Science of the Total Environment 949 (2024) 174963

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Interactions between phenanthrene exposure and historical chemical stress: Implications for fitness and ecological resilience of the sentinel species *Daphnia magna*

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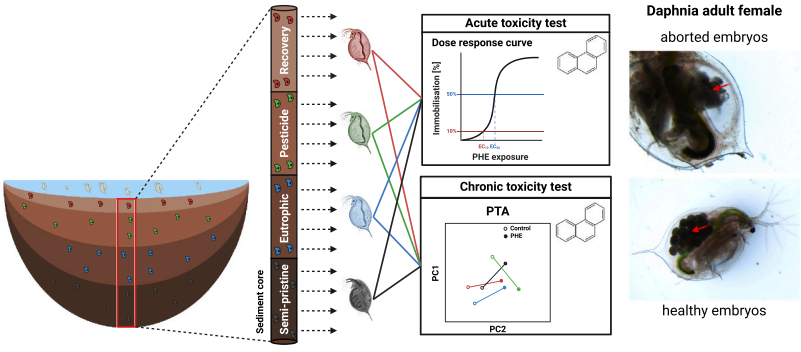
HIGHLIGHTS

- Leveraging *Daphnia* dormancy, we assessed acute and chronic PHE toxicity in strains with varied contaminant exposure histories
- PHE exposure induces developmental failure, delays sexual maturation, and reduces adult size in *Daphnia*
- Populations of *Daphnia* with chemical exposure history had greater fitness impacts than naïve ones
- We challenge the validity of current toxicity practice based on acute toxicity tested of single genotypes

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
PAHs
Waterflea
Acute toxicity

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) arise from incomplete combustion of oil, coal, and gasoline, with lipophilic properties facilitating their widespread distribution and persistence. Due to their biochemical attributes, PAHs can accumulate in animal tissues, potentially causing mutagenic and carcinogenic effects. Since the industrial revolution, PAH concentrations in the environment have risen, with lakes showing levels from 0.159 to 33,090 μg/kg sediment. Despite acute toxicity studies showing adverse effects on freshwater organisms, the long-term impacts and synergistic interactions with other pollutants remain largely unexplored.

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https://doi.org/10.1016/j.scitotenv.2024.174963
Received 27 May 2024; Received in revised form 16 July 2024; Accepted 20 July 2024
Available online 26 July 2024

Chronic toxicity
Fitness

This study investigates the impact of phenanthrene (PHE), a prominent PAH found in aquatic environments, on *Daphnia magna*, a species of significant ecological importance in freshwater ecosystems globally, being both a sentinel species for chemical pollution and a keystone organism in freshwater aquatic ecosystems. Leveraging the dormancy of *D. magna*, which spans decades or even centuries, we exposed strains with diverse histories of chemical contaminant exposure to environmentally relevant concentrations of PHE. Initially, acute exposure experiments were conducted in accordance with OECD guidelines across 16 *Daphnia* strains, revealing substantial variation in acute toxic responses, with strains naïve to chemical pollutants showing the lowest toxicity. Utilizing the median effect concentration EC10 derived from acute exposures, we assessed the impacts of chronic PHE exposure on life history traits and ecological endpoints of the 16 strains. To elucidate how historical exposure to other environmental stressors may modulate the toxicity of PHE, temporal populations of *D. magna* resurrected from a lake with a well-documented century-spanning history of environmental impact were utilized. Our findings demonstrate that PHE exposure induces developmental failure, delays sexual maturation, and reduces adult size in *Daphnia*. Populations of *Daphnia* historically exposed to chemical stress exhibited significantly greater fitness impacts compared to naïve populations. This study provides crucial insights into the augmented effects of PAHs interacting with other environmental stressors.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a family of ubiquitous environmental pollutants originating from incomplete combustion of natural or anthropogenic material. The source of these compounds can be natural (wildfires, volcanic eruptions, and coals) (Gorshkov et al., 2021; Kozak et al., 2017; Meyer et al., 2013), but it is most often linked to anthropogenic activities (Du and Jing, 2018; Liu et al., 2012). Incomplete combustion from fossil fuel, coal, gas, gasoline, wood, dispersed oil exposure, and waste incineration is the primary source of PAHs release into the environment (Abdel-Shafy and Mansour, 2016; Esteban-Sánchez et al., 2021; Gad, 2014; Kakareka and Kukharchyk, 2003; Slezakova et al., 2013). Due to the low water solubility and high lipophilic properties of many PAHs, these pollutants are persistent in the environment. Their high volatility facilitates their wide distributional range, both geographically and across environmental matrices (Friedman et al., 2014; Friedman and Selin, 2012).

Climate change contributes to the persistence and distribution of PAHs globally (Friedman et al., 2014; Kallenborn et al., 2012). Firstly, global warming induces higher volatilization of PAHs, which are globally transported by wind and precipitations, even to remote regions of the globe (Macdonald et al., 2000). Secondly, the rapid melting of glaciers and permafrost is rapidly exposing century old soil and sediment that releases PAH as a by-product of biomass biotransformation and combustion (Bogdal et al., 2011; Kosek et al., 2019). Thirdly, flood events have been shown to remobilize PAH historical contaminated sediments (Crawford et al., 2022) and fourthly, extreme droughts are the main cause of extensive wildfires that contribute to the release of PAHs in the atmosphere (e.g. Canada, Australia (Argiriadis et al., 2024; Di Virgilio et al., 2019; Kieta et al., 2022)). With increasing temperature and recurrence of extreme events forecasted for the next 30 to 50 years, the impact of PAHs and other polycyclic aromatic compounds on wildlife and humans is projected to increase dramatically (Muir and Galarneau, 2021).

Petrogenic (fossil fuel-derived) and pyrogenic (combustion-derived) PAHs have different bioavailability and, hence, different routes for bioaccumulation in wildlife (Hylland, 2006). Petrogenic PAHs are typically smaller and immediately bioavailable because they are water soluble; their main route of uptake is through ingestion of food or sediment (Douben, 2003). Conversely, pyrogenic PAHs are associated with particles, sometimes even incorporated in the structure of particles, which significantly decreases their bioavailability; their accumulation route is through binding to lipid-rich tissues, and their metabolites can be found in most animal tissues (Balmer et al., 2019; Meador et al., 1995). One of the most abundant PAHs in freshwater ecosystems is Phenanthrene (PHE); this compound is found both in the water column due to its high solubility (Abdel-Shafy and Mansour, 2016; Behera et al., 2018) and in sediment, where it binds to organic particles becoming highly persistent (Dhar et al., 2023; Du and Jing, 2018). Freshwater lentic ecosystems (lakes and ponds) are particularly susceptible to PHE pollution because they are 'receivers' on the landscape of river and urban effluents as well as land-use run-off (Huang et al., 2019; Zheng et al., 2011). This strategic position on the landscape, combined with the decadal half-life of PHE, means that lake sedimentary archives can be used to document PAHs historical pollution from the industrial revolution until modern times (Lv et al., 2020; Sutilli et al., 2020).

Volatilization and photo-oxidation of PAHs in aquatic ecosystems can result in by-products that are more or equally toxic than the parent compound to wildlife (Marwood et al., 2003). Although comprehensive reviews on the ecotoxicity of PAHs on aquatic wildlife are scarce, several adverse effects have been documented (Alegbeleye et al., 2017; Honda and Suzuki, 2020; Hylland, 2006). These include cardiotoxicity (Ainerua et al., 2020; Brette et al., 2017; Marris et al., 2020), teratogenicity (Seiler et al., 2014), and liver toxicity in fish (Haque et al., 2017); behaviour impairment and neurotoxicity in amphipods (Gauthier et al., 2016); and alteration of the cellular structure and photosynthetic activity in primary producers (Okumura et al., 2003; Othman et al., 2023). Evidence starts to emerge that the toxic effect of soluble PAHs can augment the effect of other pollutants on wildlife (Kim et al., 2018). Understanding the influence of past exposures on toxic responses to ubiquitous compounds, such as PAHs, is critical to assess species persistence under projected PAH concentrations. Our study addresses this knowledge gap by assessing the acute and chronic toxicity of PHE on populations of the sentinel species *Daphnia magna* with different histories of exposure to other pollutants.

Daphnia is a sentinel species for environmental health, traditionally used by regulators to set safe thresholds of chemicals in the environment (Abdullahi et al., 2022a). *Daphnia* is also a keystone species in freshwater aquatic food webs, where it is a grazer of algae and bacteria and preferred food of small fish and invertebrates (Abdullahi et al., 2022a; Altshuler et al., 2011). Its responsiveness to environmental pollution and its ecological relevance in freshwater ecosystems, make *Daphnia* a useful tool to assess the impact of PHE on freshwater organisms. An additional advantage of *Daphnia* is its exceptionally long dormancy, which allows us to access past populations with different history of exposure to environmental pollutants (Cuenca Cambronero and Orsini, 2018). Capitalising on the properties of *Daphnia*, we used populations resurrected from the sedimentary archives of Lake Ring, a lake with a well-paced and documented history of anthropogenic impact over the past century (Cuenca Cambronero et al., 2018). We were able to access temporal populations spanning from a semi-pristine environment to a eutrophic environment due to due to sewage inflow, and to populations exposed to high level of biocides due to agricultural run-off; the most modern population of *Daphnia* from this sedimentary archive showed lower nutrient and biocides levels (Cuenca Cambronero et al., 2018). Previously resurrected *Daphnia magna* genotypes from these temporal populations were used in this study to identify acute and chronic toxic response to relevant concentrations of PHE. We expected that

populations that had historically experienced chemical stress would have higher tolerance to PHE and, hence, express lower fitness costs that their naive counterparts. This hypothesis was based on the concept of evolved tolerance to chemical stress. Firstly, we completed a comprehensive assessment of acute toxicity on 16 *D. magna* genotypes from the four temporal populations that experienced different historical exposures to environmental stressors. Typically, acute toxicity is assessed on a single commercial strain of *Daphnia*, which may have been kept in laboratory conditions for decades (Eom et al., 2007; Hodson, 2017; Tani et al., 2021). From this initial experiment on a suite of wild *Daphnia* genotypes, we defined the median effect concentration (EC10), which we subsequently used in chronic exposures to quantify the impact of PHE on *Daphnia* fitness conditional on previous exposures to environmental stress. Our results confute the hypothesis of evolved tolerance, showing higher fitness costs in *Daphnia* populations previously exposed to chemical stress. Because of the central role that *Daphnia* plays in aquatic foodwebs, impact of PHE on this species can have cascading effects on both consumers and feeders of *Daphnia*.

2. Materials and methods

2.1. Study system

The *Daphnia* genotypes used in this study were previously resurrected from the sedimentary archive of Lake Ring, a shallow mixed lake in Jutland, Denmark (55°57′51.83″N. 9°35′46.87″E) (Cuenca Cambronero et al., 2018). The lake was semi pristine between the 1900s and 1950s (semi-pristine phase; SP); sewage inflow from a nearby town increased the lake's trophic levels leading to eutrophication from the 1950s to the 1970s (eutrophic phase; EP). Sewage was diverted from the 1980s, when run-off from agricultural land surrounding the lake caused an increase in pesticides inflow (1980–1990; pesticide phase; PP). Land use declined in modern times when the lake started recovering (Recovery Phase; RP) (Cuenca Cambronero et al., 2018; Davidson et al., 2007; Eastwood et al., 2023). Four *D. magna* genotypes from each lake phase were used in this study for acute and chronic exposures (Fig. 1).

Before the experiments, *Daphnia* genotypes were acclimated at 20 ± 1 °C, 16:8 h light-dark photoperiod, and fed ad-libitum with 0.8 mg/L of *Chlorella vulgaris* (strain CCAP 211/11B) for two generations to reduce interference from maternal effect and to synchronise reproduction among genotypes. The growth medium used was borehole water, collected from a deep aquifer well and showing stable physico-chemical properties. After purging maternal effect, clonal replicates of the 16 genotypes were exposed to phenanthrene (PHE) to assess acute and chronic toxicity.

2.2. Acute and chronic toxicity of phenanthrene (PHE)

2.2.1. Acute toxicity exposures

Following two generations of acclimation and having controlled for maternal effect, 24-h-old juveniles from the second or following broods were randomly assigned to experimental exposures. The OECD 202 guidelines were followed to perform immobilization assays and extrapolate dose response curves (OECD, 2004; Weber, 1991).

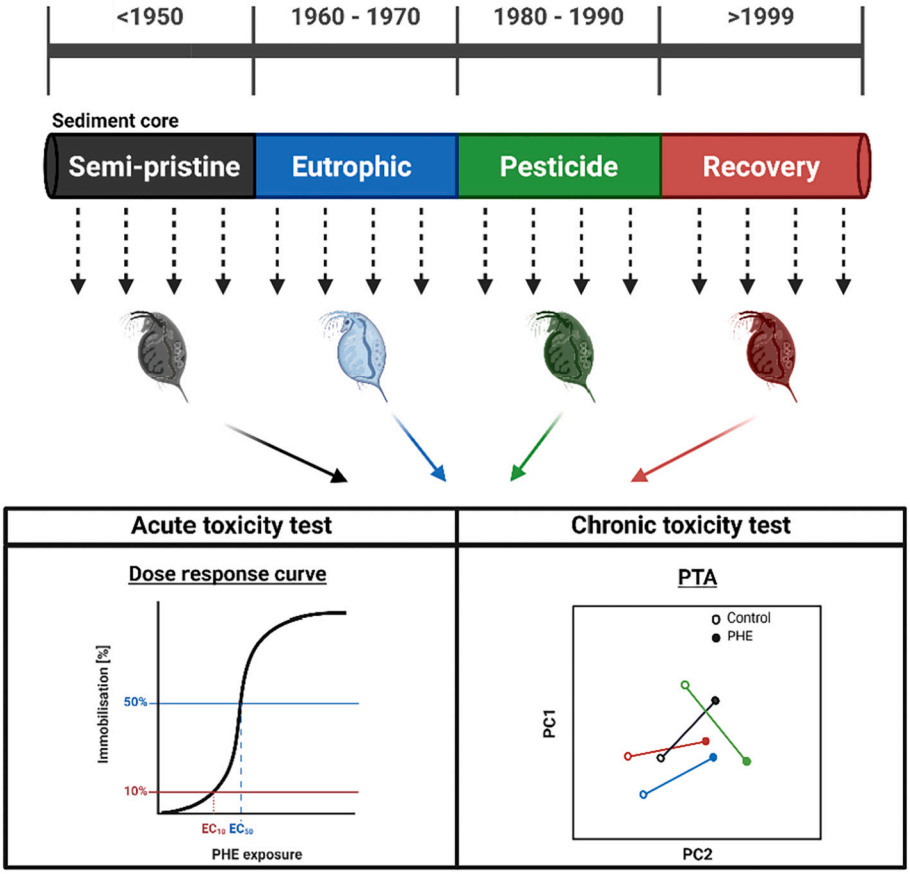

Fig. 1. Experimental design. Temporal populations of *Daphnia magna* previously resurrected from a sedimentary archive of Lake Ring were stored in standard laboratory conditions for several generations. Four lake phases were previously identified to correspond to major shifts in water quality: SP - semi-pristine phase; EP – eutrophic phase; PP – pesticide phase; RP – recovery phase. Four genotypes from each of these lake phases were used in acute and chronic toxicity tests. The mean EC10 derived from acute toxicity tests on all genotypes was used in the chronic exposures, in which fitness-linked life history traits were measured. The 16 genotypes were maintained in standard laboratory conditions (16:8-h light:dark regime; 20 °C and fed ad libitum with 0.8 mg carbon/L of *Chlorella vulgaris* daily) for two generations before commencing the acute and chronic exposures to control for maternal effect.

Immobilization assays are commonly used to assess the toxicity of chemical compounds in aquatic environments. In these assays, *Daphnia* are exposed to different concentrations of a test substance, typically over a period of 48 h. The ecological endpoint recorded in these assays is immobilization or lack of movement of the organisms, which serves as an indicator of toxicity. The exposed *Daphnia* are not fed in immobilization assays. The concentration at which a certain percentage of *Daphnia* become immobilized is referred to as effective concentration (EC). We conducted the immobilization assays on 12 biological replicates of each *Daphnia* genotype, each including 5 individuals, across five PHE concentrations: 134 μg/L; 267 μg/L; 535 μg/L; 1069 μg/L; 2139 μg/L ($N = 192$). As DMSO at a final concentration of 0.001 % was used as carrier for PHE, the control used for the immobilization assays were *Daphnia* in borehole water (no chemicals) and *Daphnia* in DMSO (0.001 %). As no significant difference was observed between control and DMSO, we only include DMSO control in the analyses and plots. Immobilization and mortality were assessed after 48 h exposure. EC50, EC25, and EC10 were calculated with GraphPad Prism version 10.0.3 using (1). Dose-response curves were plotted for individual genotypes and for temporal populations sampled from the four lake phases using GraphPad Prism version 10.0.3, using log-transformed immobilization values.

$$EC_F = \left(\frac{F}{100 - F}\right)^{\frac{1}{H}} \bullet EC_{50} \quad (1)$$

where *EC* is effect concentrations, *F* is Fraction of maximal response and *H* is the Hill Slope.

2.2.2. Chronic toxicity exposures

The EC10 mean value across the 16 genotypes (428 μg/L of PHE) from the acute toxicity test was used to perform chronic exposures encompassing the life cycle of *Daphnia* (i.e. until release of the second brood). Thirteen of the 16 genotypes used in the acute toxicity tests were exposed, while LRV 0_2, LRV 13_2, and LRII 48_1 failed early in the experiment due to unforeseen circumstances and were excluded. Clonal replicates of the 13 genotypes were randomly selected from the same cultures acclimated at 20 ± 1 °C, 16:8 h light-dark photoperiod, as used for the immobilization.

For each genotype, six clonal replicates were exposed to 428 μg/L of PHE and six were maintained in control conditions (same as the experimental exposure minus PHE) ($N = 48$). During the experiment, the growth medium was replenished every other day and spiked with the same concentration of PHE at each medium change to ensure a constant exposure to PHE across the life cycle of the organism. The experimental *Daphnia* were fed daily with 0.8 mg L^{-1} *C. vulgaris* and maintained at a constant temperature of 20 °C. On both controls and exposed clonal replicates, a suite of fitness-linked life history traits was measured for the duration of *Daphnia* life cycle (i.e. until each clonal replicate released their second brood). The life history traits measured were age at maturity (first time the parthenogenetic eggs are released in the brood pouch); size at maturity (distance from the head to the base of the tail spine); fecundity (number of juveniles across the first two broods); interval between broods (days between the first and second brood) and mortality. Size was measured after the release of the second brood, using ImageJ software (https://imagej.nih.gov/ij/).

2.3. Statistical analysis

2.3.1. Acute toxicity

An analysis of variance (ANOVA) was used to test the effect of treatment concentration, lake phase and their interaction on acute toxic response of *Daphnia* to PHE. A Post-Hoc Tukey-HSD test (Faria and Jelihovschi, 2021) was used to identify the concentrations and populations of *Daphnia* showing significant difference in toxic response.

2.3.2. Chronic toxicity

We assessed the variation for the five fitness-linked life history traits (age at maturity, size at maturity, fecundity, interval between broods and mortality) across the lake phases using a two-way ANOVA. We tested the effects of lake phase (LP) and phenanthrene treatment (PHE), as well as their interaction term (LP x PHE) on the life history traits, followed by a post-hoc analysis. A nested linear mixed effect model was used, with genotypes and replicates as random effects nested within lake-phase using the "lmerTest" package in R (Kuznetsova et al., 2017). We visualised the main effects of phenanthrene, lake-phase and their interactions using 'reaction norm' plots. We visualised the effect of PHE on mortality rate per lake phase using the Kaplan-Meier curves in Graphpad, which show the cumulative survival probability of each temporal population in control and exposed conditions (Miller, 2003). Multivariate effects were calculated using multivariate statistics (MANOVA) by combining the life history traits (age at maturity, size at maturity, fecundity, and interval between broods) as a response variable (y) and phenanthrene and lake-phase as fixed terms (y ~ phenanthrene * lake-phase).

We visualised the multivariate analysis results using phenotypic trajectory analysis (PTA) plots to describe the difference in multivariate reaction norms in terms of magnitude and direction of change following (Adams and Collyer, 2009). The magnitude of the reaction is the amount of phenotypic change between control and treatment, whereas the direction indicates a positive, negative, or linear change in fitness between control and treatment (Collyer and Adams, 2007). The R code provided by Adams and Collyer (2009) was used for the PTA.

3. Results

3.1. Acute and chronic toxicity of phenanthrene (PHE)

3.1.1. Acute toxicity

The immobilization assays (acute toxicity tests) revealed significant different toxicity among genotypes and lake phases to the five concentrations of PHE (Fig. 2; Table 1). A *post-hoc* analysis identified the semi-pristine phase population to have a significantly different toxicity response, showing an overall higher tolerance to PHE, whereas the other phases did not significantly differ in their acute toxic response to PHE (Fig. 2; Table S1). The minimum recorded EC10 was 173 μg/L and the maximum was 622 μg/L. The EC25 ranged between 281 μg/L and 828 μg/L. The EC50 ranged between 457 μg/L and 1103 μg/L. Across the three effective concentrations, LRV1_2 (recovery phase; RP) had the lowest and LRIII88_1 (semi-pristine phase; SP) the highest tolerance, respectively (Table S2). The mean EC10 value (428 μg/L) was used for the chronic exposures.

3.1.2. Chronic toxicity

PHE had a significant effect on the overall fitness of the *Daphnia* populations from the four lake phases, driven by the effects of the treatment on several individual life history traits (Table 2). The multivariate analysis revealed a significant effect of treatment (PHE) on *Daphnia*'s overall fitness, a significant different response among the lake phases to PHE and a significant interaction term between treatment and lake phases (Table 2; MANOVA). A *post-hoc* analysis revealed that the significant differences among the lake phases were driven by the semi pristine and recovery phases being significantly different from the pesticide's population (Table S3). The multivariate effects were visualised through PTA plots confirming a significant change in overall fitness between control and exposed genotypes in all lake phases (Fig. 3; M). Conversely, the lake phases did not significantly differ in the direction of fitness change between control and treatment (Fig. 3; θ).

The ANOVA analysis supported by univariate reaction norms allowed us to identify trait specific responses to PHE treatment that contributed to the overall fitness response recorded by the PTA. PHE had a significant effect on all life history traits (Table 2; ANOVA, PHE), but

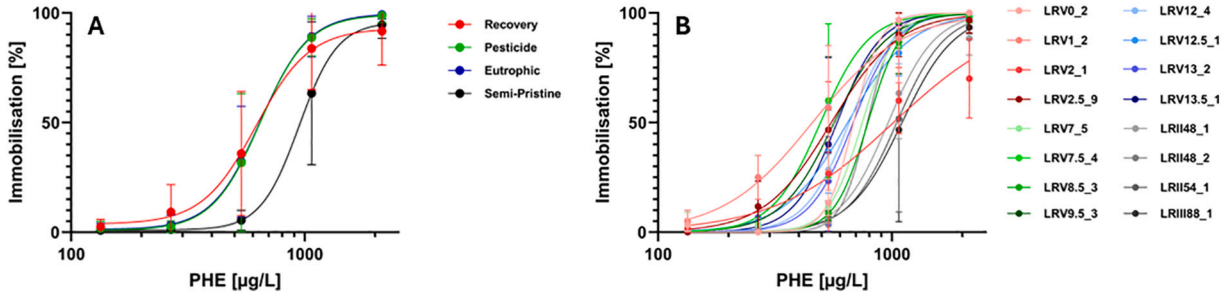

Fig. 2. Acute toxicity response to PHE. Dose response curves are shown summarising the immobilization assays per lake phase (A) (phases are as in Fig. 1) and per genotype (B). They represent the percentage of immobilized *Daphnia* following 48 h exposure to five concentrations of PHE: 134 µg/L; 267 µg/L; 535 µg/L; 1069 µg/L; 2139 µg/L; and control (no PHE). Mean and standard deviations were calculated on 4 genotypes, (each in 12 biological replicates), per lake phase ($N = 192$). The dose response curves are supported by Tables S1 and S2. The mean EC10 across the 16 genotypes (428 µg/L of PHE) was used to perform chronic toxicity exposures.

Table 1
Analysis of variance for acute toxicity tests. Univariate (ANOVA) analysis of variance measured on the acute exposures to five concentrations of phenanthrene [134 µg/L; 267 µg/L; 535 µg/L; 1069 µg/L; 2139 µg/L; and control (no PHE)], testing toxic response to different PHE concentrations (PHE), lake phases (LP) and their interaction term. The dose response curves supporting these statistics are in Fig. 2. Significant *P*-values are in bold.

	Df	F	P-val
PHE	5	459.5	**0.0001**
LP	3	9.05	**0.0001**
PHE x LP	15	2.87	**0.0003**

the most striking result was developmental failure, evident from aborted broods. In addition, we observed delayed sexual maturity, smaller size at maturity, longer time between broods and lower fecundity (Fig. 4; Table 2). PHE exposure significantly increased *Daphnia* mortality across the temporal populations (Fig. 5; Table 2). The response to PHE significantly differed among lake phases for age at maturity, fecundity, and time between clonal reproductions (Table 2; ANOVA, LP). The effect of PHE varied significantly by lake phase for age at maturity, size at maturity and interval between broods but didn't show significant differences for mortality and size at maturity (Table 2; ANOVA, LP x PHE).

4. Discussion

We hypothesized that *Daphnia* populations historically exposed to environmental stress, particularly chemical stress, would exhibit reduced fitness costs when exposed to phenanthrene (PHE) compared to their naïve counterparts. This expectation was grounded in the concept of evolved tolerance to chemical stress. Leveraging the prolonged dormancy of *Daphnia*, we tested this hypothesis by examining tolerance levels conditional on prior exposure to chemical stress using resurrection paleo-ecotoxicology. This approach allowed us to evaluate the toxic effects of PHE on the same population of *Daphnia* exposed to various environmental insults over time, mitigating the influence of different genetic backgrounds (Orsini et al., 2016).

Our study unveiled that both acute and chronic responses to PHE were influenced by previous exposure to persistent chemicals and other pollutants, with genotypes historically exposed to pollution displaying lower tolerance to PHE. Specifically, the temporal population of *Daphnia* previously exposed to biocides from agricultural runoff exhibited the lowest tolerance to PHE, whereas the naive population had the highest tolerance. Contrary to our initial hypothesis, our findings reject the notion that populations previously subjected to chemical stress possess an evolutionary advantage over naïve populations when experiencing novel chemical stressors. Despite contradicting our original hypothesis, our results align with previous research on *Daphnia*, indicating higher tolerance to recurring stress but not to novel stressors (Abdullahi et al., 2022b; Cuenca-Cambronero et al., 2021; Cuenca Cambronero et al., 2018). Paleo-ecotoxicology studies are relatively uncommon due to the specialized skills required to resurrect historical *Daphnia* populations. However, they offer valuable insights into evolutionary mechanisms of chemical tolerance. Previous paleo-ecotoxicology experiments on the same *Daphnia* temporal populations utilized in our study included a pesticide, a heavy metal, two pharmaceuticals, and an industrial chemical, albeit limited to a single genotype from each temporal population studied here (Abdullahi et al., 2022b). These studies explicitly examined the hypothesis that prior exposure to environmental stress enhances tolerance to chemical stress. However, evidence from this previous study showed that naïve *Daphnia* strains exhibited greater tolerance to novel chemical stressors. Conversely, "experienced genotypes" historically exposed to chemical stress showed tolerance to recurrent stressors. Considering that Lake Ring is predominantly rural, and thus PHE is likely not abundant in its waters, our results confirm that historical exposure to environmental stress diminishes tolerance to novel chemical stress, resulting in reduced fitness responses in 'experienced' populations, both in terms of acute and chronic toxicity responses.

Effect concentrations based on tests conducted on single commercial strains of *Daphnia* are used by regulatory agencies to define safe concentrations of chemicals in the environment. Our study challenges the OECD practice based on acute toxicity tested on single genotypes. We employed multiple genotypes to assess acute toxicity effect concentrations, uncovering significant variations in the acute toxic responses of 16 genotypes of *D. magna* to phenanthrene (PHE). Our findings indicate that relying solely on tests with single genotypes may lead to inaccurate estimates of toxicity, impacting the determination of safe environmental concentrations for chemicals. Furthermore, our results emphasize that the historical exposure of the strain used to assess toxicity can influence its response to chemicals. Failure to account for prior exposures and population level responses could introduce bias into chemical toxicity assessments. To our knowledge, our study is the first to comprehensively evaluate acute toxicity responses across *Daphnia* genotypes and populations, shedding light on a critical limitation of current toxicity assessment practices. Striking a balance between the time required for chemical testing and the number of assays regulators must evaluate is challenging. However, our results show that it is advisable to expand on the number of genotype and, potentially, on the number of species tested to have a more accurate estimate of toxicity.

Our investigation into the chronic toxic effects of exposure to phenanthrene (PHE) encompassing the life cycle of *Daphnia* revealed several adverse outcomes in *Daphnia*. These included developmental failures, evidenced by the presence of parthenogenetic eggs in the *Daphnia*'s brood pouch that failed to hatch into juveniles, as well as delays in sexual maturation and reduced size at maturity. Notably, genotypes previously exposed to chemical stress experienced significantly greater fitness costs compared to their naïve counterparts. Interestingly,

Table 2
Analysis of variance for chronic exposures. Multivariate (MANOVA) and univariate (ANOVA) analysis testing response of fitness-linked life history traits to phenanthrene (428 μg/L) in chronic exposures. The effect of treatment (PHE), Lake phase (LP) and their interaction term on five *Daphnia* fitness-linked life history traits was quantified. The life history traits assessed were age at maturity (days), size at maturity (mm), fecundity (number of offspring produced in the first two broods), interval between broods (days) and mortality. The concentration of phenanthrene used for the chronic exposures was the mean EC10 value across the 16 genotypes used in the acute toxicity test (Fig. 2).

	MANOVA			ANOVA											
				Age at maturity (day)		Size at maturity (mm)		Fecundity		Interval broods (days)		Mortality	
	df	F	P-val	F	P-val	F	P-val	F	P-val	F	P-val	F	P-val
PHE treatment (PHE)	1	316.99	**<2.2E-16**	1322.75	**<2.2E-16**	288.14	**<2.2E-16**	94.6128	**<2e-16**	146.12	**<2.2e-16**	22.255	**2.386E-06**
Lake Phase (LP)	3	6.79	**2.66E-10**	8.9604	**0,02982**	61,164	0.1060812	31.9738	**5.30E-07**	12.664	**0.005423**	3.2507	0.3546
LPxPHE	3	9.53	**1.09E-14**	24.1155	**2.36E-02**	18.5804	**0.0003338**	2.2379	0.5245	47.380	**2.89E-10**	2.7766	0.4274

there was no significant difference in mortality observed among temporal populations of *Daphnia* when exposed to PHE. This suggests that focusing solely on immobilization or mortality as ecological endpoints may not fully capture the impact of chemicals on wildlife. Adverse effects on fitness, particularly in terms of development, are of concern as they can affect the resilience and adaptation of key species like *Daphnia* to future environmental changes, with potential cascading effects on the aquatic food web.

Daphnia play a critical role in freshwater ecosystems, serving as prey for planktivorous fish and invertebrates, as well as participating in nutrient cycling and degradation as filter feeder (Lampert, 2006; Miner et al., 2012; Seda and Petrusek, 2011). Previous studies on the toxicity of polycyclic aromatic hydrocarbons (PAHs) in fish have shown reduced growth, embryonic abnormalities, and developmental failure (Carls et al., 2008; Incardona, 2017; Meador et al., 2006). PAHs have also been implicated in reproductive impacts across various species, linked to disruptions in sex hormone metabolism even at low concentrations (Behera et al., 2018; Wallace et al., 2020). Additionally, PAHs can impede the growth of phytoplanktonic species by interfering with their photosynthetic abilities (Wang et al., 2008). Therefore, our study, demonstrating the toxicity of PHE to *Daphnia* and its impacts on development, growth, and reproduction, corroborates findings of toxicity observed in both vertebrates and invertebrates studied thus far.

One limitation of our study is the lack of biomolecular data on the studied populations, which would have allowed us to link observed fitness effects with the underlying molecular mechanisms and thus the mode of action of phenanthrene on *Daphnia*. A prior paleo-ecotoxicology investigation on four genotypes of *Daphnia* with varying histories of exposure to environmental stress identified a connection between lower diversity in detoxifying genes and reduced tolerance to novel chemical stress (Abdullahi et al., 2022b). Conducting genome-wide transcriptome analysis on the *Daphnia* populations utilized in our study holds promise for identifying the molecular mechanisms of toxicity associated with one of the most common polycyclic aromatic hydrocarbons in water. Given that *Daphnia* shares genes that are ancestral in animal genomes and present across phylogenetically distant species, elucidating the molecular mechanisms of PHE toxicity could provide insights into potential toxicity targets across species, guiding further in vivo and in vitro validation of toxic effects in human surrogate models (Abdullahi et al., 2022a; PrecisionTox, 2023).

5. Conclusions

Our research has challenged the OECD practice by demonstrating that natural genotypes of *D. magna* significantly differ in their acute toxicity levels depending on the environmental stress they have previously encountered. This evidence underscores the need for an update in regulatory practices governing the safe use of chemicals.

Due to its toxicity, phenanthrene (PHE) has been classified as a Substance of Very High Concern (SVHC) and included in the Candidate List for authorization by the European Chemicals Agency (ECHA) (Doc.: ED/88/2018, Source: European Chemicals Agency, https://echa.europa.eu/). Our study on chronic exposures to PHE revealed larger fitness costs in *Daphnia* populations previously exposed to environmental stress, particularly chemical stress. Long-term exposure to PHE resulted in developmental failure, impaired growth, and delayed reproduction, indicating potential disruptions of endocrine and growth pathways. The interaction between past chemical exposure and PHE suggests a compounded effect of multiple stressors, leading to reduced tolerance to novel chemical stress with implications for species persistence. This is of particular concern given the central role that *Daphnia* plays in freshwater aquatic food webs, especially amid predictions of increasing emissions of PAHs compounded by climate change (Friedman et al., 2014) and predictions of rising concentrations of polycyclic aromatic hydrocarbons (PAHs) in terrestrial and aquatic ecosystems since the industrial revolution (Du and Jing, 2018; Vecchiato et al., 2020; Wu

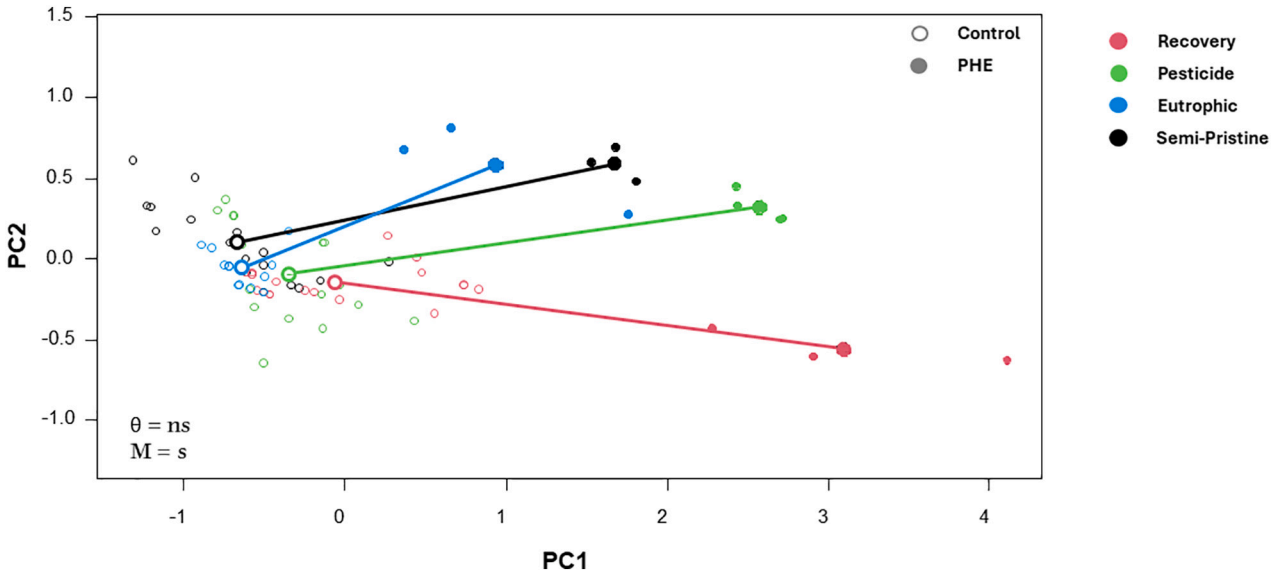

Fig. 3. Phenotypic trajectory analysis (PTA). PTA on the *Daphnia magna* genotypes from Lake Ring separated by lake phases following chronic exposure to PHE (428 µg/L). The multivariate response of five fitness-linked life history traits is shown. Open circles represent the control (nonexposed clonal 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. Differences among genotypes from different lake phases are expressed as magnitude (M) and direction (θ) of change. The statistics supporting the PTA are in Table S3. The lake phases are color-coded as in Fig. 1.

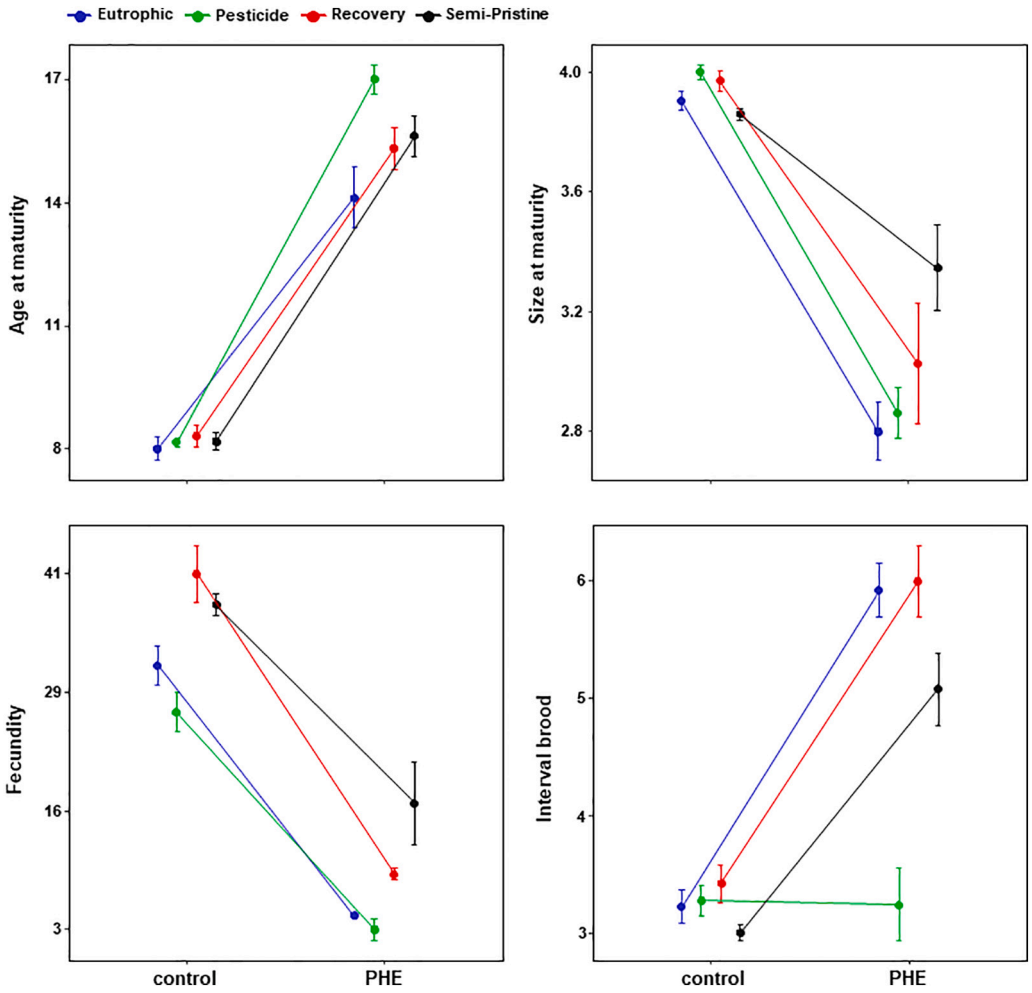

Fig. 4. Univariate reaction norms. Univariate response of four fitness-linked life history traits between control and PHE (428 µg/L) exposed *Daphnia.* Population reaction norms based on population means ($N = 48$) and SD are shown for each life history trait. The lake phases are color-coded as in Fig. 1.

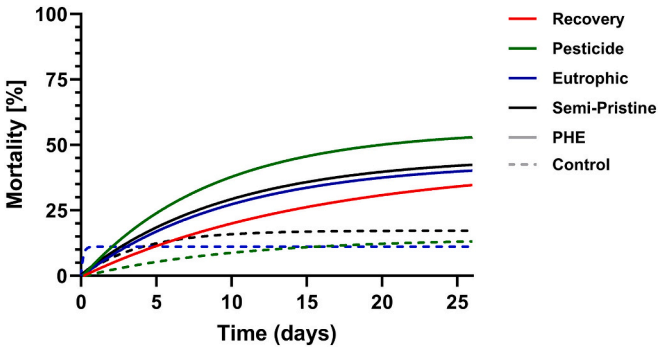

Fig. 5. Survival plots. Survival of the *Daphnia* populations following chronic exposure to PHE (428 µg/L) displayed as Kaplan-Meier curves showing cumulative survival probability of each population in control and exposed conditions. The lake phases are color-coded as in Fig. 1. Solid line: PHE exposure; Dotted line: Control treatment.

et al., 2023).

CRediT authorship contribution statement

Florian Gigl: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Muhammad Abdullahi:** Writing – review & editing, Visualization, Software, Data curation. **Marianne Barnard:** Methodology. **Henner Hollert:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Luisa Orsini:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The fitness-linked life history traits can be found in the dryad database at DOI: https://doi.org/10.5061/dryad.kh18932fr.

Acknowledgments

FG received support from the RobustNature Excellence Cluster Initiative (SynergyFund) and the Goethe University Academy for Early Career Researchers (GRADE), which enabled him to work in laboratories in Birmingham. He also received additional funding from Goethe University.

LO receives funding from the European Union's Horizon 2020 Research and Innovation program under Grant Agreement No. 965406 (PrecisionTox) and the HORIZON-IA HORIZON Innovation Actions under Grant Agreement No. 101112877 (UPSTREAM). The results and conclusions reflect only the authors' view. The European Commission cannot be held responsible for any use that may be made of the information contained therein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174963.

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