 2017). No biological pathways were impacted by OCT exposure that were distinct from the effects of AVO and OXY (Chapter 4), leaving the mechanism of this impairment unknown. This unique impairment may be representative of a specific mechanism of action resulting from the distinct functional groups present in OCT compared to AVO and OXY, and could be associated with the pattern of UVF sensitivity reported in Chapter 7 where lab *D. pulex* were more sensitive to AVO and OXY while wild *D. pulex* were more sensitive to OCT.

8.2 Standardized test assumptions

8.2.1 Multigenerational outcomes

Each experiment that tested a toxicology assumption produced results that differed from the standard test method. Chapters 3 & 4 revealed that the results of single generation exposures aren't predictive of long-term outcomes in a continuously exposed population across multiple generations, as the initial toxicity was decreased in subsequent generations. The observed acclimation of *D. magna* to UVFs follows a similar pattern that has been reported from studies of *D. magna* exposed to different toxicants (Chatterjee et al., 2019; Hochmuth et al., 2015; Leblanc, 1982; Maggio and Jenkins, 2022; Muyssen and Janssen, 2001; Poulsen et al., 2021; Song et al., 2022; Ward and Robinson, 2005), or even studies of different species (Kwok et al., 2009; Li et al., 2015; Pilakouta et al., 2020; Vedamanikam and Shazilli, 2008), indicating that acclimation to a continuous stressor is a common outcome of multigenerational exposures. These reports of

multigenerational acclimation appear to occur more frequently in shorter-lived species such as *D. magna*, *Tigriopus japonicus*, *Chironomus plumosus* and *Culicoides furens*, which is consistent with the hypothesis that shorter-lived species are better suited to acclimate to environmental stressors (Kristensen et al., 2020). As a result, characterization of risk to species that are known to be capable of acclimating to continuous stressors would benefit from studies that assess the impacts across ≥ 3 generations so that any changes can be assessed with a fully exposed germline.

8.2.2 Conspecific effects

Chapter 5 found that even small methodological deviations that are accommodated for in the design of an experiment can have an impact upon the results generated, as changing the number of *D. magna* exposed in a single test vessel caused alterations to maturation time, reproductive characteristics and survival despite scaling food and water resources to the number of organisms. These findings are consistent with previous studies that have observed subtle impacts of the presence of conspecifics within an exposure vessel on toxicity outcomes (Gust et al., 2016; Lowes et al., 2021). Although the differences found in Chapter 5 did not change the overall assessment of toxicity across generations, they do indicate that comparing data obtained from different exposure scenarios within a study without the proper controls can be confounded by even seemingly minor differences in methodology. This is particularly challenging for studies that seek to connect molecular level changes to population level responses, as smaller organisms may not yield sufficient tissues for molecular analyses when exposed individually, but group exposures can reduce data resolution if whole-organism or population level endpoints cannot be attributed towards specific individuals, preventing the formation of direct links between various

endpoints. Whenever possible, studies should seek to internally unify their methodology to mitigate these potential confounding effects.

8.2.3 Predicting mixture toxicity with individual chemicals

Changing how toxicants are assessed can also lead to differences in toxicity, as the results of Chapter 6 demonstrate that the interactions that occur within complex chemical mixtures can greatly alter the toxicity of individual components. The 5.6-fold and 9-fold reduction in the lethality of OCT and OXY, respectively, revealed antagonistic interactions between various components of the sunscreen mixtures. Previous research has observed antagonistic interactions with specific combinations of UVFs (Li et al., 2018; Park et al., 2017); however, the acute exposures of the AVO, OCT, and OXY mixture in Chapter 2 produced results similar to the effects of the most toxic UVF. The lack of UVF mixture interactions in Chapter 2 may not translate to a similar lack of interactions with a chronic exposure, which was not tested in this thesis, nor does this exclude the possibility of antagonism with other components of the sunscreen mixtures. The large reduction in toxicity that occurred in sunscreen mixtures does indicate that assessments of individual chemicals cannot be assumed to be representative of mixture effects in the absence of data providing a scientific justification for this. Investigations of other complex mixtures do not always result in antagonism, as additive or synergistic effects have been reported in investigations of other types of mixtures (Drzymała and Kalka, 2020; Martin et al., 2021; Salomão et al., 2014). In the case of UVF research, it would appear that the majority of data collected to date using exposures of individual chemicals are not directly relatable to environmental outcomes, as natural environments are expected to be contaminated by many other components of sunscreen mixtures in addition to the UVFs (National Academies of Sciences, Engineering, and Medicine, 2022). Although studies of individual UVFs are not representative of

sunscreen mixture toxicity, it is unknown whether another individual component of the mixture can be studied in isolation to adequately model the effects of sunscreen pollution; therefore, further investigations are needed.

8.2.4 Representing wild populations with laboratory organisms

In Chapter 7, it was found that both lab and wild *D. pulex* were severely impaired when cultured in non-ancestral waters, leaving them highly sensitive to chemical stressors. This finding indicates that care should be taken to use the "correct" culture waters to test aquatic organisms; therefore, studies that bring wild organisms into the lab for testing in synthetic waters are highly discouraged. The large impairments observed when *Daphnia* are cultured in a novel water along with the alterations in development observed in the presence of conspecifics reported in Chapter 5 indicate that methods have a large influence on the results of any experiment. Care should be taken to ensure that studies use the correct methods to address the question(s) under investigation, and that the specific context of the data are considered when drawing conclusions.

In addition, Chapter 7 revealed that populations that originate from unique environments do not necessarily produce the same results during experimentation. This is consistent with the findings of Chapters 3 & 4, which observed that *D. magna* populations can acclimate in response to their specific environmental conditions within a few generations spanning 2 – 4 months; therefore, the observation of differences between lab and wild *D. pulex* populations that have been isolated for several decades are unsurprising. The lack of one population being consistently superior to the other is consistent with the results reported by other lab and wild comparisons in different species, indicating that changes between isolated populations are common, but are not uniformly directional in terms of having a positive or a negative impact on organism performance (Barata and Baird, 2000; Carline and Machung, 2001; Heaton et al., 2022; Hirakawa and Salinas,

2020; Morgan et al., 2019; Robertson et al., 2016; Romero-Blanco and Alonso, 2022; Stewart Merrill et al., 2019).

The variability of processes that can cause populations to diverge such as genetic drift and selective pressures are expected to produce similarly variable differences between isolated populations (Wong, 2020). Genetically isolated populations are expected to diverge over time (Brekke et al., 2018), even when laboratory populations are inbred to minimize genetic variability (Suurväli et al., 2020). If it is desired for laboratory organisms to be representative of wild populations, then lab populations must be regularly supplemented by breeding with wild individuals to minimize the genetic deviations that can occur. Doing so would potentially create more problems for standardized testing, as outbreeding with wild organisms may impair the lab culture's suitability for rearing in laboratory conditions, as observed by the reduced viability of both lab and wild daphnids cultured in non-ancestral waters. Thus, researchers are presented a trade-off between laboratory organisms that are well suited for lab-based culturing and experimentation, or laboratory organisms that are more closely related to their wild counterparts.

8.3 Pitfalls

Toxicology research is reliant upon understanding the exact exposure doses test organisms were subjected to for each stage of experimentation. While each study presented in this thesis has quantified exposure solution concentrations to varying degrees, it would be desirable to have a greater frequency and depth of water chemistry measurements (e.g., measurements at the start and end of solution renewal in test vessels). This would provide a better understanding of how the exposure environment evolved during each experiment to counter the difficulty in working with these particular substances that are known to rapidly decrease from the nominal concentration due to adsorption (Cormier et al., 2019; Fel et al., 2019; O'Malley et al., 2021) and degradation

processes (Chou et al., 2024; Guesmi et al., 2020; Jou-Claus et al., 2024; Liu et al., 2012; Rodil et al., 2009; Wang and Kannan, 2013; Ziarrusta et al., 2018a). The difficult nature of testing the toxicity of these chemicals extended towards the ability to quantify test solution concentrations, as water chemistry data was largely limited by the ability to solicit access to reliable analytical resources, resulting in a trade-off between the cost of sample analysis and the level of in-house experience in quantifying UVFs for each possible laboratory considered. The use of radiolabelled UVFs was considered for Chapters 6 & 7 so that quantifications could be made in-house; however, this was again limited by our ability to reliably procure the required chemicals from major suppliers. Overall, these limitations served to prevent any investigations of UVF bioaccumulation or tissue distributions, which may have provided further insight into toxicity mechanisms, and allowed for a greater depth of analysis regarding the relative toxicity of each UVF by understanding the exact doses received by each daphnid.

The standardized approaches used to conduct research are defined by the resource constraints (finances, labour, time, etc.) faced by every research group. Such constraints defined methodological choices for several chapters, including the absence of a reference toxicant such as Cu during the multigenerational or lab vs wild experiments to increase confidence in the overall performance of the daphnids used with respect to other research groups. Although a reference toxicant wasn't included directly in these experiments, 48 h Cu LC50s were regularly performed on daphnid cultures throughout this thesis to monitor the culture condition prior to and during experimentation.

The testing of research assumptions does not make one immune to making assumptions of their own. Each study described in this thesis relies upon the assumptions that are tested in the other experiments, leaving each study vulnerable to the influences of assumptions that were

found to be incorrect, and the cumulative interactions between these assumptions unknown. Each of these investigations also required new assumptions to be made which have not necessarily been tested for their validity. In accordance with the current replication crisis impacting academia (Baker, 2016), the largest assumption made when conducting this research is that these results are replicable. As discussed above, the *D. magna* culture used for this thesis produced results that were similar to previously published data in some instances, and more or less sensitive in others. Due to the repeatability of results across studies discussed above, it is assumed that these differences are the result of characteristics specific to the subculture of *Daphnia* tested rather than an aspect of the methodology used for any given experiment. This thesis has provided evidence that even small changes in methodology can cause appreciable effects on the data produced; therefore, it is important that studies that assess the validity or applicability of current methodologies are independently replicated. Greater confidence in these results could be established by repeating these studies using *Daphnia* of different origins in independent laboratories that follow the same methodologies described in this thesis to the greatest extent possible, or by testing *Daphnia* populations maintained on different diets to account for the effects of nutritional status that are known to contribute towards interlaboratory variability of Cladocera studies (Cowgill, 1987).

8.4 Future directions

Chapters 3-5 assume that the acclimations that occurred in later generations of UVF exposure do not come at the cost of increasing daphnid sensitivity to a secondary stressor. All aspects of an organism's function are defined by trade-offs that are made to prioritize certain features or processes over others (Pörtner et al., 2005; Sokolova et al., 2012). The multigeneration experiment was unable to identify what biological processes were diminished in

order to increase daphnid resilience to the UVF stressors, but in the context of a constant maximum energetic budget defined by the controlled food provided to each daphnid as well as the lack of change in metabolic rates, it is illogical to expect that the observed changes did not incur a cost to the organism in some regard. Future research could investigate the effects of co-exposure to additional stressors that are known to operate through mechanisms of action that do not overlap with the mechanisms of UVF toxicity to determine to what extent the *D. magna* populations specialized in their ability to withstand UVF contamination, and what the consequences of these changes may be.

Chapter 6 is defined by a lack of supporting literature knowledge to provide additional context regarding sunscreen mixtures. The lack of environmental surveys assessing contamination of sunscreen inactive ingredients makes it difficult to assess the risk that sunscreen pollution poses to the environment due to the wide range of estimates regarding sunscreen release discussed in section 6.4.3. This study also assumes that the mixture interactions in other types of sunscreen products (e.g., lotions, creams) will be similar to those observed in the tested spray products. This research could be further expanded to investigate a greater variety of sunscreen products as well as other types of skin care products (e.g., lip balms, moisturizers) to increase confidence in the relationship between UVF toxicity in isolation and in sunscreen mixtures.

Chapter 7 makes the assumption that it was ever possible for the wild *D. pulex* population tested to produce similar results as the lab population, which may only be possible if those populations shared a recent common ancestor or originated from the same lake. It would be interesting to repeat the Chapter 7 study with the wild populations that the US EPA Newtown, Ohio *D. magna* and *D. pulex* cultures originated from to determine to what extent these differences are caused by the testing of discrete populations vs genetic changes across

populations of the same ancestry. It is also likely that repeating this experiment with different lab or wild populations would produce different results from the populations tested in this thesis to some extent; however, these findings may further support the conclusion that testing different populations can produce different results on a case-by-case basis.

In general, all future studies would also benefit from the inclusion of a broader array of endpoints that encompass more functions and/or are not commonly investigated. The endpoints recommended by *Daphnia* toxicity test guidelines (e.g., survivorship, growth, reproduction) were selected for their ease of quantification and their indisputable relevance to the fitness of the organism, but may not capture effects upon other aspects of an organism's physiology or ecological fitness. For example, OECD guidelines explicitly require that reproductive effects are determined based on the number of living offspring produced and do not refer to the assessment of other reproductive outcomes such as the release of undeveloped eggs or non-viable neonates (OECD, 2012). By including these additional measures of reproduction beginning in Chapter 3, the common occurrence of non-viable neonates as a consequence of UVF exposure was observed which was an unnoticed outcome during the Chapter 2 experiments. Science benefits from access to information; therefore, the inclusion of endpoints that can be easily captured with minimal additional effort is recommended, including the quantification of alternate reproductive endpoints such as the production of ephippia, non-viable neonates, or the failure of eggs to develop. Testing other aspects of an organism's function would provide a greater understanding its overall condition, which could be accomplished by measuring swimming activity and/or performance, the ability to detect and appropriately respond to environmental cues (e.g., light, temperature, predator or food stimuli), or assessments of offspring condition. These non-standard endpoints would come at the cost of requiring method development and validation studies, as well as

requiring additional effort during normal experimentation, but could provide additional context on other aspects of an organisms' condition.

8.5 Summary

With all assessments of the consequences of human activities, environmental impacts must be weighed against societal impacts. In the case of UVFs, an overly conservative approach that bans the use of any UVF that appears to pose a threat to the environment without first identifying suitable alternatives could result in large negative impacts to human health through increased risk of skin cancer. Thus, the ability to more accurately understand how toxicity data can be applied to natural environments will increase the confidence with which high-risk chemicals are identified. In this thesis, the toxicity of OXY using the standard test method would normally be sufficient to consider it a threat to *Daphnia*; however, the reduced toxicity observed in sunscreen mixtures along with the acclimation of exposed populations across generations reduced the threat to the extent which no immediate action appears to be required to protect freshwater environments at this time. This serves as an example of the benefits of increasing the applicability of laboratory data to the environment, which may prove useful in other cases where data is limited, or the threat appears to be borderline with respect to acceptable risk thresholds.

Conducting research in a more environmentally relevant manner is not without consequences, as the greater complexity of these studies requires more resources, reducing the rate of data generation. The benefits of standardized methods on increasing the rate and replicability of research are indisputable and irreplaceable; however, supplementing these methods with studies that test their assumptions can improve the applicability of existing and future data generated using standard approaches. Just as physicists have noted that the simple act of measuring quantum phenomena can change the observed outcomes (known as the observer

effect; Sassoli De Bianchi, 2013), all investigations of biological phenomena must be assumed to be altered somehow by the methods used to observe, measure, or experiment upon any system. As a result, it is important that the influences our methodologies have upon the results obtained are understood so that questions under investigation can be answered using the appropriate experimental design. In the context of ecotoxicology, researchers must decide whether they are investigating the general toxicity of a substance, or its environmental impacts. Pursuing either goal provides valuable data that can further our understanding of the stressor, but each goal may benefit from methodologies that are incompatible with the other. Studies that attempt to address both goals simultaneously may be unable to adequately address either aspect due to the trade-offs that are required.

The statistician George Box stated that "all models are wrong but some are useful" (Box, 1979). The pursuit of a perfect model that accurately captures all aspects of the natural world would "reasonably" lead one to conclude that only mesocosm experiments, or perhaps just *in situ* studies in real ecosystems are valid. This goes against the desire to increase the accessibility of research to increase overall scientific capacity by limiting studies to prohibitively expensive experiments, rendering regulatory bodies and government agencies unable to respond to problems of environmental importance. Instead, this thesis should be understood as a cautionary message that despite the great effort invested to date developing modern research guidelines, these methods are not infallible and still have many questions yet to be answered.

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