Ecotoxicology and Environmental Safety 282 (2024) 116751

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Combined effects of heat waves and pesticide pollution on zooplankton communities: Does the timing of stressor matter?

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ARTICLE INFO

Edited by Richard Handy

Keywords:
Multiple stressors
Microcosms
Heatwaves
Stressors asynchrony
Freshwater ecosystems

ABSTRACT

Most studies assessing the combined effects of chemical and non-chemical stressors on aquatic ecosystems have been based on synchronous stressor applications. However, asynchronous exposure scenarios may be more common in nature, particularly for pulsed stressors such as heatwaves and pesticide concentration peaks. In this study, we investigated the single and combined effects of the insecticide chlorpyrifos (CPF) and a heatwave (HW) on a zooplankton community representative of a Mediterranean coastal wetland using synchronous (CPF+HW) and asynchronous (HW→CPF and CPF→HW) exposure scenarios. CPF was applied at a concentration of 0.8 µg/L (single pulse), and the HW was simulated by a temperature increase of 8°C above the control temperature (20°C) for 7 days in freshwater microcosms. The interaction between stressors in synchrony resulted in synergistic effects at the population level (*Daphnia magna*) and additive at the community level. The partial reduction of sensitive species resulted in an abundance increase of competing species that were more tolerant to the evaluated stressors (e.g. *Moina* sp.). The asynchronous exposure scenarios resulted in a similar abundance decline of sensitive populations as compared to the synchronous one; however, the timing of stressor resulted in different responses in the long term. In the HW→CPF treatment, the *D. magna* population recovered at least one month faster than in the CPF+HW treatment, probably due to survival selection and cross-tolerance mechanisms. In the CPF→HW treatment, the effects lasted longer than in the CPF+HW, and the population did not recover within the experimental period, most likely due to the energetic costs of detoxification and effects on internal damage recovery. The different timing and magnitude of indirect effects among the tested asynchronous scenarios resulted in more severe effects on the structure of the zooplankton community in the CPF→HW treatment. Our study highlights the relevance of considering the order of stressors to predict the long-term effects of chemicals and heatwaves both at the population and community levels.

1. Introduction

Populations and communities are exposed to several, often interacting stressors. Consequently, multiple stressor research has grown substantially in the last few years (Orr et al., 2020). Available research has mainly focused on simultaneous exposure to multiple stressors. However, it has been recently highlighted that the synchronous occurrence of multiple stressors in nature is rare (Jackson et al., 2021). Considering only the simultaneous application of stressors may, thus, impair risk assessment and our ability to protect ecosystems (Meng et al., 2020a). Although temporal dynamics of multiple stressors have been largely neglected, stressors' sequence, duration, and overlap are expected to determine the effects of, and the interactions between stressors. Stressors' timing is crucial, as continuous exposure to stress can select for individuals more (or less) tolerant to additional stressors (Pawar et al., 2015), and communities assembled under stressful

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https://doi.org/10.1016/j.ecoenv.2024.116751
Received 24 July 2023; Received in revised form 21 May 2024; Accepted 15 July 2024
Available online 17 July 2024

conditions have been shown to have overall different stability characteristics when exposed to subsequent stressor pulses (Polazzo and Rico, 2021). Furthermore, past stress experiences may influence future stressors' effects on individuals and populations (Jackson et al., 2021).

Temporal dynamics of stressors are particularly relevant as several disturbances have pulsed characteristics (i.e., they are transient). Pesticides with short half-lives are a typical class of pulsed disturbance in freshwater ecosystems, which have detrimental effects on biodiversity (Beketov et al., 2013) and on the ecological status of freshwater ecosystems (Posthuma et al., 2020). Pesticides impair individual's metabolism and physiology in different ways depending on their toxic mode of action and can exert density-mediated effects that scale up to the community and ecosystem levels (Simmons et al., 2021).

Similarly to short-lived pesticides, heatwaves (HWs) are another class of pulse stressors. The World Meteorological Association (WMA) defines a HW as "five or more consecutive days of prolonged heat in which the daily maximum temperature is higher than the average maximum temperature by 5 °C (9 °F) or more" (IFRC, 2021). HWs are predicted to increase in frequency, intensity, and duration in the next 80 years (Woolway et al., 2021). This is raising concerns, as negative effects of HWs have been reported across all organism types and levels of biological organization (Polazzo et al., 2022a). Reported effects range from increased mortality rates to compositional changes (Bergkemper and Weisse, 2017). Temperature controls cells' size and metabolism, population growth, ecosysteḿs carrying capacity, and ecosystem respiration (Gillooly et al., 2001; Yvon-Durocher et al., 2011; Fussmann et al., 2014). For example, ectotherms exposed to high temperature show increased metabolism, but reduced size (Brown et al., 2004; Yvon-Durocher et al., 2011). Increased temperature enhances phytoplankton photosynthetic activity, which may lead to higher biomass at the bottom of the food web. When embedded in a community, the higher primary producers' biomass is used to fuel the temperature-increased metabolic rates of consumers, leading to a strong top-down control exerted by consumers which can reduce the overall producers' biomass standing stock (O'Connor et al., 2009).

Predicting and mitigating the effects of anthropogenic stressors, such as pesticide pollution and HWs, is a central challenge for preserving ecosystems. Interactions between pesticides and HWs appear to be particularly problematic not only because they can result in effects larger than (synergism) or smaller than (antagonism) what one could expect based on their single effects, but also because some studies show that their interactive effects depend on the application order (Dinh et al., 2016). For example, the toxicity of many micropollutants may increase for organisms that have been previously exposed to warming, following the "climate change induced toxicant sensitivity" (CITS) concept (Hooper et al., 2013; Moe et al., 2013), due to the mobilization of energy reserves to cope with the temperature increase and the limited energy available for chemical detoxification. In turn, micropollutants can reduce the heat tolerance of organisms, according to the "toxicant-induced climate change sensitivity" (TICS) concept (Hooper et al., 2013; Moe et al., 2013). Despite these concepts have been formulated more than a decade ago, a recent review by Polazzo et al. (2022a) has revealed that the effects of asynchronous exposure to HWs and pesticides have never been tested at the population or community level. The few studies investigating the relevance of the stressors application order have mainly focused on single organisms in isolation (e.g. Delnat et al., 2019, 2022; Verheyen et al., 2019; Verheyen and Stoks, 2019, 2020), and thus the propagation of such effects to higher levels of the biological organization remains unexplored.

Here, we studied the single and combined effects of an insecticide and a HW on a zooplankton community representative of a Mediterranean coastal lagoon using synchronous and asynchronous exposure scenarios. We hypothesized that (1) the synchronous exposure to both stressors will result in non-additive effects at the population and community level; and that (2) the order of the stressors considered in differed asynchronous scenarios will influence effects and post-exposure recovery at the population and community levels depending on the individual's tolerance and the affected species interactions. With this study, we highlight the need to consider the combined effects of multiple stressors and the timing of their application in the assessment of global change effects in freshwater ecosystems.

2. Materials and methods

2.1. Experimental design

To test our hypotheses, two indoor plankton-dominated microcosm experiments were conducted. The first experiment aimed at assessing the single and combined effects of multiple stressors in synchrony. It consisted of four treatments: (1) control (i.e., no insecticide, no HW), (2) exposure to the insecticide chlorpyrifos (CPF), (3) exposure to a heatwave (HW), and (4) exposure to CPF and a HW in synchrony (CPF+HW; Fig. 1). CPF was added as a single pulse of 0.8 μg/L, which is considered to be an environmentally relevant concentration given the measurements performed in a wide range of surface water ecosystems (Huang et al., 2020; Rico et al., 2021). The HW consisted of a temperature increase of 8°C above room temperature (20°C) for a period of 7 days. The HW was initiated on the same day of the CPF addition and was induced by heating-up the water of the bathtub where the treated microcosms were placed with aquarium heaters. The intended water temperature was reached approximately 12 hours after the onset of the HW while the cooling to control temperature conditions spanned for 12–20 hours. Details of the temperature measurements during the HW simulations are provided in the Supplementary Information (Figure S1).

The second experiment aimed at assessing the combined effects of HW and CPF applied in different order, considering synchronous and asynchronous exposure scenarios. It consisted of four treatments: (1) control (no CPF, no HW), (2) microcosms exposed to a HW that was initiated one week prior to the CPF addition (asynchronous: HW→CPF), (3) microcosms exposed to the HW and CPF in synchrony (CPF+HW), and (4) microcosms exposed to CPF followed by a HW, which was initiated one week after the CPF application (asynchronous: CPF→HW; Fig. 1). In this experiment, the CPF addition and the magnitude and duration of the HW were the same as described in the previous experiment. The experiment was run in parallel to the previous one, so that the controls and the CPF+HW treatment were shared among both experiments. Both experiments had 4 replicates per treatment and lasted for 72 days, with 21 days of acclimatization prior to the CPF application.

The microcosms consisted of cylindrical glass vessels (diameter 20.5 cm, height 37 cm) placed in stainless-steel bathtubs. The microcosms were filled with a total volume of 12 L of water, including 1.5 L of concentrated zooplankton collected from the Albufera lagoon (Valencia, Spain). Water was directly recovered from the shore of the lagoon, whereas zooplankton was concentrated *in situ* by passing lagoon water through a zooplankton net (mesh size: 55 μm). Zooplankton sampling was done on the 15th of October of 2020 by making linear transects over the lagoon with a boat, trying to take a representative sample of the whole zooplankton community. The collected water and zooplankton were transported to the laboratory and the experimental units were assembled on the same day. In addition, sixty individuals of *Daphnia magna* (30 juveniles and 30 adults), which are also typically found in the sampled lagoon in high abundances in other moments of the year (Romo et al., 2005), were added to each microcosm. The microcosms were exposed to a light/dark regime of 16:8 h, with a light intensity of approximately 3000 lux. Low flow aeration was set in each microcosm to simulate a soft water movement. The microcosms were checked weekly and refilled with deionized water when evaporation occurred to prevent concentration of chlorpyrifos and other water ions.

2.2. Chlorpyrifos dosing, sampling, and analysis

A CPF stock solution of 12 mg/L was prepared in methanol. Aliquots

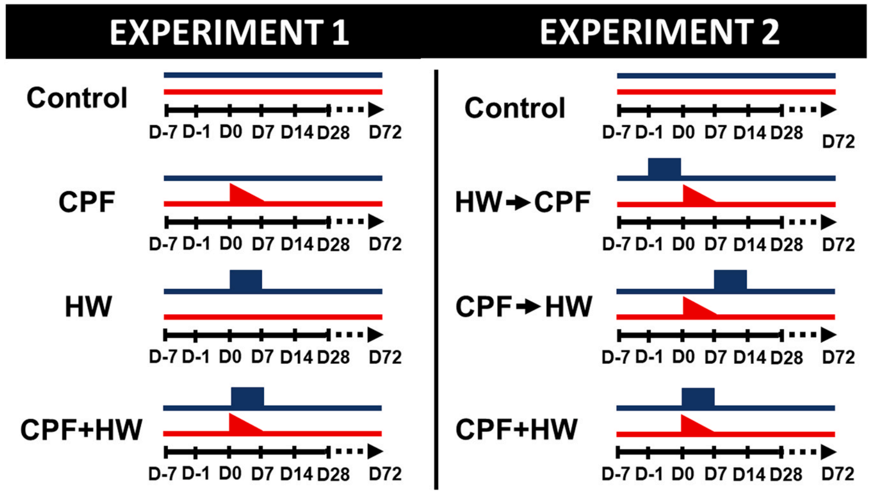

Fig. 1. Experimental design and timeline of the first (i.e., single and combined stressors in synchrony) and second experiment (i.e., synchronous and asynchronous stressors). The blue rectangle indicates the occurrence of the heatwave (HW), while the red triangle represents the chlorpyrifos (CPF) exposure. The experiments had a duration of 72 days (D72). The time zero (D0) represents the time at which the stressor was applied in the first experiment, and the time at which the CPF pulse was applied in the second experiment.

of 800 µL were added to the microcosms that were exposed to CPF to reach a final concentration of 0.8 µg/L. Similarly, aliquots of 800 µL of methanol were applied to the microcosms that were not treated with CPF following the requirements specified by the OECD (2000) guidelines.

Water samples were taken from the CPF-treated microcosms using a glass pipette and stored in amber glass bottles. Samples were frozen at −20 °C until further chemical analysis. Water samples were collected 2 hours and 1, 3, 7, 10, and 37 days after the CPF application. Samples of the microcosms that did not receive any CPF application (i.e., Control, HW) were collected 2 hours and 10 days after the start of the experiment to assure there was no cross contamination.

CPF concentrations were analysed using a gas chromatograph (GC) system (Agilent 7890 A) coupled to a mass spectrophotometer (MS) with a triple quadrupole analyser (Agilent 7000 GC/MS Triple Quad). The GC column used was an HP-5 ms (Agilent) capillary column. Chlorpyrifos was extracted using the Stir Bar Sorptive Extraction (or Twister) technique. The Twister was placed in an Erlenmeyer flask with 100 mL of water sample containing the internal standard (chlorpyrifos-d 10, 20 µL of a standard solution of 250 ng/mL) and stirred at 850 rpm overnight. After extraction, the stir bar was introduced in a glass thermal desorption tube and placed in the thermal desorption unit of the GC-MS. The limit of quantification (LOQ) and the limit of detection (LOD) of the method were 30 ng/L and 3 ng/L, respectively. The mean recovery of the method was 98 % (Relative Standard Deviation, RSD: 2 %) at 30 ng/L, and 105 % (RDS: 5 %) at 100 ng/L (n = 3). Further details of the analytical method can be found in Vilas-Boas et al. (2021).

2.3. Water quality parameters

Water physicochemical variables (i.e., temperature, dissolved oxygen, pH, electrical conductivity, and salinity) and photosynthetic pigments (i.e., chlorophyll-a and phycoerythrin) were monitored using a YSI ProDSS 626973–01 multi-meter probe at 10 cm depth on days −7, −1, 7, 14, 22, 28, 36, 42, 56, 66, and 72, relative to the CPF application. Additionally, water temperature in the microcosms exposed to the HW was monitored and recorded every 15 min during the temperature rise and decline corresponding to the HW using a YSI ProDSS 626973–01 handheld water quality meter. Details of the water temperature monitoring of the simulated HWs are provided in Figure S1.

Water samples for nutrient analysis (i.e., ammonia, nitrate, orthophosphate and total phosphorous) were taken on days −7 and 42 relative to the CPF application. Ammonia and nitrate were determined according to the method described in APHA (2005), while ortho-phosphate and total phosphorous were determined following the methods described in APHA (2005) and Mackereth et al. (1978).

2.4. Zooplankton sampling and determination

Zooplankton communities were sampled in all microcosms on days −7, −1, 7, 14, 28, 42, and 72 relative to the CPF application. Zooplankton sampling was done by collecting 1.5 L of microcosm water using a manual suction pump and by filtering the water through a zooplankton net. Subsequently, the filtered water was returned to the original microcosm. The concentrated zooplankton sample was preserved with Lugol's iodine solution (approximately 4 % v/v). Identification of zooplankton was done by using a binocular (Olympus SZx7 with objective Olympus DF PL 2X-4) for the largest zooplankton fraction (>1 mm; Cladocera, Ostracoda, Cyclopoida) and a microscope (Olympus CX41) for the smallest fraction (<1 mm; Rotifera, naupliar stages of copepods). Individuals were identified to the lowest practical taxonomic resolution level. The number of individuals per litre of microcosm water was calculated for the largest fraction after inspecting the whole sample. The abundance of the smallest fraction was calculated by recalculating the individuals counted in two sub-samples of 1 mL to numbers per litre of microcosm water.

The studied community was composed of 16 taxa. The Rotifera phylum showed the highest taxonomic richness, including eight taxa (*Polyarthra* sp., *Ascomorpha* sp., *Testudinella* sp., *Euchlanis* sp., *Lecane* spp., *Brachionus calyciflorus*, *Lepadella patella,* and *Keratella tropica*), followed by Cladocera with five taxa (*Daphnia magna*, *Moina* sp., *Diaphanosoma* sp., *Alona* sp., and *Chydorus* sp.). Copepods were classified as Cyclopoida (sub-adults and adults) and Nauplii (juveniles), while Ostracoda were not further identified.

2.5. Data analyses

The effect of the treatments on the zooplankton community was first assessed by performing a Permutation Multivariate Analysis of Variance (PERMANOVA) test based on Euclidean distances with 999 Monte Carlo permutations. The PERMANOVA analyses were performed using the CPF and the HW as independent variables for the first experiment (i.e., single and combined stressors in synchrony). Their interaction was also assessed. For the second experiment (i.e., combined synchronous and asynchronous stressors), the stressor order was used as independent variable and pair-wise comparisons (for the different stressor sequence) were assessed with the PERMANOVA. Subsequently, the Principal Response Curve (PRC) method (Van den Brink and Ter Braak, 1999) with 499 Monte Carlo Permutations was used to visualize the effects of the treatments on the zooplankton community over the experimental period as well as the main responding taxa. The PRC displays the variation between the treated communities and the controls in the different sampling times (C_{dt}) and allows the calculation of the affinity of each taxon with the PRC (b_k), thus helping to identify species whose abundance increases or decreases in response to the evaluated treatments (for further details, see Van den Brink and Ter Braak, 1999). The PERMANOVA analyses were performed using the PRIMER version 7 Software (Clarke and Gorley, 2015), while the PRC analyses were performed using the CANOCO Software, version 5 (Ter Braak, Šmilauer., 2012). In all cases data were log (x+1) transformed prior to the analysis, and a significance level of 0.05 was used to distinguish significant community-level effects caused by the evaluated treatments.

Effects of the treatments on zooplankton population abundance were only assessed for those taxa that showed the lowest or highest b_k values, as indicated by the PRC analysis. Statistically significant differences among the treatments of the first experiment (i.e., single and combined stressors in synchrony) were evaluated by a two-way ANOVA. Statistically significant differences among the treatments of the second experiment (i.e., combined synchronous and asynchronous stressors) were evaluated with a one-way ANOVA followed by a Tukey pair-wise comparison test using Bonferroni correction. Statistically significant effects of the treatments on the chlorophyll-a and phycoerythrin concentrations were also assessed using the same methods to determine a potential significant effect in the structure of the phytoplankton community. These tests were performed with the Software Jamovi 1.2.2.0 (Sahin and Aybek, 2019) assuming a significance level of 0.05. Density population graphs were made in R v4.1.2 (R Core Team, 2021) using the GGplot2 R package v.3.3.6 (Wickham, 2009).

3. Results

3.1. Effects of the single and combined stressors in synchrony

After CPF addition, measured CPF concentrations in the microcosm water varied less than 10 % from the nominal concentration (0.8 μg/L). The calculated CPF half-life (DT50) in the CPF treatment was 8.8 days, while the calculated DT50 in the CPF+HW treatment was 6.1 days (Table S1). No traces of CPF were detected neither in the controls nor in the HW treatment.

The PERMANOVA analysis showed that the effects of CPF on the community composition were significant since the moment of the pesticide application until the end of the experiment, while the effects of the HW were only significant right after the HW (day 7) and one week later (day 14), indicating post-stress recovery. The interaction between CPF and the HW was not statistically significant in any of the sampling times, indicating that the combined effects of the stressors on the community composition were additive (i.e., the magnitude of the effects was similar to the sum of effects caused by the individual stressors; Table 1). The PRC analysis showed that the first two PRCs were significant (Monte Carlo p-value: 0.002). The first PRC mainly shows the effects caused by the CPF exposure (Fig. 2A), while the second mainly displays the effects caused by the HW (Fig. 2B). In line with the PERMANOVA analysis, the first PRC shows that the impact of CPF and the CPF+HW treatment on the zooplankton community lasted for the whole experimental period, being the magnitude of the combined treatment (CPF+HW) slightly larger but not statistically different (i.e., additive effects). The first PRC also shows that the CPF and the CPF+HW resulted in an abundance decline of *Daphnia magna* and *Diaphanosoma* sp., and an abundance increase of *Moina* sp. relative to the control (Fig. 2). The second PRC mainly displays the effects caused by the HW, which were larger on day 7 and 14. The taxa that were more impacted by the HW were *Brachionus calyciflorus* and *D. magna*, which decreased in abundance, while *Diaphanosoma* sp. and *Moina* sp. experimented an abundance increase (Fig. 2).

Based on the results of the first PRC, two-way ANOVAs were calculated for the populations of *Daphnia magna*, *Diaphanosoma* sp. and *Moina* sp. to assess the single and combined effects of the evaluated stressors. *Daphnia magna* showed a significant population decline in the CPF and the HW treatments on day 7, 14 and 28 (Table 1; Fig. 3). The CPF+HW treatment also showed a statistically significant effect on the same sampling days. Based on the multiple stressor classifications defined by Piggott et al. (2015), the interaction was classified as negative synergistic on days 7 and 14, and negative antagonistic on day 28. The abundance of *Moina* sp. was generally higher in the treatments with HW (Fig. 3). The results of the ANOVA showed a statistically significant effect on day 7; however, the interaction between CPF and the HW was additive (Table 1). *Diaphanosoma* sp. was affected in the CPF and the CPF+HW treatments, with abundances dropping to zero after the CPF application. Thus, for this taxon, the interaction between the two stressors could not be properly evaluated (Figure S2).

The mean and the standard deviation of the water quality parameters measured in the control and the treatments are shown in Table S2. EC values slightly increased in the HW (2464 ± 73.5 μS/cm; mean ± SD) and the CPF+HW (2450 ± 41 μS/cm) treatments during the HW as compared to the control (2335 ± 52 μS/cm) and the CPF treatment (2294 ± 34 μS/cm). Microcosms were well oxygenated during the whole experiment, having percentages of oxygen saturation between 80 % and 100 %. After the simulation of the HW, dissolved oxygen concentrations slightly dropped in the HW and CPF+HW treatments: 85

Table 1
Results of the PERMANOVA analysis performed with the zooplankton community and the two-way ANOVA performed with the population abundances for the most responding taxa (i.e., *Daphnia magna* and *Moina sp.*) in the first experiment (i.e., single and combined stressors in synchrony). The table shows the calculated p-values for each treatment. Significant effects of chlorpyrifos (CPF), the heatwave (HW) and their interaction (CPFxHW) are indicated in bold (p-values < 0.05).

	Days relative to the chlorpyrifos application						
	D-7	D-1	D7	D14	D28	D42	D72
Zooplankton community							
CPF	0.59	0.25	**0.02**	**0.01**	**0.02**	**0.01**	**0.004**
HW	0.57	0.35	**0.002**	**0.002**	0.06	0.05	0.15
CPFxHW	0.46	0.52	0.51	0.15	0.30	0.18	0.14
Daphnia magna							
CPF	0.27	0.38	**0.02**	**<0.001**	**0.02**	0.11	0.09
HW	0.27	0.07	**0.02**	**<0.001**	**<0.001**	0.10	0.72
CPFxHW	0.90	0.52	**0.03**	**<0.001**	**0.01**	0.06	0.58
Moina sp.							
CPF	0.40	0.12	0.19	0.64	0.16	0.40	0.47
HW	0.40	0.09	**0.01**	0.09	0.14	0.29	0.25
CPFxHW	0.21	0.17	0.24	0.21	0.12	0.40	0.47

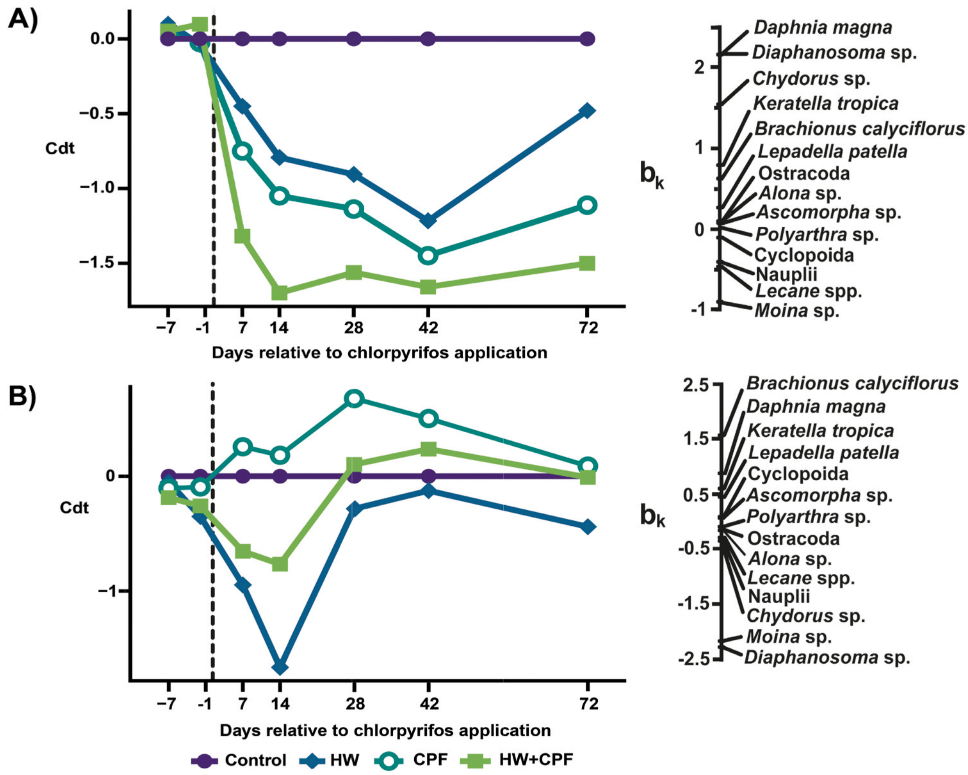

Fig. 2. Principal Response Curves (PRCs) showing the effects of chlorpyrifos (CPF), the heatwave (HW) and their combination (CPF+HW) on the zooplankton community in the first experiment (i.e., single and combined stressors in synchrony). The first PRC (A) displays the effects of CPF, while the second PRC (B) represents the effects of the HW. The C_{dt} represents the variation between the treated communities and the controls in the different sampling times, while the b_k indicates the affinity of each taxon with the PRC. The dashed vertical line indicates the moment of the CPF application. Of all variance, 35 % was explained by sampling day (displayed in the x-axis), while 26 % was explained by the treatments. Of all variance explained by the treatments, 41 % is displayed in the y-axis of the first PRC (A) and 24 % in the y-axis of the second one (B). The Monte Carlo Permutation test indicated that a significant part of the community variation is displayed in the diagrams (p-value: 0.002).

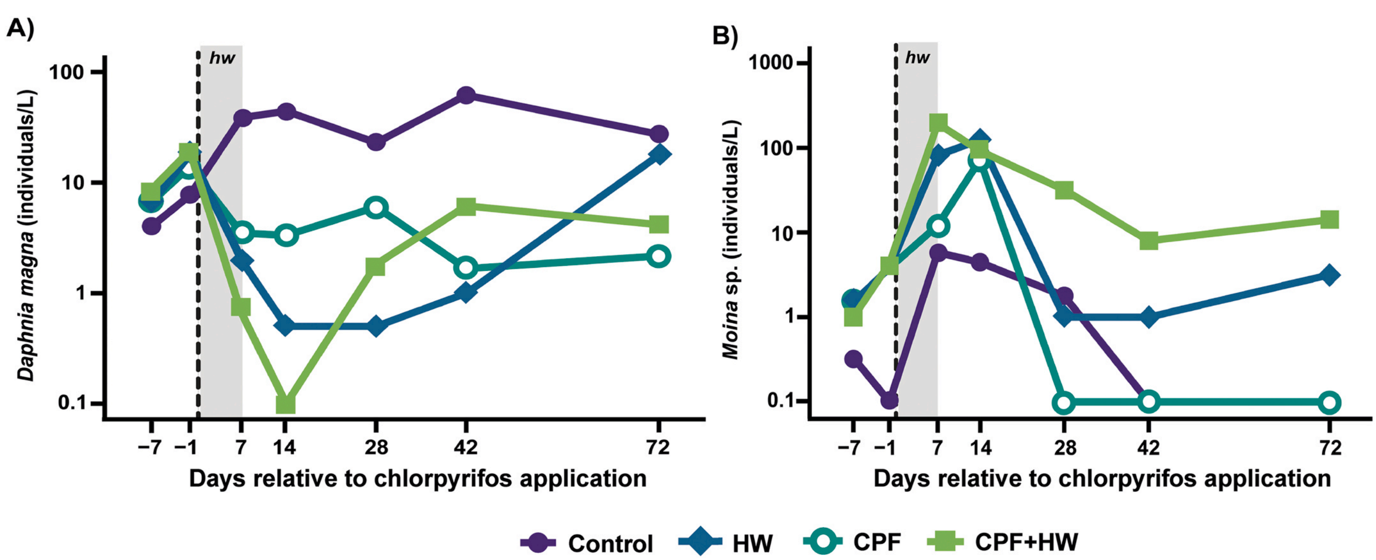

Fig. 3. Population abundance dynamics of *Daphnia magna* (A) and *Moina* sp. (B) in the first experiment (i.e., single and combined stressors in synchrony). Markers represent the mean of the population abundance per treatment (n=4). The dashed vertical line indicates the moment of the chlorpyrifos application, and the grey vertical rectangle shows the duration of the heatwave. The treatment labels indicate: CPF: chlorpyrifos; HW: heatwave; CPF+HW: chlorpyrifos and heatwave occurring at the same time. Please note that the y-axis is displayed in a logarithmic scale.

± 6.5 % and 81 ± 4.7 %, respectively. pH was similar among all treatments ranging between 7 and 8 (Table S2). The nutrient analysis showed low values of total N (considering the sum of N in ammonia and nitrate) and did not vary much from day −7 (0.36 ± 0.08 mg/L) to day 42 (0.28 ± 0.07 mg/L). Initial total P concentrations were relatively high and corresponded to typical values of eutrophic waters (Carlson and Simpson, 1996). No clear differences in nutrient concentrations were identified among treatments (Table S3).

The mean concentration of chlorophyll-a at the beginning of the experiment (day −7) was 17 ± 3.6 μg/L (mean ± SD), and the concentration of phycoerythrin was 85 ± 27.3 μg/L. Both showed a downward trend over the experimental period, reaching values of 2.7 ± 1.3 μg/L and 5.7 ± 2.8 μg/L respectively, in the last sampling day. The ANOVA performed to assess the influence of the treatments on the photosynthetic pigments showed a significant increase in the chlorophyll-a concentrations towards the end of the experiment in the CPF treatment with respect to the control, and a significant phycoerythrin increase caused by the HW on day 7. No significant effects were observed by the CPF+HW treatment (Figure S3; Table S4).

3.2. Effects of multiple stressors in asynchrony

Measured CPF concentrations in the HW→CPF and CPF→HW treatments of the second experiment fell close to the nominal concentration (variation < 10 %). In these treatments, the calculated DT50s were 8.3 and 6.0 days, respectively (Table S1).

The PERMANOVA analysis showed statistically significant differences between the HW→CPF treatment and the control on day −1, due to influence of the HW, and on days 28 and 42 (Table 2). The CPF+HW and the CPF→HW treatments showed statistically significant differences as compared to the control since the application of the stressors until the end of the experiment. The pair-wise comparisons among the different treatments showed no significant differences, except for the HW→CPF and the other treatments on D-1, probably because of the HW (Table 2). The PRC analysis showed that only the first PRC was significant (Monte Carlo p-value: 0.002). The PRC diagram shows that, although the response of the zooplankton community was similar among the three treatments, the magnitude of the effects shown in the HW→CPF treatment was lower than in the other two (Fig. 4). Also, the magnitude of the impacts of the CPF→HW stressor sequence was higher and lasted longer than the synchronous multiple stressor treatment (CPF+HW). The PRC indicated that *Daphnia magna* and *Diaphanosoma* sp. were the most sensitive taxa to the combination of both stressors in asynchrony (highest b_k values), while *Moina* sp. showed an abundance increase (lowest b_k value; Fig. 4).

Daphnia magna showed a significant population decline in all treatments as compared to the control on days 7 and 14 (Table 2; Fig. 5). However, from day 28 until the end of the experiment, there were no significant differences as regards to the control in the HW→CPF treatment (Table 2), indicating population recovery. The population abundance of *D. magna* in the CPF+HW also increased from day 14 onwards, showing no statistically significant effects as compared to the control in the last sampling day, denoting a population recovery that was achieved at a slower pace as compared to the HW→CPF treatment. Conversely, the *D. magna* population decline in the CPF→HW sequence was more severe and statistically significant until the end of the experiment, showing no recovery during the experimental period (Table 2; Fig. 5). *Moina* sp. showed a significant abundance increase in the CPF+HW and the HW→CPF treatments after the HW, and a trend towards a higher abundance in the CPF→HW and the CPF+HW treatments towards the end of the experiment (Fig. 5), although it was not statistically significant (Table 2). *Diaphanosoma* sp. showed a fast and severe population decline, yielding to extinction, so that the influence of the stressors sequence could not be properly evaluated (Figure S4).

The results of the water quality parameters in the sequential multiple stressor treatments were similar to the CPF+HW treatment, showing a slight decline in oxygen concentration and an EC increase during and shortly after the HW, and a decrease in the total P concentration over the course of the experiment (Tables S5 and S6). The concentration of chlorophyll-a and phycoerythrin showed a similar decreasing trend towards the end of the experiment (Figure S5). No statistically significant effects of the stressors sequence on these parameters were found (Table S7).

4. Discussion

4.1. Effects of single and combined stressors in synchrony

The CPF pulse led to a decline in zooplankton abundance, particularly affecting some Cladocera populations. This was expected based on the mode of action of this insecticide, which acts by inhibiting the acetylcholinesterase (AChE) enzyme in insects and crustaceans, causing over-stimulation of the nervous system, damaging nerve synapsis, and impairing muscle functioning, feeding and mobility (Matsumura, 1985). The Cladocera *Diaphanosoma* sp. and *Daphnia magna* were the most

Table 2
Results of the PERMANOVA analysis performed with the zooplankton community and the two-way ANOVA performed with the most responding taxa in the second experiment (i.e., synchronous and asynchronous stressors). The table shows the calculated p-values for the pairwise comparisons between treatments. Significant differences are indicated in bold (p-values < 0.05). The treatments indicate: HW→CPF: heatwave followed by chlorpyrifos; CPF+HW: chlorpyrifos occurring at the same time as the heatwave; CPF→HW: chlorpyrifos followed by heatwave.

	Days relative to the chlorpyrifos application (D)						
	D-7	D-1	D7	D14	D28	D42	D72
Community							
Control - HW→CPF	0.33	**0.02**	0.10	0.07	**0.04**	**0.03**	0.07
Control - CPF+HW	0.18	0.22	**0.01**	**0.004**	**0.02**	**0.02**	**0.01**
Control - CPF→HW	0.26	0.67	0.05	**0.03**	**0.02**	**0.02**	**0.02**
HW→CPF - CPF+HW	0.08	**0.02**	0.16	0.09	0.89	0.63	0.35
HW→CPF - CPF→HW	0.12	**0.04**	0.89	0.40	0.64	0.16	0.42
CPF+HW - CPF→HW	0.23	0.52	0.06	0.45	0.80	0.71	0.29
Daphnia magna							
Control - HW→CPF	1.00	1.00	**<0.001**	**<0.001**	0.07	0.20	0.08
Control - CPF+HW	0.22	**0.04**	**<0.001**	**<0.001**	**0.02**	**0.03**	0.05
Control - CPF→HW	0.05	0.18	**<0.001**	**<0.001**	**0.01**	**0.01**	**0.01**
HW→CPF - CPF+HW	0.31	0.06	0.99	0.38	0.88	0.70	0.99
HW→CPF - CPF→HW	0.07	0.24	0.95	0.38	0.50	0.24	0.64
CPF+HW - CPF→HW	0.79	0.82	0.86	1.00	0.89	0.80	0.80
***Moina* sp.**							
Control - HW→CPF	0.98	**<0.001**	0.96	0.95	0.96	1.00	1.00
Control - CPF+HW	0.49	**0.003**	**0.04**	0.40	0.81	0.76	0.81
Control - CPF→HW	0.48	0.29	1.00	0.34	0.68	0.40	0.17
HW→CPF - CPF+HW	0.73	0.40	0.10	0.70	0.98	0.76	0.89
HW→CPF - CPF→HW	0.72	**0.004**	0.98	0.62	0.92	0.40	0.23
CPF+HW - CPF→HW	1.00	0.07	0.05	1.00	1.00	0.92	0.57

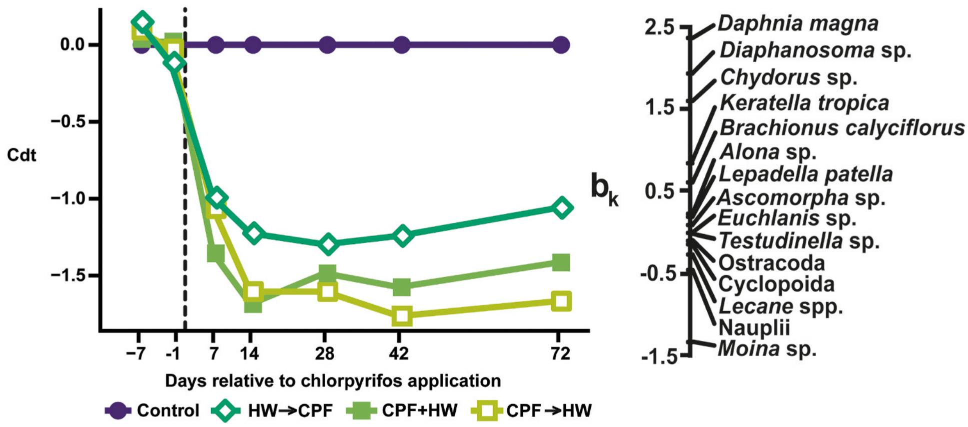

Fig. 4. Principal Response Curve (PRCs) showing the impact of chlorpyrifos and the heatwave on the zooplankton community in the second experiment (i.e., synchronous and asynchronous stressors). The C_{dt} represents the variation between the treated communities and the controls in the different sampling times, while the b_k indicates the affinity of each taxon with the PRC. The dashed vertical line indicates the moment of the chlorpyrifos application. Of all variance, 34 % could be explained by sampling day, and is displayed on the x-axis, while 26 % could be explained by the treatments. Of this variance, 41 % is explained in the y-axis (C_{dt}). The Monte Carlo Permutation test indicated that a significant part of the variance is explained by the treatments (*p*-value: 0.002). The treatments indicate: HW→CPF: heatwave followed by chlorpyrifos; CPF+HW: chlorpyrifos occurring at the same time as the heatwave; CPF→HW: chlorpyrifos followed by heatwave.

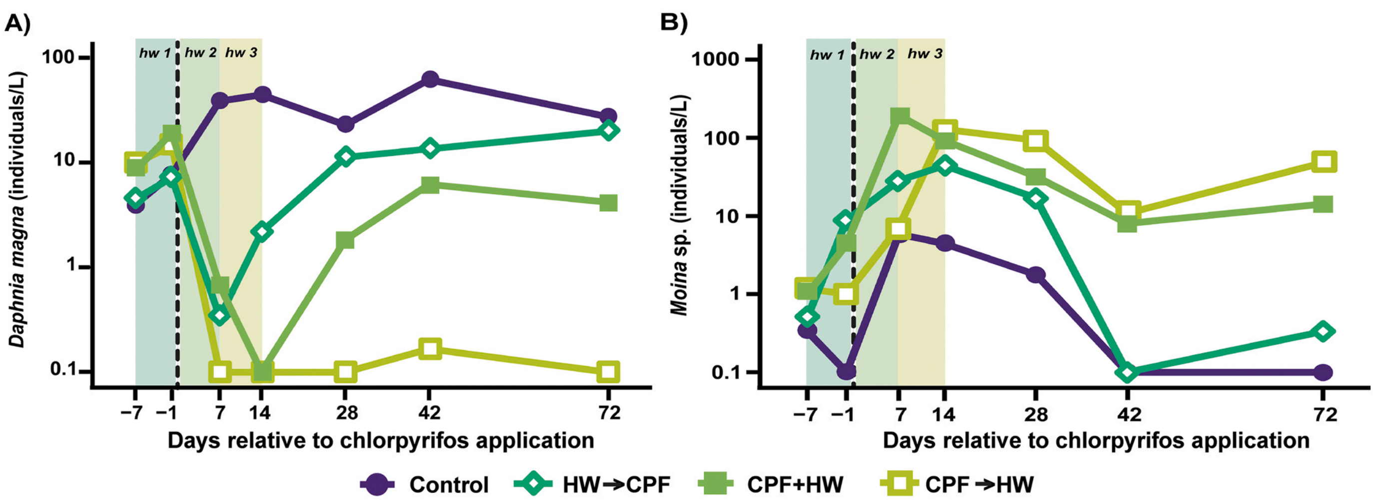

Fig. 5. Population dynamics of *Daphnia magna* (A) and *Moina* sp. (B) in the second experiment (i.e., synchronous and asynchronous stressors). Markers represent the mean of the calculated densities in each treatment (n=4). The dashed vertical line corresponds to the chlorpyrifos application. Coloured vertical rectangles indicate the heatwaves in the different treatments and are labelled according to the sequence order. The treatment labels indicate: HW→CPF: heatwave followed by chlorpyrifos; CPF+HW: chlorpyrifos occurring at the same time as the heatwave; CPF→HW: chlorpyrifos followed by heatwave. Please note that the y-axis is displayed in a logarithmic scale.

sensitive taxa to CPF, while *Moina* sp. showed a higher tolerance. Similar results were found in other laboratory and semi-field experiments performed with CPF under Mediterranean conditions, indicating consistent population and community level effects (Polazzo et al., 2022b; Vilas-Boas et al., 2021). Furthermore, the reduction in Cladocera abundance was most likely the cause of the increase in the chlorophyll-a concentration in the CPF treatment due to a reduction in the algae grazing pressure.

The HW resulted in a significant change in the zooplankton community structure. However, such change only lasted for some days after the cessation of the heat stress, suggesting that HW-induced compositional shifts may be recovered relatively fast in planktonic communities. The optimal temperature range for *Daphnia magna* has been reported to be 20–21 °C (e.g. Giebelhausen and Lampert, 2001). Temperatures near to 29 °C increase their oxygen demand, activating the anaerobic metabolism, and affecting their filtration capacity, which leads to effects on energy provision, growth and reproduction (Müller et al., 2018). On the other hand, the genus *Moina* is known to withstand high daily temperature fluctuations (5°C – 31°C), having its optimal performance at 24°C – 31°C (Hoff and Snell, 2008), while *Diaphanosoma* spp. are stenothermic thermophiles but show their optimum in the 20°C - 30°C range (Verbitskii et al., 2009). Therefore, changes in community structure caused by the HW imposed here (reaching temperatures of 28°C for 7 days) were likely driven by the boost in the growth rate of some taxa (e.g. *Moina* sp. and *Diaphanosoma* sp.) and their competitive advantage respect to species that are less tolerant to heat stress (*D. magna*). Besides structural changes in the zooplankton community, a trend towards an increase of phycoerythrin during the HW was also observed. This indicates an increase in cyanobacteria biomass and a possible change in the phytoplankton community structure.

The exposure to CPF and HW in synchrony resulted in different effects at the population and the community level. At the population level, synergistic effects of the combined stressors application on the *D. magna* population abundance were observed in the short-term, which were followed by a fast population recovery capacity when compared to the CPF treatment. The large short-term effects of the combination of both stressors can be explained by different reasons. High temperatures increase metabolic activity, respiration rates and suspended particle ingestion (Müller et al., 2018), which contribute to an increase in chemical uptake from water. This phenomenon has been described in many toxicity experiments. For example, Buchwalter et al. (2003) showed that an acute temperature increase of 4.5 °C increased CPF accumulation rates in the ephemeropteran *Cinygma* sp. and the hemipteran *Sigara washingtonensis*. On the other hand, high temperatures have been suggested to increase CPF toxicity due to the conversion of this compound into more toxic metabolites such as the CPF-oxon (Buchwalter et al., 2004; Verheyen et al., 2019) which has a higher affinity for AChE. In terms of energetic costs, organisms were probably allocating more energy away from growth or other vital functions towards detoxification (i.e., upregulation of the gluthatione-S-Transferase) and other defence mechanisms (i.e., upregulation of heat shock proteins) (Verheyen et al., 2019). Therefore, the higher chemical uptake and the formation of toxic metabolites are the most likely causes of the magnified toxicity of CPF to *D. magna* when simultaneously exposed to heat stress. On the other hand, the faster recovery of the *D. magna* population can be (partially) explained by the faster dissipation of CPF in combination with the HW. Faster water dissipation of CPF has been reported in ecosystems where microbial activity is boosted by increased temperatures or where volatilization or hydrolysis processes are activated (Racke, 1993). Such high dissipation could have resulted in higher population reproductive success compared to the CPF treatment, as juveniles, which are significantly more sensitive to CPF than adults, might have thrived better (Van den Brink et al., 2017)

At the community level, the simultaneous application of CPF and HW determined a larger compositional shift compared to the individual application of either stressor. Yet, the differences were not statistically significant, and the interaction fell within the additivity range. These results are in line with the meta-analysis performed by Jackson et al. (2016), which showed that most studies assessing the combined effects of warming and chemical contamination on communities resulted in either antagonistic or additive effects. The different outcomes observed for the community and population level confirm that multiple stressor combinations can yield different responses depending on the level of biological organization (Dinh et al., 2022; Polazzo et al., 2022c). Furthermore, the existence of functionally redundant species limit the effect of multiple stressors on ecosystem functions such as top-down control of algae (Delnat et al., 2022). For example, the depletion of *Daphnia magna* and *Diaphanosoma* sp. from the community were counterbalanced by the increase of *Moina* sp., thus overriding any indirect effects on the chlorophyll-a concentration, which was used here as a proxy for phytoplankton biomass.

4.2. Effects of multiple stressors in asynchrony

This study shows that the combination of the HW and the CPF stressors in synchrony and asynchrony resulted in similar short-term effects regarding their direction and magnitude, however different responses were observed at the population and community level in the long-term. At the population level, the HW→CPF sequence (representing CITS) was found to be the least harmful combination in the long term, allowing the most sensitive population (*D. magna*) to recover within two weeks after the CPF pulse. This is in line with the study by Meng et al. (2020b), which showed that a previous heat spike would make mosquito larvae more tolerant to a subsequent CPF pulse. According to Meng et al. (2020a), severe heat stress peaks can produce a survival selection, removing individuals that have a lower baseline AChE activity and higher sensitivity to CPF. In our experiment such individuals were likely to be juvenile daphnids, thus the HW could have selected for adults that have a higher reproductive efficiency after the heat stress. Moreover, heat stress can produce cross-tolerance, meaning that the defensive system towards the HW may lead to a better handling of CPF if both stressors activate the same physiological mechanisms (Gunderson et al., 2016). Indeed, the exposure to a previous heat spike on mosquito larvae has been proven to reduce the oxidative damage caused by CPF by the activation of heat shock proteins and antioxidant enzymes which protect the organism from stress (Meng et al., 2020b). The upregulation of these heat shock proteins allows better coping with different stressors, including warming and pesticides (Delnat et al., 2020).

The CPF→HW sequence (representing TICS) produced devastating effects on the *D. magna* population, which was not able to recover within the experimental period. In this treatment, the effects of the initial exposure to CPF were exacerbated by the following HW. Intrinsic sensitivity to chemicals is explained by the combination of toxicokinetic (uptake, biotransformation, distribution and elimination of the parent compound and its metabolites) and toxicodynamic (re-establishment of homeostasis and damage recovery) processes, which are correlated to biological traits and the presence, location and abundance of chemical receptors (Ashauer et al., 2017). Rubach et al. (2010) calculated a t_{95} (95 % depuration time) of CPF in *Daphnia magna* of 5.5 days, which suggests that during the application of the HW in this experiment, chemical depuration processes but also toxicodynamic processes were still in play. The influence of temperature on chemical toxicokinetics is well known and can be related to the Arrhenius equation. However, recent investigations suggest that chemicals with specific mode of action can affect differently toxicokinetic and toxicodynamic processes in aquatic invertebrates (Mangold-Döring et al., 2022), thus yielding less predictable responses. Meng et al. (2020a) indicated that the larger effects caused by CPF on mosquito larvae after heat stress could be explained based on the increasing oxygen demand for detoxification and internal damage repair together with a decreasing oxygen supply by the impairment of the respiration function. Thus, the results of our study suggest that the heat stress combined with the carry-over toxicity caused by the previous exposure to CPF could have affected the internal recovery rates of *D. magna* individuals, determining a population collapse.

The zooplankton community exposed to the HW→CPF treatment showed an initial significant compositional change, which recovered by the end of the experiment. Conversely, in the other two treatments (CPF+HW and CPF → HW), such recovery was not achieved, showing that the order in which communities are exposed to the multiple stressors affects community dynamics in the long term. Particularly, the community exposed to the CPF → HW showed the largest dissimilarity to the control at the end of the experiment. Differences in community structure can be mostly attributed to the magnitude of the direct effects over the sensitive species and the associated indirect effects. *Moina* sp. showed the highest population abundance increase in the CPF→HW treatment, as a result of the competition release determined by *Diaphanosoma* sp. and *D. magna*. Consistently, *Moina* sp. had the lowest population increase in the HW→CPF treatment due to the less drastic decline and the fast recovery of the *D. magna* population. Thus, interspecific competition arises here as a key factor that modulates the magnitude and duration of the effects caused by different multiple stressor sequences on populations and communities. Knillmann et al. (2013) demonstrated that the recovery time of *Daphnia* spp. populations exposed to the insecticide esfenvalerate was twice as long under a warming scenario due to the enhanced interspecific competition exerted by less sensitive taxa (i.e., *Simocephalus* spp.). On the other hand, Delnat et al. (2021) found negative sublethal effects on the mosquito larvae *Culex pipiens* (i.e., increased development time and decreased pupal mass) caused by interspecific competition with *D. magna* when combining daily temperature variation and CPF exposure. In both studies, interspecific competition did not amplify the toxicant effects, but affected the energy costs resulting in lower individualś fitness and extended long-term effects. In our experiment, cascading effects on the chloropyll-a or phycoerythrin concentration as result of the stressor sequence were not identified, although subtle effects on species composition may have occurred. This was against our expectations since some studies have indicated that changes in the dominance of Cladocera species lead to large structural changes in the phytoplankton community (O'Connor et al., 2009; Roth et al., 2022).

5. Conclusions and way forward

Pesticide exposure peaks in freshwater ecosystems may occur simultaneously or with little temporal difference as regards to heatwaves. As we have shown here, synchronous exposure to an insecticide and a heatwave can result in synergistic short-term effects on sensitive zooplankton populations, but produce additive effects at the community level, showing that the ecological impacts of multiple stressors occurring in synchrony vary depending on the level of biological organization. Furthermore, our study shows that the timing of stressors can result in different long-term effects on sensitive aquatic species. Among the stressor sequences evaluated here, we found that the CPF → HW sequence, which represents the TICS concept, resulted in the most harmful effects on sensitive zooplankton populations, while the HW → CPF sequence resulted in milder effects as compared to the synchronous scenario (CPF+HW). Moreover, we found that the differential effects of the stressor sequence on sensitive populations are propagated at the community level, affecting the dominance of different Cladocera species. Therefore, we can conclude that the timing of stressors affects the structure of aquatic populations and communities in the long-term and should be considered in the risk assessment of chemicals and for the derivation of management and conservation strategies.

Further investigations are encouraged to assess how changes in community structure due to multiple stressor sequences affect ecosystem functioning and stability. In addition, there are some areas that should be further researched to understand the generalizability of our results to other stressor combinations and ecological scenarios. First, more information is needed on the sensitivity of different species to single chemical and non-chemical stressors (e.g. thermal tolerance, salinity, etc.), including their physiological adaptation and metabolic energy demands associated to the processes of internal damage repair and recovery. In this way, our capacity to predict how a second stressor will affect the carry-over effects of the first one at the individual and population level will be improved. Second, cross-tolerance mechanisms among multiple stressors should be further investigated, including their capacity to activate similar or competing physiological processes. Correlations between multiple stressor effects on different biological endpoints (i.e., growth, feeding, reproduction) should be better evaluated, which will support our *a priori* capacity to determine non-additive effects at the population level. Finally, a deeper understanding on the interactions of the species that form the evaluated community or ecosystem are crucial to determine the persistence of effects caused by multiple stressor sequences. In this regard, the investigation of asynchronous stressors, with different durations and intensities, on freshwater species assemblages using semi-field experiments and modelling approaches will be decisive.

CRediT authorship contribution statement

Andreu Rico: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft. **Francesco Polazzo:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Ariadna Garcia:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft.

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.

Acknowledgments

This study has been supported from the CICLIC project, funded by the Spanish Ministry of Science, Innovation and Universities (RTI 2018_097158_A_C32); the H2020-MSCA-ITN ECORISK2050 project, funded by the European Union's Horizon 2020 research and innovation program (grant agreement No. 813124); and the Talented Researcher Support Programme - PlanGenT (CIDEGENT/2020/043) of the Generalitat Valenciana. A. García-Astillero thanks the research support by grant (PTA2018–016257-I) funded by the Spanish Ministry of Science, Innovation and Universities.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116751.

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