UNIVERSITY OF BIRMINGHAM

Daphnia as a sentinel species and a bioremediating agent for environmental health protection

by

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Abstract

Persistent chemicals originating from domestic and industrial activities pose significant risks to environmental and human health due to their toxicity and bioaccumulative potential, even at concentrations below approved regulatory thresholds. Conventional chemical toxicity tests disregard the impact of long-term exposures and use concentrations that organisms rarely encounter in real-world scenarios. Moreover, the potential adverse effect of chemical cocktails of unknown mixtures is rarely assessed because of the current one-chemical-at-time, hazard-focused, siloed approach to environmental and human health protection.

In my thesis, I broadened the use of the sentinel species *Daphnia magna* as a reliable diagnostic tool for water pollution and as a bioremediation agent to effectively mitigate the risks associated with chemical mixtures in the environment. Through rigorous experiments on four different genotypes of *Daphnia magna*, each with varying exposure histories to chemical pollution, I was able to quantitatively measure the uptake of four chemical compounds prioritised under the Water Framework Directive (WFD) as well as their mixtures (wastewater). In addition, I delved into the potential mechanisms of toxicity caused by these chemicals both within and across generations. To further support my research, I evaluated the ability of the same *Daphnia* genotypes to remove and uptake these chemicals and their mixtures under laboratory and semi-natural environmental conditions using mesocosms. The experiments were conducted using high-end concentrations of chemicals based on relevant concentration for surface and wastewater and aimed to evaluate the effectiveness of *Daphnia* as a remediation agent. At the laboratory scale, the removal efficiencies were 90 % for diclofenac, , 60 % for arsenic,

59 % for atrazine, and 50 % for PFOS. Validation at the prototype scale confirmed the sustained removal efficiency of diclofenac over four weeks. I discovered that previous exposures to chemical stress reduced genome-wide diversity in *Daphnia* genotypes. This, in turn, leads to reduced tolerance to novel chemical stress across generations. The decreased tolerance is due to reduced gene diversity in detoxification, catabolism, and endocrine genes in experienced genotypes. These genes are important in pathways that are conserved across species and could be potential targets for chemicals in other species, including humans. My preliminary analysis of the impact of these on the gut microbiome in *Daphnia* suggests the potential role of the bacteria in detoxification. *Daphnia* plays a crucial role in the food web of aquatic ecosystems and is a key indicator of ecosystem health. Impact of key chemicals on *Daphnia* can potentially have a cascading effect on other organism in aquatic food-webs. In this thesis, I also completed a proof-of-concept study using *Daphnia* as a natural filter to remove chemical pollutants from wastewater. Inspired by nature, this solution utilizes *Daphnia* to absorb and retain persistent chemicals, including pharmaceuticals, pesticide, and industrial chemicals, in a non-discriminatory manner. The technology has the potential to prevent harmful chemicals from entering our waterways. Doing so, it can contribute to a cleaner, more sustainable future by enabling water reuse and preventing environmental pollution.

My future work will investigate the ecotoxicity of chemical pollutants on *Daphnia* and its microbiome. I will also investigate the mechanisms of biotransformation of chemicals in the *Daphnia* body following uptake from water and wastewater. This will provide key insights into ecotoxicology using a systems biology approach and will guide the selection of *Daphnia* genotypes with the highest detoxification ability for applications in bioremediation.

Introduction: General Introduction

Author's contribution: MA conceived and wrote the chapter

Chapter 1: **Published in *Environmental Science and Technology*: Muhammad Abdullahi**; Li, X.; Abdallah, M. A. E.; Stubbings, W.; Yan, N.; Barnard, M.; Guo, L. H.; Colbourne, J. K.; Orsini, L. **Daphnia as a Sentinel Species for Environmental Health Protection: A Perspective on Biomonitoring and Bioremediation of Chemical Pollution**. *Environ. Sci. Technol.* **2022**, *56* (20), 14237–14248. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9583619/

Author's contribution: M.A and X.L share first authorship. J.K.C and L.O. share senior authorship. M.A., W.S., and M.A.-E.A. generated the data for removal efficiency of chemicals by Daphnia. X.L. and L.-H.G. collected and analysed data for the Chaobai river case study. L.O. and J.K.C conceived the framework with input from N.Y. L.O. coordinated data analysis and writing.

Chapter 2: **Published in *Molecular Ecology*: Muhammad Abdullahi**; Zhou, J.; Dandhapani, V.; Chaturvedi, A.; Orsini, L. **Historical Exposure to Chemicals Reduces Tolerance to Novel Chemical Stress in Daphnia (Waterflea)**. *Mol. Ecol.* **2022**, *31* (11), 3098–3111. https://doi.org/10.1111/MEC.16451

Author's contribution: M.A. performed the experiments. M.A. and J.Z. performed statistical analyses on fitness-linked life history traits. V.D. and A.C. performed genomics

and functional analysis. L.O. conceived the study, coordinated data analysis and writing. All authors contributed to the manuscript writing.

Chapter 3: **Unpublished: Muhammad Abdullahi,** Jiarui Zhou, Sam Benkwitz-Bedford, Stephen Kissane, Luisa Orsini: **Exposure to persistent chemicals alters gut-microbiota diversity and composition in *Daphnia magna*.**

Author's contribution: MA carried out the experiment. MA, and SBB performed statistical analysis. J.Z. developed pipeline for genome-wide transcriptional analysis. LO conceived the study and coordinated data analysis. MA wrote the chapter.

Chapter 4: **is under review in *TheScience of the total environment*: Harnessing water fleas for water reclamation: a nature-based tertiary wastewater treatment technology**

Author's contribution: MA completed chemical exposures and ran the mass spectrometry analysis under the supervision of MA-EA and LO; IS built and optimised the technology prototype and the side stream reactors; SB developed the delay differential equations under the supervision of SJ and AT. RO completed the preliminary separation of the chitin shell and the organic body in *Daphnia* under the supervision of LEM. RGL contributed intellectual property on the technology on behalf of Daphne Water Solutions Ltd. BH completed techno-commercial and market analyses on the Daphnia-technology. PT, MS, and SG contributed to the final technology design providing information on retrofitting requirements. KDD and LO conceived and coordinated the study. LO coordinated data analysis and wrote the first manuscript draft with input from MA and IS. All authors contributed to the manuscript writing and approved.

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Author's contribution: MA conceived and wrote the chapter.

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List of Abbreviations

%	-	percentage
°C	-	degree Celsius
°C/min	-	degree Celsius per minute
µg/L	-	microgram per litre

GENERAL INTRODUCTION

1.1. Global Water Crisis

Approximately two billion people worldwide have no access to clean and safe water (Gleick, 2003). About two-thirds of the world's population suffers from a severe lack of water at least once a month (Mekonnen & Hoekstra, 2016). By 2025 60% of the world's population is expected to live in water-stressed areas (Arnell, 2004; Diop, 2002; Hamilton et al., 2007; Postel, 2000). Water shortages are expected to be more severe as agricultural and industrial activities expand to feed the growing human population, with the global water supply reaching critically low levels in the next two decades (Alcamo, Henrichs, & Rosch, 2000; Tarrass & Benjelloun, 2012). The population most affected by the water crisis are often in the 'global south'; low and middle-income countries (LMICs) disproportionately suffer from water scarcity and climate change (Jain & Singh, 2010).

The global water crisis and the damage caused by climate change have accelerated the need for suitable reuse of treated water (Uhlenbrook & Connor, 2019). However, reusing water is unsafe because of persistent chemical pollutants (e.g., industrial chemicals, pharmaceuticals, and pesticides) that leach into the water supplies and that cannot be eliminated by routine wastewater treatment (Dudgeon et al., 2006; Rijsberman, 2006; Schwarzenbach, Egli, Hofstetter, Von Gunten, & Wehrli, 2010).

Persistent chemicals are of growing concern for their bioaccumulative and toxic properties, even when occurring below their regulatory approved thresholds (Abdullahi, Li, et al., 2022a). Pesticides including organochlorine (OCPs) and organophosphates, hexachlorobenzene (HCB) are widely used worldwide (Bandow, Conrad, Kolossa-Gehring, Murawski, & Sawal, 2020; Behrooz, Sari, Bahramifar, & Ghasempouri, 2009; Ben Hassine et al., 2014; Yu et al., 2014). They are demonstrated human carcinogens (Cancer, 1987; Lyon, 2014; Antonio Suppa et al., 2020) and have documented adverse effects on wildlife (e.g., (Eqani et al., 2013; Gao, Luo, Yang, Wang, & Mai, 2009; Jiang et al., 2005; K. C. Jones & De Voogt, 1999)). Among these

effects, reproductive and developmental failure, as well as neuro and cardiotoxicity have been documented (El-Nahhal & El-Nahhal, 2021; Georgiadis et al., 2018; Richardson, Fitsanakis, Westerink, & Kanthasamy, 2019; X. Wang et al., 2016). The adverse effect of these pesticides on non-target species include decreased phototaxic responses in *Daphnia* neonates (Hasenbein, Peralta, Lawler, & Connon, 2017), decline in the density of benthic invertebrates (Uddin et al., 2016), decreased hatching and embryonic deformities in Zebrafish (Rahman, Islam, Haque, & Shahjahan, 2020).

Some of the most infamous industrial chemicals are perfluorinated compounds, such as PFOS, and PFOA. Perfluorinated chemicals are widely used in various products like carpets, clothes, leather, food containers, and floor polishes for their surfactant, protection and performance properties. They are also found in fire-fighting foams and carpet spot cleaners. (Gaines, 2023; Jonas Margot, Rossi, Barry, & Holliger, 2015). They include stable precursors such as perfluorooctane sulfonate (PFOS) and perfluorooctane sulfonic acid (PFOA), are highly persistent and resistant to degradation and have been detected in aquatic environments as well as in animal and human tissue (Y. Du, Shi, Liu, Yu, & Zhou, 2009; J. P. Giesy & K. Kannan, 2002; Yeung et al., 2006). These compounds are readily absorbed through ingestion, inhalation and skin contact (Lau et al., 2007). Whereas the LC50/EC50 value of these compounds is high (40-200 mg/L;(Zheng et al., 2012)), their chronic effects can be devastating. Known adverse chronic effects of these compounds include reproductive and developmental toxicity in fish (Gerald T Ankley et al., 2005; Oakes et al., 2005; Oakes, Sibley, Solomon, Mabury, & Van Der Kraak, 2004), and mobility impairment in zebrafish embryos (Bremer et al., 2005; H. Huang et al., 2010). These compounds can also cause malformations (e.g. bent spine, inflated swim bladder, decreased heart rate) and mortality in (zebrafish; (Shi, Du, Lam, Wu, & Zhou, 2008)), delayed sexual maturation in (Daphnia; (Ji et al., 2008) and lower reproduction in (*Moina macrocopa*; (Ji et al., 2008).

It is crucial to note that the US lacks regulations on PFAS in drinking water. However, the environmental protection agency (EPA) took action by initially issuing a non-negotiable health advisory (HA) (defined as concentration in drinking water below which adverse health effects are not expected to occur) levels of 70 ng/L for PFOS in drinking water in 2016 (Cordner et al., 2019), while later in 2021, the EU set a limit of 500 ng/L in drinking water (Wee & Aris, 2023). Recently, a new controversial HA threshold of 0.02 parts per trillion (ppt) or 0.02 ng/L has been established for PFAS in drinking water, while as of March 2023, the maximum contaminant level (MCL) (defined as the guideline below which no adverse health effects are expected) for PFOS is 4 ppt or 4 ng/L (Wee & Aris, 2023). In the UK, fresh surface waters have been found to contain high levels of PFOA and PFOS. The Environment Agency (EA) discovered that the levels can be as high as 610 and 73 ng/L respectively (Ruffle et al., 2023). In 2018, the European Food Safety Authority (EFSA) established tolerable weekly intake (TWI) for PFASs. For PFOS, the TWI is 13 ng/kg/week, while in 2020, EFSA recommended a TWI of 4.4 ng/kg/week for PFOS and three other PFAS compounds (Wee & Aris, 2023).

Pharmaceuticals are also abundant in surface and wastewater. They enter waterways primarily through human and animal waste (Klatte, Schaefer, & Hempel, 2017). Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most abundant for their widespread use and partial biotransformation, which means these compounds or their by-products are not eliminated by wastewater treatment and end up in our surface waters (Świacka, Michnowska, Maculewicz, Caban, & Smolarz, 2021). Even at low doses, NSAIDs can affect reproductive success, growth, and cause locomotion defects in aquatic invertebrates (Fent, Weston, & Caminada, 2006; Kolpin et al., 2002; Pounds, Maclean, Webley, Pascoe, & Hutchinson, 2008; L. Wang et al., 2016). Anti-inflammatory drugs have also been shown to induce irreversible cytogenetic and DNA damage effects in bivalves (e.g. *D. polymorpha*, (Parolini, Binelli, & Provini, 2011)). Antibiotic resistance is a serious health concern (Aslam et al., 2021; Frieri,

Kumar, & Boutin, 2017; GLOBAL, 2015). For example, antibiotic resistance has been identified in several groups of bacteria including *Campylobacter spp.*, (Aarestrup & Wegener, 1999; Alfredson & Korolik, 2007; Hlashwayo et al., 2021; Velàzquez, Jimenez, Chomon, & Villa, 1995) , *Salmonella spp.* (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015; Threlfall, 2002; Van, Moutafis, Istivan, Tran, & Coloe, 2007; Wright, 2010), *Staphylococcus spp.*(Zieliński et al., 2020), and *E. coli,* e.tc. (Pitout, 2012) . They have demonstrated to be capable of eliciting changes in the structure, and function of the cells and tissues in vertebrates (e.g., oxidative stress, histological and cytological changes; (Catanese, Suvorov, & Vandenberg, 2015; Kiyama & Wada-Kiyama, 2015)) leading to reproductive, mutagenic, and carcinogenic effects (Cizmas, Sharma, Gray, & McDonald, 2015).

Also commonly found in surface waters and soil are heavy metal; they can enter the food chain through water, or leaching into groundwater (Fernandez-Luqueno et al., 2013). Exposure to heavy metals through food and water has very toxic effects on humans and can cause skeletal malformations, kidney failure, cerebrovascular diseases and eventually death (Bouchard et al., 2011; M. Holmstrup et al., 2010; Martin Holmstrup et al., 2011; Jomova et al., 2011; Meliker, Wahl, Cameron, & Nriagu, 2007). Contamination of freshwater resources by heavy metals have been shown to contribute to the decline of amphibians (Ficken & Byrne, 2013) and freshwater macroinvertebrates (Beasley & Kneale, 2003).

Persistent chemicals, such as industrial chemicals, pharmaceuticals and heavy metals have a documented adverse effect on macro and microinvertebrates, which are central to the food web of freshwater ecosystems. The impact on these species can have a cascading effects on the entire food web (Dang, Chauvet, & Gessner, 2005; Wallace & Webster, 1996).

1.2. Challenges of Modern Ecotoxicology

Traditionally, toxicity of chemicals has been tested as one-chemical-at-a-time, has been hazard-focused, and compartmentalized between environmental and human health (Abdullahi, Li, et al., 2022a; Carpenter, Arcaro, & Spink, 2002; Judson et al., 2011; Villeneuve & Garcia-Reyero, 2011). Model vertebrate organisms are used as surrogates to assess adverse or toxic effects in humans, whereas invertebrates with vertebrate embryos, zooplankton and algae are used as indicators of environmental toxicity (Norberg-King et al., 2018; Walker et al., 1998). Ecotoxicological assessments are routinely applied to single chemicals and test adverse effects using chemical concentrations that organisms rarely if ever experience in the natural environment (Calow & Forbes, 2003). Acute toxicity tests based on quantifying lethal doses - LC50, a concentration at which half of a test population goes extinct - are logistically advantageous. However, they do not account for effects that occur in realistic environmental conditions and derive from long-term chronic exposure to low doses of chemical mixtures (Burden et al., 2020). Chronic toxicity tests are used to assess the Maximum Acceptable Toxic Concentration (MATC) or the no-effects concentration of a given chemical (Rhodes, Adams, Biddinger, Robillard, & Gorsuch, 1995). MATC is the maximum acceptable concentration of chemicals acceptable to deem an environment safe. It is often applied to assess drinking water and food safety (Bidwell, 2020). Whereas chronic toxicity tests are better at determining the adverse effect of long term exposures, they are costly and time consuming, hence rarely applied by regulators (Price et al., 2022).

Living beings including humans are exposed to both deliberate and accidental chemical mixtures; chemicals in mixtures can originate from different sources e.g., pharmaceuticals, industrial, agricultural, and domestic use. These mixtures may contain individual components that are present at levels much lower than what is considered safe by regulatory authorities, but still contribute to the overall toxicity of the mixture (Abdullahi, Li, et al., 2022a; Kortenkamp

& Faust, 2018). Currently the assessments of risk of chemicals are limited to individual substances with the aim to reduce risks before they happen. However, this approach does not take into consideration the combined effects of exposure to multiple chemicals from different sources like pollution from home, hospitals, industries, and agriculture, which eventually poses a greater problem of protecting public health and the environment (Kortenkamp & Faust, 2018). Chemical components in mixtures are in constant interaction and are capable of eliciting risk and adverse effects in the environments, including humans. However, the current legislation is directed towards substances and is sector-specific (e.g., human and veterinary drugs, industrial chemicals and chemicals used in agriculture) and have never been evaluated as mixtures (Kortenkamp & Faust, 2018).

1.3. Daphnia as Sentinel Species

The 3 Rs (Replacement, Reduction, and Refinement), introduced by Russell and Burch (1959), aimed at achieving humane experimental techniques by promoting the use of alternative methods (such as in vitro or computer-based models) and non-sentient species to assess toxic effects of chemicals (Piersma, 2006). The traditional method of toxicity testing that uses data obtained from tests on animals to determine the potential toxicity of the substance on humans has several limitations-firstly, it may not accurately reflect the effects of low dose exposure, which is more relevant to human exposure, secondly, the use of animal models may not be predictive of human response due to differences in specie metabolism and physiology, and thirdly, the reliance on apical endpoint may not capture the full range of toxic effects such as subtle changes in gene expression and cellular function (Fischer, Milton, & Wallace, 2020). *Daphnia* is one of the key surrogate species used for ecotoxicology testing according to the OECD (Economic Cooperation and Development) guidelines (OECD, 2004, 2012). These tests aid in determining parameters, such as LC50 (defined as the concentration of a chemical that

is lethal to 50 % of the test organisms) that are crucial in determining the level of harm (Dieter Ebert, 2022). Natural water bodies can be harmed by various stressors, which can negatively impact the aquatic organism living in them. *Daphnia magna* is a commonly used model organism for testing the toxicity of theses stressors on aquatic life (Rodrigues, Pinto, Nogueira, & C. Antunes, 2023). *Daphnia* bioassays are commonly used in ecotoxicology studies due to their high fertility, ease of maintenance in laboratory, and widespread distribution, as well as their importance as bioindicators for aquatic environments, since they are sensitive to contaminants and occupy a significant position in the aquatic food webs (Rodrigues, Pinto, Martins, Formigo, & Antunes, 2021; Roig et al., 2015). According to a study by Rodrigues et al. (2023) ,the Ecological Quality Ratio (EQR) method, which compares the observed and expected abundance of macroinvertebrates in water bodies, can be used to assess the ecological quality of water and detect the effects of contaminants on aquatic organisms, including *Daphnia*.

Historically, animals have been used as sentinels to detect threats to humans (Backer, Grindem, Corbett, Cullins, & Hunter, 2001; Hazen et al., 2019; Hilborn & Beasley, 2015; Reif, 2011; Van der Schalie et al., 1999). Sentinel species are more sensitive to environmental stress than humans and other environmental species and live in environments similar to humans (Peter Rabinowitz, Matthew Scotch, & Lisa Conti, 2009). They can be used as an advanced warning system for the detection of potential chemical hazards for humans (Van der Schalie et al., 1999). Invertebrate sentinel species (e.g., *Drosophila* and *Caenorhabditis*) are often preferred to mammal substitute species because they are easy to experiment with and have 3Rs (replace, reduce, refine) compliance. In addition, they possess disease genes that are found in humans and other distant species (Mary Lauren Benton et al., 2021; Domazet-Lošo & Tautz, 2008; Evan K Maxwell et al., 2014). *Daphnia* is a unique species that has qualities that are distinct from other biomedical models. It also has similarities with surrogate species. One of its unique

traits is its parthenogenetic life cycle, which makes it easy to breed genetically identical individuals from the same genotype. This makes it an excellent candidate for a systems biology approach that can measure ecological end points and molecular biomarkers concurrently (Antonio Suppa et al., 2020),. Furthermore, *Daphnia* is an important ecological indicator and keystone species in freshwater food webs, making it a crucial organism (Nasser & Lynch, 2016). It is also used increasingly in new approach methodologies (NAMs) for chemical risk assessments and regulatory frameworks, which establish limits on hazardous substances (Joseph R Shaw et al., 2008). Lastly, *Daphnia's* ability to biotransform or bioaccumulate chemicals makes it an excellent candidate for water bioremediation applications.

Daphnia is a genus of small planktonic crustaceans historically used as a model species in ecology and evolution (Dieter Ebert, 2022). With a wide geographic distribution, *Daphnia* can be found in wide a range of freshwater environments (e.g., lakes, and ponds) (Joseph R Shaw et al., 2008). In these ecosystems, *Daphnia* occupies a central place in the food-web and plays a crucial role as keystone species (Dieter Ebert, 2022). *Daphnia* often undergoes alternate sexual and asexual reproduction. In harsh environmental conditions, *Daphnia* reproduce 'asexually', producing genetically identical offspring. Resting eggs are produced through sexual reproduction when environmental conditions are unfavorable, resulting in the production of clonal males (Cambronero & Orsini, 2018). The resting stages (dormant period of *Daphnia* embryo after egg fertilisation) (i.e., eggs encased in ephippia) are capable of surviving for extended periods in lake sediment, as they can tolerate harsh environmental conditions (e.g., freezing and drying) (Dieter Ebert, 2022). Each hatching of sexually produced resting eggs in *Daphnia* is genetically unique and will initiate the production of clones (Dieter Ebert, 2022). Extended dormancy is one of the key properties of *Daphnia*, providing access to libraries of past populations exposed to different environments (e.g. chemical pollution and climate change;(Cuenca Cambronero, Beasley, Kissane, & Orsini, 2018; Cuenca Cambronero,

Marshall, et al., 2018b; Kenji Toyota et al., 2019)). Organisms have two main ways of responding to changing environments: genetic adaptation and plasticity of fitness-related traits (Kenji Toyota et al., 2019). Genetic adaptation involves the changes in the organism's genetic makeup that enable it to better survive and reproduce in a new environment. On the other hand, 'plasticity can be defined as the phenomenon in which organisms adjust their physiological traits in response to environmental changes' (Donelson, Salinas, Munday, & Shama, 2018; Merilä, 2012; Kenji Toyota et al., 2019). Within-generation plasticity (WGP) is believed to be the primary way organisms respond to changes in their environment. This changes allows for rapid adjustments to new environmental conditions (Kenji Toyota et al., 2019). It has been suggested that plasticity is particularly important in rapidly changing environments, where genetic adaptation may not be able to keep up (Kristensen, Ketola, & Kronholm, 2020; Matesanz, Gianoli, & Valladares, 2010; Torda et al., 2017). However, Toyota et al noted that there is still much to learn about the mechanism of genetic adaptation to environmental change, and how plasticity affects long-term evolutionary responses (Kenji Toyota et al., 2019).

Transgenerational plasticity (TGP) is the ability of an individual's environment to influence the traits expressed in their offspring through non-genetic or epigenetic process. TGP can be adaptive meaning that it can enhance offspring performance and help populations to cope with environmental change (Chevin, Lande, & Mace, 2010). However, the prevalence and strength of TGP in natural systems is still a topic of debate among researchers (Kenji Toyota et al., 2019). Understanding adaptive TGP is important in predicting the consequences of parental effects on population dynamics and how species will respond to rapid environmental change (Chevin et al., 2010; Salinas, Brown, Mangel, & Munch, 2013; Torda et al., 2017; Kenji Toyota et al., 2019). By studying TGP, researchers may gain an understanding of how populations may respond to changing environmental conditions over time (Bonduriansky, Crean, & Day, 2012).

It is crucial to comprehend that the environment plays a vital role in the evolutionary process, which is defined as the factors that bring about alterations in the genetic composition of populations over time (Scheiner, 1993). It is a well-established fact that there is a correlation between an individual's phenotype and their fitness level. This correlation has a significant impact on the development of an individual's phenotype over time (Scheiner, 1993). Therefore, some organisms are better equipped to adapt to environmental stress than others. The phenomenon of phenotypic plasticity pertains to the way in which an organism's traits are expressed and how they can be impacted by environmental factors, either through a response or lack thereof (Chambel, Climent, Alía, & Valladares, 2005; Kelly, Panhuis, & Stoehr, 2011; Scheiner, 1993). If high fitness is not maintained by plasticity, there could be variations in the impact of reduced fitness on different genotypes (Walter et al., 2021). Walter et al have note that this variation may enhance the adaptive potential of the population. Consequently, the population could sustain itself through rapid adaptation (Bell & Gonzalez, 2009; Walter et al., 2020). Environmental pollution can lead to evolutionary changes in a population through various mechanisms such as natural selection (Ogata et al., 2001), genetic adaptation (Whitehead, Clark, Reid, Hahn, & Nacci, 2017; Whitehead, Pilcher, Champlin, & Nacci, 2012), and epigenetics (Harney et al., 2022). Some organisms have evolved in response to specific pollutants (e.g., killifish (Whitehead et al., 2017; Whitehead et al., 2012), invertebrates (Coors, Vanoverbeke, De Bie, & De Meester, 2009; Lopes, Baird, & Ribeiro, 2005; Pedrosa et al., 2017)). It is important to note that not all species are able to adapt to pollution and some may suffer negative effects because of exposure.

Applying resurrection ecology, both plasticity and rapid genetic adaptation have been shown in *Daphnia* (Henning-Lucass, Cordellier, Streit, & Schwenk, 2016; Luisa Orsini, Spanier, & De Meester, 2012; Stoks, Govaert, Pauwels, Jansen, & De Meester, 2016; C. Zhang et al., 2021). For example, Mary A. Rogalski (2015) discovered that metal contamination in lake

sediments can have a significant impact on the success of diapausing invertebrates like *Daphnia* after they hatch, and those that did hatch had a higher chance of dying as juveniles. In this study, it was suggested that the *Daphnia* may have evolved to withstand exposure to metals, but this adaptation could have adverse effects when they are in uncontaminated environments. Navis, Waterkeyn, Voet, De Meester, and Brendonck (2013) investigated the impact of two pesticides, carbaryl and fenoxycarb, on the hatching of dormant eggs of *D. magna*, and on the survival, growth, and reproduction of the hatched neonates (newly hatched individuals from *D. magna*). The study revealed that carbaryl did not have any negative effects on the embryonic development or hatching rate of the eggs, even at higher concentrations, unlike fenoxycarb that showed a higher dose effect on hatching. However, fenoxycarb had a significant dose-related effect on hatching and development. Cuenca-Cambronero and colleagues studied the impact of temperature, food levels, and carbamate insecticides on *Daphnia* examined both individually and in combination. To do this, the researchers analysed both present and past populations of *Daphnia* that were obtained from a sediment core with a history of human-induced stress and environmental perturbations. The researchers noticed a significant variation in the response of *Daphnia* populations when exposed to single and multiple stressors. Although the response of all populations was similar when exposed to warming alone, the population that had been historically exposed to pesticides showed a much lower level of fitness compared to other populations when warming was combined with insecticides or food limitations. This suggests that the historical exposure to pesticides may have made the *Daphnia* more vulnerable to multiple stressors (Cuenca Cambronero, Marshall, et al., 2018b). By using two scientific approaches, resurrection ecology (defined as a scientific approach used in hatching diapausing eggs that have been preserved in lake sediments for decades or even centuries) and paleolimnology (analysing sediment cores from the bottom of lakes to reconstruct past environmental conditions) Mary Alta Rogalski (2017), studied how

Daphnia- hatched form a diapausing bank- has evolved in response to heavy metal pollution over the past 50-70 years. According to the study, as the historic contamination levels increased, it was observed that *Daphnia* became more sensitive to exposure to both copper and cadmium, suggesting that the animals have evolved to become more susceptible to heavy metal pollution over time. It has been well-documented that certain organisms can adapt their behavior to mitigate the harmful effects of pollutants. Fish are a clear demonstration of this phenomenon, as they have been observed adjusting their swimming and feeding behaviors to minimize their exposure to harmful pollutants (Little & Finger, 1990; Robinson, 2009; Scott & Sloman, 2004; Weis, Smith, Zhou, Santiago-Bass, & Weis, 2001). Some organisms possess the capacity to modify their physiology to enhance their ability to withstand or process pollutants (e.g., fish (Ficke, Myrick, & Hansen, 2007), vertebrates (W. G. Du & Shine, 2015), invertebrate (I. Sokolova, 2021; I. M. Sokolova, Frederich, Bagwe, Lannig, & Sukhotin, 2012). Moreover, exposure to pollutants can cause modifications in the genetic makeup of organisms, empowering them to either withstand or disintegrate the pollutants (Muhammad Abdullahi, Jiarui Zhou, Vignesh Dandhapani, Anurag Chaturvedi, & Luisa Orsini, 2022a; Fragou, Fragou, Kouidou, Njau, & Kovatsi, 2011; Nacci, Champlin, & Jayaraman, 2010; Oleksiak, 2008; Oleksiak et al., 2011; Sharavanan et al., 2020).

Biomolecular mechanisms of response to chemical

Chemicals occurring at sublethal doses in the natural environment can induce early biomolecular signatures identified via host and microbiome responses to biological perturbations (Rosenfeld, 2017; Antonio Suppa et al., 2020). These signatures can be subsequently linked to biomarkers of toxicity that are predictive of ecological endpoints (Abdullahi, Li, et al., 2022a). Acute and chronic toxicity tests focusing on ecological endpoints are inadequate to capture these early signatures of biological perturbations (Krewski et al.,

2020). For example, routine toxicity studies often measure the adverse effects of a substance on an organism such as macroscopic abnormalities, changes in body or organ weights (Heijne, Kienhuis, Van Ommen, Stierum, & Groten, 2005). However, these measurements are not indicative of how a substance is causing those effects since they only reflect late stages of toxic action rather than components that lead to toxicity (Heijne et al., 2005).

Molecular initiating events (MIEs) (defined as the initial molecular events that lead to adverse outcomes) are the first steps in a chain of events that lead to harmful effects, and together with dose dependencies (referred to as the as the relationship between the dose of a compound and toxicological response it produces) if identified, can be used to better predict the potential harm of a toxic substance (Krewski et al., 2020). Molecular endpoints (defined as specific molecular changes that occur in response to a substance) (e.g. alterations in gene expression, protein functions, and cell signalling) (Krewski et al., 2020) allow for the detection of toxic substances in organisms and tissues at lower concentrations than traditional methods and how exposure to these toxicants affects the organism's health (Heijne et al., 2005). By detecting these endpoints earlier and at lower concentrations, it is possible to reduce the risk caused by exposure to those toxicants.

Omics techniques- a type of high-throughput technology that uses genomics, transcriptomics, and metabolomics- creates a suitable avenue in exploring the molecular mechanisms underpinning adverse responses to toxic pollutants and identify individuals at risk of developing toxicant-induced diseases (Sing et al., 2010). Transcriptomic is a tool that can help identify changes in gene expression caused by chemical exposure (Oziolor, Bickham, & Matson, 2017). These changes can lead to the production of toxic metabolites or the inhibition of important metabolic pathways (Oziolor et al., 2017). For example, a study that investigated populations of *F.heteroclitus* fish that have been adapted to living in contaminated environment showed that the gene expression patterns of control individuals (those not exposed to toxins)

in these populations were similar to those reference individuals (those living in non-contaminated environments) (Fisher & Oleksiak, 2007). This suggest adaptation or plastic response of fish to the toxicants in the environment (Oziolor et al., 2017).

The 1987 report by the National Research Council introduced the concept of biomarkers to connect external exposures to health outcomes (Council, 2012; Henderson et al., 1987). This model challenged traditional approaches of observational epidemiology and toxicology by promising to reduce uncertainty and improve predictive precision in identifying associations between exposures and disease or dysfunction (Dietert & Silbergeld, 2015). It has been proposed that the metagenome of the microbiome (defined as the genetic material of all the microorganisms that live in and on the humans body and other living organisms) should be included in the definition of gene:environment interactions (defined as the relationship between an individual's genetic makeup and their environment) (Dietert & Silbergeld, 2015). This is because it they are capable of interacting and biotransforming xenobiotics (Adamovsky et al., 2018; Dietert & Silbergeld, 2015) . This means that by considering the role of the microbiome in the progression from exposure to outcome, we can better understand how external factors such as toxins and pollutants interact with the body and its systems (Dietert & Silbergeld, 2015).

Biomarkers that only consider an organism response are incomplete because they do not take into account the microbiome, and may not accurately reflect the interactions between environmental exposures and health outcomes (Dietert & Silbergeld, 2015). However, it is not always clear whether a disruption of the gut microbiome is causing a disease, contributing to it or if it's just an effect of environmental conditions associated with that disease(Adamovsky et al., 2018). Chemical interaction in the gut microbiome can occur in two ways: directly through binding to receptors on its surface or indirectly following biotransformation by microbial enzymes present within it (Adamovsky et al., 2018). Both processes are key mechanisms prior

to what is known as molecular initiating events (MIEs) at the host chemical interface, leading toward adverse outcomes such as dysbiosis and other health-related issues hitherto (Lapanje, Rupnik, & Drobne, 2007). Demonstrating a causal relationship between chemical exposure and any changes in the microbiome being associated with an adverse outcome, rather than attributing them solely to alterations of host physiology, remains a challenge in toxicity testing (Adamovsky et al., 2018). Andrews et al. (2021) conducted a study investigating biomarkers of response and toxicity to combined immune checkpoint blockade (CICB) in patients with advance melanoma. The study found that patients who experienced immune-related adverse events (irAEs) had a higher abundance of a specific type of gut bacteria called *Bacteroides intestinalis*. Additionally, there was an upregulation of a protein called mucosa IL-1B in patient samples of colitis (inflammation of the colon) and in preclinical models. These findings suggest that targeting gut microbiota could potentially be a new therapeutic approach for reducing toxicity associated with CICB. Wu et al. (2020) compared two groups of honeybee workers: one group with a normal gut microbiota (CV) and another group with a deficient gut microbiota (GD). The researchers found that the expression of cytochrome P40 enzymes (P450s) in the midgut was higher in the CV group than in the GD group. P450s are enzymes that play a crucial role in the detoxification of xenobiotics, including pesticide. Suppa et al demonstrated the alteration of the gut microbiome in *Daphnia*, with consequences such as altered metabolism.

1.4. Expanding the Properties of Sentinel Species

Chemicals continuously release into the environment due to the inefficiency of their removal from wastewater treatment process (processes used to treat wastewater before it is released into the environment) (Kassim Olasunkanmi Badmus, Jimoh Oladejo Tijani, Emile Massima, & Leslie Petrik, 2018). Current existing treatment technologies are not effective in removing all chemicals especially those that are persistent and do not break down easily (Paromita

Chakraborty et al., 2014). The presence of these chemical in the environment can have negative effects on human, and ecosystem health because of their bioaccumulative properties (Richard Fuller et al., 2022; Polyxeni Nicolopoulou-Stamati, Sotirios Maipas, Chrysanthi Kotampasi, Panagiotis Stamatis, & Luc Hens, 2016).

Conventional and advanced effluent treatment technologies, such as membrane filtration, Fenton oxidation processes, ozonation and advanced oxidation process have been gaining more attention in recent years (Ahmed et al., 2021); however, they come with their own set of drawbacks. These treatments are energy-intensive, which means that a lot of energy is required to operate them; additionally, these technologies require many resources and are costly for operation and maintenance (Ahmed et al., 2021). Also, various strategies are used for the elimination of chemical pollutants which include physical processes such a s filtration, sedimentation, and flocculation (Ammar & Akbar, 2018; Esfahani, Mobarekeh, & Hoodaji, 2018); chemical processes like oxidation, adsorption, and ion exchange (Shen, Ding, & Zhang, 2019); biological methods of biodegradation and bioremediation (Girijan & Kumar, 2019; Shah & Shah, 2020) along with hybrid techniques all combining all three approaches for more effective removal of these compounds (Obotey Ezugbe & Rathilal, 2020; Rasouli, Abbasi, & Hashemifard, 2017). These processes are expensive to operate, require large infrastructure, and can generate toxic by-products such as bromate from ozonation for effluent treatment (Olga S Arvaniti & Athanasios S Stasinakis, 2015; Donna L Sutherland & Peter J Ralph, 2019). The use of biobased solutions such as phycoremediation, and constructed wetlands is being explored as a preferred alternative to current chemical and mechanical processes used to remove persistent chemicals from effluent water (Mengke Pei et al., 2019). However, these biobased solutions have their own limitations such as low removal efficiency for industrial-scale operations and significant space and infrastructure requirements (Mengke Pei et al., 2019). It's worth noting that Daphnia has an impressively long dormancy period that can span

hundreds of years. This remarkable trait provides a unique opportunity for creating biological archives that help researchers study dormant populations that have been exposed to different levels of pollution throughout history. By reviving these populations, scientists can analyse various strains that possess different levels of tolerance to chemical pollutants (Abdullahi, Li, et al., 2022; M. Abdullahi, J. Zhou, V. Dandhapani, A. Chaturvedi, & L. Orsini, 2022b). Recent studies have shown that *D. magna* can improve water quality of effluent water by decreasing the concentration of small particle and removing emerging contaminants (Abdullahi, Li, et al., 2022a; T Serra, Barcelona, Pous, Salvadó, & Colomer, 2022; T Serra & Colomer, 2016). It should be noted that previous attempts (e.g., (Pous et al., 2021; Pous et al., 2020)) to use *Daphnia* as a biological agent have not yielded consistent results. However, a new cutting-edge technique of selecting *Daphnia* strains based on their pollution tolerance has emerged, which has the potential to customize the strains for different types of wastewaters. This approach could offer greater flexibility in developing treatment solutions that are tailored to specific pollution levels, ultimately enhancing their effectiveness.

1.5. Thesis Outline

The aim of this thesis is to broaden the application of *Daphnia* as a diagnostic and as a bioremediation agent for water pollution, to help reduce hazard from chemical mixtures in the environment. I addressed this aim by quantifying the uptake of chemical compounds on the 'priority list' of the water framework directive (WFD) and their mixtures (wastewater) on four genotypes of *Daphnia magna* with different histories of exposure to chemical pollution. I investigate the within and cross-generational impact of these chemicals on the genotypes, identifying potential mechanism of toxicity. I use the same genotypes to quantify their capacity to remove and uptake these chemicals and their mixtures both in a laboratory setting and in a mesocosm designed to mimic semi-natural environmental conditions. The two types of

experiments are conducted to assess both the role of sentinel and remediating agent. High-end chemical concentrations from literatures were used in experiments to test *Daphnia* removal efficacy. However, these chemicals are specific to certain wastewaters or industries. The chosen concentration of each chemical was based on relevant literature for surface and wastewater.

In the **first chapter** of this thesis, I developed with colleagues a novel framework that uses *Daphnia magna* as a diagnostic early warning system for sublethal effects of chemical pollution in water. The framework proposes the application of 'omics' technologies to improve the detection of bioactive elements of chemical mixtures, determining the potential effects of untested chemicals within mixtures and identifying targets of toxicity. Also, in this study, I provide a proof of concept that *Daphnia* strains that naturally adapted to chemical pollution can serve as removal agents of ambient chemical mixtures to sustainably improve environmental health protection. In the **second chapter**, the long-term evolutionary impact of chemical pollution was studied, mediated through exposure of *Daphnia* genotypes resurrected from the sedimentary archive of a lake with a known history of chemical pollution to five chemicals to understand how historical exposure to chemicals influences adaptive responses to novel chemical stress. In this study, I measured the within- and transgenerational plasticity in fitness-linked life history traits following exposure of 'experienced' and 'naïve' genotypes to novel chemical stress. With the support and input from colleagues, I quantified the long-term evolutionary impact of chemical pollution by studying genome-wide diversity and identifying functional pathways affected by historical chemical stress. This study suggests that the revived *Daphnia* genotypes from the sedimentary archive of a lake with a known history of chemical pollution are representative of the original population and exposure of the genotypes to five chemicals is a relevant proxy for the exposure to chemical pollution in the lake. The observed reduced genome-wide diversity and reduced gene diversity at detoxification, catabolism, and endocrine genes in experienced genotypes are likely the result of historical exposure to

chemical stress. Having assessed the impact of the chemicals on the *Daphnia* physiology and survival, I started investigating the biomolecular response of the same genotype to the four chemicals studied in **Chapter 3**. It was my intention to study both the *Daphnia* molecular response and the one of its microbiome to the same chemicals. Because of time constrain, I include in this thesis the preliminary analysis on microbiome response to chemicals. In **Chapter 4,** I develop with colleagues the first prototype demonstrating the use of *Daphnia* as a biological agent for tertiary wastewater treatment. In this chapter, we show the application of a Daphnia-based technology in near real-world conditions that will lead to the future scale up of a scalable, low-cost, low-carbon, and retrofittable bio-based tertiary water treatment technology for the removal of persistent chemical pollutants and other inorganics from wastewater. My direct contribution to this chapter was the demonstration that *Daphnia* genotypes can non-selectively uptake and retain persistent chemical pollutants from water both in laboratory and near-real world conditions.

The thesis is divided into six chapters, including general introduction and a general discussion. The chapters are presented as either a manuscript, published paper or paper under review.

Introduction

In this chapter, I provide a general background on the topics studied in the thesis, including challenges in modern ecotoxicology, *Daphnia* as the modern canary in the coal mine to be both an early warning system and a remedial agent for chemical pollution. Finally, I present the framework of the thesis and describe how it contributes to achieving the thesis's overall objectives.

Author's contribution: MA conceived and wrote the chapter.

Chapter 1: **Published in *Environmental Science and Technology*: Muhammad Abdullahi**; Li, X.; Abdallah, M. A. E.; Stubbings, W.; Yan, N.; Barnard, M.; Guo, L. H.; Colbourne, J. K.; Orsini, L. **Daphnia as a Sentinel Species for Environmental Health Protection: A Perspective on Biomonitoring and Bioremediation of Chemical Pollution**. *Environ. Sci. Technol.* **2022**, *56* (20), 14237–14248.https://doi.org/10.1021/acs.est.2c01799

A framework is provided for the novel use of the sentinel species *Daphnia* as a diagnostic early warning system and a bioremediation agent for environmental health protection. My contribution in this work is predominantly in the proof-of-concept study showing that *Daphnia* uptakes chemicals from water more efficiently than other biological agents. I performed the experiments, the chemical analysis and the statistical analysis leading the work on bioremediation.

Author's contribution: M.A and X.L share first authorship. J.K.C and L.O. share senior authorship. M.A., W.S., and M.A.-E.A. generated the data for removal efficiency of chemicals by Daphnia. X.L. and L.-H.G. collected and analysed data for the Chaobai river case study. L.O. and J.K.C conceived the framework with input from N.Y. L.O. coordinated data analysis and writing.

Chapter 2: **Published in *Molecular Ecology*: Muhammad Abdullahi**; Zhou, J.; Dandhapani, V.; Chaturvedi, A.; Orsini, L. **Historical Exposure to Chemicals Reduces Tolerance to Novel Chemical Stress in Daphnia (Waterflea)**. *Mol. Ecol.* **2022**, *31* (11), 3098–3111. https://doi.org/10.1111/MEC.16451

In this chapter, we quantified the within- and transgenerational plasticity in fitness-linked life history traits following exposure of "experienced" and "naïve" genotypes of *Daphnia* to five chemicals using 'common garden' experiment. Mechanism underpinning tolerance to chemical stress was quantified by studying genome-wide diversity and identifying functional pathways

affected by historical chemical stress. I led the work on transgenerational analysis of life-history traits in *Daphnia*. The genome analysis was completed by co-authors.

Author's contribution: M.A. performed the experiments. M.A. and J.Z. performed statistical analyses on fitness-linked life history traits. V.D. and A.C. performed genomics and functional analysis. L.O. conceived the study, coordinated data analysis and writing. All authors contributed to the manuscript writing.

Chapter 3 Unpublished: Muhammad Abdullahi, Jiarui Zhou, Sam Benkwitz-Bedford, Stephen Kissane, Luisa Orsini: **Exposure to persistent chemicals alters gut-microbiota diversity and composition in *Daphnia magna*.**

In this chapter, high-throughputs multiomics approaches was applied to *Daphnia* genotypes to identify the biomolecular mechanism underpinning the difference in physiological response (**chapter 2**) to the chemical stressors via integration of host gene expression and gut-microbiota functional profile. Due to time constraints, I completed the preliminary analysis on the *Daphnia* microbiome, which I present in this chapter. The analysis of *Daphnia* transcriptome response will be completed after my thesis dissertation.

Author's contribution: MA carried out the experiment. MA, and SBB performed statistical analysis. J.Z. developed pipeline for genome-wide transcriptional analysis. LO conceived the study and coordinated data analysis. MA wrote the chapter.

Chapter 4: **Published in *science of the total environment*: Harnessing water fleas for water reclamation: a nature-based tertiary wastewater treatment technology**

This chapter is under review in *science of the total environment*: Muhammad Abdullahi, Iestyn Stead, Sophie Bennett, Rafael Orozco, Mohamed Abou-Elwafa Abdallah, Sara Jabbari, Lynne E. Macaskie, Alexandra Tzella, Stefan Krause, Bushra Al-Duri, Robert G. Lee, Ben Herbert, Peter Thompson, Megan Schalkwyk, Samuel Getahun, Karl D Dearn and Luisa Orsini.

In this chapter, we pioneer a scalable, low-cost, low-carbon, and retrofittable bio-based tertiary water treatment technology for the removal of persistent chemical pollutants and other inorganics. The technology uses the water flea *Daphnia* to non-selectively uptake and retain persistent chemical pollutants, cleaning reclaimed waters to a degree that enables water reuse. I contributed to this work by showing removal efficiency of chemical pollutants by *Daphnia* in laboratory and near real-world conditions.

Author's contribution: MA completed chemical exposures and ran the mass spectrometry analysis under the supervision of MA-EA and LO; IS built and optimised the technology prototype and the side stream reactors; SB developed the delay differential equations under the supervision of SJ and AT. RO completed the preliminary separation of the chitin shell and the organic body in *Daphnia* under the supervision of LEM. RGL contributed intellectual property on the technology on behalf of Daphne Water Solutions Ltd. BH completed techno-commercial and market analyses on the Daphnia-technology. PT, MS, and SG contributed to the final technology design providing information on retrofitting requirements. KDD and LO conceived and coordinated the study. LO coordinated data analysis and wrote the first manuscript draft with input from MA and IS. All authors contributed to the manuscript writing and approved.

Chapter 5: **Discussion and conclusion are unpublished:** In this chapter, I provide a general discussion of the main findings of the thesis and identify the strength and weaknesses of the approaches used and identify future research direction.

Author's contribution: MA conceived and wrote the chapter.

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Chapter 1:

Daphnia as sentinel species for environmental health protection: a perspective on biomonitoring and bioremediation of chemical pollution

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2.1. Abstract

Despite available technology and the knowledge that chemical pollution damages human and ecosystem health, chemical pollution remains rampant, ineffectively monitored, rarely prevented and only occasionally mitigated. We present a framework that helps address current major challenges in the monitoring and assessment of chemical pollution, by broadening the use of the sentinel species *Daphnia* as a diagnostic agent of water pollution. And where prevention has failed, we propose the application of *Daphnia* as a bioremediation agent to help reduce hazard from chemical mixtures in the environment. By applying 'omics' technologies to *Daphnia* exposed to real-world ambient chemical mixtures, we show improvements at detecting bioactive components of chemical mixtures, determining the potential effects of untested chemicals within mixtures, and identifying targets of toxicity. We also show that using *Daphnia* strains that naturally adapted to chemical pollution as removal agents of ambient chemical mixtures can sustainably improve environmental health protection. Expanding the use of *Daphnia* beyond its current applications in regulatory toxicology has the potential to improve both the assessment and the remediation of environmental pollution.

Keywords: chemical mixtures, bioremediation, monitoring, water flea, water pollution, omics

Synopsis: Chemical pollution damages human and ecosystem health. We present a framework that broadens the use of the sentinel species *Daphnia* as a diagnostic and bioremediation agent of water pollution.

2.2. Introduction

The use of animals as sentinels to detect threats to human health dates to the era when coal miners brought caged canaries into mines to provide early warning of toxic gases. The concept of the "canary in the coal mine" is based on three principles: the sentinel species i) is more

sensitive than both humans and most other animals to toxic exposure; ii) shares the same environment as humans; and iii) produces a readily detectable effect of the toxic exposure (P. Rabinowitz, M. Scotch, & L. Conti, 2009). Following the sentinel species model, hazard is typically characterised in surrogate models and extrapolated to the target species; human hazard is traditionally characterised using surrogate mammalian species, whereas ecological hazard is characterised by exposing representative species of key taxonomic groups (e.g., primary producers, invertebrates and vertebrate embryos) to the reputed hazards (Atlas, 2013).

Despite the acknowledged value of sentinel species to detect hazard, their full potential as indicators of threats to humans and the environment has not been fully realised. With the One-Medicine-One-Health initiative which began in 2010, stemming from the One-Health concept (MacKenzie & Jeggo, 2019), the nexus between humans, other animals and the environment was endorsed by physicians and veterinarians but did not lead to substantial changes in the way environmental health hazard is assessed (Kanno, 2016; Kortenkamp & Faust, 2018).

Anthropogenic chemicals used in most production processes are transported globally, and usually end up in the environment as unintentional pollutants that may harm humans and damage the environment (Brack et al., 2022; R. Fuller et al., 2022b; Naidu et al., 2021). Until the last few decades, industrial chemicals were not routinely assessed for their risk and impact on wildlife and humans (Dulio et al., 2018) and measurements of toxicity were not always part of pre-market screening for chemical safety (Brooks et al., 2020). Even the most up to date national inventories do not include chemical mixtures nor by-products and degradation products of the parent compounds that are released into the environment (Nerin, Alfaro, Aznar, & Domeno, 2013). As a result, more than 235,000 individual chemicals and 120,000 unregulated mixtures have been found in the environment (Naidu et al., 2021; T. Wang et al., 2014). Chemicals entering the environment can bioaccumulate in animal tissue and be biomagnified through the trophic chain, eventually entering our food supply, and causing

adverse health outcomes, even at low doses (e.g., (R. Fuller et al., 2022b; Pu et al., 2020; T. Wang et al., 2014)). Chemical cocktails of unknown mixtures can interact with other environmental factors (e.g., climate change, microplastics and increased salinity) collectively contributing to environmental degradation (Backhaus & Faust, 2012), and causing the premature death of 9 million people every year (16 % of deaths worldwide) (R. Fuller et al., 2022b; Landrigan & al., 2018). Chemical pollution, together with overexploitation of resources, land-use, and climate change, is one of the main causes of loss of biodiversity and led to the deterioration of 60 % of ecosystem services worldwide in the last few decades (Backhaus & Faust, 2012; Bonebrake et al., 2019; Cardinale et al., 2012).

The current one-chemical-at-a-time, hazard-focused, and siloed approach to environmental and human health protection is insufficient to address these interconnected and interdependent challenges. On one hand there is a need for a better diagnosis of the impact of chemicals on wildlife and humans. On the other, when chemicals have entered the environment, remediation may be the only solution to reduce preventable health effects and deaths. Sentinel species can play the dual role of diagnostic and remedial agents of chemical pollution. In this *Perspective*, we present a framework that expands the use of the sentinel species *Daphnia* to act both as a diagnostic early warning system and as a bioremediation tool for environmental pollution. We identify the outstanding challenges in modern (eco)toxicology that can be mitigated with the application of the framework.

2.3. Broadening The Use of Sentinel Species Daphnia

Model organisms that are distantly related to humans, such as *Drosophila melanogaster* (an insect) and *Caenorhabditis elegans* (a nematode), have historically been used both as surrogates and exemplary models in biomedical research to study fundamental biological processes as well as to understand threats to human health (Aitman et al., 2011; Apfeld &

Alper, 2018; Cheng, Baonza, & Grifoni, 2018; Pandey & Nichols, 2011). They are often preferred to mammalian surrogate species for their amenability to experimentation and their 3Rs (Replace, Reduce, Refine) compliance. In addition, they share human disease genes that are ancestral in animal genomes and shared across phylogenetically distant species (M. L. Benton et al., 2021; Domazet-Loso & Tautz, 2008; E. K. Maxwell et al., 2014). The water flea *Daphnia* shares many advantages with these model species. *Daphnia* has a short generation time enabling experimental manipulation of large populations (Miner, De Meester, Pfrender, Lampert, & Hairston, 2012), has growing genomics resources (M. Abdullahi, J. Zhou, V. Dandhapani, A. Chaturvedi, & L. Orsini, 2022a; Chaturvedi et al., 2021; J.K. Colbourne et al., 2011; L. Orsini et al., 2018), and shares many ancestral gene families with humans (J.K. Colbourne et al., 2011). *Daphnia* has additional properties that surpass traditional biomedical model species. They i) have a parthenogenetic life cycle that allows the rearing of genetically identical individuals (clones) from the same genotype, enabling the concurrent quantification of ecological endpoints and molecular biomarkers using a systems biology approach (e.g. (A. Suppa et al., 2020)); ii) are keystone species in freshwater food webs and sentinel species for water quality, making them ecological indicators (Altshuler et al., 2011; J.R Shaw et al., 2007); iii) are used in regulatory frameworks to set limits on hazardous substances for the environment and are increasingly contributing to New Approach Methodologies for chemical risk assessments (Parish et al., 2020); and iv) can biotransform or bioaccumulate chemicals (Choi, Jeon, Choi, & Kim, 2020; Jeong, Kim, & Kim, 2016), enabling water bioremediation applications (L. Orsini & Dearn, 2021) (Fig. 1).

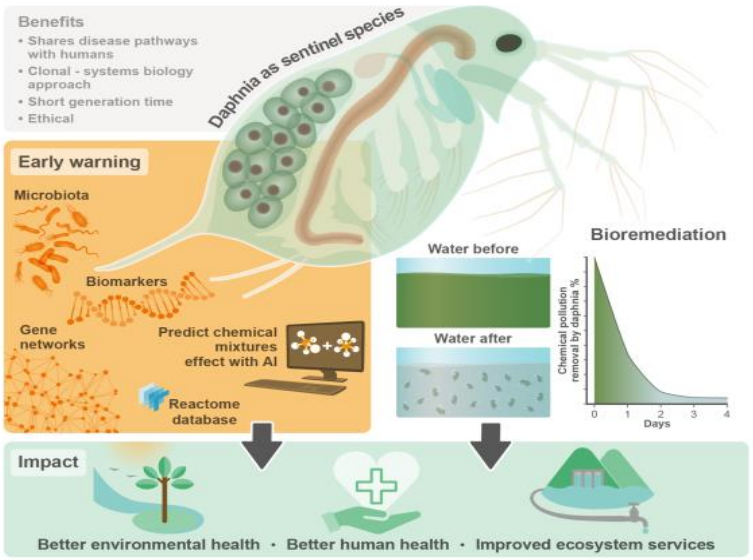

Figure 1. *Daphnia* as an early warning and remedial system. In the proposed framework, the sentinel species *Daphnia* is used both as an early warning system and as a bioremediation tool for chemical pollution. *Daphnia* clonality enables the synchronous analysis of ecological and molecular perturbations by environmental pollution (early warning). This enables the establishment of associations between sublethal doses of chemicals within mixtures and molecular biomarkers. Using the manually curated Reactome database, gene ontologies and conserved molecular functions can be identified in responsive modules across organisms, including humans. Once chemicals have entered the environment, remedial actions are needed. The sentinel species *Daphnia* has the potential to become a sustainable bioremediation agent as it removes excess nutrients from water, preventing eutrophication, and a wide range of persistent chemicals (bioremediation). By using *Daphnia* as a diagnostic and remedial agent, adverse effects for humans and the environment can be significantly reduced (impact).

2.4. Daphnia as an Early Warning System for Environmental and Human Health

We propose *Daphnia* as a diagnostic early warning system for sublethal effects of chemical pollution in water. This is achieved by measuring exposure-induced biomolecular changes and linking co-response networks of genes and metabolites (hereafter called modules) (Dugourd et al., 2021; Larras et al., 2020) to the ambient chemical mixtures. These measurements provide a cost-effective way to generate recognisable signatures of chemical exposure that potentially reflect targets of toxicity. Linking molecular-level information to the health of a subject, such as a patient or a surrogate species, is the foundation for precision medicine (Aronson & Rehm,

2015; Kim, Park, & Cho, 2013). 'Omics' data are unbiased, providing a global perspective of the molecular biological responses to environmental perturbations without *a priori* knowledge of the potential targets of toxicity (Martins, Dreij, & Costa, 2019). They also provide an early signature of dose-dependent environmental perturbations, allowing a more nuanced characterization of chemical mixture effects on biological systems (Nguyen & Wang, 2020; Sturla et al., 2014). Modules identified with 'omics' technologies can be interrogated for their conserved functionality across species based on knowledge of the evolutionary history of genes inherited from a shared common ancestor (i.e., gene orthologs) (Ankeny & Leonelli, 2011; Ros-Rocher, Perez-Posada, Leger, & Ruiz-Trillo, 2021), enabling the prediction of chemical hazard from one species to another (e.g., teratogenicity via aryl hydrocarbon receptor mediated (AhR) pathway activation (Xu et al., 2021)) and breaking the compartmentalisation between human toxicology and ecotoxicology. Furthermore, molecular biomarkers can be useful for the regulatory testing of chemicals, as they have been shown to be predictive of ecological endpoints, which are typically used for risk assessment (Taylor, Gavin, & Viant, 2018).

We propose a framework that uses non-targeted analysis to characterise real-world chemical mixtures and high throughput 'omics' technologies (e.g., transcriptomics and metabolomics) to identify co-response modules activated by these mixtures. The framework uses orthologs within conserved pathways to enable cross-species extrapolation for the early diagnosis of the potential hazards of chemical pollution, even when chemicals are present at sublethal concentrations. In this framework, *Daphnia* plays the same role that canaries played in coal mines, fulfilling its role as sentinel species. The approach can also be used as pre-market screening of new chemicals and chemical mixtures to improve chemical safety (Fig. 2). The framework uses a three-tiered approach, described in the following:

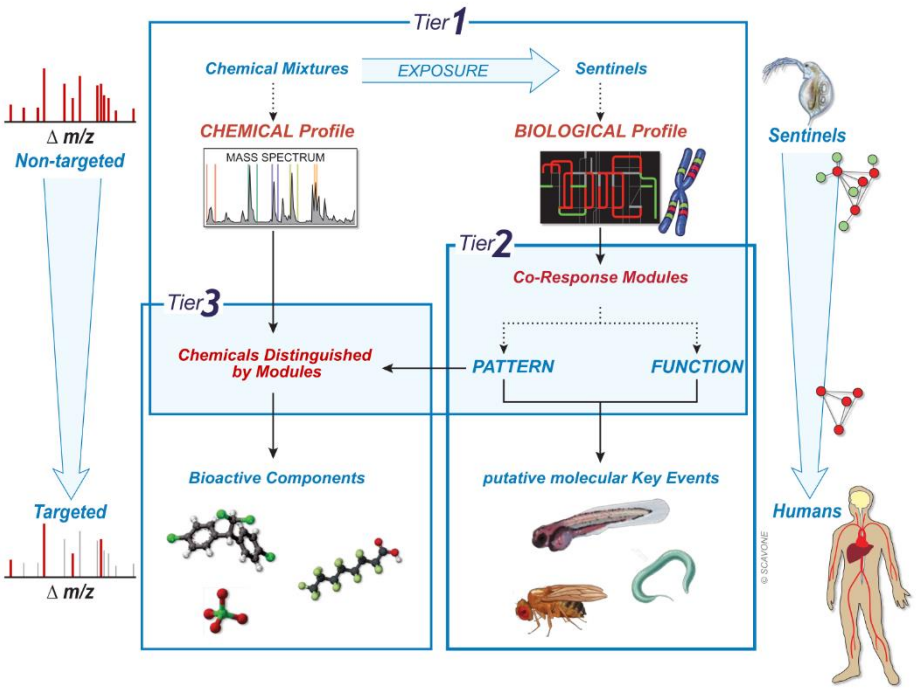

Figure 2. Three-tiered framework. The tiered approach identifies hazards of real-world chemical mixtures with putative molecular key events (putative mKEs) of the sentinel species *Daphnia.* Using functional conservation of gene and metabolites networks, the framework enables the identification of targets of toxicity across species, guiding in vivo and in vitro validations of toxic effects in human models. The approach consists of three tiers: tier 1 is the nontargeted fingerprinting of real-world environmental mixtures and of the biological effects induced by these mixtures; tier 2 identifies putative mKEs responsive to chemical mixtures; tier 3 establishes associations between bioactive chemical components within the environmental mixtures characterized in tier 1 and putative mKEs identified in tier 2.

Tier 1

The sentinel species *Daphnia* is exposed to ambient chemical mixtures (Fig. 2, Tier 1). Ideally, a non-targeted high-resolution mass spectrometry analysis is used to characterise chemical mixtures that occur in environmental media using mass-to-charge ratios of ions (Hollender, Schymanski, Singer, & Ferguson, 2017), producing a spectrum (chemical profile) of the overall detectable water chemistry. This non-targeted approach enables us to ask not if a specific substance of interest is present in a sample of water, but rather: What is in the water? The non-targeted mass-spectrometry analysis can be followed by a targeted analysis to quantify chemical compounds within mixtures (Brack et al., 2019; Brunner et al., 2020; Ccanccapa-Cartagena, Pico, Ortiz, & Reiner, 2019). A targeted analysis of multiple compounds (e.g., pharmaceuticals) can be used instead of a non-targeted analysis when the source of contamination is known (see case study below). The biological effects of the characterised chemical mixtures on the sentinel species *Daphnia* are measured following the OECD 202

guidelines (Fig. 2; Fig. S1) with the addition of an unbiased screening of the biomolecular responses using 'omics' technologies (e.g., transcriptomics and metabolomics as biomolecular profiles) (Fuertes, Jordao, Pina, & Barata, 2019; Taylor et al., 2018).

Tier 2

The biomolecular response induced by real-world chemical mixtures is measured through transcriptional and/or metabolic coordination among genes/metabolites (features) in co-response modules (Fig. 2, Tier 2) (Tohge & Fernie, 2012). The level of coordinated response of these features is determined with a correlation analysis through Pearson correlation, Spearman's rank correlation, or mutual information (Song, Langfelder, & Horvath, 2012). The co-response genes and metabolites form co-expression networks, in which highly correlated features form co-response modules that we here call **putative molecular Key Events** (putative mKEs) (Josyula et al., 2020; Kustatscher et al., 2019) (Fig. S1). These putative mKEs are different from the molecular Key Events (KEs) in an Adverse Outcome Pathway (AOP) framework (G. T. Ankley et al., 2010), which are directly linked to an adverse outcome phenotype. Instead, the putative mKEs are biological signatures of chemical exposure that identify putative targets of chemical hazard to be validated experimentally. The genes within the co-response modules are annotated using gene ontologies (e.g., GO (Harris et al., 2004)). Their functional conservation across species is established using gene orthologies (e.g., orthoDB (Kriventseva et al., 2019)). The main advantage of using co-response modules based on transcriptional/metabolic coordination is to link unknown to known, as the functions of unannotated genes and metabolites of the co-response modules are inferred based on their membership and coordination within recognisable canonical pathways using the KEGG Network database (L. Orsini et al., 2018; Tohge & Fernie, 2012). The co-response modules are mapped onto biomolecular pathways using KEGG (Kanehisa et al., 2008), PANTHER (Mi, Muruganujan, Ebert, Huang, & Thomas, 2019) or the Reactome (Jassal et al., 2020) databases,

to name a few, which all use evolutionary and functional classification of genes from organisms across the Tree of Life. This analysis places orthologs onto pathways and enables the identification of pathways that are evolutionarily conserved among distantly related species. Whereas conservation of pathways does not necessarily mean conservation of mechanisms of toxicity, such conservation provides testable hypotheses to assess conservation of targets of toxicity across species through experimental validation. With functional annotation and pathway information in place, statistical inference like gene set enrichment analysis (GSEA) (Subramanian et al., 2005) and pathway overrepresentation analysis (POA) (Khatri, Sirota, & Butte, 2012) can be performed on each module (Fig. S1). The GSEA and POA analysis identify biomolecular pathways that are enriched by genes and metabolites within each module in response to chemical exposure, more than would be expected by chance, providing mechanistic insights into the pathways that are potential targets of toxicity.

Tier 3

Significant correlations are identified between the chemical components within real-world mixtures characterised in Tier 1 and the co-response modules identified in Tier 2 (Fig. 2; Tier 3). These correlations can be established by matrix-on-matrix regression, also known as multi-block correlation analysis between the omics data (e.g., transcriptomics and metabolomics; (Picard, Scott-Boyer, Bodein, Perin, & Droit, 2021)) and chemical data (P. Mishra et al., 2021). The advantage of this approach is that multiple blocks can be analysed simultaneously in a single model so that the co-varying omics and chemical features among multiple blocks can be identified by machine learning approaches, such as sparse Partial Least Squares Discriminate Analysis (sPLS-DA; (Le Cao, Boitard, & Besse, 2011)), Multi-Omics Factor Analysis (MOFA+; (Argelaguet et al., 2020)), and Bi-order Canonical Correlation Analysis (Bi-CCA; (Dou et al., 2022)). Statistical approaches are then applied to extract significant correlations among the ones identified. The matrix-on-matrix regression is the

preferred method for non-targeted data. An alternative approach is the Weighted Gene Co-expression Network Analysis (WGCNA) that identifies chemical-associated modules using correlation analysis between the eigengene (i.e., the first principal component of a co-response module) and the chemical data (Langfelder & Horvath, 2008). The eigengene is used as a weighted average value of the gene expression or metabolite profiles in each module (Langfelder & Horvath, 2008). This approach was used in the case study shown below to validate the framework. When a co-response module that is conserved across species in Tier 2 has been associated with a specific chemical, two alternative approaches can be used to assess the hazard on biological systems: i) the chemical has been previously associated with an adverse phenotype and recorded in the Comparative Toxicology Database (Davis et al., 2019), a manually curated database of associations among biomolecular responses and chemicals as identified by experimental evidence; ii) the correlations identified are novel, therefore experimental validation is needed to move from correlations to causations. However, the correlation exercise has the main advantage of focusing experimental validations on the putative target of toxicity and on the species in which the targets are conserved. For example, vertebrate surrogate models or human cell lines derived from different tissues may be used to identify adverse effects that are associated with the putative mKEs initially identified in *Daphnia*.

The framework addresses three main outstanding challenges in modern toxicology:

Challenge 1: Adversity Endpoints

Regulations are typically applied to single chemicals and are normally set based on their observed adverse effects from toxicity testing on animals using concentrations that organisms rarely experience in the natural environment (Brescia, 2020; Rand, 1995). The focus on adversity endpoints and high chemical doses is logistically advantageous but disregards the

effects that may arise from exposures to sublethal doses (A. Blair et al., 2015). It also fails to identify early warning signatures, which use could presage and thus lead to anticipatory prevention of toxicity endpoints (Taylor et al., 2018). The AOP framework has been a positive step towards the evaluation of toxicity based on the identification of KEs that are predictive of adversity endpoints. These KEs may be observed as molecular, cellular, structural, or functional changes in biological systems induced by a molecular initiating event, allowing the identification of biomarkers of toxicity (G. T. Ankley et al., 2010). The important concept introduced by the AOP framework is the clear link between KEs and adverse outcomes at multiple biological levels of organisation (e.g., cell, organ, whole organism, ecosystem), including those that are relevant to risk assessment (G. T. Ankley et al., 2010). Yet, to date, AOPs are not routinely used for risk assessment because they are qualitative.

Our framework identifies putative mKE activated by exposure to real-world chemical mixtures, often occurring in the environment at sublethal doses, providing targets that are then used to assess exposure hazards to real-world chemical mixtures. As targets of exposure hazard may be indicative of foreseeable toxicity, early biomolecular signatures identified with the approach proposed here can subsequently be linked to biomarkers that are predictive of ecological endpoints, as previously demonstrated (Taylor et al., 2018). Putative mKEs of hazards that are proven to be predictive of adverse phenotypes therefore align with the concept of mKEs in the AOP framework (Pittman, Edwards, Ives, & Mortensen, 2018).

Challenge 2: Cummulative Effect of Chemical Mixtures

Organisms (including humans) are exposed to intentional and unintentional chemical mixtures; their individual components can be 100-fold below their regulatory approved thresholds and still contribute to the overall toxicity of the mixture (bioactivity) (Blackwell et al., 2019; Kortenkamp & Faust, 2018). Understanding the cumulative health risks caused by the

interaction among chemicals is critical to manage public health and environmental protection. Yet, chemical risk assessment is substance-driven, sector specific (e.g., pharmaceuticals, cosmetics, and biocides) and prospective - a prospective risk assessment determines, assesses, and minimises risks before they happen (Commission, 2020; Kortenkamp & Faust, 2018). Whereas intentional mixtures (formulated products) are addressed through a prospective risk assessment prior to the marketing of products, the assessment of accidental chemical mixtures is often limited to combinations of only a small number of compounds. Moreover, chemical safety legislation does not consider exposure to multiple chemicals across sectors (e.g., pharmaceuticals combined with pesticides) (Kienzler, Bopp, van der Linden, Berggren, & Worth, 2016).

Our framework enables an unbiased hazard assessment of chemical mixtures in water, which consist of chemicals pollutants from multiple sources (e.g., domestic, agricultural, industrial), and links that putative mKEs to bioactive components within these mixtures, guiding experimental validation of the targets of toxicity. This is a key advance over current practice to identify potential hazard of chemical pollution and to generate testable hypothesis to assess conservation of targets of toxicity across species. Once these substances are identified, they can then be prioritised for further testing in the context of an adverse outcome pathway and risk management.

Challenge 3: Human Toxicology and Ecotoxicology are Compartmentalised

Assessment of the risk of toxic substances to humans and the environment have been historically disconnected and compartmentalised. Vertebrate models have been used as surrogates for humans, whereas ecologically relevant algal, invertebrate and fish species have been used as surrogates for biodiversity (Kanno, 2016). The use of non-overlapping surrogate species for human and environmental toxicity has meant that cross-species extrapolation has

been applied within human toxicology and within ecotoxicology but not between these compartments (LaLone et al., 2021). Cross-species extrapolations made under the testable hypothesis that similarities among species are determined by their shared evolutionary history (i.e., "toxicity by descent") are being pursued by a European Commission research and innovation funded research programme (PrecisionTox; http://precisiontox.org), building on discoveries made in comparative genomics on the ancestry of disease-causing genes in humans (M. L. Benton et al., 2021). PrecisionTox employs a suite of biomedical model species for its investigations including *Daphnia* and is tasked with addressing the needs for a cohesive approach towards experimental design, of a mutually agreed framework to quantitatively identify functionally conserved putative mKEs among species and their link to chemical toxicity.

Our framework enables the identification of bioactive chemicals within environmental mixtures that have a measurable biomolecular effect on the sentinel species *Daphnia*, which may be indicative of hazards to other animals by identifying putative mKEs that are evolutionarily conserved. Our inclusion of an evolutionary analysis of the mKE is a key element for the modern use of *Daphnia* as a sentinel for the protection of other animal species – made possible by a high degree of pathway conservation between invertebrates and humans; human gene sets that already serve as biomarkers of chemical exposure (e.g., the US National Toxicology Program's s1500+ reference gene panel) are enriched by evolutionarily conserved genes across the animal phylogeny (J.K. Colbourne et al., 2022). The interpretation of these results for extrapolating hazards to other species reasonably assumes that the induction or malfunction of processes that are shared with humans is reflective of the chemical mixtures' mechanisms of action, but not necessarily predictive of shared adversity. The differences among species in their physiology, organ systems, adaptive responses to exposures and life history would contribute to differences in such outcomes. However, the application of this new

knowledge obtained from our framework can improve environmental health by having clearly defined protection goals. If, for example, the evolutionary analysis of the putative mKE indicated mutagenesis (or other potential mechanisms of action of greatest concern to chemical hazard assessors), this result would prioritize further investigations potentially leading to regulatory actions. Alternatively, the precautionary principle applies when setting exposure limits in Europe to substances that may cause harm to the environment. The evolutionary analysis of responsive pathways in sentinels that are associated with fundamental biological processes such as reproduction helps guide the application of this principle for protection goals that include biodiversity, while greater scientific knowledge of the actual hazards is obtained. In this way, the evolutionary conservation of response pathways enables the framework to bridge the divide between human toxicology and ecotoxicology.

We demonstrate the three-tiered approach in a case study, in which we expose one *Daphnia* strain (IRCHA clone 5; Water Research Centre, Medmenham, UK) to water samples collected from 30 sites of the Chaobai river in China (Fig. 3A).

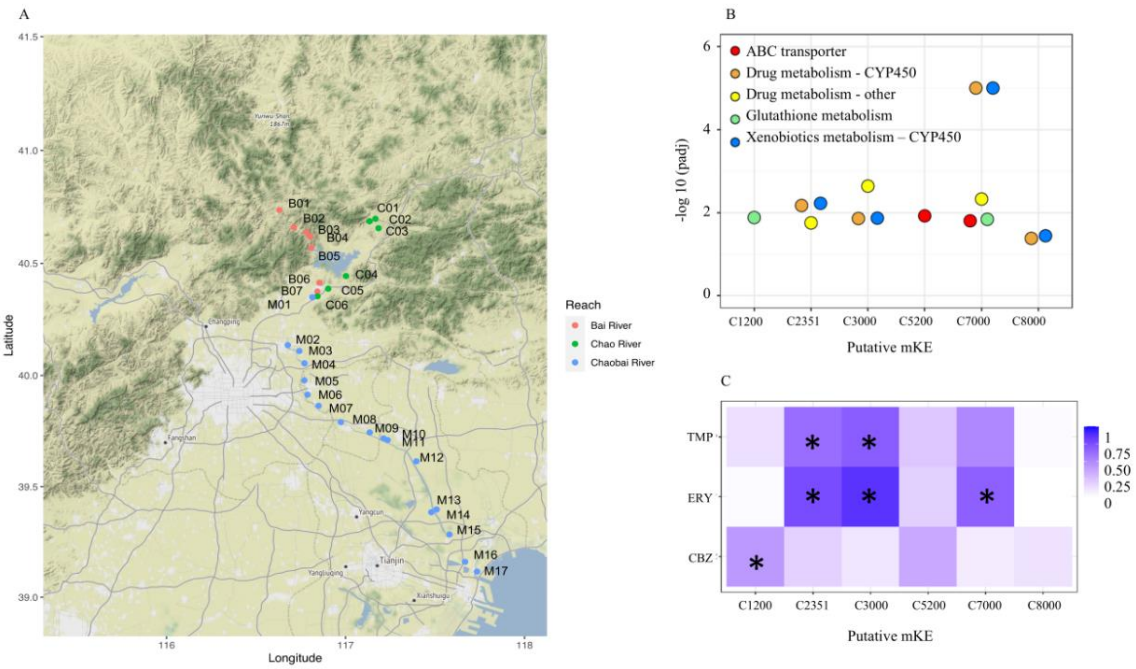

Figure 3. ***Daphnia* as a diagnostic early warning system.** (A) Map of 30 water sampling sites along the Chaobai river basin, including three main streams (B, Bai River; C, Chao River; M, Chaobai river). These 30 water samples are subjected to targeted chemical analysis to identify 22 organic compounds (mostly pharmaceuticals) listed in Table S1. (b) Six putative mKEs identified via coexpresion network analysis, are significantly enriched in xenobiotic and drug metabolic pathways identified in *D. magna* exposed to the Chaobai river water samples. The minus log-transformed adjusted P values are plotted in this figure; (C) Heatmap shows correlation between six putative mKEs and three pharmaceuticals: carbamazepine (CBZ), erythromycin (ERY), and trimethoprim (TMP). The colour coding of the correlation coefficient increases from white (0) to dark purple (1), with asterisks (*) marking the significant correlations.

The river receives industrial and domestic effluent as well as agricultural runoff (He, He, Wang, Li, & Wang, 2918). Both the Bai River and the Chao River originate from the Yunwu Mountains northeast of Beijing (sites B01-B06 and sites C01-C05; Fig. 3). The Chaobai River flows through the urban area north of Beijing and the agricultural area of Tianjin (site M01-M17; Fig. 3). Chemical profiles from the water samples were previously generated using a targeted analysis to identify polar organic pollutants, primarily pharmaceuticals (Su, Ben, Strobel, & Qiang, 2020) (Tab. S1). *Daphnia* was exposed to the Chaobai river waters following the OECD guideline 202 (OECD 202) - 24h-old *Daphnia* juveniles were exposed to the river samples without feed for 48 hours and immobilisation was recorded using OECD traditional assays. The exposed *Daphnia* were collected after 48h for RNA extraction and mRNA sequencing. Methods describing exposures, RNA data generations and data pre-processing are in the Supporting Information.

The analytical approach described in the framework above was followed for the case study. Immobilisation was not observed in any of the exposures. This may have been expected given the sublethal doses of chemical mixtures in the Chaobai river waters. Genes that passed the quality filtering were clustered into co-response modules (putative mKE) using WGCNA (Langfelder & Horvath, 2008). Conservation of these modules between *Drosophila melanogaster* and *Daphnia magna* was established using gene orthology within OrthoDB (Zdobnov et al., 2021). The orthologous genes were mapped onto biomolecular pathways using the KEGG pathway database (Kanehisa et al., 2008). From this analysis, a total of 27 co-response modules were identified. A pathway overrepresentation analysis revealed that 6 of the

27 modules were significantly enriched for xenobiotic and drug metabolism functions, namely xenobiotic/drug metabolism with cytochrome P450 (CYP450), Glutathione metabolism and ABC transporter (Fig. 3B). Using a Pearson's correlation analysis, 4 modules were identified to be significantly correlated with 3 pharmaceuticals (i.e., carbamazepine, erythromycin, and trimethoprim, P_{adj}-value < 0.05; corresponding to CBZ, ERY and TMP in Fig. 3C), which were previously detected in the sampled waters (He et al., 2918). The enrichment of xenobiotic and drug metabolic pathways that are significantly correlated with pharmaceuticals suggests that the three pharmaceuticals are bioactive and biotransformed by *Daphnia*. However, to establish mechanisms of toxicity for these three compounds within the mixture requires experimental validation, as discussed in the framework above.

Using the KEGG database, we determined that 5 pathways activated in *Daphnia* by carbamazepine, erythromycin, and trimethoprim were conserved across 7 model species (*Daphnia magna*, *Daphnia pulex*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*; Tab. S2). The orthologs compositions of these 5 pathways were highly conserved in the 7 species; *D. magna* shared more than 79 % KO terms with other species in four of the five pathways; the ABC transporter pathway was the only one showing lower conservation of orthologs across species, with 54 % similarity between *D. magna* and *C. elegans*, and 62 % similarity between *D. magna* and *D. melanogaster* (Tab. S2). The degree of functional conservation across species suggests that the targets of toxicity are shared across species. However, exposure experiments are needed to determine whether the risk of exposure are also shared.

This case study demonstrates that the three-tiered approach can link bioactive chemicals within mixtures with perturbations of functional pathways, even when adversity endpoints are not observed; and reveal whether these functional pathways are conserved across species, generating testable hypotheses to identify the targets of toxicity across species. By using

Daphnia as a ‘canary in a coal mine’, we can identify putative mKEs activated by real-world chemical mixtures before adverse outcomes occur. Using functional conservation of pathways across species, we can focus experimental validation on potential targets of toxicity in other species, greatly reducing unnecessary experimentation.

2.5. Daphnia as Biobased Solution for Water Bioremediation

Domestic and industrial processes as well as agricultural runoff are the main source of chemical pollution of surface and wastewater (P. Chakraborty et al., 2014; Han & Currell, 2017; Vasseghian, Hosseinzadeh, Khataee, & Dragoi, 2021). Once known and unknown chemicals have entered the environment, they are challenging to remove because they are not fully biotransformed or eliminated by current effluent treatments (K.O. Badmus, J.O. Tijani, E. Massima, & L. Petrik, 2018). Therefore, they end up in downstream waterways where they permeate sediment and soil and bioaccumulate through the trophic chain, eventually causing untoward health effects in humans (e.g., (R. Fuller et al., 2022b; P. Nicolopoulou-Stamati, S. Maipas, C. Kotampasi, P. Stamatis, & L. Hens, 2016; Pu et al., 2020)).

Over the last decades, both chemical and mechanical processes have been developed to remove persistent chemicals from effluent water originating from industrial processes, agricultural practices, and human and animal waste e.g., (J. Margot et al., 2013). However, these processes have high operational and energy costs, require large infrastructure, and can generate toxic by-products (e.g., bromate from ozonation for wastewater treatment) (O. S. Arvaniti & A. S. Stasinakis, 2015; D. L. Sutherland & P. J. Ralph, 2019). Biobased solutions, including phycoremediation, fungal bioremediation and constructed wetlands (plant bioremediation) are a preferred alternative to current chemical and mechanical processes to meet the net-zero carbon emission and sustainable goals of the international agenda, realised through the European Green Deal, the Zero Pollution Action Plan and the Chemical strategy for

sustainability (Commission, 2020) and are promising to remediate the environmental impact of pollutants (M. Pei et al., 2019). However, the removal efficiency of chemicals by emerging bio-based solutions is too slow for industrial-scale operations, requiring days, rather than the needed hours for industrial processes. In addition, biobased solutions can have considerable space and infrastructure requirements (e.g., phycoremediation), demanding significant investment by the private sector and resulting in environmental impact e.g., (M. Pei et al., 2019).

We present here for the first time a proof-of-concept study that elevates *Daphnia* to the role of potential alternative remedial agent for chemical pollution in water and wastewater. First, we benchmark *Daphnia* against other biological agents i.e., algae and bacteria. Secondly, we use the properties of *Daphnia* as a fast-evolving organism to environmental pollution (Abdullahi, Zhou, et al., 2022a; Chaturvedi et al., 2021) to identify strains with higher decontamination abilities that can be tailored to different wastewater sources.

Benchmarking

Influent tertiary wastewater was collected from the Finham treatment plant in Coventry (UK). After collection, the wastewater was equally split in triplicate 20 L aquaria; i) a first set of aquaria only harboured the naturally occurring bacteria population in the wastewater; ii) a second set was inoculated with a population of *Daphnia* strains from the stock collection at the University of Birmingham; and iii) a third set was inoculated with a population of algae (*Chlorella vulgaris*) from the commercially available strain SAG 211/11B. *Chlorella vulgaris* is a commonly used bioremediation agent (Nie et al., 2020), shown to remove biocides (Garcia-Galan et al., 2020) and pharmaceuticals (Hom-Diaz et al., 2017), and is, therefore, a suitable benchmark for *Daphnia*. Following 48h exposure, the abatement of 16 pharmaceuticals was quantified as compared to the initial concentrations in the wastewater quantified within 24h of

collection (Tab. S3). Removal efficiency was calculated as [influent - effluent/influent] × 100. The chemical analysis of target pharmaceuticals was conducted on the biological replicates of influent (reference) wastewater and the experimental aquaria (Tab. S3). The quantification of the 16 pharmaceuticals was completed with ultraperformance liquid chromatography (UPLC), coupled to Q-Exactive™ Orbitrap high resolution mass spectrometry following (Abdallah et al., 2019). Following solid phase extraction (SPE) of target pharmaceuticals from wastewater samples, both acidic and basic pharmaceuticals were determined using rapid polarity switching electrospray ionisation sources. Full scan MS mode at resolution of 35000 FWHM, automatic gain control (AGC) target of 1×10^6 ions at injection time of 50 ms provided the optimum parameters for high sensitivity, together with sufficient data points per peak (≥ 15) for improved reproducibility. A high-resolution accurate mass with low mass tolerance filter (< 5 ppm from authentic standards) was applied to achieve maximum selectivity with method limits of detection ranging between 0.02 - 1.21 ppb. *Daphnia* removed 7 of the 16 pharmaceuticals more efficiently than algae and bacteria and at a comparable rate the remaining 9 pharmaceuticals (Fig. 4A; Tab. S3).

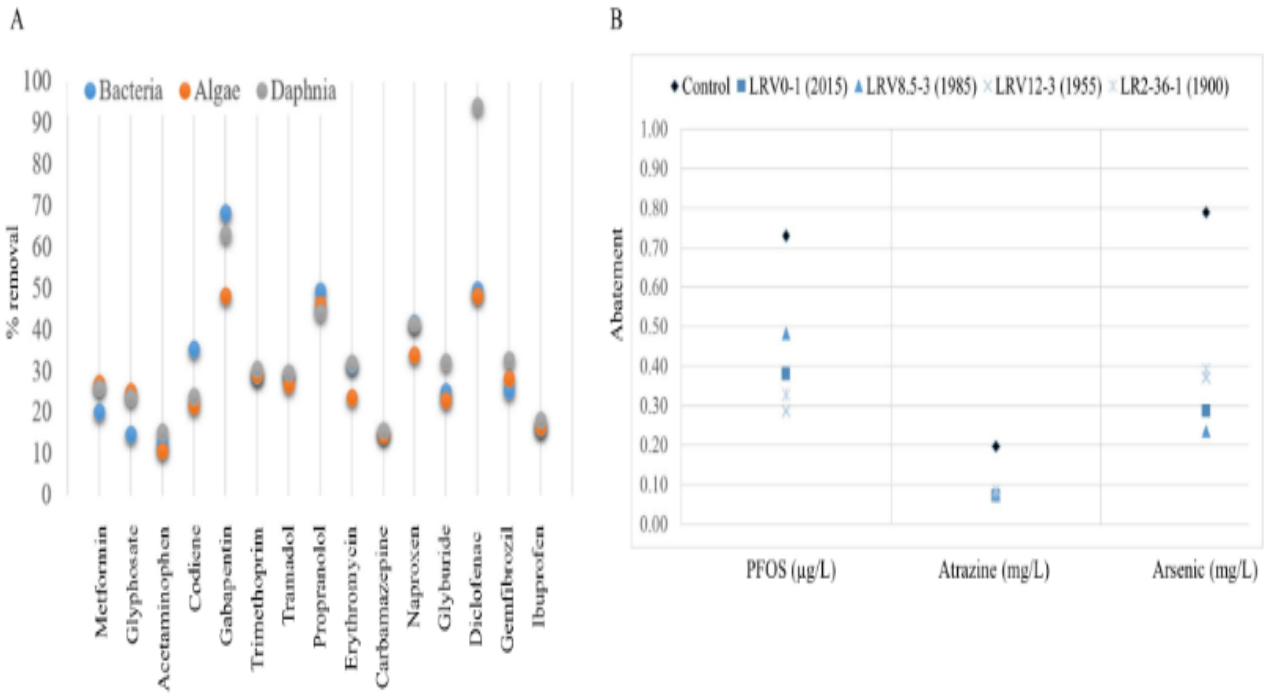

Figure 4. Bioremediation with *Daphnia*. (A) Removal efficiency of known concentrtions of 16 pharmaceuticals (ng/L) by bacteria (blue), algae (orange), and Daphnia (gray). (B) Abatement of an industrial chemical (PFOS; µg/L), a biocide (atrazine; mg/L), and a heavy metal (arsenic; mg/L) by four strains of Daphnia resurrected from a sedimentary archive with different historical environmental backgrounds: LRV0_1 (2015), LRV8.5_3 (1985), LRV12_3 (1955), and LRII36_1 (1900) (ref 26). The abatement of chemicals is shown after 48 h of exposure and compared to a control----spiked medium without Daphnia (control).

Strain-specific Chemical Removal

Having assessed that *Daphnia* survives in wastewater and abates chemicals better or equally well than other biological agents, we then tested the removal efficiency of different *Daphnia* strains in a first effort to identify strains with higher decontamination abilities. This is relevant because different wastewater sources may contain different chemical cocktails. We capitalize on our previous work, in which we studied fitness responses of four *Daphnia* strains resurrected from a sedimentary archive of a lake with a well-known history of chemical pollution (Abdullahi, Zhou, et al., 2022a; M. Cuenca - Cambronero et al., 2018). The lake experienced no chemical exposure until the 1970s, and high chemical exposure from the 1975s onward. In our previous work, we showed that genotypes that were historically exposed to chemical stress, showed reduced genome-wide diversity and lower fitness when exposed to novel chemical stress. This lower fitness was underpinned by reduced gene diversity at detoxification, catabolism, and endocrine genes. These results suggest potential lower tolerance to novel chemical stress and higher tolerance to recurring stress in experienced genotypes (Abdullahi, Zhou, et al., 2022a). Here, we study the removal efficiency of these strains - two that were naïve to chemical stress (LRV12.3 and LRII36_1) and two that experienced historical chemical stress (LRV0_01 and LRV8.5; Fig. 4), following 48h exposure to a metal (Arsenic), a biocide (Atrazine), and an industrial chemical (PFOS) (Abdullahi, Zhou, et al., 2022a). Based on historical records, all strains were naïve to Arsenic, whereas LRV_01 and LRV8.5 were likely pre-exposed to Atrazine and PFOS, even if an empirical estimates of these compounds in the lake is not available.

All chemical measurements of water exposed to the four *Daphnia* strains were done in technical duplicates. Atrazine was analysed according to the method described above by Abdallah et al. (2019) (Abdallah et al., 2019), while PFOS was quantified using the method reported by Harrad et al. (2019) (Harrad, Wemken, Drage, Abdallah, & Coggins, 2019). Briefly, water samples were extracted by solid phase extraction (SPE) using Oasis-WAX cartridges (6 mL, 150 mg, Waters). PFOS was quantified on a Sciex Exion UPLC, coupled to a Sciex 5600+ triple TOF MS. The TOF-MS is equipped with a Turbo V ion source operated in negative mode using electrospray ionization at a voltage of −4500 V operated at 450 °C. Mass spectrometric data was acquired using automatic information-dependent acquisition (IDA) with dependent product ion scan using a collision energy of −40 V. The method detection limit for PFOS was 0.5 ppb. Arsenic samples were prepared using 50 ppb germanium as internal standard. Samples prepared with 70% nitric acid were incubated at 20 ˚C for 18 hr, vortex mixed for 30 s and 100 µL aliquoted and diluted to 10 mL using DI water. The samples were quantified using a Nexion 300x ICP-MS (Perkin Elmer, Seer Green, U.K) fitted with a cyclonic spray chamber. Calibration curves spanning 1-20 ppb were constructed in DI water.

On average, *Daphnia* removed 47.3 % of PFOS, 60 % of atrazine and 60 % of arsenic. However, the strains had different removal efficiencies across the three chemicals with a maximum removal of 59 % for PFOS (LRV12.3), 65 % for Atrazine (LRV_01 and LRV8.5_3) and 70.7 % for Arsenic (LRV8.5_3) (Fig. 4B; Tab. S4). Removal efficiency observed in this study, considering fitness response to chemical exposure observed in our previous study, suggests that strains removal efficiency is likely influenced by historical exposure to chemicals. Strains historically exposed to chemical stress (e.g. LRV_01 and LRV8.5_3) show a higher removal efficiency to Atrazine and Arsenic. Conversely, LRV12.3 that is naïve to PFOS showed the highest removal efficiency. These results support our previous conclusions that strains may evolve tolerance to recurring but not novel stress. However, we previously show

that higher tolerance is associated with lower genome-wide diversity. If the patterns observed in the strains used here are validated at population level, they suggest that acquired tolerance to chemical stress is evolutionarily advantageous to recurring but not novel chemical stress and comes at a cost (Abdullahi, Zhou, et al., 2022a).

This proof-of-concept study shows that *Daphnia* has the potential to become a systemic solution for the removal of a wide range of persistent chemicals from water, preventing their diffusion through other environmental matrices (e.g., soil) and their bioaccumulation through the trophic chain. The ability to tailor strains of *Daphnia* to different wastewaters is powerful to tackle different contamination sources. With additional optimization, the *Daphnia*-based removal of chemicals from wastewater can meet the requirement of the water industry for residence times of a few hours. Additionally, whereas photobioreactors using algae need large infrastructure due to their residence time (Hom-Diaz et al., 2017), *Daphnia* populations can be retrofitted within tertiary treatment tanks. To contain the *Daphnia* populations, containment devices can be used that allow the flow-through of water while containing the *Daphnia* population (L. Orsini & Dearn, 2021). Prototype filtration devices (details are not disclosed because commercially sensitive) have been designed to retain stable *Daphnia* populations, prevent live animals from escaping from the containment volume, and collect dead *Daphnia* post-filtration (L. Orsini & Dearn, 2021). The dead animals are syphoned into a biowaste treatment process where extant technologies (e.g., oxidative catalysis) proven for other biowastes can destroy residual contaminants accumulated in the *Daphnia* body, preventing bioaccumulation and biomagnification (Briche et al., 2020). While substantial work is required to translate this proof-of-concept bioremediation solution into a market-ready technology, the use of the sentinel species *Daphnia* to remediate the effect of chemicals in the aquatic environment has the potential to maximise the shift to clean growth, enabling water reuse,

reducing resource depletion and environmental pollution, and sustaining vital ecosystem services.

2.6. Conclusion and Future Research Needs

The proposed framework has the potential to improve both the detection and the mitigation of environmental chemical pollution with a single sentinel species. The use of sentinel species to identify evolutionary conserved pathways perturbed by the same chemicals across the Tree of Life is potentially transformative to identify targets of toxicity while reducing unnecessary vertebrate animal testing. Whereas experimental validation is required to establish causation between chemicals/chemical mixtures and adversity endpoints, the framework guides experimental efforts capitalizing on the evolutionary conservation of pathways. The use of advanced computational approaches that use machine learning to identify correlations between chemicals within mixtures and putative molecular key events, can significantly improve environmental protection by identifying functional pathways that may lead to adversity before major harm happens. The ability to identify chemicals within mixtures that have an adverse outcome, enables greater precision in the regulation of chemicals as mixtures based on real-world environmental exposure.

Daphnia can also work as a sustainable bioremediation solution once chemical mixtures have entered the environment and remediation is the only solution. Some level of pollution is likely unpreventable, so effective bioremediation tools will always be needed. By expanding the use of *Daphnia* both as an early warning diagnostic and a remedial tool, we address challenges associated with the entire life cycle of chemicals and their mixtures.

National and international regulatory bodies will be understandably cautious in adopting the proposed framework. The framework must be shown to be cost effective, to provide enhanced

protection of human and environmental health, to be usable by regulators and industries, and to be comprehensible by the public. This will take time, and a period of transition where it is tested, validated, and accepted. Major EU initiatives, such as the Zero Pollution Action Plan, linked three ongoing EU projects on new approach methodologies (NAMs) to form a cluster called ASPIS, which main goal is to improve chemical safety without animal testing (https://chemicalwatch.com/370080/eu-non-animal-projects-brought-together-for-aspis-cluster). These initiatives clearly indicate a desire for novel approaches to assessing and managing chemical pollution. The transition to the novel methodologies proposed here will require changes in regulatory frameworks, following a test and acceptance phase. This will take time and resources, but the potential benefits for human and environmental health will justify the effort.

The framework can be, in principle, extended to other model species with the advantage of improving our understanding of targets of toxicity in multiple species. The use of multiple animal models and of human cell lines within the same framework can help distinguish evolutionary conserved biomarkers across the Tree of Life from biomarkers that only affect certain taxonomic groups, focusing regulatory interventions where they are most needed with reduced impact on industrial production and other human activities.

Associated Content

SI methods: Supporting methods for the Chaobai river case study.

SI Table 1: Organic pollutants in the Chaobai river

SI Table 2: KEGG pathways identified in the Chaobai river study and conserved across species

SI Table 3: Removal of 16 pharmaceuticals by different biological agents

SI Table 4: Abatement of three chemicals by different *Daphnia* strains

SI Figure 1: Step-by-step analytical pipeline of the proposed framework

2.7. Acknowledgement

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Author Contribution

MA, WS and MA-EA generated the data for removal efficiency of chemicals by *Daphnia*. XL and LG collected and analysed data for the Chaobai river case study. LO and JC conceived the framework with input from NY. LO coordinated data analysis and writing. All authors contributed to the manuscript writing.

NOTES

The authors declare no competing financial interest

DATA ACCESSIBILITY

Transcriptomics data supporting the Chaobai river case study are submitted to NCBI referring

to accession number PRJNA809147

Chapter 2:
Historical Exposure to Chemicals Reduces Tolerance to Novel Chemical Stress in *Daphnia* (water flea)

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3.1. Abstract

Until the last few decades, anthropogenic chemicals used in most production processes didn't have a comprehensive assessment of their risk and impact on wildlife and humans. They are transported globally and usually end up in the environment as unintentional pollutants causing long-term adverse effects. Modern toxicology practises typically use acute toxicity tests of unrealistic concentrations of chemicals to determine their safe use, missing pathological effects arising from long-term exposures to environmentally relevant concentrations.

Here, we study the transgenerational effect of environmentally relevant concentrations of five chemicals on the priority list of international regulatory frameworks on the keystone species *Daphnia magna*. We expose *Daphnia* genotypes resurrected from the sedimentary archive of a lake with a known history of chemical pollution to the five chemicals to understand how historical exposure to chemicals influences adaptive responses to novel chemical stress. We measure within and transgenerational plasticity in fitness-linked life history traits following exposure of 'experienced' and 'naive' genotypes to novel chemical stress. As the revived *Daphnia* originates from the same genetic pool sampled at different times in the past, we are able to quantify the long-term evolutionary impact of chemical pollution by studying genome-wide diversity and identifying functional pathways affected by historical chemical stress. Our results suggest that historical exposure to chemical stress causes reduced genome-wide diversity, leading to lower cross-generational tolerance to novel chemical stress. Lower tolerance is underpinned by reduced gene diversity at detoxification, catabolism, and endocrine genes in experienced genotypes. We show that these genes sit within pathways that are conserved and potential chemical targets in other species, including humans.

3.2. Introduction

Anthropogenic chemicals used in most production processes are transported globally, and usually end up in the environment as unintentional pollutants that harm humans and damage the environment (Z. Wang, Walker, Muir, & Nagatani-Yoshida, 2020). Until the last few decades, anthropogenic chemicals didn't have comprehensive assessments of their risk and impact on wildlife and humans (Dulio et al., 2018) and pre-market toxicity was not evaluated (Brooks et al., 2020).

Although toxicology has significantly modernised (Choudhuri, Patton, Chanderbhan, Mattia, & Klaassen, 2018), predictive frameworks rely on acute toxicity estimates that are limited in scope and do not necessarily reflect realistic exposure scenarios in situ (Kanno, 2016). These approaches also do not account for the cumulative toxicity that may arise from long-term exposures to sub-lethal concentrations of a chemical or chemical mixtures (A. Blair et al., 2015). Long term exposure to environmentally relevant concentrations of anthropogenic chemicals has been shown to cause loss of genetic diversity, with consequences on adaptive potential to novel stress (Bijlsma & Loeschcke, 2012; Fasola, Ribeiro, & Lopes, 2015; Ribeiro, Baird, Soares, & Lopes, 2012). Chemical stress has documented adverse effects on wildlife's ecological endpoints, including developmental defects and survival (Blahova, Cocilovo, Plhalova, Svobodova, & Faggio, 2020; Jantzen, Annunziato, Bugel, & Cooper, 2016), behaviour and metabolism (J. G. Xia et al., 2015), delayed growth and metamorphosis (Yoon, Park, Park, Lee, & Han, 2019), embryonic development (Balbi et al., 2018), fecundity and sexual maturity (Liu et al., 2017).

In an effort to understand the impact of long-term chemical stress on *Daphnia* fitness and susceptibility to novel chemical stress, we study the transgenerational effects of five chemicals on the priority list of international regulatory frameworks because of their widespread presence in the environment and their potential adverse effects (Chang, Adami, Boffetta, Wedner, &

Mandel, 2016; Gosset, Polome, & Perrodin, 2020; Pu et al., 2020). The chemicals used are a flame retardant (e.g., PFOS), a commonly used anti-inflammatory drug (diclofenac), the antibiotic trimethoprim, the herbicide atrazine and a heavy metal (e.g., arsenic). We use concentrations that are environmentally relevant based on literature research, opting for mid-to-high environmental concentrations found either in high income or developing countries (Graziano et al., 2006 ; Le Luu, 2019).

Daphnia plays a central role in freshwater food webs worldwide (Altshuler et al., 2011). It has a parthenogenetic life cycle, in which sexual and asexual reproduction alternate (D. Ebert, 2005). Sexual recombination results in early stage embryos that arrest their development and enter dormancy, which can be exceptionally long (e.g. up to centuries (Kerfoot & Weider, 2004). Dormant embryos that are awakened (resurrected) from dormant stages are genetically distinct and can be propagated in the laboratory via clonal reproduction, allowing the rearing of populations of isogenic individuals (clones) from a single genotype (M. Cuenca - Cambronero & Orsini, 2018). *Daphnia magna* strains were resurrected from the sedimentary archive of Lake Ring (Denmark), having a well-documented history of exposure to chemicals and other stressors (M. Cuenca - Cambronero et al., 2018; Davidson, Sayer, Perrow, Bramm, & Jeppesen, 2007). By exposing strains that have never been exposed to chemical stress (naïve) and strains that have been historically exposed to chemical stress (experienced) to the five chemicals mentioned above, we were able to study how historical exposure to safe doses of chemicals in the natural environment can influence susceptibility to novel chemical stress. We tested the hypothesis that 'experienced genotypes' were less susceptible to novel stress by showing higher fitness than 'naive genotypes' when exposed to novel chemical stress. We expected experienced genotypes to have higher detoxification abilities underpinned by enriched detoxification genes or pathways, suggesting evolution of tolerance to chemical stress.

To test these hypotheses, we quantified fitness response of two naïve and two experienced genotypes across three clonal generations to the five chemicals in common garden experiments. To link observed changes in fitness to adaptive response to chemicals, we quantified genome-wide nucleotide diversity in the four genotypes and identified enriched functional pathways diverging between experienced and naïve genotypes. To investigate the relevance of our findings in other species, we used a comparative functional analysis of protein domains to identify enriched pathways in *Daphnia* that were conserved in other species and that are potential chemical targets. This study enabled us to investigate how evolutionary responses to novel chemical stress is potentially influenced by historical exposure to chemicals. We also gained insights on how the interactions between historical and novel chemical stress may influence transgenerational adaptive responses.

3.3. Materials and Methods

3.3.1. Study System and Experimental Design

Genotypes of *D. magna* were previously resurrected (revived) from a biological archive of Lake Ring, a shallow mixed lake in Denmark (55^0 57´ 51.83´´ N, $9^0$35´46.87´´E) with a well-documented history of anthropogenic impact (M. Cuenca - Cambronero et al., 2018; Davidson et al., 2007). In the early 1900s and until late 1940s the lake was semi-pristine. In the late 1950s, it experienced eutrophication due to sewage inflow from a nearby town, which was diverted in the late 1970s; from the 1980s until the late 1990s, the lake experienced an increase in biocide run-off due to agricultural land use intensification; the lake partially recovered from high nutrient levels in modern times but still received agricultural run-off from the1999s onward. According to these records, the lake experienced no chemical exposure until the 1970s, and high chemical exposure from the 1975s onward (Fig. 1). Although the resident population of *D. magna* was likely exposed to multiple stressors over time, we here focus on tolerance to

novel chemical stress and interpret fitness and functional responses to novel chemical stress in the context of historical chemical exposure. In our previous study of the *D. magna* population from Lake Ring, we showed microevolutionary responses to recurrent chemical pollutants and that variance at fitness-linked life history traits was larger between than within temporal populations (Cuenca-Cambronero, Beasley, Kissane, & Orsini, 2018; Cuenca-Cambronero, Zeis, & Orsini, 2018; M. Cuenca - Cambronero et al., 2018; M. Cuenca - Cambronero et al., 2021).

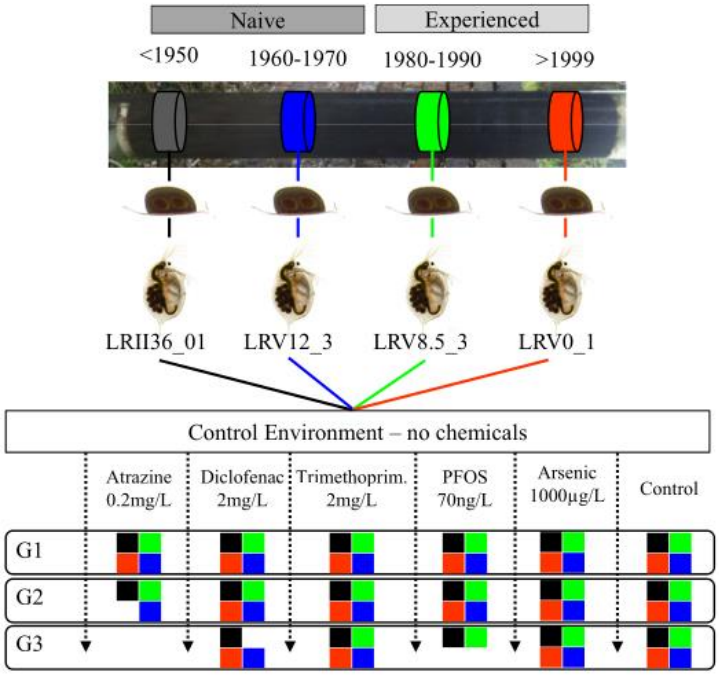


Figure 1. Experimental design. Four genotypes of *Daphnia magna*, previously resurrected from dormant embryos, were used for transgenerational exposures to five chemicals: PFOS (70 ng/L), diclofenac (2 mg/L), atrazine (0.2 mg/L), trimethoprim (2 mg/L), and arsenic (1000 µg/L). The four genotypes were resurrected from different times in the past and were either "naïve" (black-LRII36_1[<1950] and blue, LRV12_3[1960-1970]) or "experienced" (green, LRV8.5_3 [1980-1990] and red, LRV0_1 [>1999)] to chemicals. The clonal lines from the four genotypes were maintained in common garden conditions for at least two generations before the experiment to control for maternal effect. Five clonal replicates of each genotype, randomly selected from the control environment, were exposed to the five chemicals for three generations (G). coloured squares represent genotypes at each generation (G). some genotypes went extinct in G2 and G3.

For the current study, a total of 360 exposures were completed. We selected a genotype from each of the four lake phases and exposed 5 clonal replicates per genotype across three clonal generations to 6 experimental conditions (five chemicals plus control; Fig. 1). The genotypes are: LRII36_1 (<1950), LRV12_3 (1960-1970), LRV8.5_3 (1980-1990), and LRV0_1 (>1999). The former two genotypes are 'naïve' and the latter two genotypes are 'experienced' to chemical stress (Fig. 1). The concentrations of the five chemicals used are as follows, reflecting environmentally relevant concentrations in surface waters: PFOS (70 ng/L), atrazine (0.2 mg/L), trimethoprim (2 mg/L), diclofenac (2 mg/L), and arsenic (1,000 µg/L). The concentrations of PFOS and its derivatives vary dramatically between geographic areas and proximity to contamination sources (<0.8ng/L to >17mg/L; (H. H. Huang et al., 2010; Sinclair, Mayack, Roblee, Yamashita, & Kannan, 2006)) but it is typically found at concentrations of 50-100ng/L in surface waters (Bai & Son, 2021). Non-steroidal anti-inflammatory drugs such as diclofenac, acetaminophen and ibuprofen can be found at µg/L to g/L concentrations in seawater (Weigel, Bester, & Huhnerfuss, 2001) and surface waters (Fick et al., 2009), whereas their concentration is significantly lower in ground and drinking waters (ng/L; (Godfrey, Woessner, & Benotti, 2007). A concentration between 1 and 2mg/L is common in surface waters in high income economies (Fick et al., 2009). Average levels of antibiotic drugs in surface water range between ng/L and µg/L (Kummerer, 2009), with the exception of effluent originating from chemical manufacturers where concentration of antibiotic drugs can exceed 30 mg/L (Larsson, de Pedro, & Paxeus, 2007) and fish farms where they range from 1 to 6mg/L (Le & Munekage, 2004). Atrazine is one of the most used photosynthesis-inhibiting pre-emergent biocide worldwide (Prado et al., 2014), with more than 70 million Kg produced yearly (Sass & Colangelo, 2006). Typically, streams and rivers receiving agricultural run-off display concentrations of 0.2mg/L (Graziano et al., 2006). Metal contamination of groundwater is among the biggest health threats in low and middle income countries (Winkel

et al., 2011). According to the World Health Organization guidelines, the safe limit of arsenic in drinking water is 10µg/L (EPA, 2001). In high income economies, arsenic in surface and urban waters typically reaches concentrations of 30 µg/L (Barrett et al., 2018). However, higher concentrations have been recorded occasionally in high income economies (e.g. North America; (Smith et al., 2016) and frequently in low-and-middle-income countries (Chakraborti et al., 2018; Le Luu, 2019), where they range between 500µ/L and 1,000µ/L (Chakraborti et al., 2018). In some geographic regions, concentrations can exceed 3,000µg/L (e.g. Vietnam; (Le Luu, 2019).

3.3.2. Common garden experiments

We previously resurrected several genotypes of *D. magna* from Lake Ring and maintained them in the laboratory for over a year as isoclonal lines in the following standard laboratory conditions: 16:8 hr light: dark photoperiod; 0.8 mg/L *Chlorella vulgaris* fed weekly; ambient temperature: 10 ^{0}C (M. Cuenca - Cambronero et al., 2018). Before the exposure experiment for this study, clonal lineages of the four genotypes (LRII36_1, LRV12_3, LRV8.5_3, and LRV0_1) were acclimated for at least two generations to the following conditions to reduce interference from maternal effect: 16:8 h light: dark photoperiod; 0.8 mg/L *Chlorella vulgaris* fed daily; ambient temperature: 20 ^{0}C. After at least two generations in these conditions, 24h old juveniles from the second or following broods were randomly isolated and assigned to experimental conditions (Fig. 1). Each clonal generation was established following the same criteria and starting from randomly selected 24h-old juveniles from the second or following broods. Where necessary, different broods from genotypes within the same generation were used to ensure developmental synchrony among clonal lineages in the experiment. Exposures of clonal lines were conducted in individual jars of 100 ml, filled with filtered borehole water (growth medium), which was refreshed every second day. The experimental cultures were fed

daily *ad libitum* with 0.8 mg Carbon/L of *Chlorella vulgaris*. To ensure constant concentrations of the chemicals throughout the experimental exposures, the growth medium was spiked with fresh chemicals at each media change. Stock solutions were prepared for all chemicals using pure ethanol as carrier solvent. From this stock solution, dilutions of chemicals were prepared to the final concentrations listed above and a final concentration of ethanol not exceeding 0.005 ml/L. The ethanol final concentration was previously shown to have a negligible effect on *Daphnia*, with patterns in life history traits not-significantly departing from control conditions (M. Cuenca - Cambronero et al., 2018; Jansen et al., 2015).

The following fitness-linked life history traits were measured across three generations of four genotypes and 5 clonal replicates for exposed and non-exposed *Daphnia* (120 individuals across 3 clonal generations): age at maturity (first time parthenogenetic eggs are released in the brood pouch), size at maturity (distance from the head to the base of the tail spine), fecundity (sum of juveniles across 2 broods), interval between broods and mortality. Size at maturity was measured after the release of the first brood in the brood pouch using image J software (https://imagej.nih.gov/ij/). The mortality rate per genotype was determined with the survival model fit using the psm function in the 'rms' package in R v.3.6.0 (Harrell Jr, 2021). The day of mortality and mortality events were combined as a response variable while the term 'genotype' was treated as a fixed effect. The mortality curves per generation were plotted with the survplot function from the rms package in R v.3.6.0 (Harrell Jr, 2021). Genotypes were fixed across all experimental conditions and clonal generations, enabling us to control for confounding factors e.g. genetic changes occurring from one generation to the next and genetic variation among experimental exposures. Clonal replicates were nested within genotype in the statistical analyses as explained below. This design permits the analysis of within (WGP) and transgenerational plasticity (TGP), as well as the analysis of evolutionary differences among genotypes that originate from the same genetic pool.

3.3.3. Fitness Response to Chemicals

We quantified genetic, WGP, and TGP using an analysis of variance (ANOVA), and tested the effect of Generation (G), Genotype (Gen), Treatment (T), and their interaction terms on the five fitness-linked life history traits described above; a Wald chi-square test (Type III test) was used to generate an analysis of deviance table (Langsrud, 2003). Multivariate effects were calculated on the same terms using multivariate statistics (MANOVA). Both statistics were completed using the 'car' package for R v.3.6.0 (Fox & Weisberg, 2019) after checking for normality assumptions by plotting model residual versus fitted values (Q-Q plots) (A. F. Zuur, E. N. Ieno, & C.S. Elphick, 2010). Clonal replicates were fit as a random effect nested within genotype. As the four genotypes used here belong to populations separated in time that originate from the same genetic pool, a significant genotype term indicates genetic evolution of the life history trait (L. Orsini, Marshall, et al., 2016). Differences in mean trait values between an individual treatment and its control within generation are the expression of WGP. Differences in mean trait values across clonal generations are the expression of TGP. If the effect of the treatment differed significantly among genotypes (genetic effect), we would have evidence of a Gen (genotype) × T (treatment) interaction. Similarly, if the effect of the treatment differed significantly among generations, we would have evidence of G (generation) × T (treatment) interaction. If genotype means varied by generation, we would have evidence of a G (generation) × Gen (genotype) interaction. We also measured the three-way interaction term, which measures how the treatment per genotype differed across generations (Gen × G × T).

The main effects of genotype, treatment and generation plus their interaction terms on individual life history traits were visualised through univariate reaction norms, which describe

the pattern of phenotypic expression of each genotype across treatments and generations (Roff, 1997). We visualised the multivariate analysis results using phenotypic trajectory analysis plots (PTA) to describe the difference in multivariate reaction norms in terms of magnitude and direction of change (Adams & Collyer, 2009). In the PTA, reaction norms are described as multivariate vectors with varying magnitude (the amount of phenotypic change between environments) and direction (the covariation of phenotypic variables) projected onto principal components in a multivariate space (Collyer & Adams, 2007). The R code provided by Adams (Adams & Collyer, 2009) was used for the PTA analysis. We visualised the principal mode of variation and covariation among traits (trade-offs) within and across generations through a PCA analysis done with the 'prcomp' function in R followed by visualisation through fviz_pca_biplot function in the factoextra package in R v.3.6.0 on log transformed data (Kassambara & Mundt, 2020).

3.3.4. Genomics and Functional Analysis

We used clonal replicates of the four genotypes used in the exposure experiments to generate whole genome resequencing. The genotypes were cultured in standard laboratory conditions (16:8 light: dark regime, 20 ^{0}C and 0.8 mg C/L of *Chlorella vulgaris* daily) to generate sufficient material for extraction of high molecular weight gDNA (typically 1 µg/library). Two days before tissue collection, cultures were treated with a cocktail of antibiotics at a final concentration of 20 mg/L (Tetracycline-T, Streptomycin-S, Ampicillin-A) to reduce bacterial contamination from gut microbes in downstream analyses. *Daphnia* were also deprived of food for 24h before tissue collection to reduce contamination from the feedstock (algae) in downstream analyses. Genomic DNA was extracted using Agencourt DNA Advance (Beckman Coulter - A48706). gDNA was quantified using a ND-8000 Nanodrop (Thermo Fisher Scientific - ND-8000-GL). Up to 1µg of gDNA per genotype was sheared using a

Bioruptor® Pico ultrasonicator with integrated cooling module (Diagenode - B01060010), following cooling on ice for 10minutes. Sheared genomic DNA was assayed on a 2200 TapeStation (Agilent) with High Sensitivity DNA screen tapes. The sheared genomic DNA was then prepared into Illumina compatible DNA 250bp paired-end libraries using KAPA HyperPrep Kit (Roche - KK8504), without amplification. Following library construction, libraries were assayed and quantified on a 2200 TapeStation (Agilent) with High Sensitivity DNA Screen tapes. Libraries were normalised to an average concentration of 2 nM prior to pooling. Libraries were sequenced on a HiSeq2500 (Illumina) using HiSeq Rapid SBS Kit v2 200 cycles (Illumina - FC-402-4021), HiSeq PE Rapid Cluster Kit v2 (Illumina - PE-402-4002), and HiSeq Rapid Duo cBot Sample Loading Kit (Illumina - CT-403-2001) following manufacturer instructions aiming to a final depth of coverage of at least 40X per genotype.

The genome sequences were subjected to quality check by mapping raw reads onto the newly assembled reference genome of *D. magna* obtained using a hybrid assembly of long and short reads – the detailed description of this reference genome will be presented elsewhere (NCBI: SUB9530054). Mapping and quality filtered SNP variants were identified using the following steps: 1) read sequences basepair quality was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC (https://multiqc.info/); 2) Trimmomatic ver.0.33 was applied for adapter trimming and to remove low quality sequences (Bolger, Lohse, & Usadel, 2014). Paired-end reads with Q>30 and read length > 50bp were retained and mapped against the reference genome of *D. magna* using BWA-mem algorithm (H. Li & Durbin, 2010); 3) Samtools were used for format conversion, sorting, indexing and merging of mapping files onto the reference genome following (H. Li et al., 2009); 4) Picard tools were used to mark and remove PCR duplicated reads and realign the dataset with GATK (McKenna et al., 2010); 5) allelic variants were called via bcftools (https://samtools.github.io/bcftools/) after applying the samtools mpileup

command (samtools ver. 0.1.19, 45); 6) SNP were filtered with vcftools v0.1.14. Filtering criteria were as follows: minimum read depth (DP) > 10; SNP calls Quality (Phred score) for each sample Q> 30; minor allele frequency (MAF) > 5%; maximum missing values 50%; Avg. genotype quality > 50.

We studied genome-wide and chromosomal-level alpha and beta SNP diversity following methods described in Ma *et al*. (Z. S. Ma, Li, & Zhang, 2020). We quantified gene-level beta diversity between each pair of genotypes using NOISeq with no replicates mode (Tarazona et al., 2015). We identified significant differences in SNP diversity per gene between each pair of genotypes using a probability approach (Tarazona et al., 2015). To understand potential functional impact of the gene diversity differences among pairs of genotypes, we used a gene set enrichment analysis at multiple levels of functional categories including gene ontology and metabolic pathways. Firstly, we used InterProscan (P. Jones et al., 2014) that classifies genes into families, based on their protein sequence, and predicts their function based on domain information. InterProscan uses predictive models provided by several databases including Pfam, PANTHER, CDD, GO, and KEGG. We used gProfiler (Raudvere et al., 2019) for gene ontology (GO) annotations and to perform a gene set enrichment analysis, using gene homology between *D. magna* and *D. pulex* (J.K. Colbourne et al., 2011). Based on the GO annotations, we identified enriched functional pathways using gProfiler (Raudvere et al., 2019). Both for enriched pathways and gene ontologies we used p-value < 0.05 after Bonferroni correction.

3.4. Results

3.4.1. Fitness Response to Novel Chemical Stress of Naïve and Experienced Genotypes

The five chemicals had a significant effect on *Daphnia* genotypes overall fitness. This effect was driven by different combinations of individual life history traits in the five chemical exposures. In the following, we describe the overall impact on fitness (MANOVA) and unpack the contribution of individual life history traits on this overall response by interpreting the ANOVA results.

A significant three-way interaction term (Table 1; MANOVA - G x Gen x T) was observed, showing that treatment per genotype differed across generations in all chemicals, except for PFOS. The ANOVA analysis revealed that these overall patterns were driven by: i) mortality in the PFOS exposure; ii) size at maturity, age at maturity, fecundity, and mortality in the atrazine exposure; iii) size and age at maturity in the diclofenac exposure; iv) size at maturity, fecundity, and mortality in the trimethoprim exposure; and v) fecundity age at maturity in the arsenic exposure (Table 1; ANOVA - G x Gen x T).

Exposure to the chemicals induced a significant genetic response both within (Gen x T) and between (G x Gen) generations. As the genotypes originate from the same genetic pool sampled at different times in the past, significant difference in mean trait values among genotypes indicates evolutionary differences. A genotype-dependent response to treatment was observed in all chemicals, except for Atrazine (Table 1, MANOVA - Gen x T). The individual fitness-linked life history traits contributing to this response differed among treatments: i) size at maturity and mortality significantly varied between genotypes in the PFOS treatment; ii) size at maturity varied by genotype in the diclofenac exposure; iii) size at maturity and fecundity

varied among genotypes in the trimethoprim exposure; and iv) size and at maturity, and mortality varied among genotypes in the arsenic treatment (Table 1; ANOVA - Gen x T).

A significant genotype per generation effect (G x Gen) was observed across treatments, except for PFOS (Table 1; MANOVA; G x Gen). The individual fitness-linked life history traits contributing to this overall fitness response were: i) size at maturity and fecundity for PFOS; ii) size at maturity and fecundity for atrazine; iii) all traits except interval between broods for diclofenac; iv) size at maturity, age at maturity and fecundity for trimethoprim; and v) all life history traits except mortality for arsenic (Table 1; ANOVA - G x Gen).

Table 1. Analysis of variance. Multivariate (MANOVA) and univariate (ANOVA) analysis of variance, testing the effect of generation (G), genotype (Gen), treatment (T) and their interaction terms on five fitness-linked life history traits. The chemicals tested are: PFOS (70 ng/L), diclofenac (2 mg/L), trimethoprim (2 mg/L), atrazine (0.2 mg/L), and arsenic (1000 µg/L). Size at maturity (mm), age at maturity (days), fecundity (number of offspring across two broods), interval between broods 9time elapsed between broods) and mortality were measure. Each genotype is run in five clonal replicates. Significant p-values are in bold.

MANOVA				ANOVA	Size at maturity (mm)			Age at maturity (days)		Fecundity		Interval between broods		Mortality	
PFOS	Df	F	P-value	**PFOS**	Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value
Generation (G)	2	9.04	**3.2e-09 *** **	Generation (G)	2	41.68	**8.9e-10 *** **	41.94	**7.8e-10 *** **	62.40	**2.8e-14 *** **	8.10	**0.02 ***	6.48	**0.010891 ***
Genotype (Gen)	3	4.31	**7.4e-06 *** **	Genotype (Gen)	3	5.14	0.16	2.61	0.46	29.19	**2.0e-06 *** **	0.34	0.95	16.16	**0.001052 ** **
Treatment (T)	1	2.69	**0.04 ***	Treatment (T)	1	19.74	**8.8e-06 *** **	0.49	0.48	4.09	**0.04304 ***	0.03	0.87	5.18	**0.022829 ***
G x Gen	4	1.59	0.07	G x Gen	4	18.74	**0.0009 *** **	9.17	0.05707 .	33.20	**1.1e-06 *** **	1.29	0.86	5.69	0.13
G x T	2	3.07	**0.004 ** **	G x T	2	13.29	**0.001 ** **	23.12	**9.5e-06 *** **	7.44	**0.02 ***	1.72	0.42	1.87	0.17
Gen x T	3	1.94	**0.03 ***	Gen x T	3	22.85	**4.3e-05 *** **	0.54	0.91	1.28	0.73	1.92	0.59	8.98	**0.029603 ***
G x Gen x T	4	1.19	0.27	G x Gen x T	4	7.03	0.13	1.52	0.82	4.45	0.35	1.29	0.86	8	**0.038836 ***
ATRAZINE	Df	F	P-value	**ATRAZINE**	Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value
Generation (G)	2	7.07	0.84	Generation (G)	2	44.74	**1.9e-10 *** **	39.83	**2.2e-09 *** **	35.42	**2e-08 *** **	3.88	0.14	0.01	0.90
Genotype (Gen)	3	1.50	0.39	Genotype (Gen)	3	23.06	**3.9e-05 *** **	3.98	0.26	21.28	**9.2e-05 *** **	0.82	0.84	12.87	**0.004920 ** **
Treatment (T)	1	15.46	**1.4e-07 *** **	Treatment (T)	1	89.50	**< 2.2e-16 *** **	0.52	0.47	33.65	**6.6e-09 *** **	0.18	0.68	20.53	**5.881e-06 *** **
G x Gen	2	2.49	**0.02 ***	G x Gen	2	10.06	**0.006 ** **	2.35	0.31	8.69	**0.01294 ***	0.12	0.94	4.38	0.11
G x T	2	2.19	**0.04 ***	G x T	2	6.42	**0.04 ***	3.55	0.17	1.62	0.44	0.67	0.71	0.14	0.71
Gen x T	3	2.24	0.55	Gen x T	3	4.18	0.24	3.84	0.28	1.73	0.63	1.54	0.67	4.96	0.18
G x Gen x T	1	2.76	**0.04 ***	G x Gen x T	2	7.12	**0.03 ***	2.84	**0.03 ***	6.52	**0.04 ***	0.00	0.99	10.2274	**0.006014 ** **
DICLOFENAC	Df	F	P-value	**DICLOFENAC**	Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value
Generation (G)	2	14.77	**2.1e-15 *** **	Generation (G)	2	73.63	**< 2.2e-16 *** **	110.90	**< 2.2e-16 *** **	127.47	**< 2.2e-16 *** **	21.35	**2.3e-05 *** **	7.85	**0.0197461 ***
Genotype (Gen)	3	2.67	**0.002 ** **	Genotype (Gen)	3	13.25	**0.004 ** **	6.34	0.10	29.37	**1.9e-06 *** **	0.50	0.92	7.76	0.05
Treatment (T)	1	16.24	**2.4e-09 *** **	Treatment (T)	1	44.52	**2.5e-11 *** **	39.40	**3.4e-10 *** **	16.32	**5.3e-05 *** **	0.12	0.73	13.45	**0.0002447 *** **
G x Gen	5	2.37	**0.001 ** **	G x Gen	5	26.05	**8.7e-05 *** **	17.25	**0.004 ** **	56.96	**5.2e-11 *** **	3.43	0.63	14.84	**0.0110460 ***
G x T	2	10.81	**1.0e-11 *** **	G x T	2	31.09	**1.8e-07 *** **	83.62	**< 2.2e-16 *** **	21.13	**2.6e-05 *** **	5.76	0.06	21.33	**2.331e-05 *** **
Gen x T	3	2.59	**0.003 ** **	Gen x T	3	13.52	**0.004 ** **	5.42	0.14	2.00	0.57	2.82	0.42	1.97	0.58
G x Gen x T	5	3.51	**1.370e-06 *** **	G x Gen x T	5	25.37	**0.0001 *** **	17.25	**3.5e-07 *** **	8.84	0.12	5.35	0.37	0	1
TRIMETHOPRIM	Df	F	P-value	**TRIMETHOPRIM**	Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value
Generation (G)	2	16.77	**< 2.2e-16 *** **	Generation (G)	2	16.65	**0.0002 *** **	87.14	**< 2.2e-16 *** **	92.88	**< 2.2e-16 *** **	21.85	**1.8e-05 *** **	0.10	0.76
Genotype (Gen)	3	4.35	0.55	Genotype (Gen)	3	18.29	**0.0004 *** **	18.29	**0.0003827 *** **	39.90	**1.1e-08 *** **	1.35	0.72	7.01	0.14
Treatment (T)	1	1.45	0.22	Treatment (T)	1	0.25	0.62	0.34	0.56	0.07	0.79	4.20	**0.04 ***	0.94	0.33
G x Gen	6	2.31	**0.0006 *** **	G x Gen	6	21.26	**0.002 ** **	17.84	**0.006637 ** **	28.44	**7.76e-05 *** **	4.19	0.65	2.76	0.43
G x T	2	2.30	**0.023 ***	G x T	2	0.90	0.64	7.55	**0.022895 ***	4.17	0.12	1.61	0.45	0.04	0.85
Gen x T	3	3.39	**0.0001 *** **	Gen x T	3	29.47	**1.8e-06 *** **	3.78	0.29	16.34	**0.0001 *** **	2.43	0.49	2.63	0.45
G x Gen x T	6	1.75	**0.02 ***	G x Gen x T	6	16.89	**0.01 ** **	9.25	0.16	12.92	**0.04 ***	7.95	0.24	9.23	**0.02639 ***
ARSENIC	Df	F	P-value	**ARSENIC**	Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value
Generation (G)	2	14.17	**4.233e-15 *** **	Generation (G)	2	83.76	**< 2.2e-16 *** **	39.14	**3.165e-09 *** **	0.35	0.84	8.26	**0.0161066 ***	2.53	0.28
Genotype (Gen)	3	4.15	**7.332e-06 *** **	Genotype (Gen)	3	17.58	**0.0005378 *** **	134.18	**< 2.2e-16 *** **	38.44	**2.284e-08 *** **	6.25	0.10	1.01	0.80
Treatment (T)	1	1.24	0.30	Treatment (T)	1	1.81	0.18	1.91	0.17	0.03	0.85	0.08	0.77	0.00	0.98
G x Gen	6	6.08	**3.067e-15 *** **	G x Gen	6	66.16	**2.501e-12 *** **	59.61	**5.394e-11 *** **	22.31	**0.001066 ** **	27.59	**0.0001124 ***	11.66	0.07
G x T	2	3.03	**0.003557 ** **	G x T	2	10.83	**0.0044538 ** **	8.50	**0.01425 ***	2.75	0.25	1.34	0.51	7.81	**0.020177 ***
Gen x T	3	2.61	**0.002843 ** **	Gen x T	3	9.15	**0.0273738 ***	10.75	**0.01314 ***	3.24	0.36	1.34	0.72	11.64	**0.008706 ** **
G x Gen x T	6	3.23	**1.387e-06 *** **	G x Gen x T	6	11.75	0.07	38.13	**1.061e-06 *** **	33.35	**8.985e-06 *** **	1.33	0.97	0	1

Plasticity was pervasive within and across generations. Significant plastic responses to treatment (T) within generations, indicative of WGP, were observed in PFOS, atrazine and diclofenac (Table 1; MANOVA - T). These plastic responses induced: i) a significant decline in size at maturity and an increase in mortality in PFOS exposures; ii) a significant decline in size at maturity, and an increase in fecundity and mortality in the atrazine exposures; iii) a decline in size at maturity, a delay in maturation, a decline in fecundity and higher mortality in the diclofenac exposures; iv) a genotype-specific change in the time between broods in the trimethoprim exposure (Table 1; ANOVA -T; Fig. S1; Fig S2). The WGP effects on individual life history traits are summarised through univariate reaction norms in Fig. S1. The overview of mortality across treatments and generations is in Fig. S2.

Significant transgenerational plasticity (TGP) was observed in all exposures (Table 1 - G x T). These plastic responses had a significant effect on: i) size at maturity, age at maturity and fecundity in PFOS exposures; ii) size at maturity in atrazine exposure; iii) size at maturity, age at maturity, fecundity, and mortality in diclofenac exposures; iv) age at maturity in trimethoprim exposures; and v) age at maturity, size at maturity, and mortality in arsenic exposures (Table 1; ANOVA - G x T).

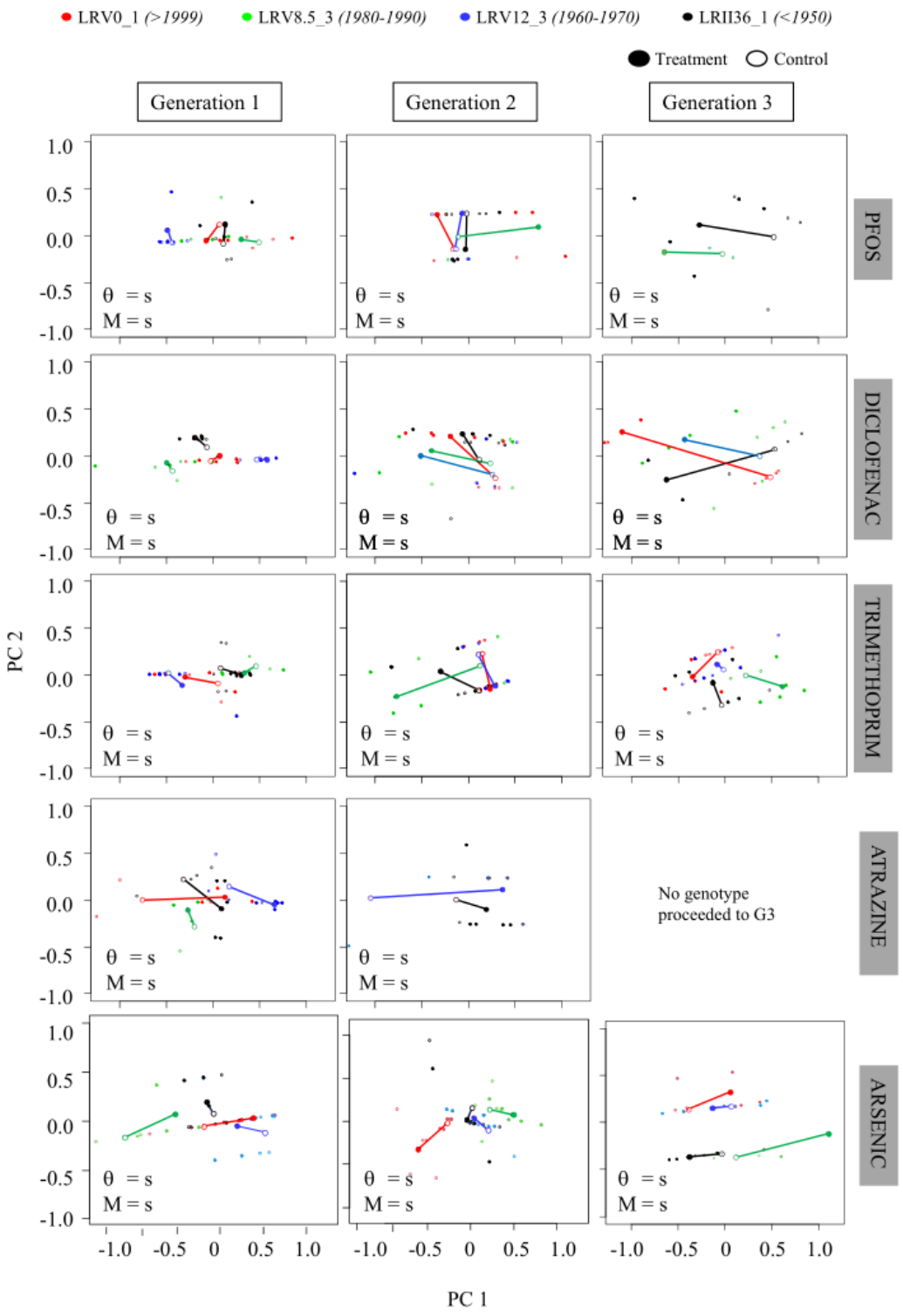
Transgenerational plastic effects visualised through PTA plots revealed an overall trend of lower mortality and smaller fitness change across clonal generations in naïve than in experienced genotypes. It also revealed different overall fitness responses of the 4 genotypes. Exposure to PFOS caused an increasing magnitude of change in all four genotypes as the generations progressed and change in direction for the naïve genotype LRII36_1 in the third generation (Fig. 2 - PFOS; Table S1). In the first generation of this exposure, the experienced genotype LRV0_1 showed a negative fitness change. In the second generation, the naïve

genotype LRII36_1 showed a negative fitness change. Two of the four genotypes (LRV0_1 and LRV12_3) went extinct in generation three (Fig. 2; PFOS). Exposure to diclofenac induced both changes in magnitude and direction of fitness across the genotypes (Fig. 2, Diclofenac). However, whereas in generation two, all genotypes experienced an increase in fitness (the treatment had higher fitness than the control), this pattern was reversed for the naïve genotype LRII36_1 in generation three, where we also observed mortality of the experienced genotype LRV8.5_3 (Fig. 2, Diclofenac). Exposure to Trimethoprim induced genotype-specific fitness changes both in direction and magnitude (Fig. 2, Trimethoprim). In the third generation, the naïve genotypes LRV12_3 and LRII36_1 experienced a positive change in fitness (Fig. 2, Trimethoprim). Exposure to atrazine imposed the highest fitness costs across the genotypes, resulting in the mortality of the experienced genotypes in generation two and of all genotypes in generation three (Fig. 2, Atrazine). This exposure induced changes both in direction and magnitude of fitness in the first generation, with the naïve genotype LRII36_1 showing the least change in fitness between the two naïve genotypes that survived the first generation (Fig. 2, Atrazine). Exposure to arsenic induced genotype-specific changes across generations; the naïve genotypes (LRV12_3 and LRII36_1) experienced the smallest overall fitness change (Fig. 2, Arsenic).

3.4.2. Detoxification Pathways and Genome-wide Diversity in Naïve and Experienced Genotypes

The genome of the 4 genotypes of *D. magna* was assembled (NCBI: SUB9530054) and the raw depth of coverage was: 84x for LRV0_1; 42x for LRV8.5_3; 49x for LRV12_3; and 45x for, LRII36_1. The genome-wide SNP-alpha diversity was comparable among the genotypes LRII36_1, LRV12_3, and LRV8.5_3, and lower in the most recent genotype (LRV0_1) (Fig. 3A; Table S2). The genome-wide alpha diversity patterns were reflected equally across

Figure 2. Phenotypic trajectory analysis. PTA on the four genotypes of *Daphnia magna* used in transgenerational exposure to five chemical classes (PFOS [70 ng/L], diclofenac [2 mg/L], atrazine [0.2 mg/L], and arsenic [1000 µg/L]), resulting from multivariate response of five fitness-linked life history traits. Open circles represent the control (nonexposed clonal replicates) and full circles represent the exposed clonal replicates. Genotype centroids are connected by reaction norms (solid lines), showing phenotypic change in direction and length. Difference among genotypes in terms of magnitude (M) and direction (Θ) of plastic response are all significant. The statistics supporting the PTA are given in Table S1. Genotypes are colour-coded as in Figure 1.



chromosomes (Fig. 3B). Beta diversity was significant in all pairwise comparisons but comparatively higher in pairwise comparisons including the LRV0_1 genotype (Table S2; P-val = 0.05).

The number of divergent genes between LRV0_1 and the other genotypes ranged between 1,093 (3.3% of the total number of *Daphnia* genes) and 1,317 (4% of the total number of *Daphnia* genes). Conversely, the number of significantly divergent genes in the pairwise comparisons involving the other genotypes ranged between 514 (1.6% of the total number of *Daphnia* genes) and 697 (2.1% of the total number of *Daphnia* genes) (Fig. 3C; Table S3). The mean gene diversity at the divergent genes was significantly lower in the experienced than in the naïve genotypes (t-test; P-val=0.03).

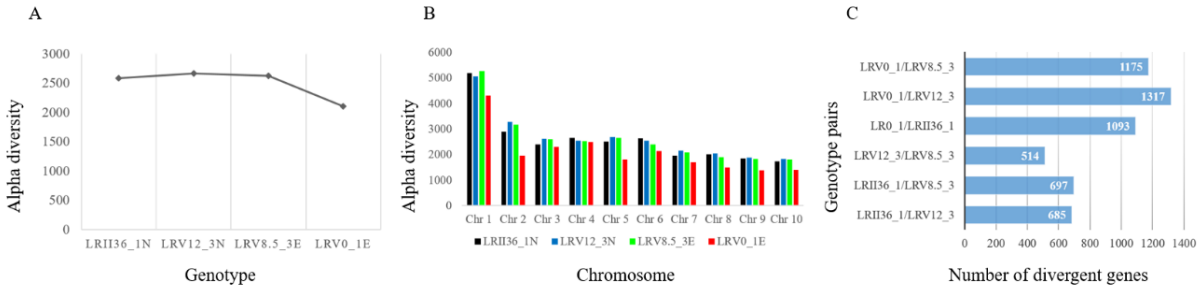


Figure 3. Genomic diversity. Alpha diversity measured at (a) genome-wide and (b) chromosome-level in the four genotypes used in this study; (c) number of significantly divergent genes between each pair of genotypes. The genotypes are colour coded as in Figure 1: LRII36_1 (<1950; black), LRV12_3 (1960-1970; blue), LRV8.5_3 (1975-1985; green) and LRV0_1 (>1999;red).

To understand potential functional impact of the divergent genes between naïve and experienced genotypes, we used a gene set enrichment analysis at different functional levels including gene ontology and metabolic pathways. Divergent genes were enriched for detoxification (Cytochrome P450), catabolites degradation (Armadillo), exoskeletal modelling (e.g. chitin-binding domain), fertilisation (Alpha-L-fucosidase) and embryonic development (Nuclear hormone receptor-like domain superfamily, Zona pellucida domain) (Table S4). Genes involved in endocrine processes (GPCR), epigenetic regulation (the Histone deacetylase family and the Elongator complex protein 1) and neuronal activities (learning and memory) were also enriched (Table S4). The gene enrichment analysis also identified significant change in genes underpinning fundamental cell functions and trafficking: oxidoreductase activity, redox and biosynthetic reactions (Dehydrogenase/reductase, Haem peroxidase), heat shock proteins (HSP70), DNA binding (Rap1 Myb domain, Zinc finger, MIZ-type), recombination (RAD50, zinc hook), and replication/cell division (Creatinase/aminopeptidase-like, Mad3/Bub1, DNA mismatch repair protein MutS, WD40), protein-protein interactions (Ankyrin), and cell trafficking (Dynamin, von Willebrand factor) (Table S4).

We used the manually curated 'Reactome' database to assess how many of the functional pathways diverging between naïve and experienced genotypes in our study were conserved across other animals, based on the conservation of the protein sequence (Table S4). These domains could be potential targets of chemical pollution in other species: i) genotoxic stress response and detoxification functions (R-HSA-176187, R-HSA-3299685, R-HSA-8849175); ii) catabolism (R-HSA-71064, R-HSA-389661, R-HSA-70370); iii) immunity genes including the major histocompatibility complex (R-HSA-6798695, R-HSA-2132295, R-HSA-2132295); iv) fat and carbohydrates metabolism (R-HSA-8964038, R-HSA-1369062, R-HSA-2024096, R-HSA-70171, R-HSA-189085,R-HSA-189200, R-HSA-1655829, R-HSA-351202, R-HSA-

1369062); and v) nervous system (R-HSA-8862803, R-HSA-375165), including the neurotransmitter acetylcholine (R-HSA-6798163). Conserved domains across species also included DNA repair (R-HSA-1221632, R-HSA-2559586, R-HSA-5632928) and elongation (R-HSA-73980); RNA binding, translation and transcription, including miRNA (R-HSA-72702, R-HSA-156827, R-HSA-203927, R-HSA-4641265); histone demethylation (R-HSA-3214842), and cell signalling, including transcription factors that bind hormones and vitamins (R-HSA-913709, R-HSA-391160, R-HSA-5663220, R-HSA-383280, R-HSA-2682334, R-HSA-2565942, R-HSA-425561, R-HSA-2682334) (Table S4). Fat and carbohydrate metabolism pathways were differentially enriched between 'naive' and 'experienced' genotypes (Table S5).

3.5. Discussion

3.5.1. Naïve Genotypes Show Higher Fitness in Response to Novel Chemical Stress

We hypothesised that experienced genotypes had an evolutionary advantage over naïve genotypes when exposed to novel chemical stress. We expected the experienced genotypes to always have higher overall fitness underpinned by enrichment at detoxification genes or pathways.

Our results show significant fitness differences among genotypes, underpinned by divergence in functionally enriched pathways for detoxification, catabolic and metabolic functions between experienced and naïve genotypes. However, naïve genotypes showed a higher tolerance to novel chemical stress, and lower fitness costs measured via ecological endpoints (e.g. mortality) and phenotypic trajectory changes, confuting our hypothesis. Previous studies

on parthenogenetic species that experienced different levels of anthropogenic stress reached similar conclusions (e.g. rotifers; (Zweerus, Sommer, Fontaneto, & Ozgul, 2017) and other *Daphnia* species; (Spaak & Keller, 2004).

The analysis of variance on the fitness-linked life history traits revealed that the evolutionary mechanisms underpinning fitness response to novel chemical stress were complex. All four genotypes expressed both WGP and TGP plasticity to cope with novel chemical stress. However, whereas WGP enabled both naïve and experienced genotypes to respond to novel chemical stress in the short term, TGP affected naïve and experienced genotypes differently. This short-term strategy was evident from the mortality of the experienced genotypes in 60% of the chemicals tested between the second and the third generation. Where mortality did not occur (e.g. trimethoprim and arsenic), the experienced genotypes showed larger fitness changes than the naïve genotypes as the generations progressed. These fitness changes occurred between the second and the third generation of exposure (PFOS, atrazine and diclofenac), indicating cumulative toxicity effects. A complex interplay between genetic adaptation, WGP and TGP has been previously observed in population-level studies of the *Daphnia* population from Lake Ring, suggesting that the genotypes used in this study are representative of the *Daphnia* subpopulations from Lake Ring (M. Cuenca - Cambronero et al., 2018; M. Cuenca - Cambronero et al., 2021; K. Toyota et al., 2019). A population-level analysis will be required to confirm the observed patterns in our study.

Highly plastic traits tend to show strong maternal effect variance and little to no genetic variance, because they are more strongly influenced by the environment, including parental environment, and because additive genetic variance may be masked by high environmental variation (Donelan et al., 2020). In our study, the average fitness of the genotypes increased in the second generation, but it declined again in the third generation, indicating transient positive

maternal effects. Transient positive maternal effects have been observed in transgenerational studies of *D. magna* exposed to gamma radiation (Parisot, Bourdineaud, Plaire, Adam-Guillermin, & Alonzo, 2015). Conversely, a persistent positive maternal effect has been observed in transgenerational studies on photoperiod length (K. Toyota et al., 2019) and endocrine disruptors (Clubbs & Brooks, 2007; Tanaka & Nakanishi, 2002). Positive maternal effect is experienced when the offspring environment perfectly matches the maternal one. In human-altered environments, such as the one linked to chemical run-off, it may be more difficult for the parental generation to detect and correctly identify novel environmental conditions that lack historical context or that increase environmental variability. In these conditions the parents may fail to respond because they lack appropriate cue-response systems (Burgess, Marshall, & 31., 2014). The transient maternal effects observed in our study could be explained by epigenetic mechanisms, as the lower diversity at epigenetic regulation genes (Histone deacetylase family and the Elongator complex protein 1) in experienced genotypes suggest. Previous epigenetic studies on cross-generation exposures of *Daphnia* to gamma radiation, identified small but significant differential methylation that could explain transgenerational transient effects in fitness (Parisot et al., 2015; Trijau et al., 2018). However, given the low genome-wide methylation in *Daphnia*, it was not possible to unequivocally link fitness and epigenetic changes (Trijau et al., 2018). Cross-generational transient fitness effects can be also explained by compensatory mechanisms, expressed through trade-offs among life history traits (M. Cuenca - Cambronero et al., 2021).

3.5.2. Higher Fitness in Naïve Genotypes is Underpinned by Higher Diversity in Detoxification

Our findings reject the hypothesis that 'experienced genotypes' have an evolutionary advantage in presence of novel chemical stress. In our seminal study on the *Daphnia* population from Lake Ring, the experienced genotypes showed a comparatively higher fitness when exposed to the same chemicals recorded in the historical environment, even if this was dependent on the severity of the stress (M. Cuenca - Cambronero et al., 2018). The limitation of our study is in the small number of clones used, which may be not representative of the local population genetic diversity, therefore providing a qualitative rather than quantitative support to our hypothesis testing. However, if the patterns observed here are validated at population level, the results of our study and of past studies on the Lake Ring *Daphnia* population suggest that the acquired tolerance to chemical stress may be evolutionarily advantageous to recurring but not novel chemical stress. It is noteworthy that one of the two naïve genotypes showed the smallest trade-offs and an overall highest fitness (LRII 36_1) when exposed to novel chemical stress. This may be explained by this genotype being resurrected from a semi-pristine environment whereas the naïve genotype LRV12.3 was historically exposed to other stressors (e.g. eutrophication). It has been shown that multiple stressors linked to anthropogenic activities can influence how organisms adapt and evolve, with evolutionary mechanisms underpinning multiple stress response being often synergistic (M. Cuenca - Cambronero et al., 2021; Jackson, Loewen, Vinebrooke, & Chimimba, 2016; Orr, Luijckx, Arnoldi, Jackson, & Piggott, 2021).

Lower tolerance to novel chemical stress was associated with reduced genome-wide genetic diversity in the modern genotype (LRV_1). These patterns suggest that genetic erosion occurred as result of multi decadal exposure to chemical stress (Diez-Del-Molino, Sanchez-Barreiro, Barnes, Gilbert, & Dalen, 2018). The theory that genetic erosion occurred in Lake

Ring has to be validated through population-level analysis of genome-wide variation. However, significant decline in genetic diversity in the most modern genotype (LRV0_1), which was exposed to chemical pollution for 10-15 years/sexual generations (starting from the 1980s and accounting for a sexual generation per year in the *Daphnia* population), agrees with experimental studies on genetic erosion, showing decline in genetic diversity following >12 generations of exposure to chemicals (e.g. (Nowak et al., 2009). Our findings on pervasive plasticity both within and between generations, align with the hypothesis of genetic erosion. Past studies have shown that plasticity can be maintained in the face of genetic erosion, but it comes with fitness costs (Luquet et al., 2011) and with reduced tolerance to environmental stress (Bijlsma & Loeschcke, 2012).

Genes significantly divergent between naïve and experienced genotypes showed lower diversity in experienced genotypes. These genes were enriched for detoxification, catabolism and endocrine processes, as well as for embryonic development and epigenetic regulation. Fat and sugar metabolism pathways were divergent between naïve and experienced genotypes. These functional differences likely explain the lower overall fitness and higher mortality of the experienced genotypes in the exposures experiment. Some of the gene domains significantly enriched in *Daphnia* were conserved across the Tree of Life and included genotoxic stress response and detoxification functions, immunity genes and genes involved in the nervous system functionality including the neurotransmitter acetylcholine. Although conservation of function does not imply identical biological outcomes, our results suggest that these pathways may be target of toxicity in other species. These discoveries can inform (eco) toxicology assessments of persistent chemicals.

Overall, our study shows that exposure to chemical stress in *Daphnia* may impose costs associated with susceptibility to novel chemical stress, potentially compromising the resilience

and adaptation to future environmental changes. Susceptibility may be influenced by the combined effect of other stressors interacting with chemicals, with potentially more severe effects. The impact on the keystone grazer *Daphnia* has important implications for aquatic food webs, given its central role in lentic freshwater environments worldwide. Functional pathways conserved across species and putatively disrupted by prolonged chemical exposure included detoxification, neuronal functions and metabolism. These pathways are potential targets in other species, including humans.

3.6. Acknowledgement

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3.7. Data Accessibility

The genome sequence data can be found at BioProject ID PRJNA727483 in the NCBI repository. The fitness-linked life history traits can be found in the dryad database at DOI doi:10.5061/dryad.4xgxd2591

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Author Contributions

MA performed the experiments. MA and JZ performed statistical analyses on fitness-linked life history traits. VD and AC performed genomics and functional analysis. LO conceived the study, coordinated data analysis and writing. All authors contributed to the manuscript writing.

Chapter 3:

Exposure to persistent chemicals alters gut-microbiota diversity and composition in *Daphnia magna*

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4.1. Abstract

Persistent chemicals are of growing concern for their bioaccumulative and toxic properties, even when occurring below their regulatory approved threshold. Their occurrence in freshwater environments is a cause of concern for species' survival and fitness. Growing evidence shows that dysbiosis, the alteration of the gut microbiota, has a role in modulating an organism's response to environmental insults, including persistent chemicals. However, most toxicity studies investigating the role of the microbiome in modulating toxicity responses use dose levels that are rarely encountered in the environment. We study the impact of environmentally relevant concentrations of PFOS, atrazine, diclofenac, and arsenic on the gut microbiota of *Daphnia magna*. We assess their impact on the *Daphnia* microbiota both as individual chemicals and as mixtures found in wastewater. Our preliminary analyses show gut microbiota dysbiosis even at approved regulatory thresholds for these chemicals.

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4.2. Introduction

Many chemicals (e.g., industrial chemicals, pharmaceuticals, and pesticides) used in domestic and industrial processes end up in the environment (Johnson, Jin, Nakada, & Sumpter, 2020; Khalil et al., 2022; Scheringer, 2009) and have documented adverse effects on both target and non-target species [(Evariste et al., 2019) (e.g. fish (Jin et al., 2017; Kan, Zhao, Zhang, Ren, & Gao, 2015), Daphnia (Antonio Suppa et al., 2020), tadpole (Knutie, Gabor, Kohl, & Rohr, 2018)]. As persistent chemicals survive wastewater treatments, they end up in lakes and rivers, making freshwater organisms particularly vulnerable to chemical exposure (Bilal, Mehmood, Rasheed, & Iqbal, 2020; Bukola, Zaid, Olalekan, & Falilu, 2015; Eggen, Hollender, Joss, Schärer, & Stamm, 2014). Whereas individual chemicals found in surface waters may be below regulatory thresholds, their unintentional mixture in the environment can lead to toxicity and hazard ((Abdullahi, Li, et al., 2022).

Traditionally, environmental toxicity is quantified by exposing algae, zooplankton, and fish embryos to individual chemicals at doses they rarely encounter in the natural environment and by extrapolating the findings in these surrogate models to other species (Schuijt, Peng, van den Berg, Dingemans, & Van den Brink, 2021). The number of whole animal toxicity studies is continuously growing, providing important insights into non-target species' response to chemical pollution (Houwenhuyse, Stoks, Mukherjee, & Decaestecker, 2021; Nichols & Davenport, 2021). However, the role of the gut microbiota in exposure toxicity is not well understood (Bestion et al., 2017; Colin et al., 2022; X.-D. Li et al., 2021; Mu et al., 2018), particularly after the biotransformation of xenobiotics (Claus, Guillou, & Ellero-Simatos, 2016). In vertebrates, dysbiosis (the imbalance among strains/genera of microbes in the gut) has been associated with health conditions, including inflammatory bowel disease, metabolic disorders, and obesity (Schippa & Conte, 2014). In invertebrates, the microbiota is essential to animals' survival and fitness (Baldassarre, Ying, Reitzel, Franzenburg, & Fraune, 2022;

Gorokhova et al., 2015; Y. Ma et al., 2023). Recent studies have shown that exposure of non-target species to ecologically relevant concentrations of chemicals induces significant fitness costs and perturbs molecular pathways with impact on (e.g., mobility, reproduction, (*Daphnia*) (Furuhagen, Fuchs, Lundström Belleza, Breitholtz, & Gorokhova, 2014), altered metabolism and development (*Danio rerio*) (Barros et al., 2022; Ping et al., 2022), cardiotox, (J. Lu et al., 2022) and neurotox effects (Elizalde-Velázquez et al., 2022). Also dysbiosis in gut microbiota have been linked to functional alterations (e.g., intestinal inflammation (Matsuoka & Kanai, 2015; Morgan et al., 2012), hepatic inflammation (Qin et al., 2014), neurological function (Rogers et al., 2016), glucose metabolism (Lippert et al., 2017).

For example, heavy metals have been linked to dysregulation and fitness cost (P. Chen, Miah, & Aschner, 2016; Jan et al., 2015; Sevim, Doğan, & Comakli, 2020). Recent studies showed the link between dysbiosis and host fitness as a result of exposure to chemicals, resulting in e.g., reduced growth, reduced antioxidant enzyme activity, alteration in the activity of hemato-biochemical parameters (Zhai et al., 2017); decreased glycolysis and lipid metabolism (J. Xia et al., 2018). Exposure to heavy metals (e.g., arsenic) have been shown to induce oxidative stress and alter detoxification metabolism in animal models, specifically mice (Coryell, McAlpine, Pinkham, McDermott, & Walk, 2018; K. Lu et al., 2014). Exposure to non-steroidal anti-inflammatory drugs of *Procambarus clarkia* to 10 mg/L of diclofenac induced alterations in gut microbiota resulting in alterations molecular functions (Zhang et al., 2022).

Recently, perfluorinated alkyl substances (PFAS) have been high on the regulatory agenda because of their highly bioaccumulative potential and persistence (Y. Du et al., 2009; J. Giesy & K. Kannan, 2002; Jonas Margot et al., 2015; Yeung et al., 2006). According to recent studies, they can induce dysbiosis in the gut microbiome community of turtles (Beale et al., 2022), humans (Thompson et al., 2022), and fish (L. Chen et al., 2018) resulting in in the inducement of intestinal inflammation and stress.

Daphnia magna plays a significant role in freshwater food webs (Dieter Ebert, 2022). Recent studies have proven that changes in the gut microbiota of *Daphnia*, like those in other biological systems, significantly affect the organism's overall health (Callens et al., 2016; Callens, Watanabe, Kato, Miura, & Decaestecker, 2018; Sison-Mangus, Mushegian, & Ebert, 2015). It has also been demonstrated that the interplay between the host's genetic makeup and environmental factors can have a significant impact on the microorganisms inhabiting the host with long-term transgenerational effect on offspring (Benson et al., 2010; Campbell et al., 2012). Recent studies showed that exposure to the herbicide Roundup below regulatory thresholds can alter the functionality of the microbiome with effect on metabolism and homeostasis (Antonio Suppa et al., 2020).

Here, we quantify the impact of PFOS, atrazine, diclofenac, arsenic, and chemical mixtures on the gut microbiota of *Daphnia magna*. We investigate the effect of these chemicals on four genotypes with different history of exposure as described in **Chapter 2**, allowing us to infer whether evolution of tolerance to chemicals can be mediated by the microbiome.

4.3 Methodology

4.3.1 Exposure of *D.magna* genotypes

Four genotypes of *Daphnia magna* were resurrected from a sedimentary archive with a well-documented history of anthropogenic impact (Cuenca Cambronero, Marshall, et al., 2018b). The sedimentary archive was pristine from the early 1900s to the late 1940s, but then, it experienced eutrophication in the late 1950s due to sewage inflow. From the 1980s to the late 1990s, the lake received biocides run-off because of increased agricultural land use in the area surrounding the lake. The lake partially recovered in modern times (> 2000) (Cuenca Cambronero, Marshall, et al., 2018b). Between 1970s to 1975s, the lake begins to experience higher exposure to chemicals. With the understanding that the resident *Daphnia magna*

population have probably been exposed to multiple stressors in the past. In this study, our focus is on exposure to chemical pollutants to study the impacts of persistent chemicals and their mixture on the microbiota of the four genotypes. Two of these genotypes (LRII36_1 AND LRV12_3) did not experience historical chemical exposure, whereas LRV8.5_3 and LRV0_1 were historically exposed to chemical pollution (see also **Chapter 2**).

Triplicates of the four genotypes were exposed to PFOS (70ng/L; CAS:2795-39-3), atrazine (0.2 mg/L; CAS:1912-24-9), diclofenac (2 mg/L; (CAS:15307-79-6) and arsenic (1000 µg/L; CAS: 7784-46-5). The genotypes were also exposed to effluent wastewater in which chemicals were measured prior to exposure (total number of exposures was 288). Prior to the exposures, clonal replicates of *D. magna* genotypes were cultured and maintained for two clonal generations in common garden conditions: 20 ± 2 ^{0}C, 16:8 light: dark photoperiod and fed daily with 0.8 mg C/L of *C. vulgaris*, following methods described in (Abdullahi, Zhou, et al., 2022a; Cuenca Cambronero, Marshall, et al., 2018a) to reduce effect from parental exposure. After the cultures have been maintained for two generations in these conditions, 24-48hr. old juveniles from either the second or third brood, were exposed to the individual chemicals and wastewater (Fig. 1)**.** Chemical-free water was used as control for individual chemical exposures. The secondary treated wastewater was sourced from the Finham wastewater treatment plant managed by Severn Trent Water, (Coventry, UK). The four genotypes were exposed to this effluent water over a period of three days 20 ^{0}C; 16:8hr. light: dark photoperiod, fed daily with 0.8 mg C/L of *C. vulgaris*. Every 24hr the *Daphnia* was flash frozen for the analysis of gut microbiota composition (Fig. 1).

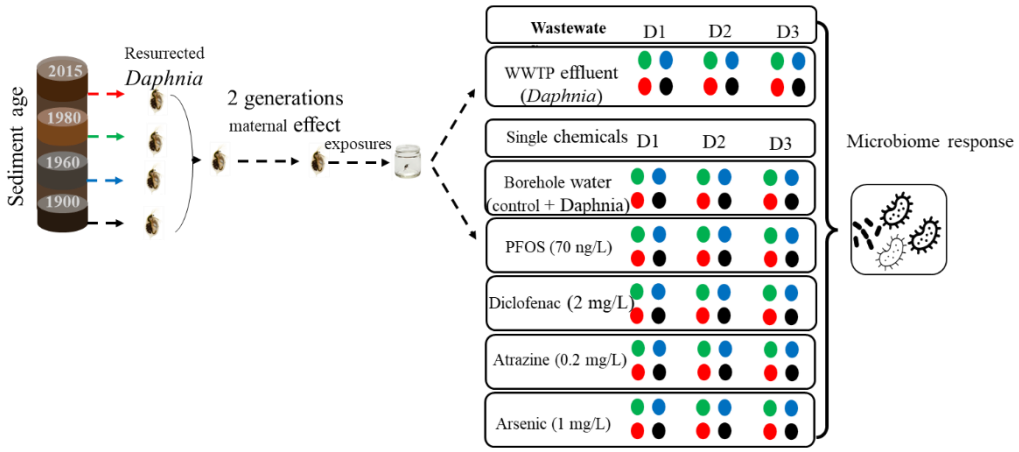

Fig.1. Experimental design. *Daphnia magna* isoclonal lines were obtained from four different genotypes, all of which were revived from dormant embryos. These genotypes were then exposed in triplicate to various substances, including wastewater effluent, PFOS (70 ng/L), diclofenac (2 mg/L), atrazine (0.2 mg/L), arsenic (1 mg/L), and a control condition (borehole water and Daphnia). Prior to exposure, the genotypes were maintained for at least two generations at a temperature of 20 ±2 oC and with a 16-8 hour photoperiod to reduce the interference of maternal effects. The gut microbiota composition was measured for each biological replicate, genotype, day, and treatment.

4.3.2. Daphnia Microbiome DNA extraction and sequencing

We extracted the gut microbiome DNA from the flash frozen tissue using the QiAMP DNA and Microbiome kit (QIAGEN) as described in the manufacturer's instruction. Extracted DNA was quantified and assessed for quality through 260/280 nm absorbance measures (Desjardins & Conklin, 2010) using a NanoDrop spectrophotometer instrument (Thermo Scientific). Amplicon libraries ('Paired end 250 bp') were obtained from the V1 region of the 16S rRNA gene (Chakravorty, Helb, Burday, Connell, & Alland, 2007), using a 2-step PCR protocol with 96 x 96 dual tag barcoding to facilitate multiplexing and to reduce cross-talk between samples in downstream analyses (Antonio Suppa et al., 2020). The first step of the PCR was conducted to target the V1 region of the 16S rRNA genes using the following primers:27f: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA;1492r:AGATCGGAAGAGCGTCG TGTAGGGAAAGAGTGT. Amplification of the target region was done in triplicate for each sample with the following cycling program: initial denaturation step for 10 s at 98 ºC, followed by 25 PCR cycles consisting of 30 s at 98 ºC, and 30 s at 64 ºC, followed by an extension step of 30 s at 72 ºC. Excess primer dimers were removed from both PCRs with High Prep PCR

magnetic beads (Auto Q Biosciences). The amplicons from PCR2 were quantitated using a 200 PRO plate reader (TECAN) using qubit dsDNA HS solution (Invitrogen). Standard curves were generated using known concentration standards on each plate to determine the sample concentration. The final PCR2 amplicons were mixed in equimolar quantities (at a final concentration of 12 pmol) using a biomek FXp liquid handling robot (Beckman Coulter). The final molarity of the pools was confirmed using a HS D1000 tapestation Screen tape (Agilent) prior to 250 bp paired-end sequencing on an illumina MiSeq platform aiming for 100,000 reads per sample and biological replicate.

4.3.3. Bioinformatics and statistical analysis

All Fastq raw reads with quality score (Q >25) were imported as qiime2 artifact using the import command in QIIME2-2022.2 (Bolyen et al., 2019). In other to obtain amplicon sequence variants (ASVs), the imported qiime2 artifacts were quality filtered, trimmed, joined using paired-end reads information using the plugin 'DADA2' in QIIME2 (Callahan et al., 2016). The following trimming parameters was used: trim-left-f&r = 20, trunc-len-f&r = 235. After these steps, a feature table per sample count and representative DNA sequences were obtained, and subsequently visualised to understand how many sequences were associated with each sample. Both the feature table and the table with the individual representative sequences were subsequently merged using the '**qiime feature-table merge**' and '**qiime feature-table merge-seqs**' plug-in in QIIME2. The q2-fragment-insertion (Janssen et al., 2018), was used to generate a rooted phylogenetic tree of the feature sequences. We conducted taxonomic classification of the gut-microbiota community in *D. magna* with QIIME2 Naïve Bayes q2-feature-classifier plug-in, which was trained against a preformatted reference reads from the SILVA database and the taxonomy files 'Silva 138, with 99% OTUSs of full length sequence" (Yilmaz et al., 2014). The pre-formatted reference files were downloaded from Data resources

— QIIME 2 2023.2.0 documentation. Taxonomic classification of the feature-table was then conducted using the 'qiime feature-classifier classify-sklearn' command, and then visualizing the resulting taxonomic assignments with the '**qiime metadata tabulate**' command. We removed ASVs present in only a single sample, and ASVs with a total abundance of less than 10 from the feature table with the command '**qiime feature-table filter-features**'. Subsequently, all ASVs that share the same taxonomic assignment were collapsed into a single feature to the genus level using the '**qiime taxa collapse**' command. For the downstream analysis, the filtered features-table as well as the feature table taxonomic information, sample metadata, phylogenetic tree were imported into R (R. D. C. Team, 2009) version (4.2.3), and merged into a phyloseq object using the qza_to_phyloseq function (McMurdie & Holmes, 2013).

All libraries were rarefied before quantifying α-diversity and β-diversity. Differences in α-diversity among treatments were assessed using the non-parametric Kruskal-Wallis chi-squared test in R version 4.2.3 (R. D. C. Team, 2009). The α-diversity for Shannon (diversity) and Simpson (evenness) are visualised in boxplot for the four chemical compounds including wastewater and control (borehole water medium). Subsequently, the results from the Kruskal-Wallis test was subjected to a post-hoc analyses with Dunn's Multiple comparison test using the 'dunnTest' function from the FSA package (Ogle, Wheeler, & Dinno, 2020) in R version 4.2.3. We tested whether the gut communities varied by treatment, genotype, day of exposure and their interaction terms using the adonis2 function in the vegan package (Oksanen et al., 2013) in R (999 permutations). The PERMANOVA (Anderson, 2005) model was fitted with the treatment, genotypes, and day as factors, while the calculated weighted-unifrac distance were fitted as a response variable, nesting genotypes within replicates. The post-hoc analysis on the PERMOVA analysis was calculated using the "pairwise.perm.manova" function in the R RVAiMemoire package (Hervé & Hervé, 2020). Taxonomic bar-plots were obtained using

the top 10 most abundant genera using the 'tax_glom' function in the R phyloseq package (McMurdie & Holmes, 2013). The taxonomic resolution setting was set to merge taxa less than the top 10 using a user defined function. 'Stacked bar plot' were obtained using the 'ggplot' function in the R ggplot2 package (Wickham, Chang, & Wickham, 2016).

4.4. Results

4.4.1. Alpha diversity

We used two diversity metrics namely Shannon (diversity), and Simpson (evenness), to determine whether the richness of the gut microbiota changed with exposure to individual chemicals and chemical mixtures in wastewater. A diversity of the microbiota (α-diversity) differed significantly between control and treatments (Shannon $p < 0.005$; Simpson $p < 0.02$) (Fig. 2). The post-hoc analysis revealed a significant difference in α-diversity between arsenic and diclofenac (Shannon; $p < 0.018178$, Simpson; $p < 0.04617$), atrazine and diclofenac (Shannon; $p < 0.027427$, Simpson, $p < 0.043928$; and diclofenac and wastewater (Simpson; $p < 0.021631$, Shannon; $p < 0.019477$).

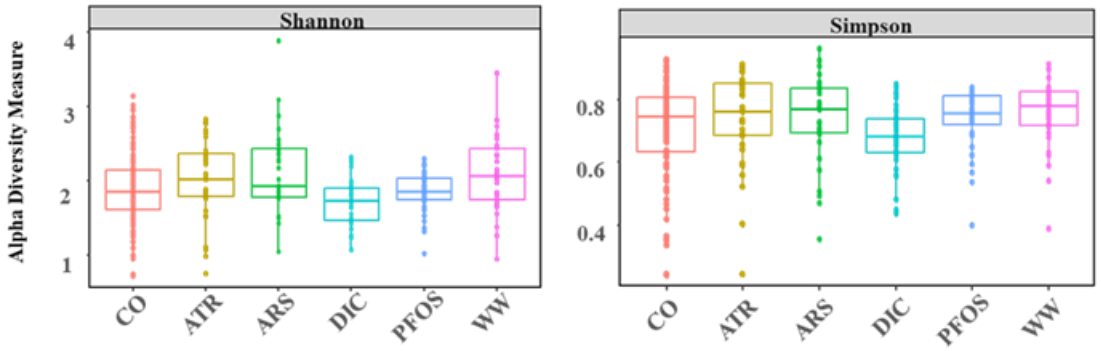

Fig. 2: Two alpha diversity indices, including Shannon diversity and Simpson evenness, were used to evaluate the within-treatment diversity for the four *D. magna* genotypes exposed to ecologically relevant concentrations of the following chemicals; PFOS (70ng/L), diclofenac; DIC (2mg/L), atrazine; ATR (0.2 mg/L), arsenic; ARS (1 000 µg/L) and wastewater; WW, including control borehole water; CO.

4.4.2. Beta diversity

The PERMANOVA analysis showed a significant response to treatment in all exposures, including individual chemicals and wastewater (Table 1). This means that the microbiota had a significantly different composition in the treated *Daphnia* than in the controls. The microbiota composition significantly differed among genotypes in all treatments, whereas varied by day only in PFOS and diclofenac among genotypes in all treatments (Table 1). A significant treatment x genotype effect was observed in Atrazine and Arsenic treatments (Table 1). A post hoc analysis on the weighted unifrac distance revealed that the genotype **LRV0_1: LRII36_1**(arsenic; p = 0.048, atrazine; p = 0.006, diclofenac; p = 0.04, PFOS; p = 0.006, wastewater; p = 0.01), **LRV12_3:LRV0_1** (arsenic; p = 0.006, atrazine; p = 0.006, diclofenac; p = 0.01, PFOS; p = 0.01, wastewater; p = 0.01), **LRV_8.5_3:LRV0_1** (arsenic; p = 0.006, atrazine; p = 0.006, diclofenac; p = 0.006; PFOS; p = 0.006, wastewater; p = 0.006) and **LRV8.5_3:LRII36_1** (diclofenac; p = 0.04), significantly diverged from each other, whereas **LRII36_1 : LRV12_3** did not.

4.4.3. Taxonomic composition plot

The result from the PERMANOVA analysis showed that exposure to atrazine had a significant effect on the gut microbiome composition of *Daphnia* genotypes, (Table 1; Treatment, Fig. 3a). Following exposure to atrazine, we observed that the microbiome composition was impacted by a significant ‘genotype’ term, and a ‘genotype x treatment’ interaction term (Table 1; Genotype, Treatment x Genotype). However, only a significant ‘genotype’ term was observed, ‘Treatment x Genotype’ did not affect the microbiome composition (Table 1).

Our study revealed that PFOS exposure had a significant impact on the microbiome composition of *Daphnia* genotypes (Table 1; Treatment, Fig. 3a). However, the 'Treatment x Genotype' interaction term did not significantly affect the microbiome composition (Table 1; Treatment x Genotype, Fig. 3a). Furthermore, our study revealed a significant influence of exposure duration on the microbiome composition in *Daphnia* genotypes, while the interaction term of 'Treatment x Day' did not (Table 1; Day, Fig. 3a). In this study, we observed that the microbiome was impacted by a significant 'genotype' effect, whereas 'Treatment x Genotype' interaction effect did not (Table 1; Treatment x Genotype).

1

Weighted-Unifrac		Atrazine			PFOS			Diclofenac			Arsenic			Wastewater	
Effect	**Df**	**R-squared**	***P-value***	**Df**	**R-squared**	***P-value***	**Df**	**R-squared**	***P-value***	**Df**	**R-squared**	***P-value***	**Df**	**R-squared**	***P-value***
Treatment	1	0.01811	**0.017***	1	0.03634	**0.001 *** **	1	0.07276	**0.001 *** **	1	0.04607	**0.001 *** **	1	0.11988	**0.001 *** **
Day	2	0.01691	0.106	2	0.0283	**0.02 ***	2	0.02284	**0.041 ***	2	0.01435	0.154	2	0.01385	0.163
Genotype	3	0.1356	**0.001 *** **	3	0.10984	**0.001 *** **	3	0.08429	**0.001 *** **	3	0.07208	**0.001 *** **	3	0.07132	**0.001 *** **
Treatment:Day	2	0.01	0.355	2	0.00596	0.713	2	0.00716	0.572	2	0.02585	**0.026 ***	2	0.00528	0.731
Treatment:Genotype	3	0.04203	**0.005*** **	3	0.01266	0.479	3	0.00766	0.806	3	0.0285	**0.046 ***	3	0.01593	0.314
Day:Genotype	6	0.0291	0.396	6	0.02919	0.413	6	0.0349	0.244	6	0.03657	0.201	6	0.01886	0.81
Treatment:Day:Genotype	6	0.01701	0.865	6	0.00908	0.998	6	0.01131	0.991	6	0.02057	0.765	6	0.01856	0.814

Table 1. PERMANOVA. Permutational Multivariate Analysis of Variance using Weighted-Unifrac distance metrics. We tested for the effect of treatment (Atrazine, PFOS, Diclofenac, Arsenic, and Wastewater), genotype, time of exposure (day), and their interaction terms. Significant effects are in bold.

Our study showed that exposure to diclofenac has a significant impact on the microbiome composition of the different *Daphnia* genotypes as confirmed in (Table 1; Treatment, Fig. 3b). However, the microbiome composition was not significantly affected by the 'Treatment x Genotype' interaction term (Table 1; Treatment x Genotype). Our study revealed a significant influence of exposure duration on the microbiome composition of the *Daphnia* genotypes while the interaction term of 'Treatment x Day' did not (Table 1; Day). In this study, we observed that the microbiome composition was significantly impacted with regards to the 'genotype' effect, whereas 'Treatment x Genotype' interaction term did not (Table 1; Treatment x Genotype).

The PERMANOVA analysis revealed that the exposure to arsenic had a significant impact on the gut microbiome composition of *Daphnia* genotypes, (Table 1; Treatment, Fig. 3a). Following exposure to arsenic, we observed that the microbiome composition was impacted by a significant 'genotype' term, and a 'genotype x treatment' interaction term (Table 1; Genotype, Treatment x Genotype, Fig. 3a). The result from the PERMANOVA showed that the microbiome composition was impacted by a significant 'Treatment x Day' interaction term following exposure to arsenic, but 'Day' did not affect the microbiome composition (Table 1; Treatment x Day, Fig. 3a).

The PERMANOVA analysis showed an impact of wastewater exposure on the gut microbiome composition in *Daphnia* genotypes, as confirmed by both distance metrics. (Table 1; Treatment, Fig. 4). We observed that the microbiome composition was impacted by a significant 'genotype' term, and a 'genotype x treatment' interaction term did not (Table 1; Genotype, Treatment x Genotype, Fig. 4).

As shown in (Fig. 3a, 3b), the dominant genera in the gut microbiota of the four *Daphnia* genotypes across all the treatments are *Limnohabitans* and *Acinetobacter*. We observed that

the genus *Emticia* completely disappeared in the 'naïve' genotype; LRV12-3 and the 'experienced' genotype; LRV8.5-3, while the genus *Chryseobacterium* disappeared in both the 'naïve': LR2-26-1, LRV12-3 and the 'experienced': LRV8.5-3, LRV0-1 genotypes after exposure to wastewater. Also, the genus *Hydrogenophaga* disappeared in one of the 'naïve' genotypes: LR2-36-1, and one of the experienced genotypes: LRV8.5_3, when exposed to wastewater, and in the 'naïve' genotype: LR2_36_1, and the 'experienced' genotype: LRV0_1, when exposed to diclofenac (Fig.3b). The genus *Flavobacterium* disappeared in only the 'experienced' genotype: LRV0_1, after exposure to diclofenac, PFOS, and atrazine (Fig.3a, 3b). Our results showed that the genus *Sphingobium*, and *Pseudomonas* were consistently decreasing in abundance among the four genotypes when exposed diclofenac (Fig. 3a, 3b)**.** The genus *Limnohabitans* showed a decreased in abundances across the duration of exposure in the four genotypes when exposed to diclofenac, PFOS, and atrazine (Fig. 3a, 3b).

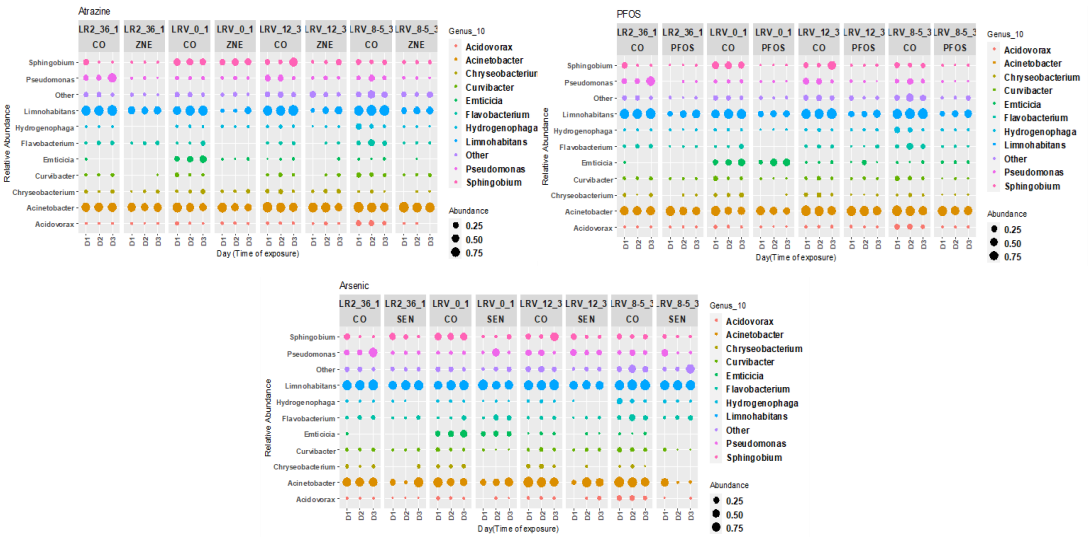

Fig. 3a. Gut microbiota after exposure to PFOS, atrazine, and arsenic- co: control, ZNE: atrazine, SEN: arsenic. The relative abundance of the top ten bacteria genus identified from exposure to treatment, as listed in Fig 1, are shown for the four *Daphnia* genotypes across biological replicates and days. The PERMANOVA statistics in Table 1 support this plot.

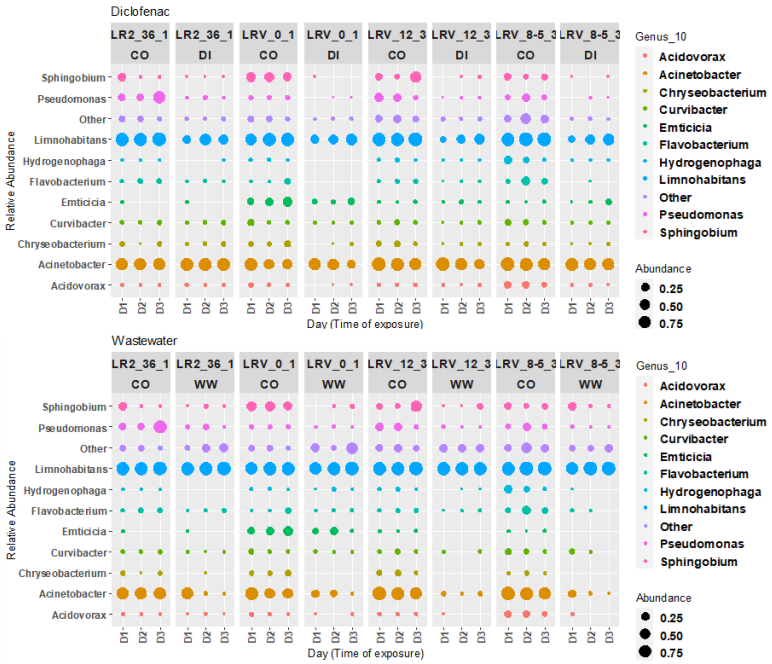

Fig. 3b. Gut microbiota after exposure to diclofenac, and wastewater- co: control, DI: diclofenac, ww: wastewater. The relative abundance of the top ten bacteria genus identified from exposure to treatment, as listed in Fig 1, are shown for the four *Daphnia* genotypes across biological replicates and days. The PERMANOVA statistics in Table 1 support this plot.

4.5. Discussion

Changes in the diversity of the gut-microbiome in Daphnia

We found that exposure of *Daphnia* to low doses of persistent chemicals and their mixtures affected the microbiota richness and composition. Diclofenac treatment showed the most severe impact on the gut microbiome with the highest reduction in **α**-diversity. This reduction was at the expense of *Sphingobium*, *Pseudomonas*, *Hydrogenophaga*, *Flavobacterium*, and *Limnohabitans* genera which were most impacted by this treatment. The increased diversity observed in wastewater treatment compared to other treatments was caused by the increased abundance of the unclassified genera (other). Alpha diversity (α-diversity) gives an overall summary of the structure of a community or ecosystem in terms of its species richness (observed), evenness (Simpson) or both (Shannon) (Willis, 2019). Alpha diversity is a very important functional biomarker for assessing both metabolic and gastrointestinal health (Plassais et al., 2021). Numerous studies have established a correlation between the reduction of alpha-diversity and various health conditions (Gupta et al., 2020), such as inflammatory

bowel disease (Opstelten et al., 2016), obesity and metabolic disorders (Dao & Clément, 2018; Le Chatelier et al., 2013). Diclofenac has been reported to cause numerous adverse effects (e.g. mortality and reproductive decline) in non-target species (vertebrates (Oaks et al., 2004), fleas and fish (Lee, Ji, Kho, Kim, & Choi, 2011)), particularly at a concentration higher than what is usually found in the environment. Previous research concluded that the gut microbiome is crucial for the survival and fitness of *Daphnia magna* (Sison-Mangus et al., 2015). We have shown in our previous study that exposure to diclofenac induces a significant plastic response on fecundity (Abdullahi, Zhou, et al., 2022b). In this study, alteration in the alpha diversity of the gut microbiome in the presence of diclofenac corroborates with a recent study reporting the toxic effect of diclofenac on freshwater crayfish (*Procambarus clarkii*) (Y. Zhang et al., 2021). From our findings, the Shannon and Simpson diversity of the wastewater treatment did not significantly differ with the control (Fig. 2). The observed significant difference in the microbiome α-diversity between diclofenac and wastewater was caused by the decrease in the abundance and disappearance of *Emticia*, *Chryseobacterium*, *Acinetobacter*, and *Acidovorax* genus in wastewater treatment. Wastewater effluents and their components mixtures affects the microbiota of freshwater species even at environmentally regulated threshold (Luan, Liu, Fang, Chu, & Xu, 2020; Millar, Surette, & Kidd, 2022). Previous studies have assessed the toxicological impact of wastewater effluent in the environment (Tamminen et al., 2022). Yet very few studies have investigated the impact of wastewater effluent on gut microbiome of the host (e.g., (Mehl et al., 2021)). In our study, it is possible that the decrease in the abundance of the following genera: *Emticia*, *Chryseobaterium*, *Acinetobacter* and *Acidovorax* of the gut microbiome of the *Daphnia* genotype following exposure to wastewater effluent arise from stress due to physiological response of the host (Stothart, Palme, & Newman, 2019), or altered diet (Mehl et al., 2021).

Changes in the gut microbiome composition

Changes in microbiota composition were genotype-specific (Table 1). The genotypes showing the largest adverse effect of individual chemicals on their microbiota composition were LVR0_1 and LRV8.5_3. This was induced by the disappearance of the *Sphingobium, Pseudomonas, Hydrogenophaga, and Flavobacterium* genus in LRV0_1 following exposure to diclofenac (Fig. 3b), the disappearance of *Emticia, Chryseobacterium, and Acidovorax* in LRV8.5-3, following exposure to arsenic (Fig. 3a). These genotypes have been historically exposed to chemical pollution (Fig.1). This suggest that the reduced tolerance to novel chemicals in 'experienced' genotypes is not only underpinned by host genetic defects involving detoxification, and catabolism ((Muhammad Abdullahi, Jiarui Zhou, Vignesh Dandhapani, Anurag Chaturvedi, & Luisa Orsini, 2022b), but also microbiome composition contribute to the fitness, reproduction and tolerance against novels stressors in *Daphnia* (Akbar et al., 2022). Also, the significant alteration in the bacteria genera observed in these genotypes at low dose exposure indicates that the painkiller drug (diclofenac) and (heavy metal) alters the gut microbiome. *Daphnia* genotypes LRII36_1 and LRV12_3 (naïve) showed the least change in microbiota composition and richness. This pattern is explained by the lower genetic diversity due to reduced genes for detoxification and metabolism in the 'experienced' genotypes compared to the 'naïve' genotype (see chapter 2) ((Abdullahi, Zhou, et al., 2022b). The microbiome plays a significant role in a host's response to external stressors, both directly and indirectly (Hall et al., 2018). At the same time, the host's actions can also affect the microbiome, which then indirectly impacts the host's physiological response and life-history traits (Spor, Koren, & Ley, 2011; Wong et al., 2015). The presence of *Flavobacterium*, *Limnohabitans*, and *Pseudomonas* bacteria genera in the *Daphnia* gut is a well-established fact (Akbar et al., 2020; Cooper & Cressler, 2020; Macke, Callens, De Meester, & Decaestecker, 2017). Additionally,

the host's genetic environment plays a significant role in shaping the gut microbiome of Daphnia (Macke et al., 2020; Sullam, Pichon, Schaer, & Ebert, 2018).

4.4. Conclusion

Our results show that exposure to individual chemicals and chemical mixtures causes gut-microbiome dysbiosis in *Daphnia* under realistic environmental conditions. Because *Daphnia* is a very important species in the food web of aquatic ecosystems and an ecosystem bioindicator organism, the impact on *Daphnia* may have a cascading effect on other organisms in freshwater. In addition to this indirect effect through the trophic chain, an analysis of pathway conservation across the Tree of Life may reveal if the same pathways are potential targets in other species. Due to time constraints, this analysis was not completed but it warrants exploring.

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AUTHOR CONTRIBUTIONS

M.A. performed the experiments. M.A. and S.B.B performed bioinformatics and statistical analyses. S.K. performed genomic library construction. L.O. conceived the study, coordinated data analysis and writing. All authors contribute to writing.

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Chapter 4:

Harnessing Water Fleas for Water Reclamation: A Nature-based Tertiary Wastewater Treatment Technology

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5.1. Abstract

Urbanisation, population growth, and climate change have put unprecedented pressure on water resources, leading to a global water crisis. Water reclamation is urgently needed for sustaining people's societal, economic, and environmental future. However, water reuse is unsafe unless persistent chemical pollutants, such as pharmaceuticals pesticides and industrial chemicals, are removed from reclaimed water. Whether wastewater effluent must meet clear discharge standards for nitrogen, phosphorus, organic matter, suspended solids, and pathogens removal of persistent chemicals is not routinely regulated. However, regulations on persistent chemical pollutants are emerging, together with higher awareness of the public for potential adverse effects of persistent chemicals on human and animal health. State-of-the-art technologies for the reduction of persistent chemical pollutants in wastewater typically involves high operational and energy costs and potentially generates toxic by-products (e.g., bromate from ozonation). Nature-based solutions are preferred to these technologies for their lower environmental impact. However, so far, bio-based tertiary wastewater treatments have been inefficient for industrial-scale applications. Moreover, they often demand significant financial investment and large infrastructure, undermining sustainability objectives. Here, we present a scalable, low-cost, low-carbon, and retrofittable nature-inspired solution that could be integrated into current wastewater treatment systems to remove persistent chemical pollutants. The technology uses the water flea *Daphnia* to non-selectively uptake and retain persistent chemical pollutants (pharmaceutical, pesticides and industrial chemicals). It provides an additional polishing step to the treatment of final wastewater effluent, producing higher quality effluent than current treatments, and meeting requirements to produce water appropriate for reuse in irrigation, industrial application,

and household use. By preventing persistent chemicals from entering waterways, this technology has the potential to maximise the shift to clean growth, enabling water reuse, reducing resource depletion and preventing environmental pollution.

Keywords: water reclamation, biotechnology, tertiary wastewater treatment, pharmaceuticals, pesticides, PFOS

5.2. Introduction

Urbanisation, population growth, unsustainable food production and climate change have put unprecedented pressure on water resources, leading to a global water crisis (Joseph, Ryu, Malano, George, & Sudheer, 2020; Mancosu, Snyder, Kyriakakis, & Spano, 2015; Mishra, Kumar, Saraswat, Chakraborty, & Gautam, 2021). Sustainable use of water resources including the reclamation of previously used water are needed for sustaining people's societal, economic and environmental future. However, persistent micropollutants in treated wastewater (pharmaceuticals, pesticides, and industrial chemicals), originating from domestic and industrial processes, escape conventional wastewater treatment and prevent its safe reuse (K'Oreje K et al., 2016; Rimayi, Odusanya, Weiss, de Boer, & Chimuka, 2018). Inefficient wastewater treatment contributes significantly to the high number of chemicals found in the environment (Naidu et al., 2021; T. Wang et al., 2014). In particular, wastewater effluent discharged into rivers is one of the main routes of distribution of chemical pollutants, as rivers are the main component of water reservoirs, irrigation and aquifer recharges. Through the use of surface water for these activities, micropollutants make their way to humans through the food chain and water supply, adversely affecting the health of million people every year (R. Fuller et al., 2022a). Contamination of water resources not only impacts human health, but also contributes to loss of biodiversity and the deterioration of ecosystem services worldwide (Backhaus, Snape, & Lazorchak, 2012; Cardinale et al., 2012).

Wastewater treatment only removes a small proportion of micropollutants (Blum et al., 2017; D. L. Sutherland & P. J. Ralph, 2019) through adsorption onto activated sludge (Tran, Reinhard, & Gin, 2018). Tertiary treatments designed to reduce micropollutants, such as ozonation and chlorination have high operational and energy costs, require large

infrastructure, and can generate toxic by-products (e.g., bromate from ozonation; organo-chlorine compounds from chlorination) (Jahan, Li, & Pagilla, 2021; X. F. Li & Mitch, 2018). Moreover, the removal efficiency of these tertiary treatment technologies is affected by the hydrophobicity and ionization characteristics of chemical pollutants (J. Ma, Dai, Chen, Khan, & Wang, 2018), meaning that the substrate determines the contaminants that can be removed. Often, tertiary treatment technologies must be combined to remove a wide range of chemical pollutants (Skouteris, Saroj, Melidis, Hai, & Ouki, 2015). Biological tertiary wastewater treatments have been advocated to manage wastewater sustainably, enabling water reuse and allowing the recovery of valuable resources such as nutrients and energy (Duque, Campo, Val Del Rio, & Amorim, 2021). However, these solutions are too slow for industrial-scale operations, requiring days rather than the needed hours to treat wastewater effluent, and require large infrastructure, undermining sustainability objectives (Wollmann et al., 2019).

We have pioneered and prototyped a scalable, low-cost, low-carbon, and retrofittable nature-inspired tertiary treatment technology for municipal wastewater. It uses *Daphnia* (waterfleas) to non-selectively remove different classes of chemical pollutants from secondary treated wastewater, improving wastewater effluent that meets current discharge standards for pathogens and other inorganics (e.g., nitrogen and phosphorous). *Daphnia* populations are retrofitted in containment devices within secondary clarifiers to polish effluent before final discharge. Once installed, the technology is largely self-sustaining, thanks to the ability of this species to reproduce clonally. *Daphnia*'s exceptionally long dormancy (hundreds of years) enables the resurrection of dormant populations that have experienced different historical pollution pressures. Our pioneering research on the waterflea *Daphnia* enabled us to leverage these properties and source

strains with different tolerance to chemical pollutants to be employed in the technology development (Abdullahi, Li, et al., 2022b; Abdullahi, Zhou, et al., 2022b). In a proof-of-concept study, we benchmarked the *Daphnia*'s removal of 16 pharmaceuticals from wastewater against other biological agents, i.e., algae and bacteria in laboratory conditions (Abdullahi, Li, et al., 2022b). Here, we show removal efficiency of an industrial chemical (e.g., Perfluorooctanesulfonic acid (PFOS), a pesticide, a heavy metal and a pharmaceutical by *Daphnia* strains, both as individual chemicals and as mixtures in real wastewater. We show that chemicals taken up by *Daphnia* are not released back into the water, showing bioaccumulation of heavy metals in its tissue. We demonstrate the technology at prototype scale, using volumes of water comparable to the wastewater generated by a single household for applications in municipal wastewater treatment. The technology has the potential to maximise the shift to clean growth, enabling water reuse, reducing resource depletion and environmental pollution.

5.3. Materials and Methods

5.3.1. Exposure of Daphnia strains to Chemical pollutants in Controlled Laboratory Conditions

Daphnia *magna* strains used for the technology development were revived (resurrected) from multiple sedimentary archives with a well-documented history of anthropogenic change. To protect commercially sensitive information, the proprietary strains are only labelled with DM (*Daphnia magna*) and the approximate age of the strain. For this study, we used four *Daphnia* strains of different ages, spanning a century: DM1900; DM1960; DM1980; and DM2015.

Our first action was to assess chemical removal efficiency of different chemical pollutants by strains of *Daphnia magna* with different histories of exposure to chemicals. For this assessment, we exposed four strains, in triplicates over 3 days, to borehole water spiked with known concentrations of an industrial chemical (PFOS: 70ng/L; CAS:2795-39-3), a biocide (atrazine:0.2 mg/L; CAS:1912-24-9), a pharmaceutical (diclofenac: 2 mg/L; (CAS:15307-79-6) and a heavy metal (arsenic 1 mg/L; CAS: 7784-46-5), as well as to secondary treated wastewater. The chosen concentrations of individual chemicals were identified from literature research to be environmentally relevant and observed in surface and/or wastewater. The borehole water is collected from a deep aquifer well and its chemical properties checked quarterly. It has been stable for the past 10 years and it is used as growth medium for our cultures and exposure experiments.

Prior to the exposures, clonal replicates of the four *Daphnia* strains were maintained for at least two clonal generations in common garden conditions 20 ± 2^0C, 16:8hr light: dark photoperiod and fed daily with 0.8 mg C/L of *Chlorella vulgaris* - to control for maternal effect following (Abdullahi, Zhou, et al., 2022b; M. Cuenca - Cambronero et al., 2018). Following this acclimation phase, 24 h-old juveniles from the third or following broods were exposed to known concentrations of individual chemicals and to wastewater (Fig. 1). The exposures were conducted over three days to assess whether the chemicals were excreted or retained by *Daphnia*. This was a critical step for the use of *Daphnia* as biological filter. The *Daphnia* density (10 *Daphnia* in 100 ml of borehole water) in each experimental exposure and biological replicate was chosen to ensure that the spiked borehole water or wastewater were filtered completely every 24 h – this was based on previously tests showing the filtering capacity of *Daphnia magna* (Pau et al., 2013; T. Serra, Muller, & Colomer, 2019).

A total of 216 exposures were completed to quantify the removal efficiency of the four classes of chemicals by the four genotypes of *Daphnia* (Fig. 1). Chemical-free water was used as control for individual chemical exposure, whereas wastewater without *Daphnia* was used as reference for exposure to real wastewater. The four chemicals used in individual exposures were quantified in wastewater before and after exposure to *Daphnia*. The secondary treated wastewater was sourced from the Finham wastewater treatment plant managed by Severn Trent Water, serving a population equivalent of 430,470 (Coventry, UK). All exposures were conducted at 20 ± 2 ^{0}C, 16:8hr light: dark photoperiod. *Daphnia* in the individual chemical exposures were fed *ad libitum* with 0.8 mg Carbon/L of algal suspension (*Chlorella vulgaris*). *Daphnia* in wastewater exposures fed on organics already present in wastewater.

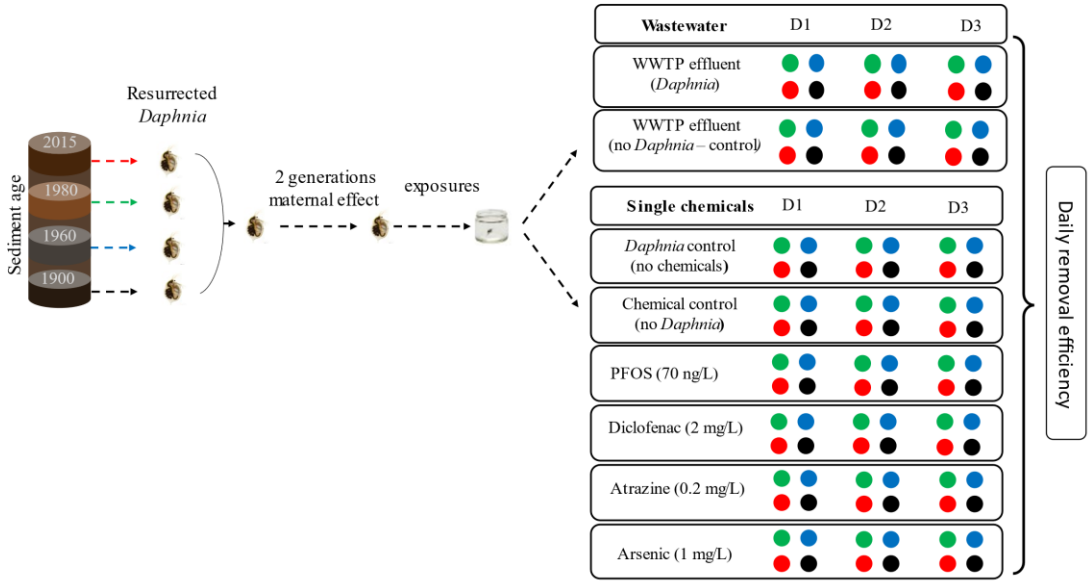


Fig. 1. Experimental design for laboratory exposures. Resurrected *Daphnia magna* genotypes from lake sediment deposits were maintained in controlled laboratory conditions (20 ± 2 ^{0}C, 16:8hr light: dark photoperiod and fed daily with 0.8 mg C/L of *C. vulgaris*) for two generations before exposure to single chemicals and wastewater secondary treated effluent. Two generations in these conditions were used to control for material effect. Single chemicals were quantified following exposure of four *D. magna* genotypes over 3 days to PFOS - 70ng/L; Diclofenac - 2mg/L; Atrazine - 0.2mg/L and Arsenic - 1mg/L.The same four *D. magna* genotypes were also exposed to wastewater effluent and the same four chemicals were quantified both in the starting effluent and after exposure to *Daphnia*.

5.3.2. Quantifying Chemicals in Laboratory Experiments

Following exposures, individual chemical concentrations were quantified in the growth medium (borehole water spiked with chemicals and wastewater) using mass spectrometry following (Abdallah et al., 2019; Harrad et al., 2019) as explained in the Supplemental Methods (Appendix A). The concentration of PFOS, atrazine, and diclofenac, both in the medium from individual chemical exposures and in wastewater were quantitated with ultraperformance liquid chromatography (UPLC) coupled with high-resolution mass spectrometry following (Abdallah et al., 2019; Abdullahi, Li, et al., 2022b). Arsenic concentration in both water and *Daphnia* tissue was quantified by using a Nexion 300x ICP-MS (Perkin Elmer, Seer Green, U.K) fitting with a cyclonic spray chamber. Calibration curves spanning 1-20 ppb for borehole water and 1-10 ppb for *Daphnia* were constructed in DI water. For quantification of arsenic in the *Daphnia* tissue 50 ppb germanium was infused as an internal standard. Ideally, all chemicals would have been quantified in the *Daphnia* tissue to understand mechanisms of chemical uptate and transformation. However, understanding these mechanisms is not needed for the technological application. Moreover, time consuming protocol optimisation is required.

5.3.3. Removal Efficiency at Laboratory Scale

The removal efficiency of the chemicals from the growth medium (borehole water spiked with known chemical concentrations and wastewater in which chemicals were quantified before the exposures) by *Daphnia* was quantified as explained in Appendix A (section 1.1.) and calculated as [starting concentration − final concentration /starting concentration] ×100. On these quantitation we applied an analysis of variance (ANOVA)

with the Satterthwaite's method (lmerTest package; (Kuznetsova, Brockhoff, & Christensen, 2017)) to assess if removal efficiency varied by genotype, day and their interaction term, nesting clonal replicates within genotype. Before applying the ANOVA analysis, the data were tested to meet the parametric assumption of the model by plotting the model residual vs fitted values (Q-Q plots) with the "car" package (Fox & Weisberg, 2018; A. F. Zuur, E. N. Ieno, & C.S. Elphick, 2010).The removal efficiency was plotted using the ggplot2 package (Gentleman, Hornik, & Parmigiani, 2009), with the standard error calculated using the 'summarySE' function in Rmisc package in R (R. C. Team, 2022).

5.3.4. Chemical Pollutants Removal at Prototype Scale

Having identified the strains with the highest removal efficiency in the laboratory exposures, we tested removal efficiency of a population of these strains in outdoor conditions for four weeks. We built a closed environment of 300L (hereunder 'prototype'), holding borehole water spiked with diclofenac at the same concentration used in laboratory exposures (2 mg/L). Diclofenac is among the most common chemicals found in effluent wastewater. The amount of spiked borehole water was equivalent to wastewater produced daily by a single household of 3.5 people. Diclofenac was spiked at the beginning of each week and removal efficiency measured daily, 5 times a week for 4 weeks, on 3 replicated samples of 100ml randomly sampled from the top 1m of water. Removal efficiency was quantitated with ultraperformance liquid chromatography coupled with high-resolution mass spectrometry as described above, using optimised methods (Abdallah et al., 2019).

Dissolved oxygen and water temperature were recorded daily in the prototype using a HANNA sensor (H19146). The prototype was protected from the rain with a tarpaulin and topped up every other week to compensate for evaporation. This strategy was applied to mimic a real-world open system (secondary clarifier) in which water levels are constant and in which the technology will eventually be retrofitted. The average temperature of the tank was regulated by a thermostat. However, fluctuations occurred due to the prototype being outdoors, reflecting realistic operational conditions. An algal suspension of *Chlorella vulgaris* was added daily to the prototype at a concentration comparable to the laboratory conditions described above. These conditions mimic a constant flow of organic matter entering the secondary clarifiers in a real-word wastewater treatment process. To mimic the flow of a secondary treatment tank in a wastewater treatment, the prototype was aerated using a small submersible pump that recirculated the water at a flow rate of 1900 L/hr through a diffused nozzle maintaining laminar flow conditions at a macroscopic scale. These conditions were consistent with secondary clarifiers in wastewater treatment works. Diclofenac removal efficiency was calculated as in the laboratory experiments above. We tested whether removal efficiency significantly differed among weeks by using the lmer Test package (Kuznetsova et al., 2017), nesting days within weeks.

5.3.5. Engineering a Self-Sustaining Technology Prototype

For future installations in wastewater treatment works, we engineered and tested a system comprising of a live and a back-up environment. The live environment consists of filtration vessels retrofitted within secondary clarifiers for effluent polishing, whether the back-up environment consists of tandem bioreactors comprising a feedstock (algae *Chlorella vulgaris* CCAP 211/11b) growing chamber linked to algal medium (BBM;

Appendix B; Table S1), and a *Daphnia* chamber (Appendix B; Fig. S1). This system produces *Daphnia* used to seed the secondary clarifiers and as a back up system in case of shock events.

The vessels in the live environment are interconnected by a manifold system, and float in the top metre of the prototype. These vessels allow for the movement of water through porous meshes, sustaining *Daphnia* populations, and the collection of biowaste (dead *Daphnia* at the end of their life cycle). The latter happens via a system of interconnected valves that isolate the individual devices while the biowaste is funnelled into further treatment. Possible treatments of his biomass are discussed in section 4.3.

Three vessels of 20L capacity each were introduced in a 300L outdoor prototype and seeded with *Daphnia* to assess removal efficiency at prototype scale. As *Daphnia* is self-sustaining via clonal reproduction, the *Daphnia* population density is expected to increase exponentially until it reaches carrying capacity (Bruijning, ten Berge, & Jongejans, 2018). Following the *Daphnia* population introduction in the prototype, the number of *Daphnia* was allowed to reach the same density of the laboratory exposures described in section 2.1 before removal efficiency of diclofenac was quantified. During the experiment, the *Daphnia* density was monitored in each vessel at the beginning and the end of each week over four weeks by collecting triplicate 100ml-samples. Daphnia individuals were counted in the sampled volume and the density in the prototype approximated from these counts.

The controlled semi-automated back-up environment is maintained in controlled lighting, temperature and fluid transfer as described in the Supporting Methods (Appendix A; section 1.2). To understand the impact of shock events on the *Daphnia* population

dynamics with consequences on removal efficiency in real world environments, we developed a model to capture the dynamics of both juvenile *Daphnia* (*J(t)*) and adult *Daphnia* (*A(t)*) that uses delay differential equations to capture the maturation time (τ) of juveniles. This model is described in the Supplemental Methods (Appendix A, section 1.3).

5.3.6. Techno-Economic Assessment of the Daphnia-based Technology

The *Daphnia*-based technology performance was benchmarked against a range of existing tertiary wastewater treatment technologies, including established and emerging technologies through a desk techno-economic analysis. The competitor technologies assessed were ultraviolet irradiation, ozonation, chlorination, activated carbon, and multi-media filters. The operating and performance parameters used for this analysis, subject to availability, were: i) contaminant removal, capital expenditure (CAPEX), ii) operational expenditure (OPEX), iii) by-products generation, iv) energy used, and v) carbon footprint. The same criteria were used to benchmark the technology against emerging biological tertiary wastewater treatment technologies including DRAM microbial technology, membrane biofilm reactors (MBfR), rotating biological contactors (RBC), phytoremediation, packed bed reactor (PBR), and photobioreactors.

5.4. Results

5.4.1. Exposure of Daphnia strains to Chemical pollutants in Controlled Laboratory Conditions

We quantified removal efficiency of the four *Daphnia* strains in water spiked with known concentrations of the four chemical pollutants belonging to four chemical classes. The removal efficiency of PFOS and diclofenac in individual chemical exposures varied

significantly by *Daphnia* genotype, whereas the four genotypes did not significantly differ in their removal efficiency of arsenic and atrazine (Table 1A; Fig. 2). The average removal efficiency across the three days of exposure was 90% for Diclofenac, 50% for PFOS, 59% for atrazine and 60% for arsenic (Fig. 2). The historical *Daphnia* strain DM1900 showed the highest removal efficiency for diclofenac (95%) and PFOS (65%) (Fig. 2). The removal of the four individual chemicals did not significantly differ across the three days, suggesting that once taken up by *Daphnia,* the chemical pollutants were not excreted back into the water (Table 1A).

We quantified the removal efficiency of the four strains in wastewater. Whereas PFOS and diclofenac were found in the wastewater sampled in the UK, atrazine and arsenic were not detected. This was not known at the beginning of the experiment. PFOS in the sampled wastewater was found at an average concentration of 35ng/L, whereas diclofenac was found at an average concentration of 530µg/L. The removal efficiency of PFOS from wastewater did not significantly differ among genotypes. However, the genotypes removal efficiency varied significantly by day, with some genotypes (e.g., DM1980)

Table 1. ANOVA. An analysis of variance (ANOVA) is used to test whether the removal efficiency of individual chemicals spiked in borehole water (A) and of the same chemicals from wastewater (B) varied by genotype, day and their interaction term. Clonal replicates were nested with genotype. Atrazine and Arsenic were not found in the wastewater sampled from the Finham treatment plant (UK).

	A. Individual compounds									B. Wastewater			
		PFOS		Diclofenac		Atrazine		Arsenic		PFOS		Diclofenac	
Effect	DF	F	*Pr*	F	*Pr*	F	*Pr*	F	*Pr*	F	*Pr*	F	*Pr*
Genotype	3	3.81	**0.04**	3.87	**0.04**	2.10	0.16	0.81	0.51	3.13	0.07	327.08	**<0.001**
Day	2	0.10	0.91	1.65	0.23	3.49	0.07	0.48	0.63	5.13	**0.02**	84.60	**<0.001**
Genotype:Day	6	2.60	0.08	1.00	0.47	1.31	0.33	1.58	0.23	7.28	**0.002**	10.05	**0.002**

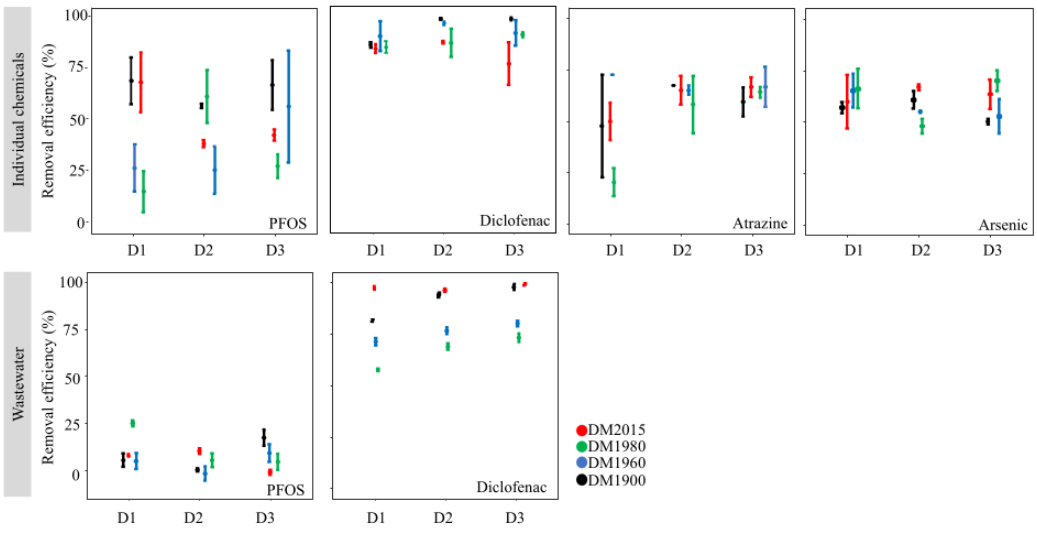


Fig. 2. Removal efficiency of chemical pollutants at laboratory scale. Removal efficiency (%) of 4 individual chemical compounds from borehole water and from wastewater: Atrazine and Arsenic were not found in the wastewater sampled from the Finham treatment plant (UK). Error bars show variance among biological replicates in the experiments. The four *Daphnia* genotypes are colour coded: DM2015 (red); DM1980 (green); DM1960 (blue); and DM1900 (black).

having higher removal in day one and lower in days 2 and 3, and other genotypes (e.g., DM1900) having increased removed over time (Table 1B; genotype:day; Fig. 2). The four genotypes differed significantly in their removal efficiency of diclofenac from wastewater (Table 1B; genotype). The removal efficiency differed significantly across the three days of the experiment, with two out of four genotypes showing a higher removal from day 1 to day 3 (Table 1B; genotype: day; Fig. 2; DM1900 and DM 2015). The average removal efficiency was lower for both chemicals when they occurred in mixtures (wastewater) than in individual chemical exposures, especially for PFOS (Fig. 2).

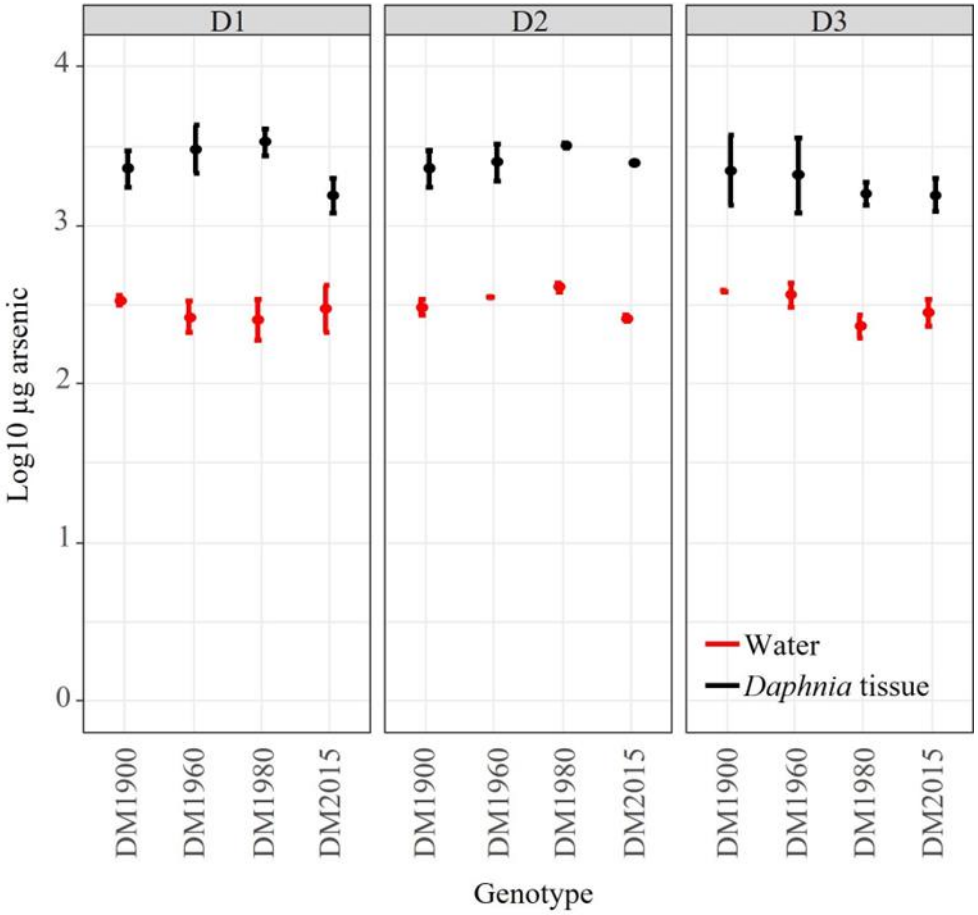
5.4.2. Mechanisms of Chemical Removal: Proof of Concept

Our water analysis revealed that chemicals removed by *Daphnia* were not excreted back into the water. Using the heavy metal arsenic, we quantified the amount of chemical present in the *Daphnia* tissue to assess whether the amount removed from the water corresponded to the one accumulated in the *Daphnia* tissue, providing a first indication of the mechanisms of chemical removal by the biological agent. We worked under the hypothesis that if the amount of chemical removed from water corresponded to the amount found in the *Daphnia* tissue, the compound was bioaccumulated. Conversely, if the amount of chemical removed from water was different from the amount measured in the *Daphnia* tissue, it wound indicate incomplete metabolization and potential generation of by-products. The amount of arsenic removed from water was not significantly different from the concentration of arsenic recovered from the *Daphnia* tissue across the three days, confirming that the arsenic removed from water was bioaccumulated in the *Daphnia* tissue, as expected for a heavy metal (Fig. 3). The quantification of the other chemicals in the *Daphnia* tissue would have been useful but protocols are not yet established.

5.4.3. Prototype-Scale Demonstrator

We tested the removal efficiency of diclofenac by a population of *Daphnia* strains in the outdoor prototype over a period of 4 weeks. A population of *Daphnia* comprising an equal proportion of DM1900 and DMV2015 was used. The highest removal efficiency in the prototype was 90%, with an average across all weeks of 78% (Fig. 4). The technology performance was constant across the four weeks

Fig. 3. Removal and bioaccumulation of arsenic from spiked water. Removal of arsenic from spiked borehole water and arsenic quantified in the tissue of four *Daphnia* genotypes across three days: DM1900, DM1960, DM1980 andDM2015. The two data series are not significantly different (chi-square Pearson correlation P = 1)



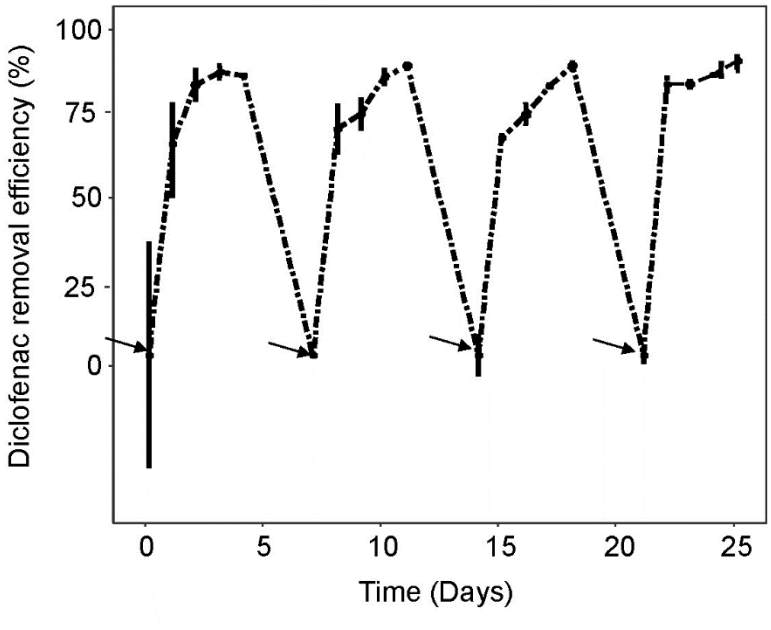


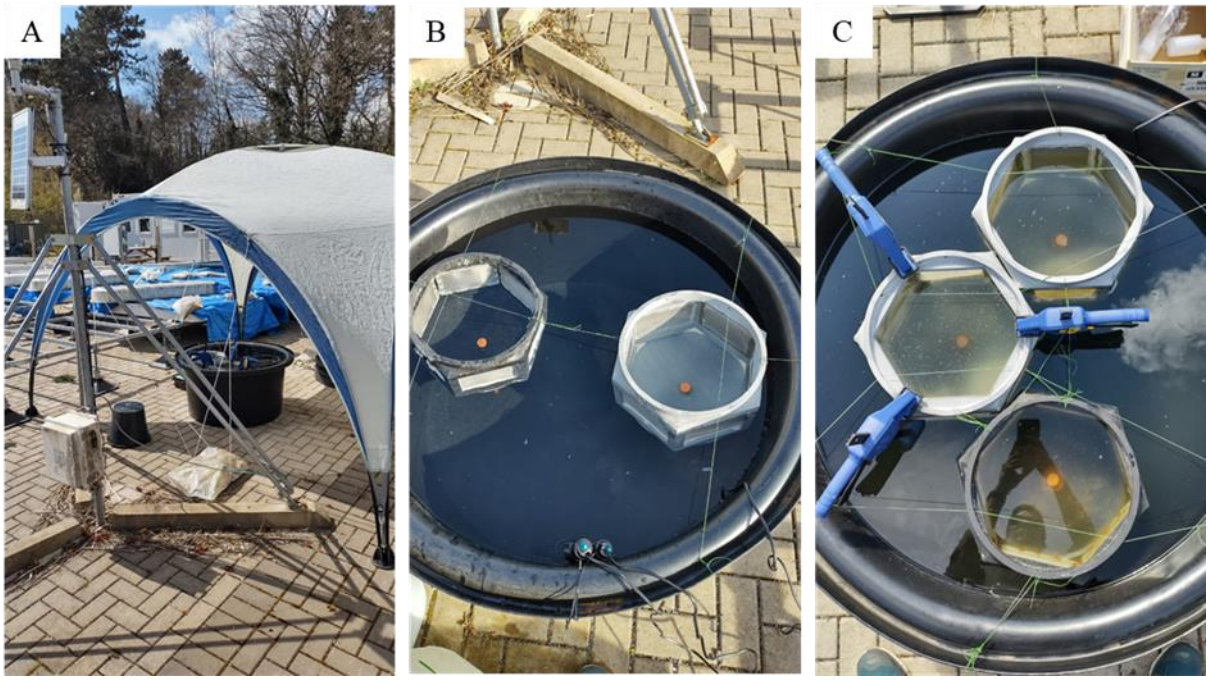
Fig. 4. Sustained performance at prototype scale. Removal efficiency of diclofenac (arrows indicate spiking times) spiked in a prototype-scale environment resembling volumes of wastewater produced daily by a single household of 3.5 people. Removal efficiency was assessed with mass spectrometry analysis and calculated as follows: [starting concentration − final concentration /starting concentration] ×100.

regardless of the outdoor conditions (the removal efficiency did not significantly differ between weeks; ANOVA; P = 1) (Fig. 4). The sustained removal efficiency was accomplished by a thriving population of *Daphnia* for the duration of the trial, expressing an oscillation around an average density of 101 individuals/L (Appendix A, Fig. S2A).

The prototype was designed to mimic, as much as possible, operation conditions of secondary clarifiers in wastewater treatment works (Fig. 5A). To this end, a diffused nozzle maintained a laminar flow of water (Fig. 5B). The water temperature averaged 21°C, with daily oscillations between 17°C and 24°C (Fig. S2B). Dissolved oxygen was on average 6 ppm, ranging between 4 - 9 ppm (Fig. S2B). Visual inspection of the water in the prototype showed no evidence of leakage of the filtration devices (Fig. 5C). At the end of the trial period a visual inspection of the filtration devices base confirmed the collection of debris (Fig. 5C), including *Daphnia* chitin exoskeleton that was shed at each

moult cycle, confirming that the design allows for the collection of biowaste (dead *Daphnia*

Fig. 5. *Daphnia*-based wastewater technology prototype. The *Daphnia*-based technology was tested at prototype scale in outdoor conditions (A). A tarpaulin protected the prototype from rainfall. Filtration devices were suspended in the top 1 m of the prototype tank to assess the technology performance over 4 weeks (B). At inoculation, the vessels are clear. Over time, the self-sustaining population of *Daphnia* grows to carrying capacity populating the vessels (C). The water flows freely through the walls of the vessels that are made of porous meshes. The solid base collects biowaste (dead *Daphnia* and shredded carapace) that is syphoned out to further treatment by using a system of interconnected valves (the red plug at the bottom of the vessel is the connection to the valve system).



and other debris) without disturbing the live *Daphnia* population in the filtration vessels.

The live *Daphnia* population is contained in vessels consisting of a solid frame and a porous mesh that allows for the movement of water, while retaining the live *Daphnia* population and allowing for the collection of biowaste (dead *Daphnia*) at the base of the vessel (Fig. 6). The interconnected valves at the bottom of the vessels allow the isolation of the individual devices while the biowaste is funnelled into further treatment, as discussed in section 4.3 below. A backup environment consists of tandem bioreactors for

the on-site production of *Daphnia*, supported by the feedstock (algae) (Fig. 6). This backup environment has the critical function to generate *Daphnia* to seed the initial population in the live environment and to replenish this population in case of shock events. In the next result section, we present the first modelling results to determine the impact of shock events on the *Daphnia* population.

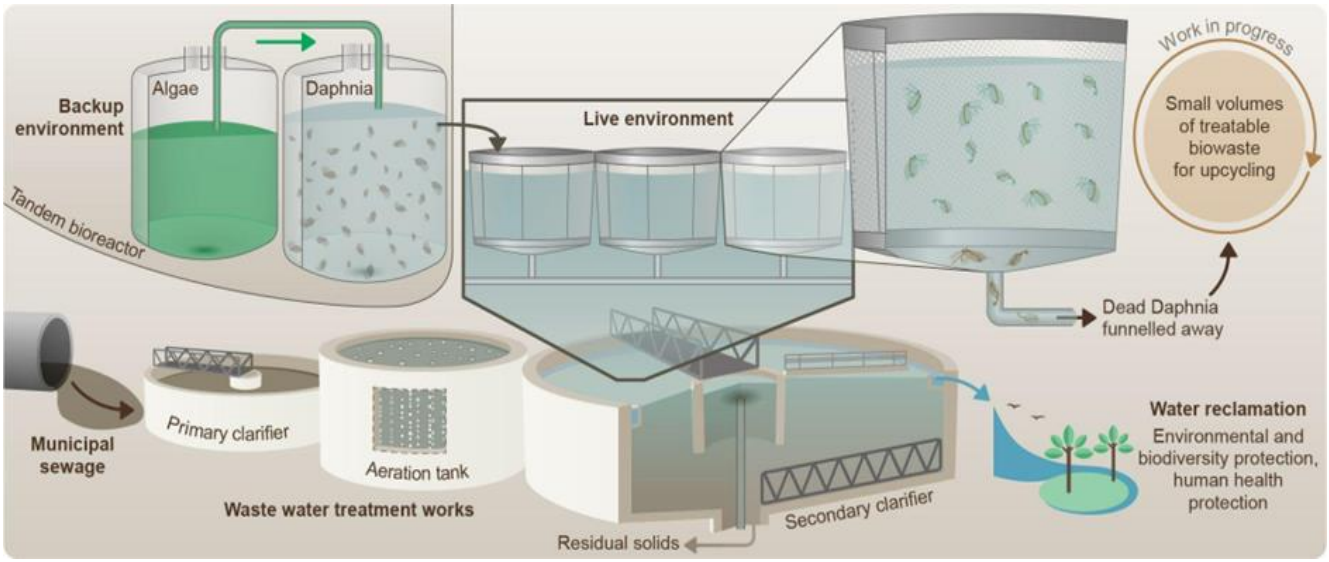


Fig. 6. Conceptual flow diagram of the water bioremediation process. The *Daphnia*-based water bioremediation technology consists of a live environment and a back-up environment. Modular interconnected devices are introduced in secondary clarifiers (live environment) to sustain a population of *Daphnia* that removes chemical pollutants, generating clean effluent. At the end of the life cycle and after having bioaccumulated/biotransformed persistent pollutants, dead *Daphnia* settle at the bottom of the containment devices and are syphoned into a waste treatment process of upcycling. The backup environment consists of a tandem *Daphnia*/algae bioreactor used to seed the initial population of *Daphnia* in the live environment and to top-up this population in case of shock events.

5.4.4. Modelling Daphnia Population Dynamics to Prevent the Impact of Shock Events

To ensure constant performance of the technology, and based on filtration efficiency in a laminar flow environment, we estimated that the *Daphnia* population density should be between 100 and 200 individuals/L. The health and stability of the *Daphnia* population can be affected by shock events (e.g., extreme temperature, pulses of chemical pollutants above accepted thresholds), affecting the removal efficiency of chemical pollutants. Predicting population crashes is critical to ensuring constant chemical removal. We derived a first set of simulations to understand how changes in population dynamics can cause a loss of pollutant removal performance (Supplemental Methods, section 1.3). We therefore simulated the *Daphnia* population dynamics in a closed environment using a delay differential equation model, which accounts for juvenile and adult stages of the *Daphnia* population (Fig. S3). While future work will facilitate calibration of the model against data collected under a variety of environmental conditions, the model is already able to qualitatively distinguish between two scenarios; i) the first is where the balance of birth and death rates results in oscillatory dynamics, but ultimately the population is able to reach a stable positive equilibrium where chemical micropollutants are removed efficiently (Fig. S3, solid line); ii) in the second scenario, an excess inflow of pollutants results in increased death rate and a population crash (Fig. S3; dashed line); in this scenario, the resident population would be unable to remove chemical pollutants. For operational practice, it will be essential to use calibrated models to identify dynamics leading to population crashes before they happen, guiding the top-up of the *Daphnia* populations from the side stream reactors in real-world environments.

5.4.5. Techno-Economic Assessment of the Daphnia-based Technology

The techno-economic analysis served to benchmark the *Daphnia*-based technology against both established mechanical/chemical processes and emerging bio-based solutions. This exercise evidenced that our technology presents several technical, commercial and sustainability advantages over established and emerging treatments at comparable removal efficiency benchmarked on available data on individual chemicals. The advantages of the *Daphnia*-based technology include the removal of a wide range of chemical pollutants; capital expenditure (CAPEX) and operational expenditure (OPEX) several orders of magnitude lower than conventionally adopted technologies, both mechanical and bio-based; low infrastructure requirements and non-toxic by-products generation (Table S2 and S3). The analysis also revealed that the environmental impact of the technology in terms of odour, visual and noise impact was negligible and smaller than the one of both mechanical/chemical (Table S2) and bio-based solutions (Table S3).

5.5. Discussion

5.5.1. Removal Efficiency and Mechanism of Micropollutants Removal

Our previous research showed that *Daphnia* can remove 7 out of 16 pharmaceuticals more efficiently than algae and bacteria and the remaining 9 at a comparable rate in controlled laboratory conditions (Abdullahi, Li, et al., 2022b). Here, we demonstrated the removal efficiency by four carefully selected *Daphnia* strains of four chemical pollutants belonging to found distinct classes: pharmaceuticals, pesticides, heavy metals and

industrial chemicals. We showed their removal efficiency both in single chemical exposures and in mixtures found in secondary treated wastewater. We found significant differences in removal efficiency among *Daphnia* strains. This difference can be explained by different histories of exposure, and hence tolerance, to chemical pollution. The strains used in our study were revived from sedimentary archives of lakes with known gradients of chemical pollution. The removal efficiency of these strains aligns with their evolved tolerance to recurring chemical stress, confirming previous findings (Abdullahi, Zhou, et al., 2022b). The selection of *Daphnia* strains based on their evolved tolerance to pollution is unique to our innovation. Our understanding of the ecological and evolutionary properties of the biological agent allows the tailoring of *Daphnia* strains to different wastewater sources, providing unprecedented flexibility for optimising treatment solutions to reach highest efficiency for specific wastewater sources.

We started exploring the mechanisms of removal of micropollutants by *Daphnia*, by focusing on the heavy metal arsenic. As compared to organisms that passively sorb micropollutants, *Daphnia* in an active filter feeder with higher metabolic capabilities than unicellular organisms. As the chemicals taken up by *Daphnia* were not released back into the water and did not cause mortality, as direct observations of the laboratory exposures and the outdoor prototype showed, we expect that biotransformation plays a role at least for the transformation of some chemical pollutants. Conversely, heavy metal or highly persistent chemicals (e.g., PFOS), are more likely to be bioaccumulated within the *Daphnia* tissue; we demonstrated that the amount of arsenic in the *Daphnia* tissue was not significantly different from the amount of arsenic removed from the exposure medium (borehole water spiked with known concentrations of arsenic), confirming mechanisms of bio-uptake for heavy metals in this species (Tan, Fan, & Wang, 2012). A similar

analysis of the *Daphnia* tissue will be needed to fully understand the biotransformation mechanisms of other chemical pollutants by this biological agent (Jeon, Kurth, Ashauer, & Hollender, 2013). To date, limited information is available on the mechanisms of removal of freshwater invertebrates because of technological limitations in detecting biotransformation products and parent compounds at low concentrations within the tissue of these organisms. Recent advances in liquid chromatography high-resolution mass spectrometry (e.g., Orbitrap) are promising for metabolite profiling due to their sensitivity and selectivity (Abdallah et al., 2019). Yet, lack of reference standards for metabolites in non-model species makes the quantification of parent compounds and metabolites in these species challenging. It is noteworthy that whereas we envision to tackle these challenges in the future, understanding these mechanisms is not necessary to the *Daphnia*-based technology applications. Critically, the system of valves at the bottom of the containment devices in the live environment, funnels away dead *Daphnia* preventing the release of chemicals back into the water.

An area often overlooked in studies assessing bio-based applications for water treatment is the effect of chemical mixtures in wastewater on removal efficiency. Studying the response to individual chemicals as well as mixtures is vital because combined effects of chemicals may produce cumulative or synergistic effects that are more than or different from the sum of individual effects (Sprinkle & Payne-Sturges, 2021). In our study, we demonstrated comparable removal efficiency of diclofenac in single chemical and mixture (wastewater) exposures. Conversely, the removal of PFOS was lower in wastewater. The recovery of PFOS from wastewater was lower than the concentrations used in the individual chemical exposures. This, together with the use of a different reverse phase extraction cartridge to capture mixtures, may have affected the

quantification of this compound in wastewater. It is also possible that the removal efficiency of PFOS in wastewater was dampened by synergistic effects with other chemicals (Ahrens & Bundschuh, 2014; Yang et al., 2019). In this instance, carefully selecting *Daphnia* strains with evolved tolerance to PFOS and other commonly occurring chemicals is likely to improve the removal efficiency of PFOS from wastewater. The results of our study provide important insights areas of further development for the innovation.

5.5.2. Technology Performance, Risk and Mitigation

The *Daphnia* technology presents performance, economic and engineering advantages. By non-selectively taking up different chemical pollutants, the technology overcomes the limitation of some state-of-the-art technologies affected by the hydrophobicity and ionization characteristics of chemicals (J. Ma et al., 2018). By providing a retrofittable solution it negates the need for major infrastructure modifications, which are required for other bio-based solutions (e.g. phycoremediation; (Škufca et al., 2021). The technology is sympathetic to current secondary clarifier designs, which have been identified through co-development with the water industry. Whereas variations of the engineering design for secondary clarifiers with variation in tank profile, inlet and outlet geometry and scraping technologies (Trianni, Negri, & Cagno, 2021), may require adaptation of the containment devices in the live environment, the inherent agility and scalability of our technology, as well as the positioning of the containment devices in the top 1m of the treatment tanks, enables its installation into most treatment plants without impacting the existing infrastructure. Modifications of the containment devices may be required for off-grid and small works installations. However, the modularity of the system permits these adjustments without major modifications of the wastewater plants and the technology.

Because of these properties the *Daphnia*-based technology has potential for applications in low- and middle-income countries, meeting key sustainable development goals.

The outdoor prototype served to demonstrate that the removal efficiency of the proxy chemical (diclofenac) was comparable to removal efficiency measured in the laboratory and was sustained over time, regardless of the unstable outdoor climatic conditions. The next step in the technology development is the further upscaling in a continuous open-flow environment holding real wastewater. This process will likely be stepwise, with validation in an intermediate scale prototype before installation is attempted within wastewater workflows. We expect success of upscaling based on prototype-scale demonstrators and co-development with end-users. Should the technology fail in upscaling, we will revert to smaller scale off-grid or smaller wastewater treatment applications, which are in demand for rural areas and smaller treatment plants. These smaller scale applications also meet the demand of low- and middle-income countries, as well as of higher income countries where less than 80% of the population is connected to public urban wastewater treatment systems (e.g., Albania, Croatia, Slovenia and Poland (Fatimah et at., 2022)).

Extreme climates can potentially affect *Daphnia* performance impacting the removal efficiency of persistent chemicals. It is possible to overcome these limitations by either using strains naturally adapted to different climates - similar approaches have been used in phytoremediation (Ferro, Gentili, & Funk, 2018) - or by reverse engineering *Daphnia* strains. The latter approach is possible as shown by reverse genetics application in *Daphnia* (Fatimah, Adhitama, Kato, & Watanabe, 2022; Nakanishi, Kato, Matsuura, & Watanabe, 2014).

With respect to the potential release of the bioremediation agent into waterways, while the system is designed to prevent such release, *Daphnia* do not pose an environmental threat. Additionally, if released accidentally, *Daphnia*'s chance of survival in running water is very low because they are lake-dwelling species. In case of other shock events that can affect the chemical removal efficiency due to a crash of the resident *Daphnia* population, the innovation uses side stream reactors as a backup and top-up system.

5.5.3. Outstanding Challenges and Future Technological Developments

5.5.3.1. Waste Management

We designed a manifold system regulated by a valve system that enables the dead *Daphnia* collection (biowaste) without disturbing the live population, as demonstrated in our prototype. This biowaste consists of an organic matrix (dead *Daphnia*) and persistent inorganics that are not biotransformed by *Daphnia* (e.g., heavy metals). Incineration of this biomass is feasible given the estimated modest average of 1 to 2 tonnes of biomass per clarifier/wastewater plant/year (Environment, 2019). For a fully circular system that enables the reuse of the biomass produced yearly, sustainably sourced 2D photocatalysts are an exciting new avenue for the reduction of persistent chemicals without the production of toxic by-products (Pérez-Álvarez et al., 2022). The development of a sustainable system to treat the refuse biomass is underway but will require significant efforts and optimisation.

5.5.3.2. Pushing Sustainability and Circularity

Pushing the concept of circularity that underpins our technology, we explored the market needs for other valuable by-products of the technology. One such product is chitin, which comprises the exoskeleton of *Daphnia*. Market research identified a potential market for chitin and its derivative chitosan into existing supply chains by serving applications in agriculture, textiles, food preservation, filtration, bioprinting, and as fuel cell catalysts (Environment, 2019). In response to these findings, a proof-of-concept was completed to test the separation between the organic matrix and the protein-chitin shell for onward valorisation. This preliminary work suggests that significant removal of the organic soft body is achieved at 240°C in hot compressed water and preserves the chitin structure (Fig. S4). However, the impact of the treatment on the deacetylation, deproteinization and demineralization of the chitin fraction and how this may impact deacetylation to chitosan for onward valorisation are yet to be determined. At least the separation of the organic and chitin material in hot compressed water at high temperatures ensures that no residual pollutants are presence in the chitin matrix.

5.5.3.3. Plug-in and Forget

The population dynamics modelled through the delay differential equations was able to distinguish population dynamics of a thriving population from the one of a population nearing a tipping point, eventually affecting the technology performance. Our next challenge is the encapsulation of this population model into a user-friendly interface to enable the application of the technology in a commercial environment by prompting a top-up of the population from the back-up environment before the *Daphnia* population reaches critical low density in the live environment, affecting chemical removal.

5.6. Conclusion

The *Daphnia*-based technology presented here provide a potentially ground-breaking, carbon-neutral process for removal of persistent chemical pollutants, such as pharmaceutical, pesticides, industrial chemicals, and heavy metals, from wastewater. Preventing the discharge of these chemicals in the environment will prevent environmental deterioration and impact on biodiversity. The low carbon footprint of the technology, combined with prevention of pollution of surface water provides a practical solution to increasingly stringent regulations (e.g., Urban Wastewater Directive on micropollutants removal; European Directive 2008/1/EC for pollution prevention and control; EU chemical strategy for sustainability 2020). By providing an add on polishing step to the traditional wastewater treatment, the technology contributes to deliver higher quality effluent, decreasing additional treatment requirements to produce water appropriate for reuse applications e.g., irrigation, industrial applications and use household use, such as toilet flushing.

5.7. CRediT authorship Contribution Statement

MA and IS – Investigation; formal analysis; writing; visualisation; SB – software; RO – Investigation; M A-E – resources, supervision; SJ, LEM AT - visualisation; software; supervision; SK, B A-D, RGL, BH, PT, MS and SG – Resources; KDD and LO – conceptualisation; methodology; writing; supervision; funding acquisition. All authors – writing.

5.8. Data Availability Statement

The mass spectrometry data for laboratory exposures and prototype performance can be found at the dryad entry: https://datadryad.org/stash/share/IqavWPfhp8nPycJbV_Jw76y8JDJdtnX3AMDzYjdTvX0. The codes used to generate the ANOVA analysis and the delay differential equations are available at the github entries (DWS/Anova.rmd at main · madbullahi/DWS (github.com) and DWS/MathscriptsDaphnia2022.zip at main · madbullahi/DWS (github.com).

5.9. Declaration of Competing Interests

LO and KDD declare potential financial conflict of interest as majority shareholders of Daphne Water Solutions Limited, a start-up which aims at commercialising the Daphnia-based technology. The data and engineering designs presented in this manuscript were realised through grants awarded to LO and KDD in their academic roles and are part of the technology proof of concept prior to commercialisation.

5.10. Acknowledgements

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Discussion and Future Requirements

In Chapter 1, We present a novel framework that expands the application of *Daphnia* beyond its current usage in ecology, evolution, and toxicology. Our research helps address the critical challenges of monitoring and preventing chemical pollution by leveraging *Daphnia* as both a diagnostic agent and a bioremediation agent to reduce hazards associated with chemical mixtures in the environment. The framework's innovation lies in the utilisation of machine learning to identify bioactive components in ambient mixtures that disrupt functional pathways in *Daphnia*. Additionally, the framework utilises evolutionary-conserved pathways across species to hypothesise and assess the toxicity of targets across species through experimental validation. The framework presented here is an effective method for evaluating the safety of chemicals prior to their release into the market. This important practice plays a critical role in mitigating chemical pollution and its negative effects on human and ecosystem health. Our framework utilises the sentinel species, *Daphnia*, to identify bioactive components in chemical mixtures and accurately identify the targets of toxicity. These bioactive components are linked with the potential molecular initiating event (mKE) (events that are believed to be the first step in the chain of events that leads to adverse effects in living organisms), enabling us to identify the targets of toxicity that require experimental validation with precision. By identifying mKEs that are evolutionarily conserved, we can better understand the potential hazard of environmental mixtures to other animals. Although our framework can predict potential chemical mixture hazards for various species, it's crucial to understand that common mechanisms of action do not always equate to common adverse

consequences. Our framework has the potential to enhance environmental health by establishing precise protection goals and providing valuable insights. In cases where the potential harm of a substance is uncertain, it is advisable to err on the side of caution and take preventative measures instead of waiting for conclusive evidence of harm.

Monitoring and comprehending the effects of anthropogenic chemicals is crucial. These chemicals can have unintended, long-lasting effects on non-target organisms, even at concentrations below regulatory standards. We made a first effort to understand the long-term impact of five chemicals on the 'priority list' of international regulatory frameworks in Chapters 2 and 3. In Chapter 2, we study the transgenerational impact of the chemicals on the life-history fitness traits of *Daphnia* genotypes. The five chemicals tested significantly affected the overall fitness of the *Daphnia* genotypes, and this effect was driven by different combinations of the life history traits in the five chemicals. A significant three-way interaction term was observed, showing that treatment per genotype differed across generations for all chemicals except for PFOS. In this chapter, we also discovered that long-term exposure to low doses of chemicals has an impact on the genetics of the *Daphnia* genotypes. We observed that the 'experienced' genotype LRV0_1 has a lower genetic diversity compared to LRII36_1, LRV12_3 and LRV8.5_3. In this work, the lower genetic diversity of the most recent genotype (LRVO_1) implies that it has a reduced ability to adapt to changes in environmental conditions, such as the detoxification of novel chemical stressors. Our findings suggest that the evolutionary history of the strains may have influenced the response of the genotypes to chemical stressors. Specifically, the genotypes historically exposed to chemicals showed reduced tolerance to novel stress compared to those not. This implies that exposure to certain

chemicals in the past may have resulted in the selection of genotypes that can adapt well to those chemicals but may not be as competent in dealing with unfamiliar chemical stressors. The caveat of this study is the limited number of strains used-any conclusion should be validated at the population level. For example, in the final part of the discussion, you find: Overall, our study shows that exposure to chemical stress in *Daphnia* may impose costs associated with susceptibility to novel chemical stress, potentially compromising the resilience and adaptation to future environmental changes. Susceptibility may be influenced by the combined effect of other stressors interacting with chemicals, with potentially more severe effects. The impact on the keystone grazer *Daphnia* has an important implication for aquatic food webs, given its central role in the lentic freshwater environments worldwide. Functional pathways conserved across species and putatively disrupted by prolonged chemical exposure included detoxification, neuronal functions, and metabolism. These pathways are potential targets in other species, including humans

In Chapter 3, we investigate the impact of these same chemicals on the microbiome of *Daphnia magna*. Our preliminary analyses show gut microbiota dysbiosis even at approved regulatory thresholds for these chemicals. Because *Daphnia* is a very important species in the food web of aquatic ecosystems and an ecosystem bioindicator organism, the impact on *Daphnia* may have a cascading effect on other organisms in freshwater. In addition to this indirect effect through the trophic chain, an analysis of pathway conservation across the Tree of Life may reveal if the same pathways are potential targets in other species. Due to time constraints, this analysis was not completed but it warrants exploring. I have intended to use a systems biology approach to further explore the impact

of these chemicals on host response and understanding the links between microbiome and host functional pathways.

The framework in Chapter 1 also presents for the first time a proof-of-concept study that elevates *Daphnia* to the role of a potential alternative remedial agent for chemical pollution in water and wastewater. We have developed a technology for tertiary wastewater treatment that utilises *Daphnia* and offers a scalable solution for efficiently removing persistent contaminants. This comes in response to the growing global demand for such solutions, driven by increasingly strict regulations such as the Urban Wastewater Directive for removing micropollutants, European Directive 2008/1/EC for pollution prevention and control, and European Directive 2004/35/CE for preventing and mitigating environmental damage. Furthermore, the utilisation of Daphnia-based technology effectively tackles the prevalent issues in the water industry concerning the elimination of trace amounts of phosphorus and other nutrients, which is crucial in averting the eutrophication of downstream water resources. This technology also eliminates other inorganic elements, making it an excellent solution to address current water industry challenges. We have successfully developed the engineering infrastructure necessary to deploy Daphnia-based technology at a prototype scale, which perfectly mimics the wastewater produced by a single household. Our prototype includes freshwater spiked with proxy chemicals, and our laboratory experiments have demonstrated that this technology is highly effective in removing persistent chemical pollutants from wastewater, even though its efficiency may vary for some chemicals. Going forward, our partnership with the water industry will be focused on scaling up this technology in a continuous open-flow environment using real wastewater. The performance of *Daphnia* can be affected by extreme climate conditions, whether too cold

or too warm. One way to overcome this limitation is by utilizing strains naturally adapted to different climates or reverse engineering *Daphnia* strains. The bioremediation system is engineered to avoid any chance of the agent being discharged into water bodies. Furthermore, the *Daphnia* species employed in the procedure do not pose any ecological risk. Even if accidentally released, it is improbable for them to thrive in flowing water because they mostly inhabit lakes. We have developed a system that uses valves to collect dead *Daphnia* and other forms of biomass from water treatment. This system is capable of handling both organic and inorganic matter, including non-transformed PFOS. Furthermore, the innovation has additional backup and top-up systems utilizing side stream reactors that can address any unexpected event that may affect chemical removal efficiency due to a decrease in the *Daphnia* population. Understanding the mechanism of bioaccumulation or biotransformation in *Daphnia* regarding persistent chemicals is crucial. While progress is being made, the development of standardised mass spectrometry libraries for the targeted chemicals has posed a challenge. The selection of *Daphnia* strains based on their evolved tolerance (as shown in Chapters 2 and 3) to pollution is unique to our innovation. Our understanding of the ecological and evolutionary properties of the biological agent allows the tailoring of *Daphnia* strains to different wastewater sources, providing unprecedented flexibility for optimizing treatment solutions to reach the highest efficiency for specific wastewater sources. According to independent market research, this technology's estimated amount of biomass at a European scale is 2,567,610 tons per year. This amount is equivalent to what is generated by approximately 28,529 wastewater treatment plants. We are confident that by implementing a benchtop reactor, we can effectively eliminate persistent chemicals from the biomass produced by our technology. This will allow us to efficiently reuse the

biomass, maximising its potential. A promising approach to efficiently reduce persistent chemicals without producing harmful by-products is using 2D photocatalysts made from eco-friendly materials like graphite and urea. These photocatalysts can be customised to absorb UV or visible light, making them highly versatile and suitable for diverse applications. If this photocatalytic process proves to be successful, it will allow for a completely circular system. Using delay differential equations, we have developed a population dynamics model that can effectively distinguish between a thriving population and one that is nearing a tipping point. This is crucial as it has the potential to impact technology performance. Our next goal is to simplify this model into a user-friendly interface. This will allow for the seamless operation of the technology by prompting a population top-up from the backup environment before the *Daphnia* population reaches critical low density in the live environment.

Future directions

i. Finding the link between functional pathways that correlate with gut bacteria alteration in *Daphnia* and their conservations across species can reveal toxicity targets in other species and guide the regulation of chemicals with toxic effects.
ii. The functional analysis at the population level needs to be expanded to validate patterns.
iii. On the technology side, we need to demonstrate the effectiveness of the technology in an open-flow environment using actual wastewater. This process

will likely be stepwise, with validation in an intermediate-scale prototype before installation is attempted within wastewater workflows.

iv. Finally, understanding the mechanisms of biotransformation of chemicals will provide unique insights into toxicology but also a way to select more tolerant strains in the technology application

Appendix A: Supplementary information for Chapter 1

Supporting Methods for the Chaobai River case study

Daphnia magna 24h-old juveniles (IRCHA clone 5; Water Research Centre, Medmenham, UK) were exposed to 30 water samples from the Chaobai river in triplicates. The exposure assays followed the OECD 202 guidelines. After 48h of exposure, immobilisation was recorded, and mobile juveniles were flash frozen for RNA extraction and mRNA sequencing from exposed *Daphnia* and from clonal replicates maintained in control conditions. Total RNA was extracted using the RNA Advance Tissue kit (Beckman Coulter) applied to flash-frozen tissue following the manufacturer's instructions. Extracted RNA was quantified using a Nanodrop-8000 Spectrophotometer (ThermoFisher ND-8000-GL) and integrity assessed on the Agilent Tapestation 2200 (Agilent G2964AA) with High Sensitivity RNA Screen Tapes (Agilent 5067- 5579). Total RNA (1µg) was poly(A) selected using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs E7490L) and then converted in mRNA libraries using a NEBNext Ultra Directional RNA Library Prep Kit (New England Biolab E7420L) and NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), following the manufacturer guidelines. Sample handling was performed with the Biomek FxP workstation (Beckman Coulter A31842). Constructed libraries were assessed for quality using the Tapestation 2200 (Agilent G2964AA) with High Sensitivity D1000 DNA Screen Tape (Agilent 5067-5584). Multiplexed libraries (100-bp paired end) were sequenced on a HiSeq4000 by the Beijing Genomics Institute (BGI) to obtain 5M reads per sample. Sequenced reads quality was assessed using fastqc (v0.11.5) (Brown,

Pirrung, & McCue, 2017), followed by multiqc (v1.5) (Ewels, Magnusson, Lundin, & Kaller, 2016). Transcripts were mapped onto the *D. magna* reference transcriptome (L. Orsini et al., 2018; L. Orsini, Gilbert, et al., 2016) using default settings in Salmon (version 0.8.2, Patro et al. 2017). The reads were then trimmed using Trimmomatic 0.32 (Bolger et al., 2014) with the following parameters: 1) Illumina adapter cutoff with two seed mismatches; 2) palindrome clip threshold of 30 and a simple clip threshold of 10; 3) Phred quality score >30; 4) minimum trimmed reads length of 50 bp. The read count matrix of mapped transcripts was summarised at gene level and further analysed in R (version 4.0.3). Low count genes (genes with read count < 10/sample) were removed. Read counts were normalised by the size factor defined in the DESeq2 package (version 1.30.0; (Love, Huber, & Anders, 2014)). A total of 2,796 genes were significantly differentially expressed in at least one site. These genes were clustered on co-responsive modules using WGCNA (Langfelder & Horvath, 2008) to identify putative molecular key events (mKEs). For each mKE, we identified conserved orthologs between *Drosophila melanogaster* and *D. magna* using OrthoDB (Zdobnov et al., 2021). We mapped genes onto pathways using the KEGG pathway database (Kanehisa et al., 2008). Pathway overrepresentation analysis was done using the Fisher's exact test and enriched pathways with a Benjamini-Hochberg corrected P_{adj}-value < 0.05 were retained. The general workflow of data analysis is illustrated in Figure S1.

Supplementary Table 1.

List of organic pollutants and their concentration range in water samples from the Chaobai River as reported in (Su et al., 2020). The site names are corresponding to those in Figure 3A. The compound names and abbreviations; the CAS numbers (CAS No.); the limit of quantification of each compound (LOQ; ng/L); the concentration range reported by [122] for each compound in the Chaobai River basin, with the site with the highest concentration in parentheses; the number of sites at which the chemical was detected (above LOQ) are shown.

Compound (abbreviation)	CAS No.	LO (ng/L)	Range (ng/L) (site)	No sites
Atenolol (ATE)	29122-68-7	1.82	0-5.84 (M06)	1
Azithromycin (AZN)	83905-01-5	0.34	0-4.99 (M06)	3
Bezafibrate (BF)	41859-67-0	0.41	0-10.86 (M06)	13
Caffeine (CAF)	58-08-2	1.40	0-64.69 (M06)	29
Carbamazepine (CBZ)	298-46-4	0.36	0-35.23 (M11)	25
Clarithromycin (CLA)	81103-11-9	0.74	0-4.60 (M06)	3

Erythromycin (ERY)	114-07-8	0.86	0-593.68 (M16)	27
Metoprolol (MET)	37350-58-6	1.08	0-52.73 (M06)	10
Roxithromycin (ROX)	80214-83-1	0.63	0-29.48 (M06)	5
Sulfadiazine (SDZ)	68-35-9	1.31	0-22.62 (M06)	4
Sulfamethoxazole (SMX)	57-68-1	1.37	0-260.20 (M06)	12
Trimethoprim (TMP)	738-70-5	0.69	0-132.18 (M16)	16
Ciprofloxacin (CIP)	85721-33-1	0.79	0-7.42 (C03)	3
Chlortetracycline (CTC)	64-72-2	0.99	0-1.58 (M08)	1
Doxycycline (DOX)	564-25-0	1.03	0-18.69 (M17)	5
Enrofloxacin (ENR)	93106-60-6	0.55	0-6.62 (C03)	6
Lomefloxacin (LOM)	98079-51-7	0.52	0-5.09 (C03)	2
Norfloxacin (NOR)	70458-96-7	1.12	0-3.87 (C06)	1
Oxytetracycline (OTC)	79-57-2	0.93	0-2.09 (B07)	1
Propranolol (PROP)	526-66-6	0.66	0-4.99 (M12)	1
Sulfamerazine (SMR)	127-79-7	1.62	0-4.96 (C06)	1

Tetracycline (TET)	60-54-8	1.37	-	0

Supplementary Table 2

KEGG pathways conserved across species based on the KEGG orthology (KO) between *Daphnia magna* and six model species (*Daphnia pulex*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*). The total number of orthologous groups (and the percentage over the total ortholog group within a given pathway) shared between *D. magna* and other species are shown.

Index	**map00480**	**map00980**	**map00982**	**map00983**	**map02010**
Pathway Description	Glutathione metabolism	Metabolism of xenobiotics by cytochrome P450	Drug metabolism - cytochrome P450	Drug metabolism - other enzymes	ABC transporters
No orthologs in *Daphnia magna*	21	5	6	19	13
No orthologs shared with *Daphnia pulex*	19 (90%)	5 (100%)	6 (100%)	19 (100%)	12 (92%)
No orthologs shared with *Danio rerio*	19 (90%)	4 (80%)	5 (83%)	19 (100%)	12 (92%)
No orthologs shared with *Drosophila melanogaster*	18 (86%)	4 (80%)	5 (83%)	15 (79%)	8 (62%)
No orthologs shared with	19 (90%)	5 (100%)	5 (83%)	17 (89%)	7 (54%)

Caenorhabditis elegans					
No orthologs shared with *Mus musculus*	20 (95%)	5 (100%)	6 (100%)	19 (100%)	13 (100%)
No orthologs shared with *Homo sapiens*	20 (95%)	5 (100%)	6 (100%)	19 (100%)	13 (100%)

Supplementary Table S3

Concentration (ng/L) of 16 pharmaceuticals in wastewater at the time of sampling (Reference) and following treatment with Bacteria, Algae or *Daphnia*. For each treatment, three biological replicates were generated (R1, R2, and R3) with ultraperformance liquid chromatography (UPLC), coupled to Q-Exactive™ Orbitrap high resolution mass spectrometry. This table supports Figure 4A in the main manuscript file.

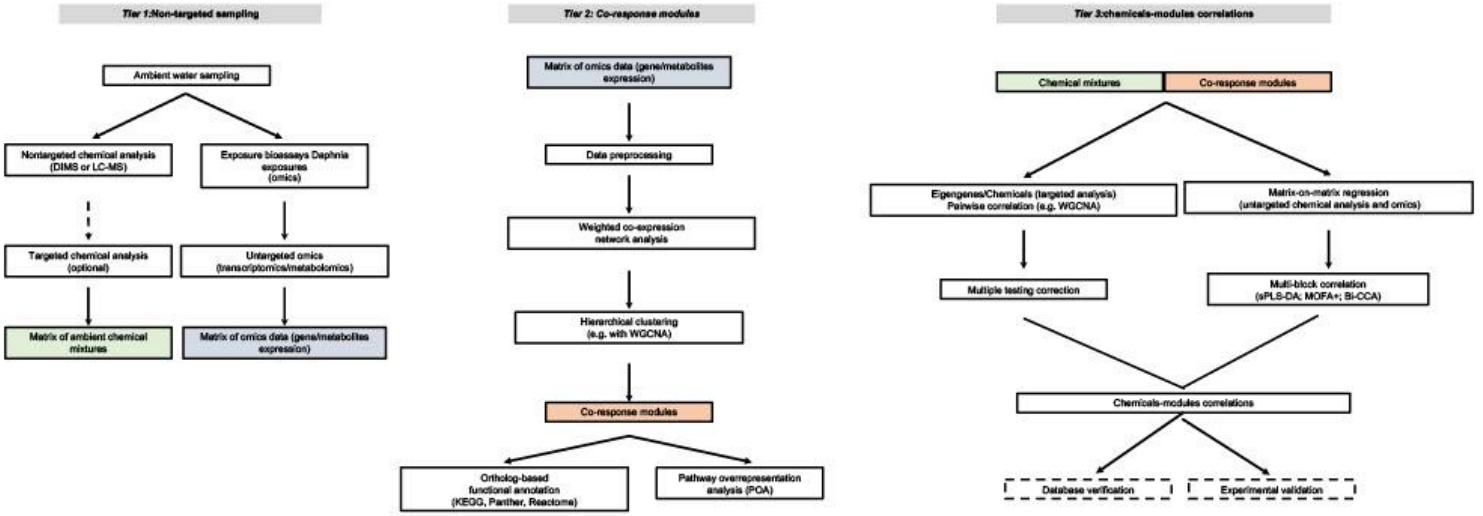
Conc(ng/L)/treatment	**Reference**				**Bacteria**				**Algae**				***Daphnia***			
Name	**R1**	**R2**	**R3**	**AV**	**R1**	**R2**	**R3**	**AV**	**R1**	**R2**	**R3**	**AV**	**R1**	**R2**	**R3**	AV
Metformin	658	784	742	728	563	549	628	580	517	582	494	531	506	568	539	538
Glyphosate	82	107	76	88	78	72	76	75	63	69	66	66	63	75	64	67
Acetaminophen	890	768	819	826	709	715	743	722	708	763	737	736	667	681	744	697
Codeine	197	234	153	195	128	119	130	126	149	138	170	152	140	172	131	148
Gabapentin	36	32	27	32	14	7	9	10	11	15	23	16	9	12	14	12
Trimethoprim	417	387	462	422	323	301	277	300	291	310	297	299	301	285	291	292
Tramadol	436	388	476	433	303	326	288	306	311	338	302	317	309	283	322	305
Propranolol	36	21	42	33	18	17	15	17	18	15	20	18	21	16	18	18
Erythromycin	76	58	59	64	47	49	36	44	56	45	46	49	50	39	42	44
Carbamazepine	806	761	784	784	686	641	664	664	682	656	674	671	672	680	633	662
Naproxen	128	147	154	143	90	76	84	83	88	93	102	94	79	90	82	84
Glyburide	303	268	243	271	199	216	195	203	228	216	182	209	197	174	181	184
Ibuprofen	10783	11023	10881	10896	9172	8869	9370	9137	9514	8633	9156	9101	9017	8952	8773	8914
Diclofenac sodium	121	129	92	114	58	54	60	57	59	56	62	59	52	53	39	48
Gemfibrozil	743	858	785	795	539	581	661	594	574	612	522	569	521	553	530	535

Supplementary Table S4

Controlled laboratory exposures of four *Daphnia* strains (LRV_01; LRV8.5-3; LRV12.3; and LR236_1) to PFOS (µg/L), atrazine (mg/L) and arsenic (mg/L). Influent is the concentration of each compound (note the different units) spiked in the growth medium and effluent is the final concentration of each chemical after exposure to *Daphnia* for 48h. Control –spiked medium without Daphnia. This Table supports Figure 4B in the main manuscript file.

PFOS (µg/L)	Influent	Effluent
Control	0.73	0.67
LRV0-1	0.73	0.38
LRV8.5-3	0.73	0.48
LRV12-3	0.73	0.29
LR2-36-1	0.73	0.33
Atrazine (mg/L)		
Control	0.18	0.20
LRV0-1	0.18	0.07
LRV8.5-3	0.18	0.08
LRV12-3	0.18	0.07
LR2-36-1	0.18	0.08
Arsenic (mg/L)		
Control	0.79	0.79
LRV0-1	0.79	0.29
LRV8.5-3	0.79	0.23
LRV12-3	0.79	0.37
LR2-36-1	0.79	0.39

Figure S1. Step-by-step analytical pipeline for the proposed framework. In Tier 1 water samples are collected from different sources. An untargeted chemical analysis is applied to the water samples to quantify chemical mixtures, optionally followed by a targeted chemical analysis. *Daphnia* is exposed to the water samples in a battery of OECD bioassays, at the end of which tissue is collected for omics data analysis. Biochemical matrices are the output of Tier 1. Network analysis (e.g. WGCNA (Langfelder & Horvath, 2008)) is applied to omics and chemical data to identify co-response modules. The KEGG (Kanehisa et al., 2008), Panther (Mi et al., 2019) and Reactome database (Jassal et al., 2020) are then used for functional annotation of these modules. Enrichment of response modules within functional pathways is achieved with a pathway overrepresentation analysis (POA). In Tier 3, correlations between response modules identified in Tier 2 and chemicals in mixtures identified in Tier 1 are established. These correlations can be established following two analytical processes: 1) matrix-on-matrix regression analysis with machine learning to establish significant correlations. This approach is preferred for untargeted data; and 2) correlation between the first principal component of co-expression module eigengenes and targeted chemical analysis data using e.g. WGCNA. Once significant correlations are established between modules and chemicals, these can be validated through search in public databases (if they are already known) or experimentally (if they are novel). This figure complements Figure 2 in the main text.



Appendix B: Supplementary information for Chapter 2

Table S1. PTA results. Phenotypic trajectory analysis testing magnitude (magnitude) and direction (θ) of change across genotypes and generations for exposures to five chemicals: PFOS (70 ng/L); Diclofenac (2 mg/L); Trimethoprim (2 mg/L); Atrazine (0.2 mg/L) and Arsenic (1,000 µg/L). Missing data reflect genotypes' extinction. Significant *P-values* are in bold. The statistics in this table support Figure 2.

	Generation 1				Generation 2				Generation 3			
	Magnitude	P_{mag}	Θ	P_{θ}	Magnitude	P_{mag}	θ	P_{θ}	Magnitude	P_{mag}	θ	P_{θ}
PFOS	0.0007	**0.002**	1572.35	**0.001**	0.0600	**0.001**	1537.10	**0.001**	0.0000	**0.002**	0.00	**0.001**
DICLOFENAC	0.0068	**0.001**	1164.53	**0.001**	0.0125	**0.002**	274.51	**0.001**	0.0003	**0.002**	1418.90	**0.001**
TRIMETHOPRIM	0.0075	**0.001**	2481.88	**0.001**	0.0197	**0.001**	425.39	**0.001**	0.0197	**0.001**	425.39	**0.001**
ATRAZINE	0.0423	**0.001**	1720.02	**0.001**	0.0000	**0.002**	0.00	**0.001**	n/a		n/a	
ARSENIC	0.0178	**0.001**	1675.63	**0.001**	0.0197	**0.001**	216.50	**0.001**	0.0882	**0.001**	6353.09	**0.001**

Table S2. Alpha and beta diversity. Chromosomal-level and genome-wide alpha diversity; and chromosomal-level and genome-wide beta diversity between each pair of genotypes used in the study (LRII36_1; LRV12_3; LRV8.5_3; and LRV0_1) supported by a Wilcoxon test.

	Alpha diversity					Beta diversity					
	LRV0_1	LRV8.5_3	LRV12_3	LRII36_1		LRV0_1 x LRV8.5_3	LRV0_1 x LRV12_3	LRV0_1 x LRII36_1	LRV8.5_3 x LRV12_3	LRV8.5_3x LRII36_1	LRV12_3 x LRII36_1
Chr1	4307	5266	5064	5192	Chr1	1.13	1.12	1.13	1.08	1.06	1.09
Chr2	1964	3176	3282	2891	Chr2	1.26	1.27	1.23	1.05	1.09	1.10
Chr3	2313	2605	2616	2408	Chr3	1.10	1.11	1.11	1.05	1.11	1.11
Chr4	2489	2524	2553	2662	Chr4	1.10	1.14	1.10	1.11	1.11	1.11
Chr5	1813	2653	2695	2519	Chr5	1.24	1.23	1.21	1.07	1.12	1.11
Chr6	2137	2406	2555	2630	Chr6	1.13	1.13	1.14	1.10	1.10	1.09
Chr7	1701	2079	2159	1966	Chr7	1.14	1.15	1.14	1.05	1.10	1.09
Chr8	1496	1911	2045	2009	Chr8	1.19	1.18	1.18	1.07	1.07	1.05
Chr9	1384	1837	1889	1847	Chr9	1.18	1.19	1.18	1.07	1.07	1.08
Chr10	1403	1819	1825	1734	Chr10	1.15	1.16	1.15	1.06	1.07	1.08
genome-wide	2100.70	2627.60	2668.30	2585.80	genome-wide	1.16	1.17	1.16	1.07	1.09	1.09
mean	2101	2628	2668	2586	mean	1.16	1.17	1.16	1.07	1.09	1.09
st. dev.	1880	2364	2429	2325	P-Val	0.05	0.05	0.04	0.02	0.02	0.02

Table S3. Divergent genes between pairs of genotypes. Number of genes showing significant differences in SNP diversity between each pair of genotypes. The *D. magna* gene ID; the number of SNPs found in each gene in the pairwise comparison; the gene length in bp; the start and end position of the gene; the P-value supporting divergent number of SNPs per genes; and the Chromosome where the genes sit, are shown.

See Abdulahi *et al.*_TableS3.xls

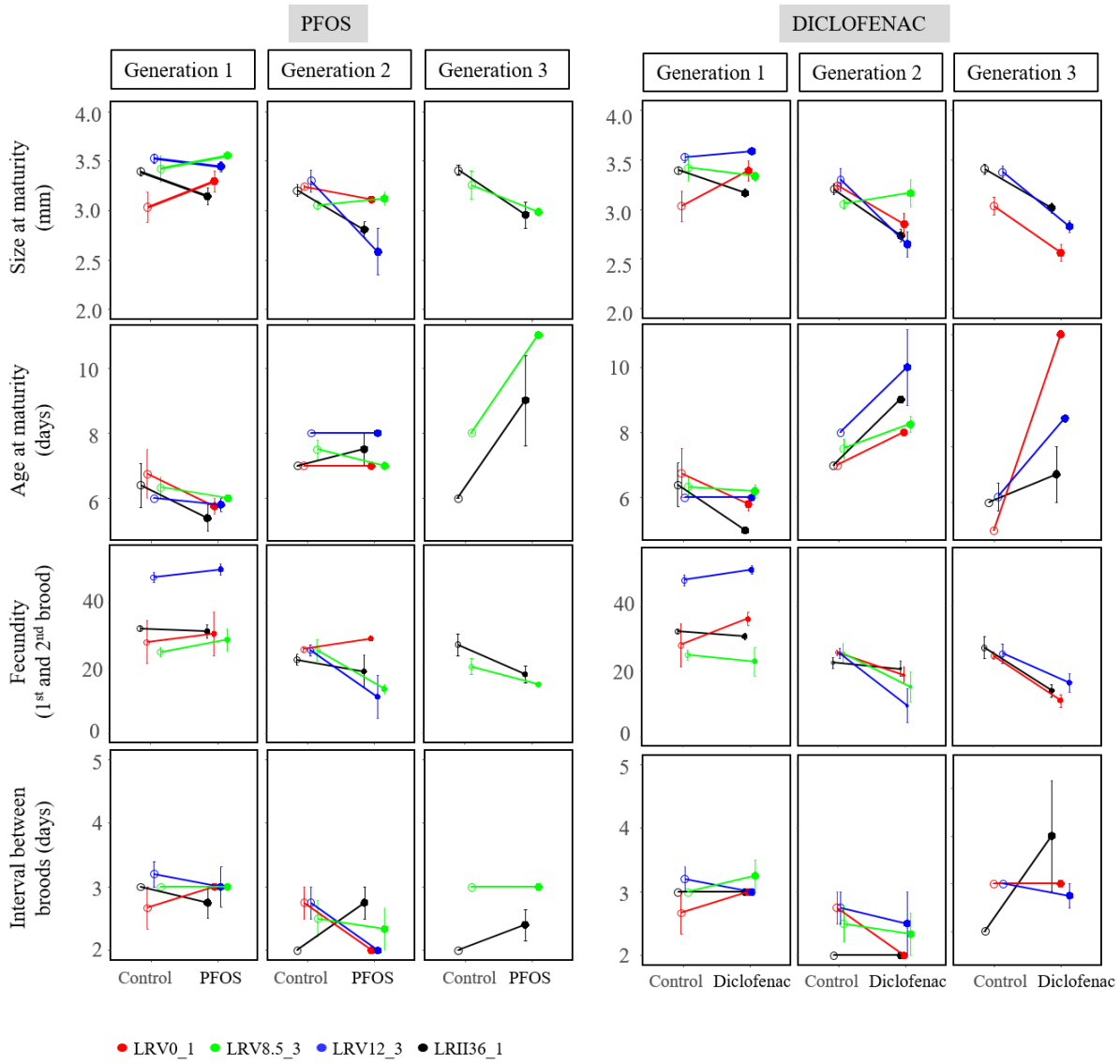
Table S4. Domain functional analysis. List of *D. magna* genes (*D. magna*_gene ID) enriched in the pairwise genotype analysis identifying divergent genes; gene sequence length (bp); online database searcher for onthology (database); the functional analysis is based on curated databases, including Interproscan, Pfam, and Panther; start and end location of each gene; Interproscan ID; GO terms (GO ID); functional pathways conserved across the Tree of Life are listed from the Reactome database.

See Abdullahi *et al*_Table S4

Table S5. Pathway analysis. List of pathways enriched between genotypes from the chemical-free and chemical-loaded environment.

Pathway function	Pthway ID	Adj P-val	Domain size
Lysosome	KEGG:04142	0.00	122
Glycan degradation	KEGG:00511	0.01	122
Lipids metabolism	KEGG:00130	0.02	46

Figure S1. Cross-generational fitness burden of chemical pollution. Univariate reaction norms of fitness-linked life history traits after exposure to five chemicals: [PFOS (70ng/L); diclofenac (2mg/L); trimethoprim (2 mg/L); atrazine (0.2 mg/L) and arsenic (1,000 µg/L)] across three clonal generations. Size at maturity (mm), age at maturity (days), fecundity, interval between broods (time elapsed between broods averaged over two broods) are shown. Average and SD per genotype across five biological replicates are shown. Genotypes are color-coded as in Figure 1: LRII36_1 (<1950; black); LRV12_3 (1960–1970; blue); LRV8.5_3 (1975–1985; green) and LRV0_1 (> 1999; red). Where reaction norms are not displayed, the genotypes went extinct in the treatment.



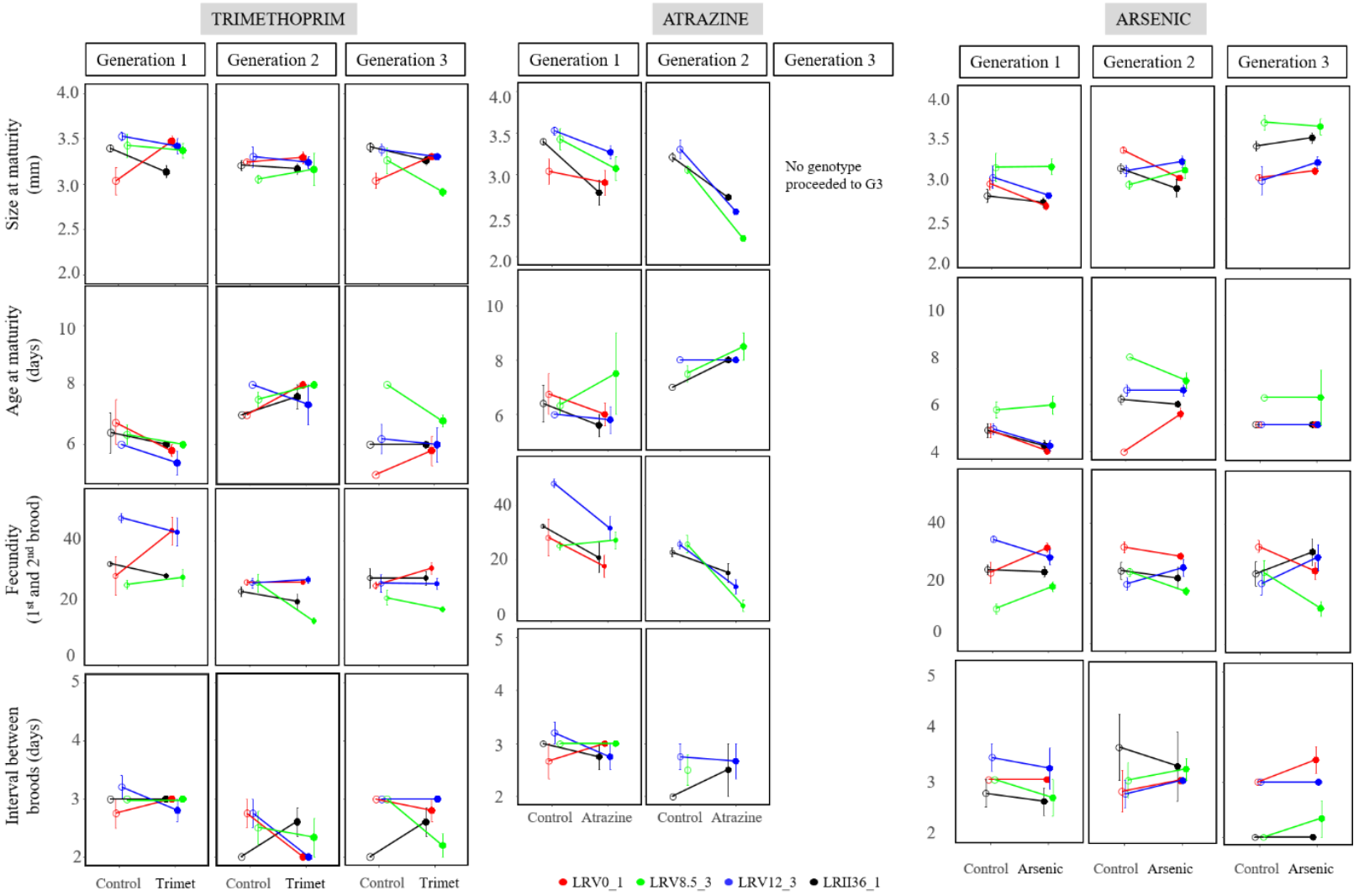
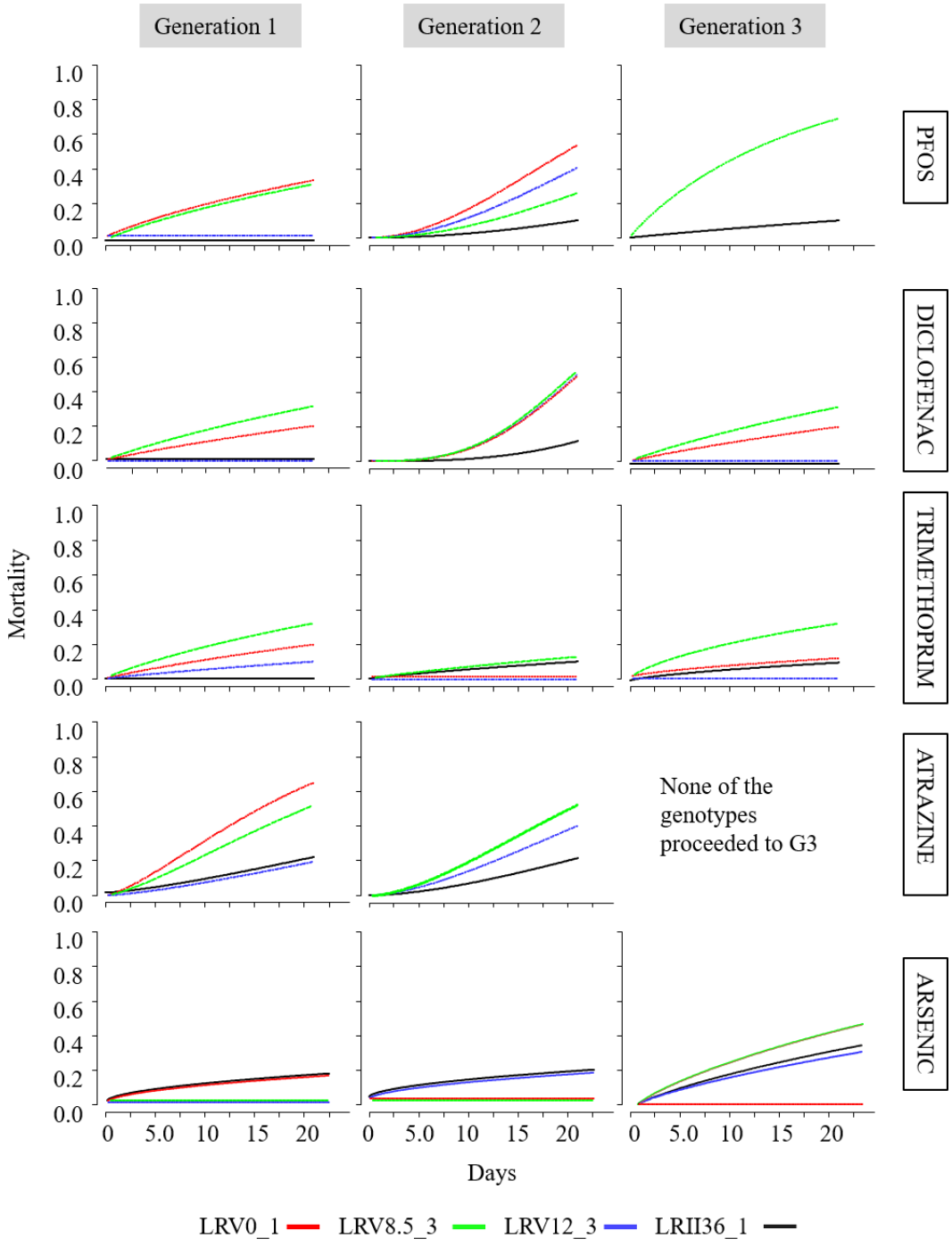

TRIMETHOPRIM
ATRAZINE
ARSENIC
Generation 1
Generation 2
Generation 3
Size at maturity (mm)
Age at maturity (days)
Fecundity (1st and 2nd brood)
Interval between broods (days)
No genotype proceeded to G3
Control
Trimet
Atrazine
Arsenic
LRV0_1
LRV8.5_3
LRV12_3
LRII36_1

Figure S2. Cross-generation survival plots. Survival plots per genotype and generation for exposure to PFOS (70ng/L); diclofenac (2mg/L); trimethoprim (2 mg/L); atrazine (0.2 mg/L) and arsenic (1,000 µg/L). The plots are obtained with a survival model fit via the "psm" function in the "rms" R package V.3.3. A separate model was fitted to each treatment. Genotypes are color-coded as in Figure 1: LRII36_1 (<1950; black); LRV12_3 (1960–1970; blue); LRV8.5_3 (1975–1985; green) and LRV0_1 (> 1999; red).



Appendix C: Supplementary information for Chapter 4

SUPPORTING METHODS

Single chemical analysis and micropollutants mixtures in controlled laboratory experiments

Following exposures in laboratory-controlled conditions, the concentration of chemicals was quantified using mass spectrometry. The water samples were spiked with internal standards [M4PFOS(13(C4)octane sulfonate), carbamazepine-d10 (CAS: 132183-78-9), chlorophenol-4-d4 (CAS:285132-91-4), and germanium]. The wastewater samples were filtered to remove excess suspended matter and then spiked with 50 *n*g of an internal standard mixture including 13C-PFOS and D4-chlorophenol. The SPE cartridges were all conditioned before sample extraction. Briefly, Oasis wax, used for preconcentrating of PFOS in the water samples, was preconditioned by passing a sequence of 5 ml of 0.1% NaOH in methanol and equilibrated with 5 ml MilliQ water. Oasis HLB was used for preconcentrating diclofenac and atrazine in the water samples. The MCX cartridges were optimised for preconcentrating PFOS and then used both for preconcentrating PFOS and diclofenac from the tertiary effluent wastewater samples. The preconditioning of the oasis HLB and MCX SPE cartridges was done with 2 ml methanol and equilibrated with 2 ml deionized Milli Q water at a flow rate of 1 drop/sec. After preconcentration of the samples, PFOS was eluted with 4 ml methanol followed by 4 ml of 0.1 % NaOH. Diclofenac and Atrazine were eluted with 5 ml methanol followed by 5 ml of 5 % NaOH. The MCX pre-concentrated cartridges were washed with 3 ml of 0.5 % formic acid (HCOOH) in MilliQ water. After drying, the pre-concentrated target analytes were eluted with 5 ml methanol followed by 5 ml of 5% NH_4OH in methanol (Abdallah et al., 2019). Eluents were evaporated to incipient dryness under a gentle stream of nitrogen using a TurboVap II

evaporator [Biotage, Sweden] and then reconstituted into 150 µl of 8:2 water/methanol mixture. The pharmaceutical's concentration was determined using rapid polarity switching electrospray ionisation sources. Full scan MS mode at resolution of 35000 FWHM and automatic gain control (AGC) target of 1E6 ions with injection time of 50 ms provided the optimum parameters for high sensitivity and improved reproducibility with ≥15 data points per peak. A high-resolution accurate mass with low mass tolerance filter (<5 ppm from authentic standards) was applied to achieve maximum selectivity with method limits of detection ranging between 0.02 - 1.21 ppb. The quantitation of PFOS was done using the methods described in (Harrad et al., 2019). PFOS was quantified on a Sciex Exion UPLC, coupled to a Sciex 5600+ triple TOF MS. The TOF-MS was equipped with a Turbo V ion source operated in negative mode using electrospray ionization at a voltage of −4500 V operated at 450°C. Mass spectrometric data were acquired using automatic information-dependent acquisition (IDA) with dependent production scan using a collision energy of −40 V. The method detection limit for PFOS was 0.5 ng/L. Arsenic samples were prepared using 50 ppb germanium as the internal standard. Samples prepared with 70% nitric acid were incubated at 20°C for 18 h and vortex-mixed for 30s. Aliquots of 100 µL were diluted to 10 mL using DI water. The samples were quantified using a Nexion 300X inductively coupled plasma mass spectrometer (ICP−MS) (PerkinElmer, Seer Green, U.K.) fitted with a cyclonic spray chamber. Calibration curves spanning 1−20 ppb were constructed in DI water.

Side stream tandem bioreactors

The controlled semi-automated back-up environment is used to seed the live environment and as a top up system in case of shock events. It consists of a tandem bioreactor The feedstock reactor is illuminated with full spectrum light at 8000 Lux, aerated and heated at 20ºC. The

Daphnia reactor is illuminated with a cool white LED set on 16:8 hour light:dark regime at an intensity of 800 Lux. When the feedstock vessel reaches a stable growth, the *Daphnia* reactor is inoculated. The algae typically reach a stable growth when they express an optical density (OD) of 0.5 ± 0.2 at wavelength of 440 nm on a spectrophotometer (Young, Reed, & Berges, 2022). Filtered borehole water over 2µm filters was used in the *Daphnia* reactor, starting from an initial inoculum of a few individual *Daphnia*/L. This inoculum can vary between 10 and 50 individuals/L. Alternative growth media can be used e.g. artificial lake water and COMBO (Table S2; (Kilham, Kreeger, Scott, Goulden, & Herrera, 1998)). With increasing density of the *Daphnia* population, the volume of feedstock dispensed increases accordingly. An optimal feeding regime ranges between 0.1mgC/*Daphnia*/d to 0.2mgC/*Daphnia*/d. The optical density (OD) of algae at 440 nm can be directly converted into g L^{-1} by Y = 0.211 (OD) where Y is the concentration in g L^{-1} (Young et al., 2022). The tandem bioreactor performance is monitored regularly by measuring the algae OD and counting the number of *Daphnia* in 100mL volume, sampled from the *Daphnia* bioreactor. Once the *Daphnia* population density reaches 200 *Daphnia*/L, a portion of the volume (typically 1L twice a week) is harvested and replenished with borehole water or other appropriate growth medium, guaranteeing a continuous production of *Daphnia*. The *Daphnia* produced in the side stream reactor is used to seed secondary clarifiers at first installation of the technology as a top-up system in case of shock events that affected the resident *Daphnia* population.

Modelling impact of shock events on the Daphnia population dynamics

With a view to understanding how removal of micropollutants in a live environment may be affected by shock events (e.g., pulses of chemicals), we have developed a model to capture the dynamics of both juvenile *Daphnia* (*J(t)*) and adult *Daphnia* (*A(t)*) that uses delay differential

equations to capture the maturation time (τ) of juveniles. Juveniles and adults have different filtering capacity, hence including both in the model is critical to fully understand the overall population dynamics, and the chemical removal efficiency. The resulting equations are given by:

$$\begin{aligned}
\frac{dN}{dt} &= (N_0 - N)D - \phi_J(N)J - \phi_A(N)A, \\
\frac{dP}{dt} &= (P_0 - P)D - \phi_J(P)J - \phi_A(P)A, \\
\frac{dJ}{dt} &= e_A\phi_A(N)A - e_A \exp\left(-\int_{t-\tau}^{t} \delta_J\big(P(s)\big)\, ds\right)\phi_A\big(N(t-\tau)\big)A(t-\tau) - \delta_J(P)J, \\
\frac{dA}{dt} &= e_A exp\left(-\int_{t-\tau}^{t} \delta_J\big(P(s)\big)\, ds\right)\phi_A\big(N(t-\tau)\big)A(t-\tau) - \delta_A(P)A,
\end{aligned} \tag{1}$$

where $N(t)$ and $P(t)$ denote the nutrients (bacteria, algae, and organic matter) and pollutant concentration in the secondary clarifiers at time t. The constants N_0 and P_0 denote the concentration of the growth-limiting nutrient and death-inducing pollutant introduced to the system. The constant *D* is the dilution rate of the system. The functions ϕ_J and ϕ_A represent the specific per-capita nutrient/pollutant uptake of the juvenile and adult populations, and δ_J and δ_A represent the specific per-capita death rates which ultimately may be influenced by shock events to the system. The growth rate of the juveniles involves the adult efficiency factor e_A and e_A^{-1} describes the amount of nutrients per adult organism needed to generate one juvenile. The exponential term multiplying *A(t-τ)* represents the fraction of juveniles that survive for the time period to complete the conversion process to adults.

We use the following functions and parameters to illustrate the potential qualitative behaviour of *Daphnia* population dynamics in a closed environment resembling the prototype:

$$\phi_J\big(N(t)\big) = \phi_J N(t),$$

$$\phi_A\big(N(t)\big) = \phi_A N(t),$$

$$\delta_J\big(P(t)\big) = \delta_{J,}$$

$$\delta_A\big(P(t)\big) = \delta_A + \delta_{AP}P(t),$$

$A(t) = 1, J(t) = N(t) = P(t) = 0$, for $t \in [-\tau, 0], \tau = 6, \phi_J = 10, \phi_A = 20, \delta_J = 0.4,$

$$\delta_A = 0.1, \delta_{AP} = 10, e_A = 1, D = 0.5, N_0 = 100,$$

with $P_0 = 7$ (solid line) and $P_0 = 10$ (dashed line).

Table S1. Bold Basal Medium (BBM). Medium used to grow algae (*Chlorella vulgaris*) as feedstock for *D. magna* in the tandem bioreactors. The inorganic components are dissolved in deionised water.

Compound	**Empirical formula**	**Stock concentration (gL^{-1})**
Potassium di-Hydrogen orthophosphate	KH_2PO_4	17.50
di-Potassium Hydrogen orthophosphate	K_2HPO_4	7.50
Magnesium Sulphate	$MgSO_4 \cdot 7H_2O$	7.50
Sodium Nitrate	$NaNO_3$	25
Calcium Chloride	$CaCl_2 \cdot 2H_{20}$	2.50
Sodium Chloride	NaCl	2.50
EDTA Tetrasodium salt	$EDTA – Na_4$	50
Potassium Hydroxide	KOH	31
Ferrous Sulphate	$FeSO_4 \cdot 7H_2O$	4.98
conc. Sulfuric Acid	H_2SO_4	10ml
Boric Acid	HBO_3	11.42
Zinc Sulphate	$ZnSO_4 \cdot 7H_2O$	14.12
Manganese Chloride	$MnCl_2 \cdot 4H_2O$	2.32
Copper Sulphate	$CuSO_4 \cdot 5H_2O$	2.52

Table S2 COMBO medium. Growth medium for *Daphnia magna* in the tandem bioreactors. This medium could be swapped for filteredt lake or borehole water. The inorganic components are dissolved in deionised water. The COMBO may be further enriched with Biotin (d-biotin) and B12 (cyanocobalamin).

Compound	**Empirical formula**	**Stock concentration (gL^{-1})**
Calcium Chloride di-Hydrate	$CaCl_2 \cdot 2H_2O$	36.67
Magnesium Sulphate Heptahydrate	MgSO4 · 7H2O	36.97
Potassium Phosphate Dibasic	K_2HPO_4	8.71
Sodium Nitrate	$NaNO_3$	85.01
Sodium Bicarbonate	$NaHCO_3$	12.60
Sodium Metasilicate Nonahydrate	$Na_2SiO_3 \cdot 9H_2O$	28.42
Boric Acid	H_3BO_3	24
Potassium Chloride	KCl	7.45

Table S3. Techno-economic analysis chemical/mechanical processes. The strengths and weaknesses of six established technologies were compared to the *Daphnia*-based technology. These are ranked according to a red-amber-green criteria where red represents poor performance, amber represents mid-range performance, and green represents strong performance. Where data were not available, the cell is blank.

Techno-Economic Indicators		UV	Ozonation	Activated Carbon	Chlorination	Reverse Osmosis	Multi-Media Filtration	DWS
CAPEX (£/m^3/d)								
OPEX (£/m^3)								
Energy Consumption (KWh/m^3)								
Carbon Intensity (kg CO_2e/m^3)								
Hydraulic Retention Time								
Land Area Required (m^2/m^3/d)								
Contaminant removal	Organics (BOD, COD)							
	Pharmaceuticals (ibuprofen)							
	Pharmaceuticals (naproxen)							
	Pharmaceuticals (atrazine)							
	Pharmaceuticals (diclofenec)							
	Inorganics (nitrogen & phosphorus)							
	Bacteria							
	Virus							
	Protozoa							
Odour Impact								
Noise Impact								
Visual Impact								
Reliability								
By Products								
Employment (Employees/m^3/d)								
Public acceptance								
Complexity								

Table S4. Techno-economic analysis biological wastewater treatments. The strengths and weaknesses of seven bio-based technologies were compared to the *Daphnia*-based technology. These are ranked according to a red-amber-green criteria where red represents poor performance, amber represents mid-range performance, and green represents strong performance. Where data were not available, the cell is blank. *MBR = membrane biofilm reactor, RBC = rotating biological contactor, HSF = horizontal subsurface flow, FWS = Free water sur-face, PBR = packed bed reactor, DWS = Daphne Water Solutions*

Techno-Economic Indicators		Dram (Epona)	MBR	RBC	HSF	FWS	PBR	Photobioreac-tor (algae)	DWS
CAPEX (£/m^3/d)									
OPEX (£/m^3)									
Energy Consumption (KWh/m^3)									
Carbon Intensity (kg CO_2e/m^3)									
Hydraulic Retention Time									
Land Area Required (m^2/m^3/d)									
Contaminant removal	Organics (BOD, COD)								
	Pharma-ceutical (Ibo-profen)								
	Pharma-ceutical (Naproxen)								
	Pharma-ceutical (Atrazine)								
	Pharma-ceutical (Diclo-fenac)								
	Inorganics (N+P)								
	Bacteria								
	Virus								
	Protozoa								
Odour Impact									
Noise Impact									
Visual Impact									
Reliability									
By Products									
Employment (Employees/m^3/d)									
Public acceptance									
Complexity									

Figure S1. Experimental design for laboratory exposures. Resurrected *Daphnia magna* genotypes from lake sediment deposits were maintained in controlled laboratory conditions (20 ± 2 ^{0}C, 16:8hr light: dark photoperiod and fed daily with 0.8 mg C/L of *C. vulgaris*) for two generations before exposure to single chemicals and wastewater secondary treated effluent. Two generations in these conditions were used to control for material effect. Single chemicals were quantified following exposure of four *D. magna* genotypes over 3 days to PFOS - 70ng/L; Diclofenac - 2mg/L; Atrazine - 0.2mg/L and Arsenic - 1mg/L. The same four *D. magna* genotypes were also exposed to wastewater effluent and the same four chemicals were quantified both in the starting effluent and after exposure to *Daphnia*.

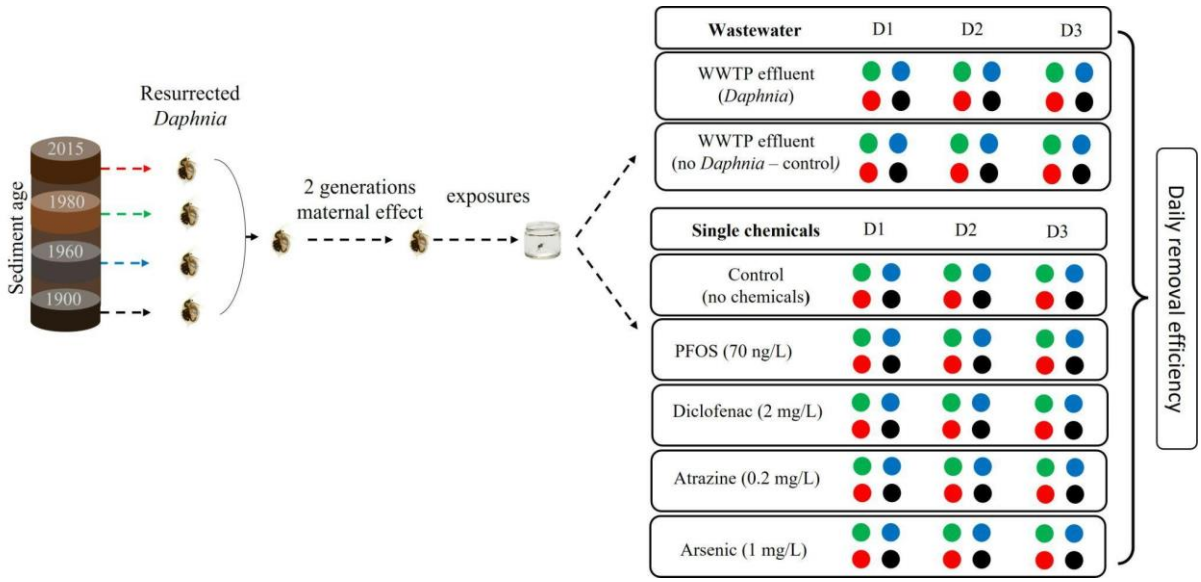


Figure S2. Flow diagram for the *Daphnia*/algae tandem bioreactor. Algae growth medium (Bold Basal Medium, BBM described in Table S1) is introduced in the algae vessel that has been inoculated with a culture of *Chlorella vulgaris* through an automated system. When the optical density of the algae reaches a stable value of 0.5 ± 0.2 OD measured at 440 nm, the algae population has reached a carrying capacity. At this point, the isolation valve is activated and a regular flow of medium passes from the algae to the *Daphnia* vessel, where the *Daphnia* population is maintained at a constant density of 150/200 individuals/L. The density is monitored regularly by counting the number of *Daphnia*/100ml. When the *Daphnia* population density has reached a steady state, it can be harvested at regular intervals for seeding or topping up the live environment through the 'harvest valve'.

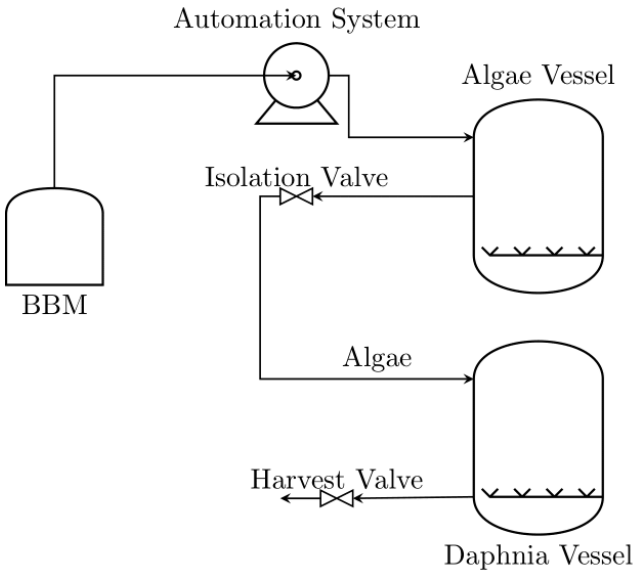


Figure S3. Environmental parameters in the outdoor prototype. Temperature and oxygen (A) measured daily in the outdoor prototype; and *Daphnia* population density (individuals/L) monitored weekly (B).

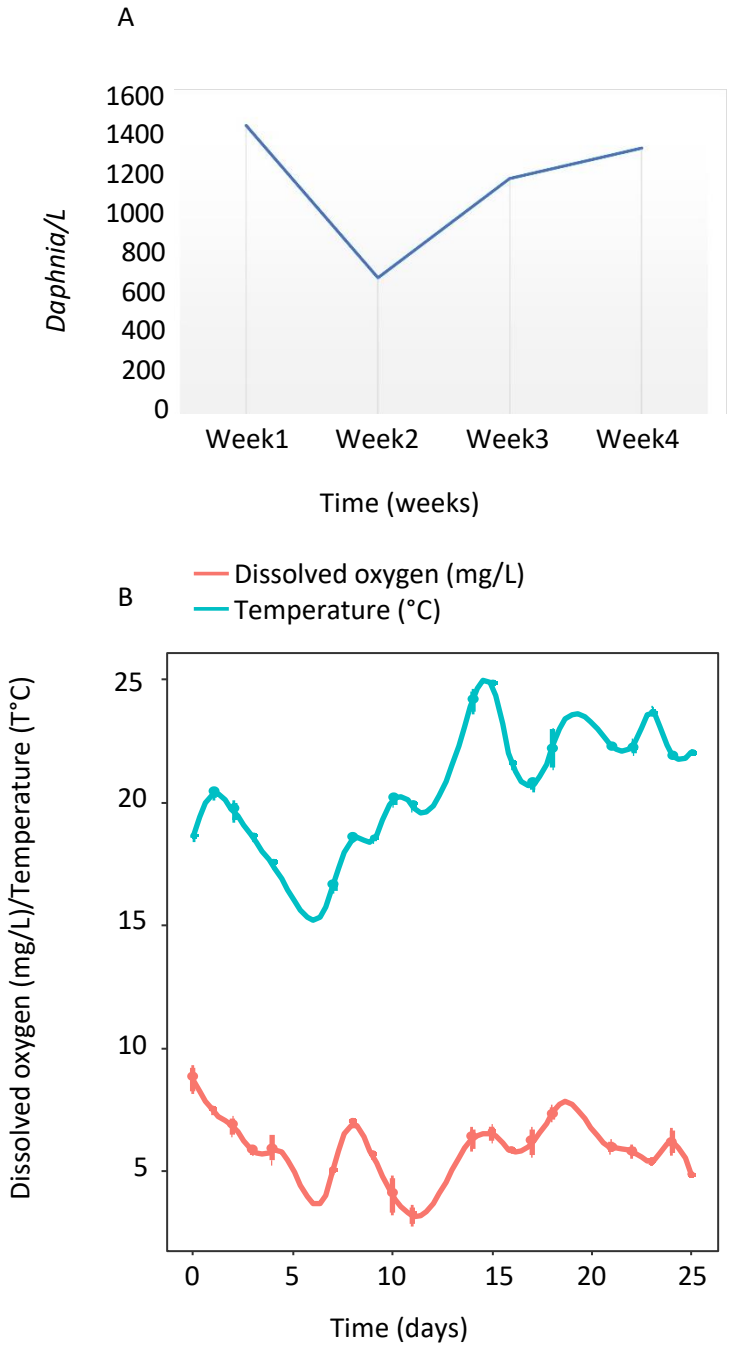


Figure S4. *Daphnia* population dynamics in a closed environment. Simulation results from a delay differential equation population dynamics model illustrating qualitatively how a change in environmental conditions, here represented by different inflows of pollutants, can affect the *Daphnia* population over time: high pollutants result in a population collapse (dashed red line); regular pollutant loads expected in wastewater treatment plants for municipal wastewater result in an oscillating equilibrium of the population over time (solid blue line). This oscillating dynamic is determined by death and birth rate.

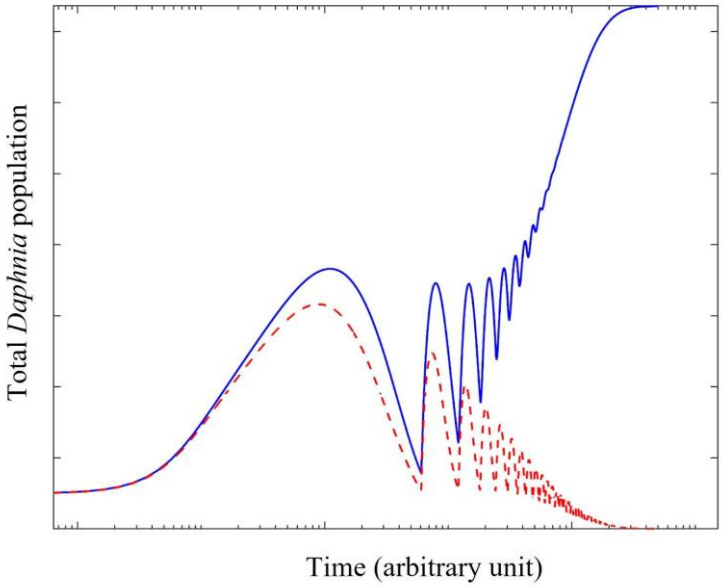


Figure S5. Process of separation of the soft body from the chitin shell. A) Concentrated aqueous suspension of *D. magna*. B) Thermal hydrolysis reaction residues after filtration at 180°C, 220°C and 240°C. C) *D. magna* residues after reactions at 220°C and D) 240°C. The darkest areas in panel C show organic material still embedded in the chitin fraction. The organic matter is almost completely absent in panel D.