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Biological and Ecotoxicological Responses of *Daphnia pulex* to 2,4-D and Malathion Contaminated Laboratory Microcosm

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Abstract:

The enormous growth of industrialisation, urbanisation, and agricultural practices has drastically increased the accumulation of contaminants in the environment, often hampering the activity of non-target aquatic species. Cladoceran species are considered valuable biomarkers for ecotoxicity studies because they are easy to culture, have transparent bodies, and are highly sensitive to chemical pollution. The study aimed to determine the acute and chronic toxicity of sublethal concentrations of selected pesticides, i.e., Malathion 50 EC (a.i. Malathion) and 2,4-D ethyl ester 38% EC (a.i. 2,4-D), on the biological characteristics of *Daphnia pulex*. The 24h and 48h LC_{50} values for 2,4-D and Malathion were (3.5 mg/L and 2.3 mg/L) and (0.76 µg/L and 0.25 µg/L), respectively. Malathion led to reductions in clutch size, heart rate (bpm), population density (individuals per litre), and body length of *D. pulex*. Meanwhile, exposure to 2,4-D resulted in a significant decrease in heart rate (bpm) and clutch size as the concentration increased, compared to the control group. However, the body length and population size of *D. pulex* were not so affected at lower concentrations of 2,4-D in chronic exposures. Approximately 20% of the Daphnia in the exposure groups switched to producing ephippia, resulting in reduced newborn Daphnia (Figure 4d). The study suggests that *D. pulex* is a reliable marker for toxicity caused by sublethal doses of Malathion and 2,4-D. However, they exhibit varying sensitivity levels throughout the 11-day exposure period. The findings indicate that the tested pesticides have noticeable impacts on the evaluated parameters of *D. pulex*.

INTRODUCTION:

Water bodies are often the ultimate destination for waste products released into the environment, with agricultural activities a major contributor to their degradation (Deus and Bakonyi 2012). Pesticides are chemical substances that are used to destroy or prevent any pests, e.g., Insects, rodents, fungi and weeds. Approximately 1.8 billion

people worldwide are engaged in agriculture, and most rely on chemical pesticides to protect their crops (Alavanja 2009). Pesticides that are applied to agricultural fields are often leached into nearby water bodies and cause toxicity to aquatic organisms. These toxic pesticides also lead to the ageing of lakes by causing eutrophication (Scholz et al. 2012). Over the past century, pesticides have become a crucial part of agricultural systems worldwide, leading to a significant increase in crop yields and food production (Alexandratos and Bruinsma 2012). Although pesticides have been primarily designed to target organism toxicity, some non-target plant and animal species are also severely affected by their application (Jayaraj et al. 2016). A non-selective herbicide like Glyphosate reduces the growth of nitrogen-fixing bacteria in soil (Santos and Flores 1995), whereas 2,4-D hinders the conversion of ammonia into nitrates with the help of soil bacteria (Frankenberger and Tabatabai 1991).

The Cladoceran, also known as water fleas, is a group of microcrustaceans (Phylum Arthropoda), ranging from 0.2 mm to 6 mm in body size (Thackeray 2022). *D. pulex* is a small crustacean and a commonly found zooplankton species. They have a generational period of 3-10 days (Harrow 2020). *D. pulex* is the most commonly and widely distributed species of Cladocera, ranging from about 0.2 mm to 5.0 mm in body size. It serves as a good model organism for toxicological studies because of its small size, ease of cultivation, short life cycle, and higher sensitivity towards pollutants like pesticides, heavy metals, etc. They are an important component of the freshwater ecosystem and food web. It is commonly found in a wide range of aquatic habitats. In oligotrophic lakes, it has a light-pigmented body, whereas it may become bright red in hypereutrophic water bodies because of the production of haemoglobin.

The present study aimed to determine the toxicity of sublethal concentrations of two pesticides, namely 2,4-D ethyl ester 38% EC (a.i. 2-4 D), and Malathion 50 EC (a.i. Malathion), on the selected biological characteristics of the Cladoceran species, i.e., *D. pulex.* The study concluded that *D. pulex* is a reliable biomarker for toxicity caused by sublethal doses of the selected pesticides. However, they exhibit varying sensitivity levels throughout the 11-day exposure period. These findings highlight the significant impacts of the tested pesticides on the physiological and behavioural parameters of *D. pulex*, underscoring the need for careful monitoring of these substances in aquatic environments. Many chronic toxicity studies focus on single compounds, single endpoints (often reproduction only), or standard model species such as *Daphnia magna*. This research enhances existing literature by providing comparative, mechanistic, or species-specific insights that enhance the interpretation of ecological risk.

1. MATERIAL AND METHODS

2.1 Culture and maintenance of *D. pulex*

The experiments were carried out in the Laboratory. The daphnids used in the tests were collected by manual sampling using a plankton net of mesh size 60µm from the Yamuna River and were cultured in a 5-litre capacity plastic tub filled with 4 L of RO water in the laboratory under suitable environmental conditions. As *D. pulex* is a sensitive organism, the culture was kept under a controlled temperature of about 22 ± 2 °C and photoperiod (14:10 h light/dark). The pH of the culture water was 7.8–8.0, and the dissolved-oxygen level was maintained at 4.6 ± 0.2 mg/L by gently agitating the culture with a glass rod two or three times a day. The alkalinity and hardness observed were about 146 ± 1.7 mg/L and 115 ± 4.0 mg/L, respectively. The animals were fed once a day with a mixture of Chlorella vulgaris ($1–2 \times 10^5$ cells mL^{-1}) cultivated under 12h light and harvested by centrifugation (Connon, 2007), and yeast was prepared by dissolving a pinch of dry yeast powder in 200 grams of warm water containing about 1500 cells per ml. The culture medium was partially replaced with fresh RO water twice a week to minimise chloride build-up during the culturing phase (Woodley et al. 2023; Long eco water).

2.2 Chemicals

Commercial formulations instead of technical-grade active ingredients were deliberately chosen to mimic field exposure. 2,4-D ethyl ester 38 % EC (trade name “Noweed”, Batch #N001, 38 g a.i. per 100 mL, Northern Insecticides Pvt. Ltd., Muzaffarnagar, India). Malathion 50 EC (trade name “Mal-50”, Batch #MAL-2306, 50 g a.i. per 100 mL, GreenChem Agro, Ghaziabad, India). Both products were purchased from a licensed pesticide retailer and stored at 4 °C in the dark. Active-ingredient contents were verified against the manufacturers’

Certificates of Analysis (≥ 98 % of label claim). Stock solutions were prepared in de-ionised water immediately before each test; nominal concentrations therefore refer to active ingredient (a.i.).

2.3 Selecting test organisms

For test purposes, neonates (< 24 h old) from the second or third broods were selected. Gravid females were gently pipetted from the stock culture 24 h before the test and transferred to a 1 L glass beaker containing culture medium; this ensured synchronised release of neonates. The newly released neonates were collected within 12 h, examined under a stereomicroscope, and those bearing ephippia or any morphological anomalies were discarded as ephippia formation indicates prior stress.

2.4 Range-finding test

A preliminary range-finding test was conducted to bracket lethal concentrations. For 2,4-D ethyl ester 38 % EC, test solutions of 50, 20 and 10 mg/L^{-1} were prepared from a 100 mg/L^{-1} stock solution; for Malathion 50% EC, 20, 10 and 2 µg/L^{-1} were prepared from a 50 µg/L stock. Each concentration and the control were run in triplicate (10 neonates per replicate). After 24 h, 100 % mortality occurred at the two highest concentrations for each pesticide, whereas the lowest concentration showed partial or no mortality. These results guided the definitive LC_{50} tests as recommended in OECD Guideline 202.

2.5 Acute toxicity test (OECD 202)

For the definitive 48 h acute test, six concentrations of 2,4-D (0.50, 1.00, 3.00, 5.00, 7.00, 10.00 mg L^{-1}) and six concentrations of Malathion (0.06, 0.10, 0.20, 0.50, 0.80, 5.00 µg L^{-1}) plus a control were prepared. All dilutions were made in ASTM hard-water medium (re-constituted; hardness 160 mg $CaCO_3$ L^{-1}) to enhance reproducibility. Three replicates per concentration, each with 10 neonates (< 24 h), were maintained in 100 mL glass beakers containing 80 mL of test solution. Because of the short duration, the animals were not fed, and the medium was not renewed. Tests were run at 20 ± 2 °C with constant illumination (600 lx). Mortality and immobility were scored at 24h and 48 h; daphnids failing to swim within 15 s of gentle agitation were considered immobile. Water-quality parameters (pH, DO, conductivity, and hardness) were measured at 0 and 48 h.

2.6 Chronic toxicity test (OECD 211)

Four sub-lethal concentrations, selected as fractions of the 48 h LC_{50}, and a control were tested for 11 days. For 2,4-D: 0.08, 0.10, 0.50, 0.80 mg/L; for Malathion: 0.04, 0.06, 0.08, 0.10 µg L^{-1}. Each concentration and control comprised 10 individually-housed neonates in 50 mL glass vials containing 40 mL test solution. The medium was renewed every 48 h and freshly dosed from stock. During the 11 days, animals were fed daily with 0.5 mL of *C. vulgaris* suspension (≈ 5×10^4 cells) and yeast solution, and debris was carefully removed at each renewal.

The initial number of individuals per treatment was n = 10. Due to mortality during chronic exposure, the final sample size used for each endpoint is 8±1. End-points recorded were heart rate (bpm) measured using a digital camera at 100X magnification. Individual organisms were acclimated for 5 minutes before recording. Heartbeats were counted for one minute and represented vas beats per minute. Each individual was measured three times, and the mean value was used for analysis. Video recordings were analysed in slow motion to improve accuracy. All recordings were conducted at 20 ± 1°C under controlled laboratory conditions to minimise temperature-related variability. To reduce observer bias, heartbeats were counted independently by two observers, and the average value was used, clutch size (number of neonates per brood), body size (dorsal length from the top of the head to the base of the tail-spine, using ImageJ), and cumulative population size (total live offspring per female).

2.7 Statistical analysis

The 24 h and 48 h LC_{50} values and their 95 % fiducial limits were estimated by probit regression (GraphPad Prism 10, "log-probit" model, slope ± SE reported). Goodness-of-fit was assessed with the χ^2 test supplied by the software. For the chronic test, data were checked for normality (using the Shapiro–Wilk test) and homogeneity of variances (using the Levene test). Significant differences between control and treatments for each endpoint were analysed by one-way ANOVA followed by Dunnett's post-hoc test ($\alpha = 0.05$). Where assumptions were violated,

data were log-transformed. All statistics were performed in GraphPad Prism 10, and values are reported as mean ± SE unless stated otherwise.

2. RESULTS

3.1 Acute toxicity

The acute-exposure assay revealed pronounced differences in the susceptibility of *D. pulex* to the two commercial formulations. For Malathion 50 EC, concentration–response curves were steep, yielding 24-h and 48-h LC_{50} values of 0.76 µg L^{-1} (95 % CL 0.432–1.4 µg L^{-1}) and 0.25 µg L^{-1} (95 % CL 0.175–0.345 µg L^{-1}), respectively (Figure 1a). In contrast, exposure to 2,4-D ethyl ester 38 % EC produced LC_{50} values of 3.5 mg L^{-1} (95 % CL 2.65–7.56 mg L^{-1}) at 24 h and 2.3 mg L^{-1} (95 % CL 2.96–3.5.04 mg L^{-1}) at 48 h (Figure 1b). Thus, on an active-ingredient basis, Malathion was several orders of magnitude more lethal than 2,4-D under the same test conditions.

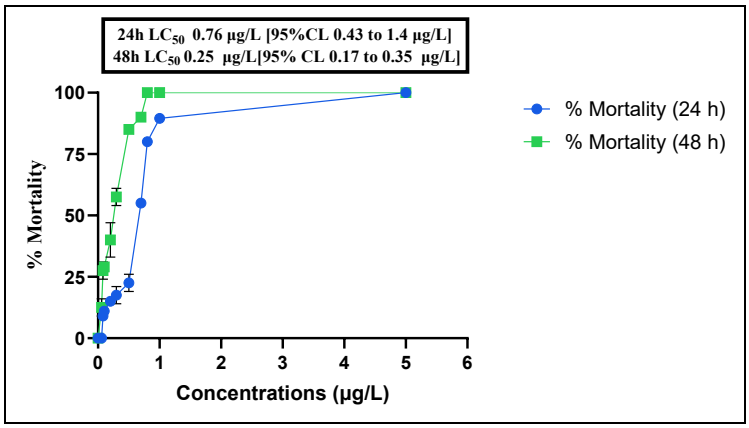

Fig. 1a 24h and 48h LC_{50} with 95% CL for insecticide Malathion 50 EC (a.i.: Malathion).

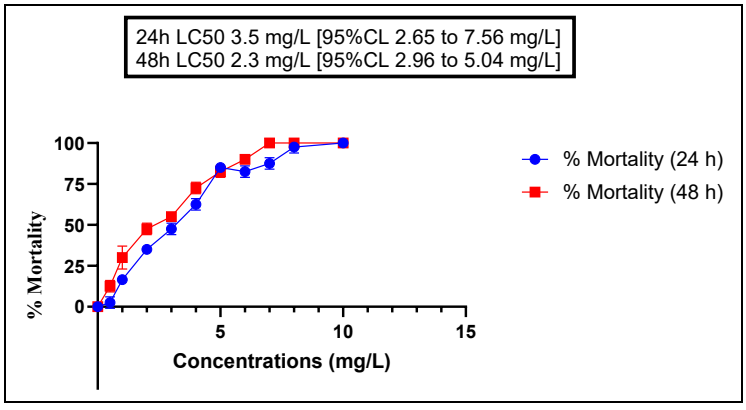

Fig. 1b 24h and 48h LC_{50} with 95% CL for herbicide 2,4-D ethyl ester 38% EC (a.i. 2-4 D)

3.2 Chronic toxicity (11 d)

3.2.1. 2,4-D ethyl ester 38 % EC

Sub-lethal, 11-day exposures corroborated the acute patterns. When daphnids were reared in sub-mg/L^{-1} concentrations of 2,4-D, their physiological and reproductive traits declined only at the upper end of the range. Heart rate fell steadily with concentration, dropping from a control mean of 195 ± 9.4 bpm to 124 ± 8.1 bpm at 0.80 mg L^{-1} (Figure 2a). Clutch size decreased modestly at 0.10 mg L^{-1} and more sharply at 0.80 mg L^{-1}, falling to 12 ± 1.2 neonates per brood versus 16.5 ± 1.2 in controls (Figure 2b; Figure 4a). Body length was unchanged

at 0.08 mg L^{-1} but shortened significantly at 0.80 mg L^{-1}, while total population output declined by 22 % at the highest concentration (Figures 2c, 2d). Control mortality never exceeded 10 %.

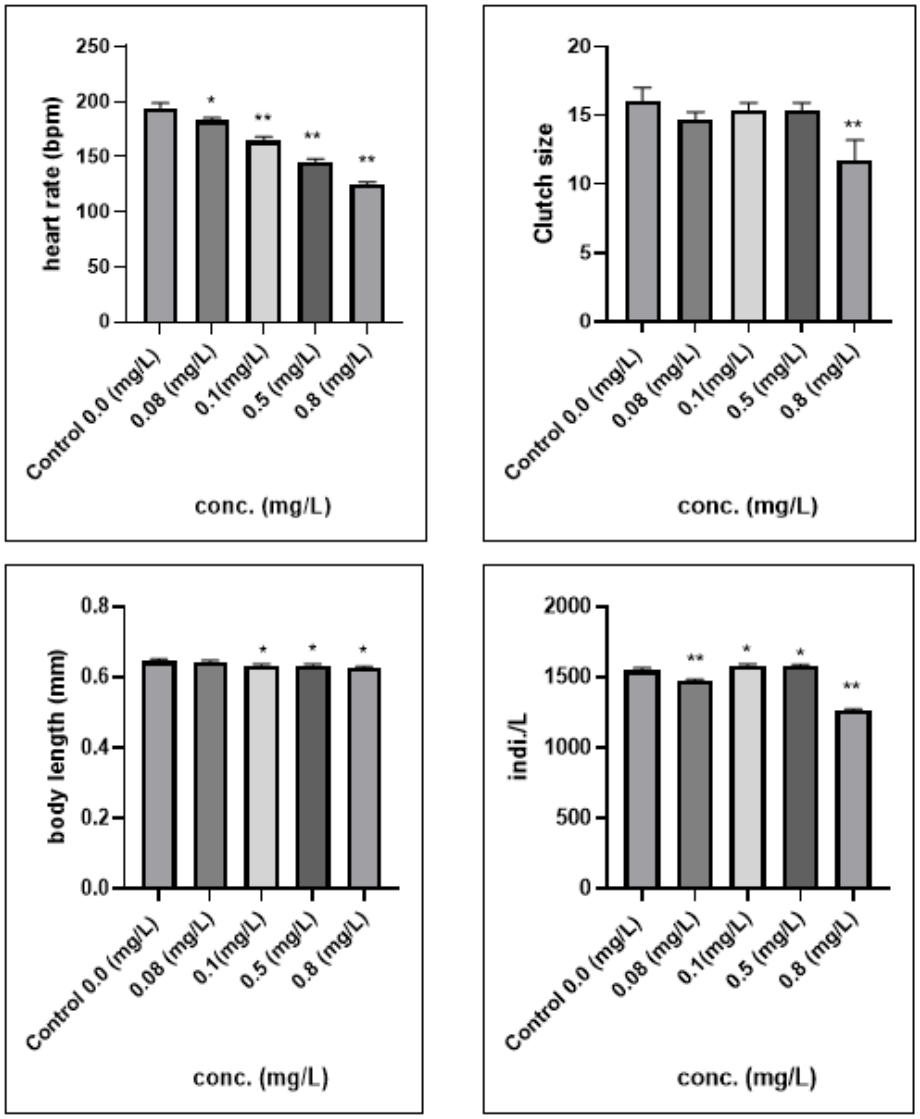

Fig. 2 Population size, body length, heart rate, and clutch size of *D. pulex* (mean ± SD) after 11 days of exposure to different concentrations of 2,4-D ethyl ester 38% EC (a.i. 2-4 D) in chronic toxicity tests. The *asterisk* (*) indicates the value is significantly different from the control ($p < 0.05$, Dunnett's test, degrees of freedom=18)

2.2.2. Malathion 50% EC

Malathion caused far stronger sub-chronic effects. Heart rate fell progressively across the exposure series and reached 98 ± 8.6 bpm at 0.10 µg L^{-1}, almost half the control value (Figure 3a). Clutch size declined at every concentration, dropping to 4 ± 0.8 neonates per brood at 0.10 µg L^{-1} (Figure 3b; Figure 4b, 4c). Body length was significantly reduced even at the lowest tested concentration, averaging 0.368 ± 0.028 mm at 0.10 µg L^{-1}, compared with 0.645 ± 0.008 mm in the control group (Figure 3c). Population size mirrored these trends, falling by roughly one-third at 0.06 µg L^{-1} and by 72 % (450 ± 9.1), ind/L at 0.10 µg L^{-1} (Figure 3d). Together, these data indicate that Malathion impairs cardiac activity, growth, reproduction and overall population performance of *D. pulex* at concentrations several orders of magnitude lower than those required for comparable effects from 2,4-D**.**

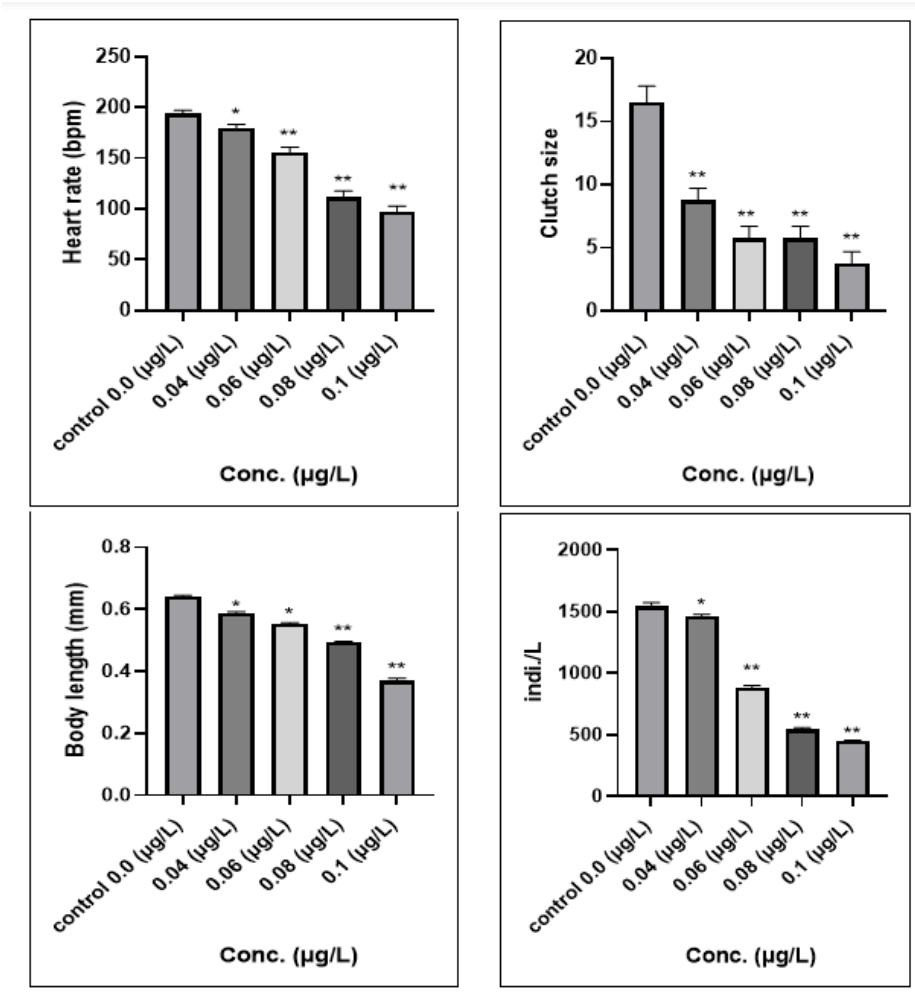

Fig. 3 Population size, body length, heart rate, and clutch size of *D. pulex* (mean ± SD) after 11 days of exposure to different concentrations of Malathion 50 EC (a.i.: Malathion) in chronic toxicity tests. The *asterisk* (*) indicates the value is significantly different from the control ($p < 0.05$, Dunnett's test, degrees of freedom =18)

Fig. 4 a) *D. pulex* from the culture before treatment

b) *D. pulex* after treatment, having 5 eggs

c) *D. pulex* after treatment with the highest conc. of Malathion 50 EC (a.i. Malathion) showing three eggs

d) *D. pulex* with ephippia

3. DISCUSSION

The present study represents a hazard-based laboratory assessment rather than a realistic exposure scenario and compares the acute and chronic toxicity of two widely used formulations, Malathion 50% EC and 2,4-D ethyl ester 38 % EC, to *D. pulex* in the controlled laboratory conditions. Our data show unequivocally that Malathion is orders of magnitude more toxic than 2,4-D, both in short-term lethality (24–48 h LC_{50}) and in longer-term, sub-lethal impairment of growth, reproduction and population output.

4.1 Acute toxicity in the context of published ranges

The 24-h LC_{50} of 0.76 µg/L and 48-h LC_{50} of 0.25 µg/L for Malathion place *D. pulex* squarely inside the US-EPA "highly toxic" category (<1 µg/L) and align with the 1–10 µg L^{-1} window reported for *D. magna* and *M. macrocopa* (Wong et al.1995; Tomlin 2006; Relyea 2009). By contrast, our 2,4-D LC_{50}s—3.5 mg L^{-1} (24 h) and 2.3 mg L^{-1} (48 h)—lie toward the lower tail of the very broad 20–422 mg L^{-1} range compiled by the USEPA (2019) for cladocerans, yet remain higher than many historical values for 2,4-D esters in *D. magna* (e.g. 132 mg L^{-1}, EFSA 2014; 416 mg L^{-1}, Milam et al. (2005). Rassoulzadegan and Akyurtlakli (2002) reported EC50 values for technical malathion (48 h) by the probit method as 80 and 28 µg/L, and for commercial malathion (48 h) as 3.0 µg/L. Lilius et al. (1994) reported the 48h LC_{50} values as 353 µg/L.

Formulation is a key explanatory variable. Ester forms of 2,4-D are consistently more toxic than acid or amine salts: LC_{50}s for acids/salts run from 25 to 643 mg ae L^{-1}, whereas esters range from 2.2 to 11.8 mg ae L^{-1} (Tomlin

2006; RED 2005). Our use of an ethyl-ester product, therefore, accounts for the lower LC_{50} relative to amine-salt studies.

4.2 Chronic effects and mechanistic considerations

Sub-lethal, 11-day exposures reinforced the acute ranking of hazard. In *D. pulex*, Malathion concentrations as low as 0.06 µg/L^{-1} curtailed heart rate, clutch size and somatic growth, and 0.10 µg/L slashed population output by 72 %. These endpoints coincide with the acetylcholinesterase (AChE) inhibition and metabolic reallocation described by Hernandez et al. (2014). Mesocosm work shows that such individual-level effects can cascade to community shifts, e.g. declining cladocerans and phytoplankton blooms (Groner and Relyea 2011), highlighting the ecological significance of our findings. Hernandez et al. (2014) studied the toxic effects of malathion on the survivorship and reproductive variables of *D. pulex* and *Diaphanosoma birgei.* They recorded that for either cladoceran species, the increase in malathion concentration resulted in a reduction in survivorship and fecundity.

In contrast, 2,4-D produced detectable impacts only at the highest tested level (0.80 mg/L). Heart rate fell by 36 %, clutch size by 27 %, and body length by 12 %, with a net 22 % reduction in population size. These thresholds echo "no-effect" outcomes reported for *D. magna* and *Ceriodaphnia silvestrii* at ≤0.1 mg/L (Kashian and Dodson 2002; Moreira et al. 2023) but converge with (Silva et al. 2020) observation of clutch suppression in *C. silvestrii* above 60 mg/L.

Body-length depression in our Malathion series (0.645 → 0.368 mm) mirrors the 11-day reduction reported for *D. pulex* at 0.25 mg/L methyl-parathion (Fernandez et al. 1993), suggesting that organophosphate-induced metabolic costs impinge directly on somatic growth. The fact that ephippia appeared in ~20 % of 2,4-D–exposed females further indicates a stress-driven switch toward resting-egg production, a pattern also noted under glyphosate and mixed herbicide regimes. Ephippia formation in cladocerans is widely recognised as a survival strategy under adverse conditions. However, this response is not exclusively triggered by toxicant exposure. Factors such as population density, food limitation, photoperiod, temperature fluctuations, and culture history can also induce sexual reproduction and ephippia production. Therefore, while pesticide exposure may have contributed to the observed response, alternative explanations cannot be excluded under the present experimental design. A limitation of the present findings is that additional drivers of ephippia formation, including density-dependent effects and nutritional status, were not studied separately. Future studies incorporating factorial designs that manipulate these variables would help clarify the specific role of pesticide-induced stress in ephippia induction.

4.3 Implications for risk assessment

Taken together, the data confirm that short-lived spikes of Malathion, even at sub-µg/L levels routinely detected in run-off, pose an acute and chronic threat to cladoceran assemblages, while equivalent exposures to 2,4-D ester are less immediately hazardous but could induce reproductive deficits at higher, yet still environmentally plausible, concentrations. Because both pesticides co-occur with elevated temperatures and fluctuating pH in subtropical water bodies, future work should incorporate factorial designs to disentangle interactive effects. Field mesocosms that track community composition and nutrient cycling would be particularly valuable for translating laboratory toxicity thresholds into ecosystem-level risk metrics.

4. CONCLUSION

In this study, we have demonstrated that exposure of *D. pulex* to 2,4-D ethyl ester 38% EC (a.i. 2-4 D) and Malathion 50% EC (a.i.. Malathion) can drastically impact their biological characteristics. The results demonstrated significant adverse effects on survival and physiological performance, indicating that even low concentrations can negatively influence cladoceran populations. The findings from the above studies highlight the harmful impacts of pesticide exposure on *D. pulex*. Acute toxicity tests revealed that even short-term exposure to certain chemical compounds can lead to high mortality rates, with varying LC_{50} values depending on the chemical formulation. Chronic exposure, on the other hand, demonstrated equally important effects, including alterations in biological characteristics such as heart rate, body size, clutch size, and population growth. The results show that *D. pulex* accurately indicates both the sublethal and lethal effects of 2,4-D and Malathion exposure, highlighting its usefulness as an affordable and dependable bioindicator for evaluating agrochemical pollution in

aquatic environments. However, certain limitations must be acknowledged. The studies were carried out in controlled laboratory settings that do not completely mimic the complexity of natural environments, including variations in physicochemical factors, ecological interactions, and the processes involved in pesticide breakdown. Therefore, future investigations should emphasise long-term assessments of toxicity across multiple generations, the analysis of the cumulative effects of pesticides, and the inclusion of biochemical and molecular indicators to clarify toxicity mechanisms. Additionally, conducting mesocosm and field-based validation research is advisable to connect laboratory results with actual ecological contexts.

Author contribution: All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were done by Samiksha Agarwal. The first draft of the manuscript was written by Samiksha Agarwal and Saltanat Parveen. All authors read and approved the final manuscript. Conceptualisation: [Samiksha Agarwal, Saltanat Parveen]. Methodology: [Samiksha Agarwal, Saltanat Parveen]. Formal analysis and investigation: [Samiksha Agarwal]. Writing-original draft preparation: [Samiksha Agarwal]. Writing- review and editing [Samiksha Agarwal, Saltanat Parveen]. Funding acquisition: [No]. Resources: [Samiksha Agarwal]. Supervision: [Saltanat Parveen]. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest: No potential conflict of interest exists among the authors.

Availability of data and materials. Data would be made available on reasonable request.

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