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

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ABSTRACT

Industrialization, urbanization, and agricultural practices have substantially increased the accumulation of contaminants in the environment, often adversely affecting nontarget aquatic species. Cladoceran species are considered valuable bioindicators in ecotoxicity studies because they are easy to culture, have transparent bodies, and are highly sensitive to chemical pollution. This study aimed to determine the acute and chronic toxicity of two selected pesticides, namely Malathion 50 EC (a.i., Malathion) and 2,4-D ethyl ester 38% EC (a.i., 2,4-D), to *Daphnia pulex*. The 24- and 48-h median lethal concentration (LC₅₀) values for 2,4-D were 3.5 and 2.3 mg L⁻¹, respectively, and those for Malathion were 0.76 and 0.25 µg L⁻¹, respectively. Malathion reduced clutch size, heart rate, population density (individuals per liter), and body length in *D. pulex*. As the 2,4-D concentration increased, heart rate and clutch size decreased significantly compared with those in the control group. However, body length and population density were not significantly affected at lower concentrations of 2,4-D during chronic exposure. Approximately 20% of the daphnids in the exposure groups switched to producing ephippia, resulting in fewer newborn individuals. The study suggests that *D. pulex* is a reliable bioindicator of toxicity caused by sublethal doses of Malathion and 2,4-D. However, its sensitivity varied over the 11-day exposure period. The findings indicate that the tested pesticides significantly affected the evaluated parameters of *D. pulex*.

1. INTRODUCTION

Water bodies are often the ultimate destination for waste products released into the environment, and agricultural activities contribute significantly to their degradation (de Deus & Bakonyi 2012). Pesticides are chemical substances used to control or eliminate pests such as 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 applied to agricultural fields are often leached into nearby water bodies, causing toxicity to aquatic organisms. These toxic pesticides also contribute to lake aging 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 & Bruinsma 2012). Although pesticides are primarily designed to target pests, some non-target plant and animal species are also severely affected by their application (Jayaraj et al. 2016). A nonselective herbicide such as glyphosate reduces the growth of nitrogen-fixing bacteria in soil (Santos & Flores 1995), whereas 2,4-D hinders the conversion of ammonia

to nitrates by soil bacteria (Frankenberger & Tabatabai 1991).

Cladocerans, also known as water fleas, are a group of microcrustaceans (phylum Arthropoda) ranging from 0.2 to 6 mm in body size (Thackeray 2022, Agarwal et al. 2026). *D. pulex* is a small crustacean and a commonly occurring zooplankton species. It has a generation time of 3–10 days (Harrow 2020). *D. pulex* is the most common and widely distributed cladoceran species, ranging from approximately 0.2 to 5.0 mm in body size. It is an excellent model organism for toxicological studies because of its small size, ease of cultivation, short life cycle, and high sensitivity to pollutants such as pesticides and heavy metals. It is an important component of freshwater ecosystems and food webs and occurs in a wide range of aquatic habitats.

The present study aimed to determine the acute and chronic 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), based on selected biological characteristics of *D. pulex*. The 24 and 48 h LC₅₀ values for 2,4-D were 3.5 and 2.3 mg L⁻¹, respectively, and those for Malathion were 0.76 and 0.25 µg L⁻¹, respectively. Malathion reduced the clutch size (number of eggs per female), heart rate (bpm), population density (individuals per liter), and body length (mm) of *D. pulex*. Exposure to 2,4-D also caused significant decreases in heart rate and clutch size with increasing concentration compared with those in the control group. However, body length and population density were not significantly affected at lower concentrations of 2,4-D during chronic exposure. Approximately 20% of the daphnids in the exposure groups switched to producing ephippia, resulting in fewer newborn individuals. The study suggests that *D. pulex* is a reliable bioindicator of toxicity caused by sublethal doses of the selected pesticides. However, the sensitivity of *D. pulex* varied over the 11-day exposure period. These findings highlight the significant effects of the tested pesticides on the physiological, behavioral, and reproductive parameters of *D. pulex* and 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 provides comparative, mechanistic, and species-specific insights that improve the interpretation of ecological risk.

2. MATERIALS AND METHODS

2.1. Culture and Maintenance of *D. pulex*

The experiments were conducted in the laboratory. The daphnids used in the tests were collected manually from the

Yamuna River using a plankton net with a mesh size of 60 µm and were cultured in a 5 L plastic tub containing 4 L of reverse osmosis (RO) water under suitable environmental conditions. Because *Daphnia pulex* is a sensitive organism, the culture was maintained at 22 ± 2 °C with a 14:10 h light/dark photoperiod. The pH of the culture water was maintained at 7.8–8.0, and the dissolved oxygen concentration was maintained between 4.5 and 5.0 mg L⁻¹ by gently agitating the culture with a glass rod two or three times a day. Alkalinity and hardness were 145 ± 5 and 110 ± 10 mg L⁻¹, respectively. The cultured cladocerans were fed once daily with a 5 mL mixture of *Chlorella vulgaris* (1 × 10⁶ cells mL⁻¹) and yeast (*Saccharomyces cerevisiae*), prepared by dissolving a pinch of dry yeast powder in 200 mL of warm water containing approximately 1,500 cells mL⁻¹ in a 1:1 ratio. The culture medium was partially replaced with fresh RO water twice a week to minimize chloride buildup during the culture period (Woodley et al. 2023, Long Eco Water 2025).

2.2. Chemicals

Commercial formulations rather than technical-grade active ingredients were deliberately chosen to simulate field exposure. 2,4-D ethyl ester 38% EC (trade name Noweed, Batch No. N001, 38 g a.i. per 100 mL, Northern Insecticides Pvt. Ltd., Muzaffarnagar, India) and Malathion 50 EC (trade name Mal-50, Batch No. MAL-2306, 50 g a.i. per 100 mL, GreenChem Agro, Ghaziabad, India) were used in the study. Both products were purchased from a licensed pesticide retailer and stored at 4 °C in the dark. The active ingredient contents were verified against the manufacturers' Certificates of Analysis (≥98% of the label claim). Stock solutions were prepared in deionized water immediately before each test; therefore, the nominal concentrations refer to the active ingredients (a.i.).

2.3. Selection of Test Organisms

For the experiment, non gravid and active individuals from the F1 broods were selected and were examined under a stereomicroscope. Neonates bearing ephippia or showing morphological anomalies were discarded because ephippia formation indicates prior stress.

2.4. Range-Finding Test

A preliminary range-finding test was conducted to determine the range of 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, test solutions of 20, 10, and 2 µg L⁻¹ were prepared from a 50 µg L⁻¹ stock solution. Each concentration and the control were tested in triplicate, with 10 neonates per

replicate. After 24 h, 100% mortality was observed at the two highest concentrations of each pesticide, whereas the lowest concentration resulted in partial mortality. These results were used to determine the concentration ranges for the definitive LC₅₀ tests according to the Organisation for Economic Co-operation and Development (OECD) Test No. 202 (OECD 2004).

2.5. Acute Toxicity Test

For the definitive 48 h acute test, six concentrations of 2,4-D (0.50, 1.00, 3.00, 5.00, 7.00, and 10.00 mg L⁻¹) and six concentrations of Malathion (0.06, 0.10, 0.20, 0.50, 0.80, and 5.00 µg L⁻¹), plus a control, were prepared following OECD Test No. 202 (OECD 2004). All dilutions were made in American Society for Testing and Materials (ASTM) hard-water medium (reconstituted; hardness, 160 mg CaCO₃ L⁻¹) to enhance reproducibility. Three replicates were maintained for each concentration, with 20 individuals (<24 h old) per replicate in 500 mL glass beakers containing 80 mL of test solution. Because of the short test duration, the animals were not fed, and the test medium was not renewed. The tests were conducted at 20 ± 2 °C under constant illumination (600 lx). Mortality and immobility were assessed at 24 and 48 h. Daphnids that failed to swim within 15 s of gentle agitation were considered immobile. Water-quality parameters, including pH, dissolved oxygen (DO), conductivity, and hardness, were measured at 0 and 48 h.

2.6. Chronic Toxicity Test

Four sublethal concentrations, selected as fractions of the 48-h LC₅₀, and a control were tested for 11 days using a modified OECD 211 protocol. For 2,4-D, the concentrations were 0.05, 0.10, 0.50, and 1.00 mg L⁻¹, whereas for Malathion, they were 0.04, 0.06, 0.08, and 0.10 µg L⁻¹. Each concentration and the control comprised 20 individually housed neonates in 50 mL glass vials containing 40 mL of test solution. The medium was renewed every 48 h and freshly prepared from the stock solution. During the 11-day exposure period, the animals were fed daily with 0.5 mL of *C. vulgaris* suspension (approximately 5 × 10⁴ cells) and yeast solution, and debris was carefully removed at each medium renewal.

The initial number of individuals per treatment was *n* = 20. Because of mortality during chronic exposure, the final sample size used for each endpoint was 18 ± 1. The recorded endpoints included heart rate, clutch size, body size, and population size (total number of individuals). Heart rate was measured in beats per minute (bpm) using a digital camera at 100× magnification. Individual organisms were acclimated for 5 min before recording. Heartbeats were counted for

1 min, and each individual was measured three times; the mean value was used for analysis. Video recordings were analyzed in slow motion to improve accuracy. All recordings were conducted at 20 ± 1 °C under controlled laboratory conditions to minimize temperature-related variability. To reduce observer bias, heartbeats were counted independently by two observers, and the average value was used. Clutch size was recorded as the number of eggs per female. Body size was measured in ImageJ as the dorsal length from the top of the head to the base of the tail spine. Population size was recorded as the total number of individuals.

2.7. Statistical Analysis

The 24- and 48-h LC₅₀ values and their 95% fiducial limits were estimated by probit regression (GraphPad Prism 10, log-probit model; slope ± standard deviation [SD] reported). Goodness of fit was assessed using the χ² test provided in the software. For the chronic test, data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was assessed using Levene's test. Significant differences between the control and treatment groups for each endpoint were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test (α = 0.05). When the assumptions were violated, the data were log-transformed. All statistical analyses were performed using GraphPad Prism 10, and values are reported as the mean ± SD unless stated otherwise.

3. RESULTS AND DISCUSSION

3.1. Acute Toxicity

For Malathion 50 EC, the concentration–response curves were steep, yielding 24 and 48 h LC₅₀ values of 0.76 µg L⁻¹ (95% confidence limits, 0.43–1.4 µg L⁻¹) and 0.25 µg L⁻¹ (95% confidence limits, 0.17–0.35 µg L⁻¹), respectively (Fig. 1(a)). In contrast, exposure to 2,4-D ethyl ester 38% EC produced 24 and 48 h LC₅₀ values of 3.5 mg L⁻¹ (95% confidence limits, 2.65–7.56 mg L⁻¹) and 2.3 mg L⁻¹ (95% confidence limits, 2.0–5.04 mg L⁻¹), respectively (Fig. 1(b)). Thus, on an active-ingredient basis, Malathion was several orders of magnitude more lethal than 2,4-D under the same test conditions.

3.2. Chronic Toxicity (11 d)

3.2.1. 2,4-D Ethyl Ester 38% EC

Sublethal 11-day exposures corroborated the acute patterns. When daphnids were reared at 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 decreased steadily with increasing concentration, from a control

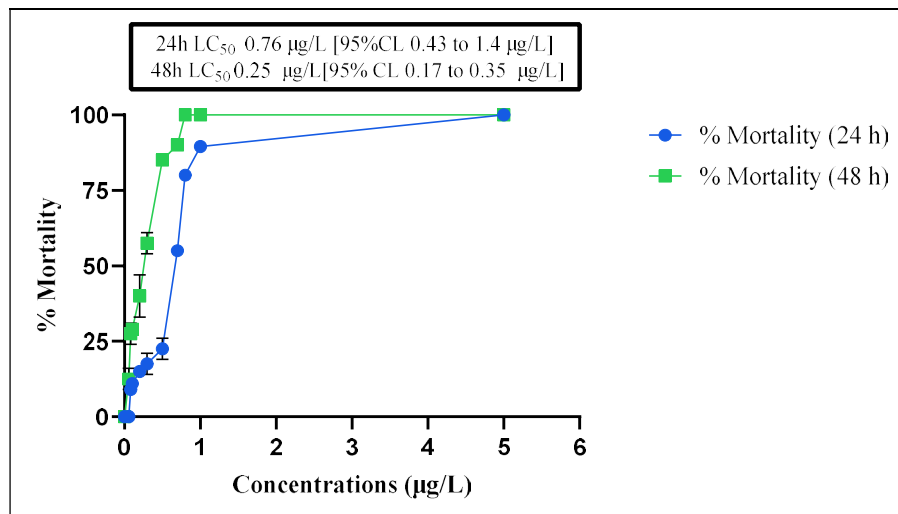


Fig. 1(a): 24 and 48 h LC₅₀ values with 95% confidence limits (CL) for the insecticide Malathion 50 EC (a.i., Malathion).

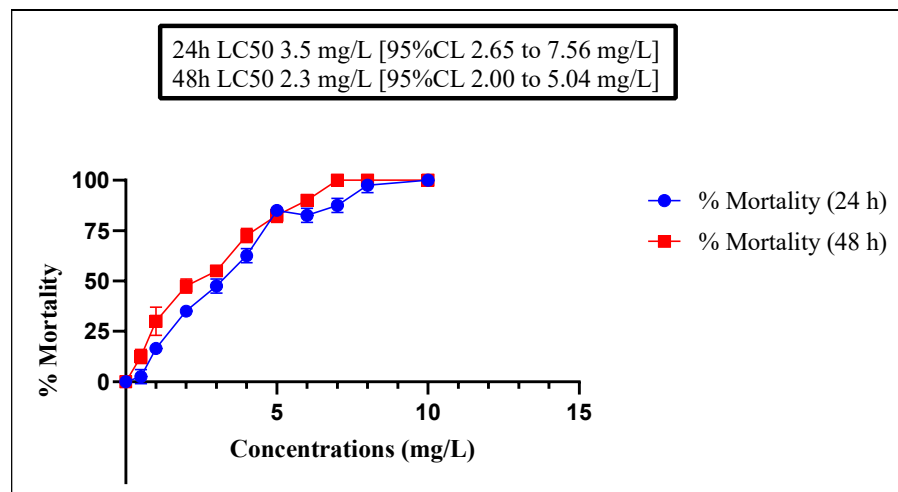


Fig. 1(b): 24 and 48 h LC₅₀ values with 95% CL for the herbicide 2,4-D ethyl ester 38% EC (a.i., 2,4-D).

mean of 195 ± 9.4 bpm to 124 ± 8.1 bpm at 1.00 mg L^{-1} (Fig. 2(a)). Clutch size also decreased at the highest concentration, reaching an average of 12 ± 1.2 neonates per brood at 1.00 mg L^{-1} compared with 16.5 ± 1.2 in the control (Fig. 2(b), Fig. 4(a)). Body length was significantly reduced at the highest concentration, reaching an average of 1.628 ± 0.002 mm at 1.00 mg L^{-1} compared with 1.645 ± 0.008 mm in the control. Total population output decreased by 18% to $1,267 \pm 6.4$ individuals L^{-1} at the highest concentration (Fig. 2(c) and 2(d)). Control mortality did not exceed 10%.

3.2.2. Malathion 50 EC

Malathion caused more pronounced subchronic effects. Heart rate decreased progressively across the exposure series

and reached 98 ± 8.6 bpm at 0.10 µg L^{-1} , approximately half the control value (Fig. 3(a)). Clutch size decreased at every concentration, reaching 4 ± 0.8 neonates per brood at 0.10 µg L^{-1} (Fig. 3(b), Fig. 4(b) and (c)). Body length was significantly reduced at the two highest concentrations, reaching an average of 1.268 ± 0.028 mm at 0.10 µg L^{-1} compared with 1.645 ± 0.008 mm in the control (Fig. 3(c)). Population size followed the same trend, decreasing by approximately 42% at 0.06 µg L^{-1} and by 70% to 450 ± 5.5 individuals L^{-1} at 0.10 µg L^{-1} (Fig. 3(d)). Together, these data indicate that Malathion impaired cardiac activity, growth, reproduction, and overall population performance in *D. pulex* at concentrations several orders of magnitude lower than those required to produce comparable effects with 2,4-D.

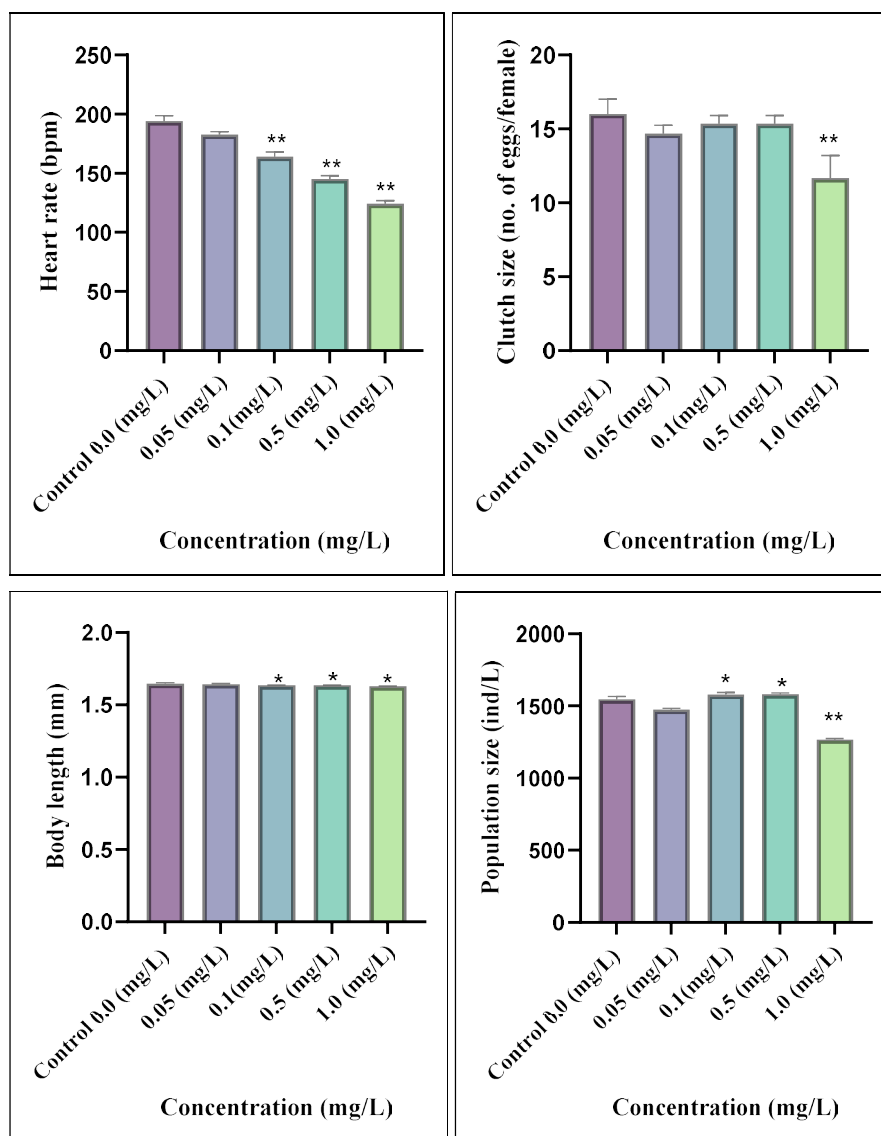


Fig. 2: Heart rate, clutch size, body length, and population size of *D. pulex* (mean $\pm$ SD) after 11 days of exposure to different concentrations of 2,4-D ethyl ester 38% EC (a.i., 2,4-D) in chronic toxicity tests. Asterisks indicate significant differences from the control: * $p < 0.05$ and ** $p < 0.001$ (Dunnett's test, 18 degrees of freedom).

3.3. Discussion

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

3.3.1. Acute Toxicity in the Context of Published Ranges

The 24 h LC_{50} of $0.76 \mu\text{g L}^{-1}$ and 48 h LC_{50} of $0.25 \mu\text{g L}^{-1}$ for Malathion place *D. pulex* within the U.S. Environmental Protection Agency (US EPA) (2006) highly toxic category ($<1 \mu\text{g L}^{-1}$) and align with the $1\text{--}10 \mu\text{g L}^{-1}$ range reported for *D. magna* and *M. macrocopa* (Wong et al. 1995, Tomlin 2006, Relyea 2009). In contrast, the 2,4-D LC_{50} values of 3.5 mg L^{-1} (24 h) and 2.3 mg L^{-1} (48 h) lie toward the lower end of the broad $20\text{--}422 \text{ mg L}^{-1}$ range compiled by the US EPA (2019) for cladocerans and are substantially lower than historical values reported for 2,4-D esters in *D. magna*, for example, 132 mg L^{-1} (EFSA 2014) and 416 mg L^{-1} (Milam et al. 2005). Rassoulzadegan and Akyurtlakli (2002) reported

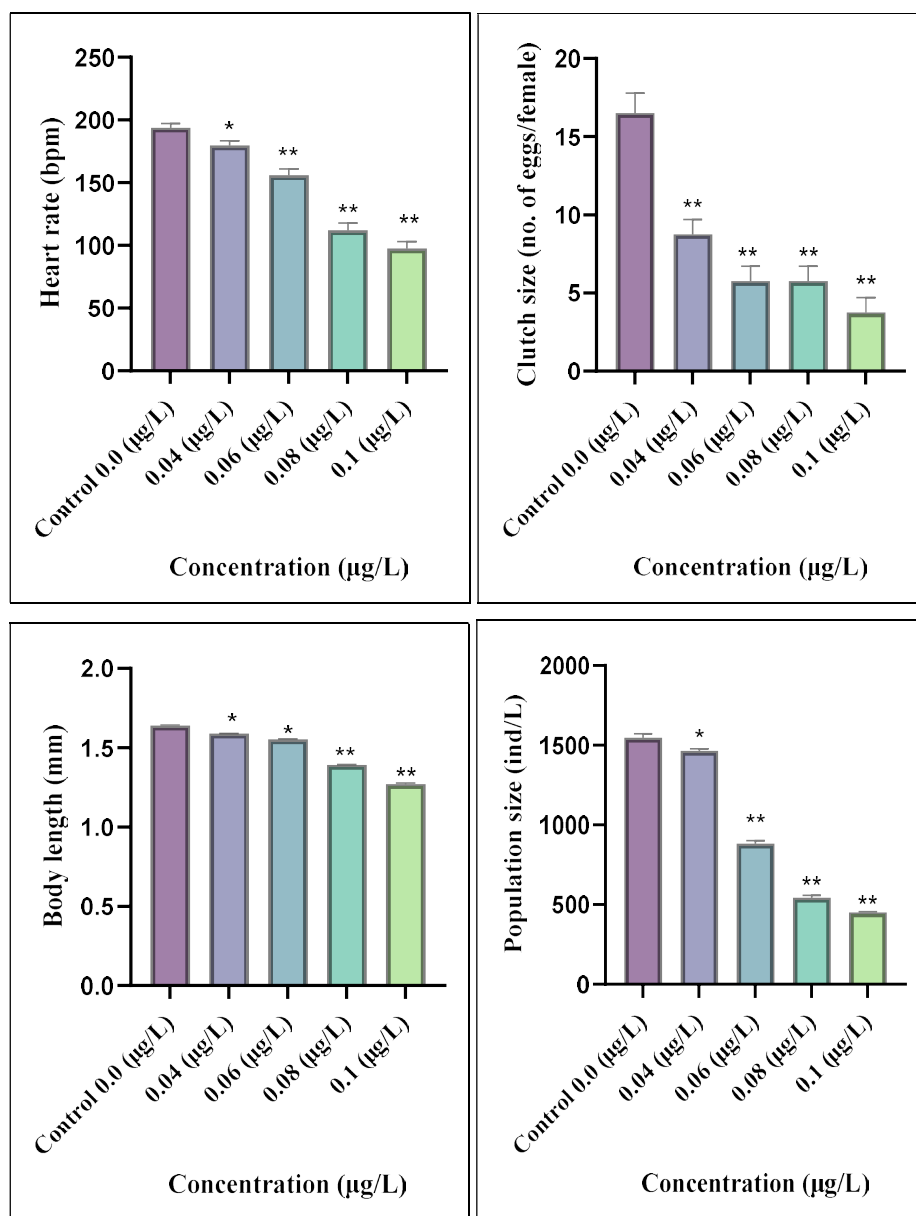


Fig. 3: Heart rate, clutch size, body length, and population size of *D. pulex* (mean $\pm$ SD) after 11 days of exposure to different concentrations of Malathion 50 EC (a.i., Malathion) in chronic toxicity tests. Asterisks indicate significant differences from the control: * $p < 0.05$ and ** $p < 0.001$ (Dunnett's test, 18 degrees of freedom).

48 h median effective concentration (EC_{50}) values of 80 and 28 $\mu\text{g L}^{-1}$ for technical Malathion and 3.0 $\mu\text{g L}^{-1}$ for commercial Malathion using the probit method. Lilius et al. (1994) reported a 48 h LC_{50} value of 353 $\mu\text{g L}^{-1}$.

Formulation is a key explanatory variable. Ester forms of 2,4-D are consistently more toxic than acid or amine salts. LC_{50} values for acids and salts range from 25 to 643 mg acid equivalent (a.e.) L^{-1} , whereas those for esters range from 2.2 to 11.8 mg a.e. L^{-1} (Tomlin 2006, US EPA

2005). Therefore, the use of an ethyl ester product in the present study may account for the lower LC_{50} value relative to studies involving amine salts.

3.3.2. Chronic Effects and Mechanistic Considerations

Sublethal 11-day exposures reinforced the acute ranking of hazard. In *D. pulex*, Malathion concentrations as low as 0.06 $\mu\text{g L}^{-1}$ reduced heart rate, clutch size, and growth, whereas 0.10 $\mu\text{g L}^{-1}$ reduced population output by 70%. These endpoints coincide with the acetylcholinesterase (AChE)



Fig. 4: (a) *D. pulex* from the culture before treatment; (b) *D. pulex* after treatment, with five eggs; (c) *D. pulex* after treatment with the highest concentration of Malathion 50 EC (a.i., Malathion), with three eggs; and (d) *D. pulex* with ephippia.

inhibition and metabolic reallocation described by Bravo-Hernández et al. (2014) and Agarwal and Parveen (2026). Mesocosm studies show that such individual-level effects can cascade into community-level changes, including declining cladoceran populations and phytoplankton blooms (Groner & Relyea 2011), highlighting the ecological significance of the present findings. Bravo-Hernández et al. (2014) studied the effects of Malathion on the survival and reproductive variables of *Daphnia pulex* and *Diaphanosoma birgei* and reported that increasing Malathion concentrations reduced survival and fecundity in both cladoceran species.

In contrast, 2,4-D induced a clear concentration-dependent decline in all physiological and population-level parameters evaluated. Heart rate progressively decreased with increasing concentration, reaching 124 ± 8.1 bpm at 1.00 mg L^{-1} . Similarly, clutch size decreased with increasing concentration, with the lowest reproductive output of 12 ± 1.2 neonates per brood at the highest tested concentration. Body length also declined progressively with increasing exposure, reaching an average of 1.628 ± 0.002 mm at 1.00 mg L^{-1} . Consistent with these individual-level effects, total population output decreased by approximately 18% at the highest concentration, reaching 1.267 ± 6.4 individuals

L^{-1} . Overall, the progressive reductions in heart rate, clutch size, body length, and population size demonstrate the concentration-dependent sublethal effects of 2,4-D exposure. These thresholds are consistent with the no-effect outcomes reported for *D. magna* and *Ceriodaphnia silvestrii* at $\leq 0.1 \text{ mg L}^{-1}$ (Kashian & Dodson 2002, Moreira et al. 2023) but converge with the observation by Silva et al. (2020) of clutch suppression in *C. silvestrii* above 60 mg L^{-1} .

The reduction in body length in the Malathion exposure series, from 1.645 to 1.268 mm, mirrors the 11-day reduction reported for *D. pulex* exposed to 0.25 mg L^{-1} methyl parathion (Fernandez-Casalderrey et al. 1993), suggesting that organophosphate-induced metabolic costs directly affect somatic growth. The occurrence of ephippia in approximately 20% of 2,4-D-exposed females further indicates a stress-induced shift toward resting-egg production, a pattern also observed under glyphosate and mixed-herbicide regimes. Ephippia formation in cladocerans is widely recognized as a survival strategy under adverse conditions. However, this response is not exclusively triggered by toxicant exposure. Population density, food limitation, photoperiod, temperature fluctuations, and culture history can also induce sexual reproduction and ephippia production. Therefore, although

pesticide exposure may have contributed to the observed response, alternative explanations cannot be excluded under the present experimental design. A limitation of the present study is that other drivers of ehippia formation, including density-dependent effects and nutritional status, were not investigated separately. Future studies incorporating factorial designs that manipulate these variables would help clarify the specific role of pesticide-induced stress in ehippia induction.

3.3.3. Implications for Risk Assessment

Taken together, the data indicate that short-lived spikes of Malathion, even at sub- $\mu\text{g L}^{-1}$ levels routinely detected in runoff, pose an acute and chronic threat to cladoceran assemblages, whereas 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 mesocosm studies that track community composition and nutrient cycling would be particularly valuable for translating laboratory toxicity thresholds into ecosystem-level risk metrics.

4. CONCLUSIONS

This study 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 substantially affect its biological characteristics. The results showed significant adverse effects on survival and physiological performance, indicating that even low concentrations can negatively affect cladoceran populations. Acute toxicity tests revealed that short-term exposure to the tested pesticides can cause high mortality, with LC_{50} values varying according to the chemical formulation. Chronic exposure also caused important effects, including changes in heart rate, body size, clutch size, and population growth. The results show that *D. pulex* is a reliable indicator of the sublethal and lethal effects of 2,4-D and Malathion exposure, supporting its use as an affordable and dependable bioindicator for evaluating agrochemical pollution in aquatic environments. However, the experiments were conducted under controlled laboratory conditions that do not fully represent the complexity of natural environments, including variations in physicochemical factors, ecological interactions, and pesticide-degradation processes. Future studies should include long-term, multigenerational toxicity assessments, analyses of cumulative pesticide effects, and biochemical and molecular indicators to clarify toxicity mechanisms. Mesocosm and field-based validation studies are also needed to relate laboratory results to actual ecological conditions.

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