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Sequential pesticide exposure: Concentration addition at high concentrations - Inhibition of hormesis at ultra-low concentrations

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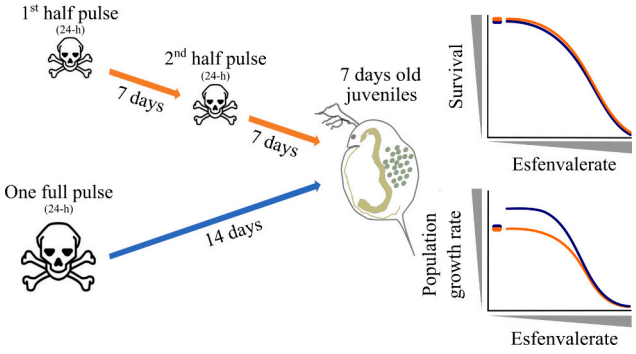
HIGHLIGHTS

- We compared effects of sequential vs. single-pulse exposure on *Daphnia magna*.
- Mortality of sequential exposure could be predicted by concentration addition.
- Population growth rate at high concentrations also followed concentration addition.
- At low concentrations single exposure caused a hormetic response.
- Sequential exposure suppressed the hormetic responses in population growth rate.

GRAPHICAL ABSTRACT



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ABSTRACT

Sequential pesticide exposure is a common scenario in both aquatic and terrestrial agricultural ecosystems. Predicting the effects of such exposures is therefore highly relevant for improving risk assessment. However, there is currently no information available for predicting the effects of sequential exposure to the same toxicant at both high and low concentrations. Here we exposed one-week-old individuals of *Daphnia magna* to the pyrethroid Esfenvalerate for 24 h and compared the effects with individuals treated twice with half the concentration after 7 and 14 days. We showed that at the concentrations close to the LC_{50}, both the survival and population growth rate from the two half-pulses were consistent with the concentration addition approach. At low (1/10th to 1/100th of the LC_{50}) and ultra-low concentrations (1/100th to 1/1000th of the LC_{50}), survival was around 100 %, while the population growth rate showed a hormetic increase following the one-pulse exposure but not for the two-pulse exposure. We hypothesize that this hormetic effect is due to lower systemic stress (SyS) after pesticide exposure in combination with only one rebound stress pulse. Our study suggests that while the lethal effects of sequential exposure are according to the concentration addition model, the sublethal effects at low and ultra-low concentrations need to consider hormetic effects.

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1. Introduction

Aquatic organisms are exposed to diverse pesticide scenarios, ranging from single short-term pulses to repeated exposures. Short-term pulses of pesticides can induce latent effects (EFSA, 2013), especially at low concentrations (Liess and Schulz, 1996), as mechanistically quantified by Liess and Groning (2024). Sequential pesticide exposure commonly occurs both in terrestrial (Botias et al., 2015; Geiger et al., 2010) and aquatic ecosystems (Kreuger, 1998; Liess et al., 2021; Liess et al., 1999).

Extending the standard effect assessment to include sequential exposure scenarios provides a more realistic risk assessment, as these scenarios approach field conditions. The effects of such sequential exposures are influenced by toxicant concentrations, exposure duration (Hoang et al., 2007), and recovery period between exposure (Schunck and Liess, 2022). For instance, a recovery period of 2 to 6 h between two 1-h pulses of carbamate compounds significantly reduced the toxicant's effect compared to a single 2-h pulse in midges (*Chironomus riparius*) at EC_{20} concentrations (Kallander et al., 1997). Similarly, Naddy and Klaine (2001) found that *Daphnia magna* can survive a highly toxic Chlorpyrifos exposure at concentrations near EC_{50} if there is sufficient time (72 h) for recovery between successive exposures in contrast to situations with less recovery time (0 to 48 h).

In order to assess the toxicity of repeated diazinon pulses with different recovery periods, Ashauer et al. (2010) employed the Threshold Damage Model (TDM). They discovered that an insufficient recovery period of 2 and 7 days for *Gammarus pulex* between successive LC_{30} pulses of diazinon increased individual sensitivity resulting in a notable "carry-over" toxicity effect. Specifically, their findings indicated that TK-TD models can explain the increased mortality following the second pulse of the same concentration and duration due to carry-over toxicity. The time frame required for full recovery and to prevent any carry-over toxicity effects was determined to be 28 days (Ashauer et al., 2010). However, this study focused only on high concentrations and did not identify the effects of sublethal concentrations. Also, they did not compare the results with a single full pulse, so the cumulative effects of both pulses could not be concluded. Furthermore, the study was limited only to survival analysis.

Traditional toxicity assessment methods, such as concentration addition (CA) (Loewe, 1926) and effect addition (EA) (Bliss, 1939), which are effective for evaluating the toxicity of mixtures, have not been applied to sequential exposure scenarios. Although CA effectively predicts the mixture toxicity of chemicals with similar or different modes of action, there are instances where the predictions of mixture toxicity by CA and EA may overlap (Escher et al., 2020).

As highlighted in the preceding discussion, there is very limited understanding of the effects of sequential exposure to the same toxicant, and no studies have examined these effects at sub-lethal concentrations. To address this gap, we compared the effects of two half-pulses – with a 7-day recovery period – to one full-pulse of esfenvalerate at high (0.316–3.16 μg/L), low (0.0316 and 0.1 μg/L) and ultra-low (0.001 and 0.01 μg/L) concentrations on *D. magna*. This study aims (i) to predict the nature of sequential exposure effects (additive, synergistic, or antagonistic) on survival and (ii) population growth rate across a range of concentrations.

2. Materials and methods

2.1. Study design

We investigated the effects of sequential exposure to the insecticide esfenvalerate by comparing one-pulse exposure with two-pulse sequential exposure. The cumulative concentration of two-pulse exposure was equivalent to one-pulse of the toxicant. We used 270 neonates of *Daphnia magna*, aged ≤24 h, obtained from the "Aachen V" clone, cultured by the working group System-Ecotoxicology at the Helmholtz Center for Environmental Research – UFZ, Leipzig, Germany. After 7 days of acclimation, organisms were exposed to one full-pulse and 1st half-pulse of esfenvalerate for 24 h. Under the one-pulse exposure scenario, organisms were exposed to eight concentrations including 0, 0.001, 0.01, 0.0316, 0.1, 0.316, 1, 3.16 μg/L. For the two-pulse exposure, concentrations were halved to simulate the one-pulse equivalent, resulting in the following concentrations: 0, 0.0005, 0.005, 0.0158, 0.05, 0.158, 0.5, and 1.58 μg/L. Organisms in the latter setup were exposed again after a recovery period of 7 days. Thus, the combined concentrations of two-pulse exposure were similar to that of the one-pulse exposure. Each treatment included 15 replicates, and the experiment was repeated thrice, with each round spanning 21 days. Mortality was monitored daily for 14 days after exposure and dead organisms were immediately removed from the experiment. Neonates were counted and removed every second day.

2.2. Experimental conditions

Daphnia culture was maintained in 2.0 L beakers with 1800 mL of artificial *Daphnia* medium (ADaM) (Klüttgen et al., 1994). The culture was maintained at 20.0 ± 1.0 °C temperature with a 16:8 h day/night cycle. All experiments were conducted under the same temperature and photoperiod conditions as the culture. Neonates used in the experiment were from 3rd to 5th brood taken from 3- and 4-week-old mothers. The medium for both culture and experiment was changed three times a week. All experiments were conducted with single organisms in 100 mL vessels containing 80 mL of ADaM. Only, during exposure to the toxicant, the volume of the contaminated medium was 50 mL. Daphnids in the culture and experiment were fed with green algae *Desmodesmus subspicatus* and an additional feed of yeast on weekends. The algal concentration fed to Daphnids was 0.5×10^9 cells ind^{-1} day^{-1} for 1st week, 1.15×10^9 cells ind^{-1} day^{-1} for 2nd week, and 1.35×10^9 cells ind^{-1} day^{-1} for the 3rd week.

2.3. Exposure to toxicant and chemical analysis

We used the pyrethroid Esfenvalerate (CAS) 66,230–04-4, purity 99.8 % for contamination, concentration 100 μg/mL of acetonitrile, obtained from HPC Standards GmbH, Am Wieseneck 7, 04451 Borsdorf, OT Cunnersdorf, Germany. The stock solution of esfenvalerate (concentration: 100 μg/L) was prepared using acetonitrile that was further diluted in ADaM to prepare different concentrations. The Esfenvalerate concentrations we used, range from a maximum concentration, one order of magnitude higher than the frequently detected field concentration of 0.1 μg/L (Cooper et al., 2003) to the lowest concentration equal to the regulatory acceptable concentration (RAC) of 0.0005 μg/L (EFSA, 2014). Low (0.0316 and 0.1 μg/L) and ultra-low (0.001 and 0.01 μg/L) concentrations were defined based on 50 % lethal effect concentration (LC_{50}). Concentrations 2–3 orders of magnitude lower than the LC_{50} were classified as ultra-low, while that 1 order of magnitude lower were categorized as low concentrations. The concentration of acetonitrile was consistent across all treatments. To mitigate uncertainty regarding the toxicant's effect, solvent controls were prepared with an equivalent amount of acetonitrile. The concentration of acetonitrile both in treatments and controls was maintained at 0.01 mg/L, well below the criteria set for maximum acute concentration (1145 mg/L), and continuous concentration (413.9 mg/L) that cause any harmful effects (Zhang and Jin, 2001). For the two-pulse exposure, acetonitrile was also administered in two pulses in control as well as treatments. After 24 h of exposure to Esfenvalerate, each organism was transferred individually from the contaminated medium to fresh medium. The organisms were carefully moved to the clean medium using glass pipettes (250 mm diameter) with only a minimal amount of contaminated medium (0.5–1 mL) being included. Great care was taken during the transfer to avoid any physical disturbance. For chemical analysis, exposure concentrations of esfenvalerate were analyzed using GC–MS by SGS Analytics

Germany GmbH - Höhenstraße 24–70,736 Fellbach. The measured concentrations of each exposure concentration were not deviating >20 %. Concentrations below the detection limit (0.0005, 0.001, 0.005 µg/L) were verified using higher concentrations as stock solutions for serial dilutions (Table 1).

2.4. Statistical analysis

All the statistical analyses and graphical representations were performed using R-Studio version 2022.12.0 + 353.pro20 for Windows (Team, R, 2020) and the basic R version 4.2.2 for Windows (Team, R.C, 2020). To compare esfenvalerate tolerance under different exposure scenarios, we calculated the LC_{50} (median lethal concentration) after 7 and 14 days for both one-pulse and two-pulse exposures using the software INDICATE (version 2.3.1; https://www.systemecology.de/indicate/). This software applies the classic five-parameter log-logistic model LL5 to generate concentration-response curves (Liess et al., 2016). The average survival of three replicates per treatment was calculated, and the data for all days were analyzed to identify any temporal changes in survival. Subsequently, we compared the LC_{50} values of esfenvalerate for one-pulse and two-pulse exposure conditions. Neonates were counted for each treatment for both one-pulse and two-pulse exposure conditions. Reproduction was observed for 21 days according to OECD guidelines (OECD, 2012). To evaluate the population growth rate, we calculated the intrinsic rate of increase (r) for each concentration in both exposure scenarios at 7 and 14 days post-contamination using the Euler-Lotka equation (Euler, 1970; Lotka, 1913).

$$1 = \sum_{x=0}^{\Omega} l_x m_x e^{-rx}$$

Ω represents the oldest age class in the population, while x is the current age class. The probability of surviving to age x is indicated by l_x, m_x refers to the number of neonates per mother per day, r is the per capita rate of increase for the population per day and e represents a mathematical constant with a value which is approximately 2.718.

The population growth rate for both exposure scenarios on different days was compared using two-sample paired *t-tests*. Additionally, the weekly neonate count for each concentration was subjected to comparative analysis. To identify uncertainty due to experimental variations, we included 95 % confidence intervals for survival and population growth rate related to experimental repetitions.

3. Results

3.1. Survival of D. magna in one-pulse and two-pulse exposure

In both exposures, decrease in survival was observed only at high concentrations (0.316, 1 and, 3.16 µg/L). Survival was calculated as percentage, showing a decrease from day 1 to day 14 post-contamination. In the single exposure, the survival rate remained constant from day 7 to day 14, indicating that there was no latent mortality after day 7. For the two-pulse exposure, we evaluated the individual effects of each half-pulse. The difference in mortalities followed by 1st half (black dotted line) and 2nd half pulse (blue line) indicates the individual effects of both pulses (Fig. 1). A black dotted line indicates *Daphnia* survival in the absence of a second pulse effect. At day 14 post-contamination, both the one-pulse (blue) and two-pulse (orange) exposures showed similar survival rates, as depicted in box plots (see Fig. 1). The recovery period between the two pulses was not sufficient to completely eliminate the effect of the first pulse, causing the organisms to remain sensitive to the second pulse. As a result, the effects were additive rather than antagonistic. Dose-response curves for both exposures followed the principle of concentration addition, with LC_{50} values of 0.92 µg/L for the one-pulse exposure and 0.99 µg/L for the two-pulse exposure after 14 days from the initial exposure (Fig. 1).

Table 1
Nominal and measured concentrations (µg/L) of Esfenvalerate analyzed during different experimental rounds.

S. no	Nominal concentration	Measured concentration
1	0.01	<0.01
2	0.0158	0.02
3	0.0316	0.03
4	0.05	0.06
5	0.1	0.11
6	0.158	0.13
7	0.316	0.31

3.2. Population growth rate

The population growth rate was significantly increased by one-pulse exposure compared to the controls and two-pulse exposure (p-value <0.001, df = 22) at low (1/10th to 1/100th of the LC_{50}) and ultra-low (1/100th to 1/1000th of the LC_{50}), sublethal concentrations 1 to 3 orders of magnitudes below the LC_{50} (Fig. 2). However, no significant difference was observed in the two-pulse exposure compared to controls. Additionally, at high concentrations (0.316, 1, and 3.16 µg/L), the population growth rate followed concentration addition under both exposure scenarios. A hormetic effect, characterized by high population growth at low and ultra-low concentrations compared to controls, was only observed in one-pulse exposure but not in the two-pulse exposure (Fig. 2).

3.3. Temporal effects on population growth rate

We conducted a comparison of population growth rate trends from day 14 to day 21 both in one-pulse and two-pulse exposures. A significant rise in population growth rate was observed only in the one-pulse exposure from day 14 to day 21 at 0.001 µg/L (paired t-test, p-value <0.05, df = 4), 0.316 µg/L (p-value <0.1, df = 4), and 1 µg/L (p-value <0.01, df = 4) (Fig. 3). This increase was more pronounced when ultra-low and low concentrations were combined (0.001, 0.01, 0.0316, and 0.1 µg/L), showing a significant change from day 14 to day 21 (p-value <0.001, df = 11). In contrast, no significant increase was observed

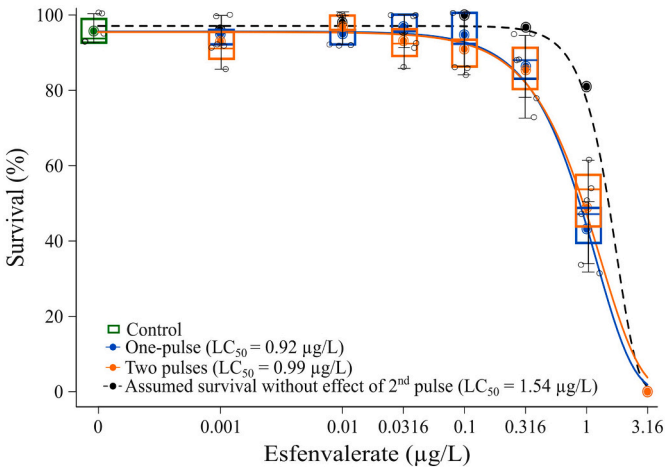

Fig. 1. Survival of *D. magna* at day 14 after a 24 h exposure to esfenvalerate under one-pulse and two-pulse exposure scenarios. In two-pulse exposure, the 2nd half pulse was given after a recovery period of 7 days following 1st half pulse. The dashed black line represents the assumed survival of two-pulses if no additional mortality would be caused by the 2nd pulse. The points (blue, orange, and black) show the average survival rate from three experimental repetitions for both exposure scenarios. The solid lines represent the concentration-response curves for one full pulse (blue) and two half pulses (orange), while the boxes show the individual data distribution for each round.

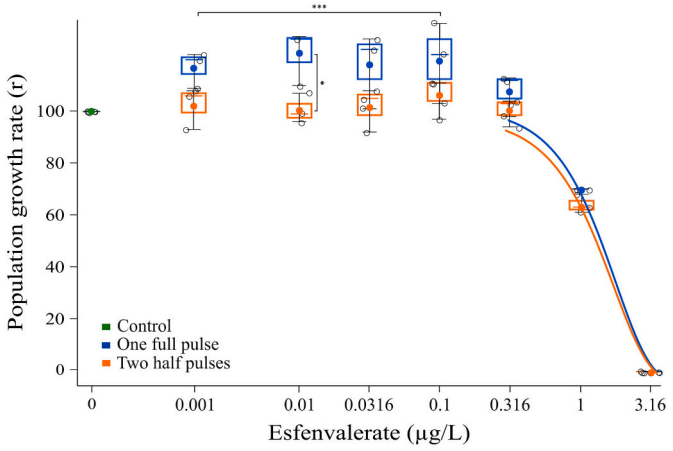

Fig. 2. Population growth rate of *D. magna* 14 days after a 24-h exposure to esfenvalerate under one-pulse and two-pulse exposure scenarios. Population growth rate was calculated for three experimental repetitions for each concentration from the day of the first brood until the end of the experiment. Black points represent the data points for experimental repetitions. Blue and orange points represent the mean value for each concentration. The significance level is displayed as "*" for *p*-value <0.05 and "***" for *p*-value <0.001.

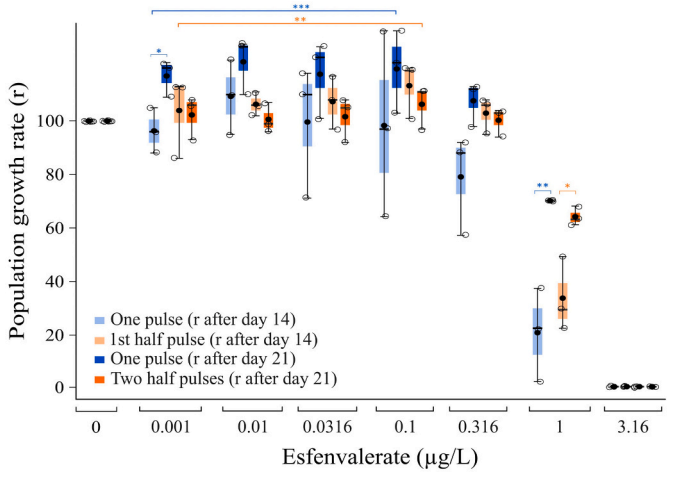

Fig. 3. Population growth rate trends of *D. magna* under 1 and 2 pulse exposure scenarios. After 14 days (second pulse), both one-pulse and two-pulses were equal in terms of concentrations. Population growth rate (r) was calculated for each concentration at 14 and 21 days. Black points represent the mean value for each concentration and asterisks indicate significance levels: "*" for *p*-value <0.05, "**" for *p*-value <0.05, and "***" *p*-value <0.001.

under two-pulse exposure from day 14 to day 21 at individual concentrations except at 1 µg/L (*p*-value <0.05, df = 4), suggesting that repeated pulses suppress the population growth rate increase. However, when ultra-low and low concentrations were combined (0.001, 0.01, 0.0316, and 0.1 µg/L), a significant decrease from day 14 to day 21 was observed (*p*-value <0.01, df = 11; Fig. 3).

4. Discussion

We investigated the lethal and sublethal effects of two-pulse exposures at half concentration and compared them with one-pulse exposures at full concentration after 7 and 14 days. We focused on two endpoints in *D. magna*: survival and population growth rate. Our results indicate that while survival of the two-pulse exposure scenarios followed concentration addition and was equivalent to the one-pulse scenario, the population growth rate only aligned with the CA approach at concentrations close to the LC_{50}. In particular, we found for the first time that two half-pulses at low (1/10th to 1/100th of the LC_{50}) and ultra-low (1/100th to 1/1000th of the LC_{50}) concentrations canceled out the hormetically enhanced growth rate of a single full pulse in *D. magna* (Fig. 2). Such results have not yet been shown as no other studies have investigated the effect of two-half pulses at such ultra-low concentrations.

Studies reporting sequential exposure to similar toxicants at high concentrations close to the LC_{50} show that the second pulse induces higher mortality (Ashauer et al., 2010), a finding also confirmed by our study. However, we additionally compared the overall effects of two-pulses to one-pulse and observed additive effects according to the concentration addition model. Accordingly, the higher mortality of the 2nd pulse can be explained by the log-logistic shape of the concentration-response relationship. Kallander et al. (1997) discovered that two 1-h pulses of organophosphorus compounds produced the same level of toxicity as a continuous 2-h pulse at EC_{20} concentrations. They attributed this equal toxicity to the lack of recovery following the first pulse. However, their comparison was based on equal exposure duration. No prior studies have reported the effects of sequential exposure to the same toxicant using concentration addition. Almalki et al. (2018) found additive effects of sequential exposure on the tissue content of specific neurotransmitters in the brain at high concentrations. However, they used two different chemicals (ethanol and methamphetamine).

Further, we observed an increase in population growth rate following a one-pulse exposure to ultra-low and low concentrations (0.001–0.1 µg/L), indicating a clear hormetic response compared to controls. Since long, it has been demonstrated that organisms can benefit from such minimal toxicant levels (hormesis) (Schulz, 1888), leading to increased growth and reproduction (Liess et al., 2019; Schunck and Liess, 2023; Stanley et al., 2013). In our study, hormesis from one-pulse exposure only became apparent in the second week after contamination. This is consistent with the observation that at ultra-low toxicant concentrations a pesticide-induced rebound stress occurs during the first week after pulse contamination (Liess and Groning, 2024). From then, a pesticide induced reduction of system stress (SyS) appears (Liess et al., 2019). Accordingly, in the first week after contamination, no hormesis is present in either approach due to the rebound stress. In the 2nd week, hormesis can then develop in the approach without contamination. Whereas in the setup with another contamination, a further rebound stress occurs and prevents the formation of hormesis. Correspondingly, in two-pulse exposure, the second pesticide rebound stress prevents the expression of hormesis in the second week.

5. Conclusion

We conclude that the concentration addition approach (CA) can accurately predict the mortality caused by repeated sequential exposure to similarly acting pesticides at high concentrations close to the LC_{50}. Additionally, sequential exposure at low and ultra-low concentrations may inhibit the hormetic increase in population growth rate. Taking these effects into account enables a more realistic risk assessment of pesticides.

CRediT authorship contribution statement

Imrana Mushtaq: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Naeem Shahid:** Writing – review & editing, Visualization, Supervision, Formal analysis, Conceptualization. **Ayesha Siddique:** Writing – review & editing, Formal analysis, Conceptualization. **Matthias Liess:** Writing – review & editing, Writing – original draft, Supervision, Resources, Formal analysis, 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

Data will be made available on request.

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