



# Combined stress of an insecticide and heatwaves or elevated temperature induce community and food web effects in a Mediterranean freshwater ecosystem

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## ABSTRACT

Ongoing global climate change will shift nature towards Anthropocene's unprecedented conditions by increasing average temperatures and the frequency and severity of extreme events, such as heatwaves. While such climatic changes pose an increased threat for freshwater ecosystems, other stressors like pesticides may interact with warming and lead to unpredictable effects. Studies that examine the underpinned mechanisms of multiple stressor effects are scarce and often lack environmental realism. Here, we conducted a multiple stressors experiment using outdoor freshwater mesocosms with natural assemblages of macroinvertebrates, zooplankton, phytoplankton, macrophytes, and microbes. The effects of the neonicotinoid insecticide imidacloprid (1 µg/L) were investigated in combination with three temperature scenarios representing ambient, elevated temperatures (+4 °C), and heatwaves (+0 to 8 °C), the latter two having similar energy input. We found similar imidacloprid dissipation patterns for all temperature treatments with lowest average dissipation half-lives under both warming scenarios (DT<sub>50</sub>: 3 days) and highest under ambient temperatures (DT<sub>50</sub>: 4 days) throughout the experiment. Amongst all communities, only the zooplankton community was significantly affected by the combined treatments. This community demonstrated low chemical sensitivity with lagged and significant negative imidacloprid effects only for cyclopoids. Heatwaves caused early and long-lasting significant effects on the zooplankton community as compared to elevated temperatures, with *Polyarthra*, *Daphnia longispina*, Lecanidae, and cyclopoids being the most negatively affected taxa, whereas *Ceriodaphnia* and nauplii showed positive responses to temperature. Community recovery from imidacloprid stress was slower under heatwaves, suggesting temperature-enhanced toxicity. Finally, microbial and macrofauna litter degradation were significantly enhanced by temperature, whereas the latter was also negatively affected by imidacloprid. A structural equation model depicted cascading food web effects of both stressors with stronger relationships and significant negative stressor effects at higher than at lower trophic levels. Our study highlights the threat of a series of heatwaves compared to elevated temperatures for imidacloprid-stressed freshwaters.

## 1. Introduction

Nowadays, climate change has become one of the greatest threats for nature (Malhi et al., 2020). Apart from a gradual increase in the average global surface temperatures, there are multiple facets of climate change that incorporate temporary extreme events, such as heatwaves,

droughts, heavy precipitations, and storms (IPCC, 2021). Nevertheless, the different expressions of climatic change are expected to negatively affect various types of ecosystems (IPCC, 2021). Given the increasing number of congruent predictions about more intense and more frequent heatwaves (Meehl and Tebaldi, 2004; Perkins et al., 2012; Woolway et al., 2021), and observations of rapidly rising temperatures, there is

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growing concern about how climatic changes, particularly heatwaves, may affect freshwaters (Macaulay et al., 2021a; Polazzo et al., 2022b; Roth et al., 2022; Hermann et al., 2023).

Future warming in the Mediterranean region is projected to surpass global warming rates by 25 % in addition to an increasing series of heatwaves per year, and both are posing a risk to freshwater ecosystems (Zittis et al., 2016; Lionello and Scarascia, 2018). Since a large group of organisms that inhabit these aquatic ecosystems are temperature-sensitive ectotherms, they can be pushed beyond their thermal tolerance range by raising temperatures (Huey and Stevenson, 1979; Walberg and Green, 2021). Owing to the considerable influence of temperature on various life-history traits of these organisms (O’Gorman et al., 2017; Bonacina et al., 2023), such as reproduction, growth, development, and life expectancy (Atkinson, 1994; Brown et al., 2004; Daufresne et al., 2009; Salo et al., 2017; Betini et al., 2020; Zohary et al., 2021), higher temperatures may result in an increased risk of freshwater species and population extinction and ergo in biodiversity loss (Reid et al., 2019; Martínez-Megías and Rico, 2022).

Securing the world’s food production for an expanding global population (about 9.7 billion in 2050, (UN, 2022)) in the face of ongoing climate change, poses a fine line between the demand for more sustainable agriculture and intensive pesticide use (Lykogianni et al., 2021). Pesticides and other agrochemicals became an important input for the agri-food sector, enhancing crop yields and food production (FAO; Alexandratos and Bruinsma, 2012). However, the application of, for instance, the first and most widely used neonicotinoid, imidacloprid (Jeschke et al., 2011), contributed to global concern of adverse effects on non-target organisms (Wood and Goulson, 2017; Hladik et al., 2018; Yamamuro et al., 2019). Neonicotinoids act via the postsynaptic nicotinic acetylcholine receptors of the organisms’ nervous systems (Taillebois et al., 2018) and have been proven to be particularly toxic to aquatic insects and some copepods (Sumon et al., 2018; Chará-Serna et al., 2019; Merga and Van den Brink, 2021; Van de Perre et al., 2021).

Picturing the complexity of how different climate change-related temperature scenarios and chemical stressors may act in isolation and combined on different levels of biological organization, it is obvious that more empirical data from environmentally realistic, large-scale studies are needed. Insights coming from field and semi-field studies will provide a better understanding of multiple stressor effects and the underlying mechanisms which drive them (De Laender, 2018; Orr et al., 2020). Recent studies demonstrated that interactions between temperature and chemicals may attenuate or exacerbate toxicity via increased uptake, excretion, degradation and metabolization rates (Mangold-Döring et al., 2022; Huang et al., 2023). However, it remains challenging to extrapolate these findings from individual-level to higher levels of biological organization which include intra- and inter-specific species interactions (Piggott et al., 2015; Jackson et al., 2016; Schäfer and Piggott, 2018). Mesocosms enable experimental research with high ecological and environmental complexity by integrating species interactions across different trophic levels (Paiva et al., 2021). As more severe effects on species appeared for an insecticide under daily fluctuating temperature rather than constant temperature (Vasseur et al., 2014; Verheyen and Stoks, 2019), experimental designs that consider daily and seasonal temperature fluctuations instead of constant temperature regimes may prevent underestimations of chemical effects. It appears obvious then, that there is a lack of knowledge on multiple stressor effects of different, environmentally realistic climate change scenarios and chemicals on freshwater ecosystems with multiple trophic levels and natural food web assemblages (Polazzo et al., 2022b; Vijayaraj et al., 2022). Considering the capability of marine, terrestrial and freshwater ecosystems to recover from pulse disturbances (Hillbrand and Kunze, 2020), it remains questionable to what extent and how fast different ecological components of a freshwater ecosystem recover from multiple stress associated with climate change and chemical exposure.

Here, we used outdoor mesocosms located in the Mediterranean

region (central Spain) to empirically test our hypothesis that an environmentally relevant concentration of imidacloprid and two different temperature-related climate change scenarios, as single and combined treatments, will significantly affect the structure and dynamics of communities and populations of different trophic levels of a freshwater ecosystem. We studied these multiple stressor effects during the treatment phase, when both temperature and chemical stressors were applied simultaneously, and assessed recovery in the following post-treatment phase, when no stressors were applied. Because multiple stressor effects on one trophic level may propagate through the entire food web, ecological cascade effects were investigated using a structural equation model.

## 2. Materials and methods

### 2.1. Experimental design

The outdoor mesocosm-experiment was conducted at the IMDEA Water Institute, in Alcalá de Henares (central Spain). After a three-month period of acclimatization, the experiment lasted for about three months from the 19th of April (day -7) through the 19th of July 2021 (day 84). We used a fully randomized, 2 × 3 factorial design with a chemical treatment of 0 (i.e., control) and 1 µg/L of the neonicotinoid insecticide imidacloprid (Admire®, 70 % active ingredient, Bayer), the latter being representative of the global average concentration of 0.73 µg/L detected in aquatic ecosystems (Sánchez-Bayo et al., 2016), as well as ambient, elevated temperatures, and three repeated heatwaves as temperature treatments (see 2.2). All six treatments were replicated four times, resulting in 24 mesocosms, hereafter referred to as cosms. The “transportable temperature and heatwave control device” (TENTACLE) was used for temperature manipulation and temperature recording in all cosms (Hermann et al., 2022). TENTACLE allows water temperature monitoring and manipulation of up to three different climate change-related scenarios, including ambient and elevated temperature fluctuations and heatwaves. Following the acclimatization phase, all treatments were simultaneously applied at the start of the experiment. The three-month acclimatization period was considered to be sufficient time for the mesocosms to reach a homogeneous state. Homogeneity of the mesocosms was assessed by sampling all ecosystem endpoints investigated prior to the start of the treatment phase.

All cosms were round-shaped glass fiber bowls with a diameter and total depth of 1.2 m and with a total volume of about 1000 L. Each cosm contained a 0.3 to 0.4 m layer of silty-sand sediment from the vicinity of the institute, and was filled with 850 L of freshwater from an artificial pond of the research facility. To compensate for water evaporation in the cosms, we filled plastic buckets biweekly with tap water and exposed it for several days to natural outdoor conditions (to ensure low chlorine concentrations in the cosms) prior to refilling the cosms to equal water volumes of 850 L. To colonize the cosms with phytoplankton and zooplankton during the acclimatization period, we collected the communities (55 µm net) from the same artificial pond and distributed them in equal shares over all cosms. Additionally, we evenly cut the standing stock of the macrophytes (*Myriophyllum spicatum*, *Elodea nuttallii*) in the cosms which was visually confirmed by inspecting the area covered by macrophytes. Although macrophyte development was not further quantified in the course of the experiment, we can report from memory that continuous growth was observed in all cosms. The cosms were stocked with equal amounts of macroinvertebrate species, collected independently of size and sex from the Henares River near Humanes, Guadalajara (Spain). To gain a similar initial macroinvertebrate composition in the mesocosms, we evenly distributed 1-litre jars filled from large buckets in which the collected macroinvertebrate species were homogenised by mixing.

We installed four traps on the bottom of each cosm enabling macrofauna colonization to later assess diversity and abundance. Two traps were filled only with pebble stones and two with leaves and pebble

stones. Additionally, macrofauna feeding was assessed in each cosm by providing a litter bag made out of nylon laundry bags (Essentials, Netherlands) with a size of 17 × 11 cm and a large mesh size ( $\leq 0.5$  cm). These bags were filled with 2 g of dried leaf litter from branchless leaves of *Populus* spp. We placed six small HDPE-bottles (100 mL, opening diameter 31 mm, VWR), filled with the same sediment as in the bottom of the cosms, on a plastic tray and submerged them in each cosm to assess imidacloprid concentrations in sediment. To assess microbial leaf litter decomposition (MLLD), 144 nylon bags (500  $\mu\text{m}$  mesh size, size 14 × 11 cm) were prepared and filled with 2 g *Populus* spp. leaves similar as for the macrofauna feeding. We hung sets of six of them with a nylon string in each cosm above the sediment. A wooden stick placed on top of all cosms facilitated the hanging of leaf litter bags, samplers, extra autologgers, and the temperature sensors of TENTACLE.

## 2.2. Temperature control and manipulation with TENTACLE

Temperatures of the ambient, elevated and heatwaves treatments were continuously recorded, and the latter two temperature treatments were controlled by TENTACLE. Ambient, daily, temperature fluctuations formed the temperature baseline for the simulated elevated temperatures and heatwave scenario. The average temperature in all ambient cosms was calculated at a regular interval of 3 min and used as reference for the elevated temperatures treatment. Thus, the temperature treatment followed the daily ambient temperature fluctuations with a + 4 °C offset. The temperature increase is representative for the estimated future warming scenario of 4.4 °C (IPCC, 2023). Lastly, a target temperature of 24, 28, and 32 °C was set for the first, second, and third heatwave, respectively. This target temperature was chosen with a + 8 °C offset to the varying mean ambient temperatures in the field (16, 20, and 24 °C) which were determined several days before the heatwave occurrence based on temperature records. By doing so, an equal amount of energy (+4 °C) was added under both temperature-manipulated scenarios over the experimental time. The duration of the repeated heatwaves was set to 7 days, which is comparable to the average duration of recorded lake heatwaves with  $7.7 \pm 0.4$  days (Woolway et al., 2021). After an occurring heatwave episode, the temperature decreased back to ambient conditions for 7 days before the next heatwave was simulated. The temperature treatment phase spanned from the 26th of April (day 0) through the 7th of June (day 42) followed by the recovery phase with no heating applied from the 7th of June (day 42) through 19th of July 2021 (day 84).

## 2.3. Imidacloprid spike and analyses in water and sediment

Imidacloprid was applied on days 0, 14, and 28 using a stock solution of 2 g/L prepared in Milli-Q water. As the cosms had a water volume of about 850 L, we prepared 1 L bottles with Milli-Q water, spiked them with 650  $\mu\text{L}$  of the prepared imidacloprid stock, and distributed the bottle volume equally over the whole water surface of the cosms that received the chemical treatment. Then, we stirred the water column gently with a wooden stick during each spiking event to achieve a homogeneous imidacloprid distribution.

Imidacloprid concentrations in the aqueous phase were measured in all cosms 4 h (i.e. day 0) after the pesticide application, and on days 3, 10, 14, 17, 24, 28, 31, 38, and 45 relative to the first imidacloprid application by taking 5 L of depth-integrated water samples. Before returning the sample back to the cosm, a subsample of approximately 3 mL was collected with a 4 mL amber glass vial (BGB, 130,401-W) and directly transferred to the analytical lab for analyses.

Imidacloprid concentrations in the sediment phase were assessed by collecting one HDPE-bottle with a sediment sample on days 11, 25, 39, 53, 67, and 81 relative to the first imidacloprid application. All samples were analysed by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS (QqQ)) on an Agilent 1290 Infinity II liquid chromatography system coupled to mass spectrometry with a 6495C

triple quadrupole analyser (Agilent Technologies, USA). For further details of the chemical analytical analyses see Huang et al. (2021). Imidacloprid fate in water and sediment were determined by means of exponential models assuming first-order kinetics. Dissipation half-lives ( $DT_{50}$ ) were calculated by  $\ln(2)/k$ , with  $k$  as the dissipation rate constant of the least squares fit. Time weighted average (TWA) concentrations were calculated by the sum of the imidacloprid concentrations of each measured time point, multiplied by the time period of observation since the last measurement and finally divided by the total time of observation.

## 2.4. Physico-chemical water quality variables

Water quality variables comprising dissolved oxygen (DO) concentration and saturation, pH, total dissolved solids (TDS), electrical conductivity (EC) were measured weekly in situ by using a portable multi-meter (YSI Pro DSS 626,973-01) and water quality test meter (Pancellent) on day -4, 3, 10, 17, 24, 31, 38 during treatment phase (i.e., day 0 to day 42) and biweekly on day 52, 66, and 80 during recovery phase (i.e., day 42 to 84). We conducted all measurements in the morning and evening, while ecosystem productivity was the calculated difference in DO concentrations from diurnal oxygen cycles (Kersting and van den Brink, 1997). In each of the three different temperature treatments, a hobo logger was submerged in one cosm to measure water temperature as backup as well as irradiance at the same depth of about 0.5 m as the temperature sensors of TENTACLE. Nutrients were sampled weekly during the treatment phase on day -4, 3, 10, 17, 24, 31, 38 and biweekly during the recovery phase on day 52, 66, and 80 by taking a 5 L of depth integrated water samples. From this sampled volume, 250 mL amber plastic bottles (Scharlab) were filled for the analyses of nitrate ( $\text{NO}_3^-$ ), orthophosphate ( $\text{PO}_4^{3-}$ ), and total phosphorus ( $P_{\text{tot}}$ ) using UV-Visible spectroscopy and standard procedures set by Mackereth et al. (1978). To mineralize samples for  $P_{\text{tot}}$  analysis, 4.5 mL of a sample was mixed with 0.8 mL of potassium persulphate solution ( $\text{K}_2\text{S}_2\text{O}_8$ , 50 g/L in Nano Pure Water) at 120 °C for 30 min.

## 2.5. Benthic macroinvertebrates and zooplankton

Benthic macroinvertebrates diversity and abundance was assessed on day -7, 7, 21, 35, 49, 63, and 77 by gently collecting all four traps by means of a 500  $\mu\text{m}$  sampling net and transferring the trapped sample to a plastic bucket filled with water of the corresponding cosm. Subsequently, the traps and samples were thoroughly washed over a series of sieves (5, 2, 1, and 0.5 mm) and finally transferred to a species identification tray. Macroinvertebrates were identified by naked eye to the family or lowest possible taxonomic level. Following species identification and counting, the sample was returned to the associated cosm and all traps were resubmerged.

Zooplankton was sampled from each cosm on days -4, 10, 24, 38, 52, 66, and 80 using a PVC tube. Depth-integrated samples from the cosms were collected until a total sample volume of 5 L was obtained. The total sample was concentrated into a 100 mL polyethylene bottle using a 55  $\mu\text{m}$  zooplankton net, preserved with lugol solution, and stored in the laboratory under dark conditions for later species identification. A binocular (Olympus SZX7) was used to examine and count all individuals from the taxa belonging to Cladocera, Copepoda, and Ostracoda (macro-zooplankton). A subsample of 1 mL was collected from the concentrated sample to analyze the micro-zooplankton, and final counts were recalculated to individuals per liter. The micro-zooplankton individuals (mainly Rotifera and nauplii) were counted and identified using a microscope (Olympus CX41).

## 2.6. Phytoplankton

Phytoplankton taxa and abundances were investigated by taking water samples on day -1, 10, 24, 38, 52, 66 and 80 relative to the first

imidacloprid application in addition to chlorophyll-a and phycoerythrin concentration measurements performed with the same portable multi-meter device used for measuring the water quality variables. Hence, chlorophyll-a and phycoerythrin measurements were carried out together with the physico-chemical water quality measurements. We collected phytoplankton by filling 250 mL amber plastic bottles (Scharlab) from a 5 L depth integrated water sample obtained with the PVC tube. Phytoplankton taxa and counts were assessed in a 1 mL subsample by means of an inverted microscope and a Sedgewick-Rafter counting cell (Graticules Optics).

## 2.7. Leaf litter degradation by macroinvertebrates, and microbes

Submerged litter bags were collected after 14, 28, 42, 56, and 84 days of exposure to investigate leaf litter degradation by macrofauna as an indicator for feeding. Subsequently, all remaining leaf material was transferred from a bag to aluminum bowls and dried for at least 24 h at 60 °C to determine leaf dry weight and loss compared to the initial dry weight. Finally, litter bags were refilled with the same initial amount of new dried leaf material and immersed in the associated cosm. Two replicates of hanging nylon bags were collected from each cosm to investigate MLLD as an indicator for microbial activity on the same days as the litter bags and dried in the same manner.

## 2.8. Data analysis

The importance of temperature, imidacloprid, sampling day, and their interaction effects on imidacloprid fate, ecological, and physico-chemical properties of the freshwater ecosystem were studied using generalized linear mixed models (GLMMs). Fixed effects included imidacloprid, temperature, and sampling day (i.e., main effect). Cosms were treated as a random effect in the intercept-only models to account for the variance specific for each cosm. We compared residual errors (sigma), marginal and conditional  $R^2$ -values, AIC- and BIC-values to achieve best model fit and analyzed the contribution of the random effect to explained variances. All abundance data was ln-transformed, and the skewness of the data distribution was calculated to determine whether a log-transformation of all other dependent variables was required (Webster and Oliver, 2007). Visual inspection for normal distribution of residuals was also performed using histograms and Q-Q-plots. The results of the best model fit with parameter estimates were evaluated using Satterthwaite's correction method in an ANOVA table with F-tests and p-values. Estimated marginal means (EMMs) and their contrasts were calculated to determine significant effects by Tukey p-value and confidence level adjustment method. Standardized effect sizes (Cohen's d) were calculated (Nakagawa and Cuthill, 2007) for further comparison of significant effects. The applied GLMM structure was:

$$O_{p,q,r,s,n} = \mu + C_p + T_q + D_r + C_p \times T_q + C_p \times D_r + T_q \times D_r + C_p \times T_q \times D_r + MC_s + \varepsilon$$

where  $O_{p,q,r,s,n}$  represents the  $n$ -th observation of a response variable with  $\mu$  as the mean (i.e. intercept) and  $\varepsilon$  as the error component, under  $p$ -th chemical treatment ( $C$ , two levels),  $q$ -th temperature treatment ( $T$ , three levels),  $r$ -th sampling day ( $D$ , levels were response variable dependent), and  $s$ -th microcosm test system ( $MC$ , twenty four levels).

The macroinvertebrates, zooplankton, and phytoplankton community structure dynamics were analyzed with the principal response curves (PRCs) method using CANOCO program version 5 (Van den Brink and Ter Braak, 1998, 1999; Ter Braak and Smilauer, 2012) for the analysis of significant effects caused by the temperature treatments, chemical and their interactions. Prior to the community analyses, macroinvertebrate and zooplankton data was  $\ln(2x + 1)$ , and the phytoplankton data  $\ln(0.001x + 1)$  transformed (see Van den Brink

et al., 2000 for rationale). The PRC technique is based on redundancy analysis (RDA) and extracts responses of the community composition to the stressors in time from the total variation in community composition. The PRC comprises all treatments and the interaction with time as explanatory variables, while sampling day is included as covariate. Thus, the y-axis describes each treatment versus the control as a regression coefficient ( $c_{dt}$ ) against time on the x-axis. Species weights ( $b_{ik}$ ) present the affinity of each individual taxon within the community response. The significance of the amount of treatment variation displayed in the PRC diagram was tested by Monte Carlo permutation test by permuting whole time series of the mesocosms (Van den Brink and Ter Braak, 1999). Significance of effects at each isolated sampling day were assessed by conducting Monte Carlo permutations under the RDA option by including only the samples from that particular sampling date.

Finally, a structural equation model (SEM, Shipley, 2016) was applied, based on available information on known food web interactions (Polazzo et al., 2023), to examine causal ecological relationships and food web effects of imidacloprid and temperature on the macroinvertebrate, zooplankton, and phytoplankton communities. The macroinvertebrate community was divided into functional groups (i.e., detritivores, shredders, grazers and predators) with regards to the observed endpoints macroinvertebrate feeding and MLLD. Besides direct stressor effects, ecological cascade effects were investigated, as well as ecological relationships with macroinvertebrate feeding and microbial leaf litter decomposition. It is noteworthy that all observed nodes were recorded directly at the end of the treatment period and the sum of all individual taxa of the communities were calculated based on abundances to generate a uniform data matrix. A maximum likelihood estimation method was used to obtain the best SEM fit and reversed causality was tested in terms of our a priori hypothesis regarding propagating effects across different trophic levels. Chi-squared test, goodness of fit index (GFI), comparative fit index (CFI), standardized root mean square residual (SRMR), and root mean square error of approximation (RMSEA) were used to assess model quality. The univariate GLM- and SEM-analyses (lavaan package, Rosseel, 2012) were performed with R (R Core Team, 2020) with a significance level  $\alpha=0.05$ .

In all analyses and for both treatment and recovery phase, temperature effects were investigated by contrasting elevated temperatures (E) or heatwave (HW) scenarios against ambient conditions (Amb) across non-chemical and chemical treatments if not otherwise indicated. Additionally, both temperature treatments were compared to each other across non-chemical and chemical treatments. Chemical effects were investigated by contrasting the imidacloprid treatment against the chemical control across all temperature scenarios.

## 3. Results

### 3.1. Temperature and underwater irradiance

During the treatment phase, water temperatures ranged between 13.1 and 27.6 °C (Amb), 13.0 and 31.8 °C (E), and 13.2 and 35.7 °C (HW) (Fig. 1). Throughout the whole experiment (i.e., treatment and recovery phase), the average ambient water temperature was 22 °C and ranged between 13 °C and 30 °C (Fig. 1). With the exception of a few temporary declining temperature periods, the ambient water temperature gradually increased during the experiment. In the treatment phase (Fig. 1), elevated temperatures followed, except of the very beginning, the ambient daily pattern with a 4 °C ( $\pm 0.5$  °C) offset, while the programmed heatwaves reached the set base temperatures of 24, 28, and 32 °C. During the recovery phase no more heating was supplied (Fig. 1), so cosms with manipulated elevated temperatures or heatwaves returned to daily ambient temperature fluctuations. The recordings of irradiance under water showed the highest maximum and average illumination for Amb (11.9 and 2.9  $\mu\text{W}/\text{cm}^2$ ), followed by E (11.4 and 2.7  $\mu\text{W}/\text{cm}^2$ ), and the lowest for HW (9.6 and 2.1  $\mu\text{W}/\text{cm}^2$ ).

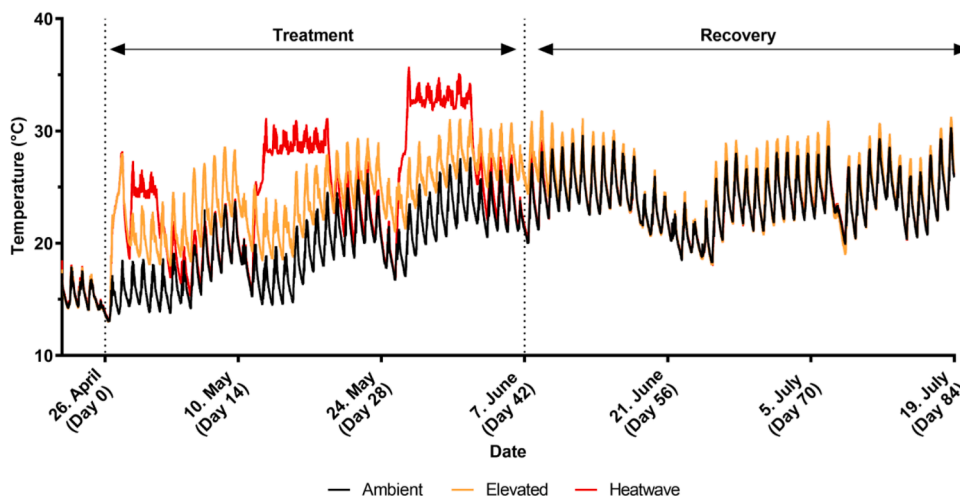


Fig. 1. Temperature treatments recorded in the mesocosms subjected to three different scenarios (i.e. ambient (black), elevated (orange), and heatwaves (red) in the course of the experiment. The dotted lines indicate the start and the end of the treatment phase followed by the recovery phase without temperature manipulation.

### 3.2. Imidacloprid concentrations in water

Imidacloprid concentrations in water were on average 129 % of the nominal concentration during the three spiking events. Imidacloprid dissipation in water ( $R^2$ : 0.96 – 0.99) was relatively fast across all

Table 1

DT<sub>50</sub>-values with 95 % confidence intervals (C.I.) and time-weighted averages (TWA) after 1st (10 d), 2nd (10 d), and 3rd (17 d) imidacloprid spiking (µg/L) in water for ambient (Amb), elevated (E), and heatwave (HW).

		Water		
		DT <sub>50</sub> (days)	95 % C.I.	TWA <sub>10d,10d,17d</sub> (µg/L)
1st spiking	Amb <sub>1st</sub>	4.67	4.09 – 5.36	0.40
	E <sub>1st</sub>	4.01	3.58 – 4.50	0.34
	HW <sub>1st</sub>	4.40	3.94 – 4.92	0.40
2nd spiking	Amb <sub>2nd</sub>	4.11	3.55 – 4.77	0.40
	E <sub>2nd</sub>	3.73	3.00 – 4.67	0.38
	HW <sub>2nd</sub>	3.48	3.15 – 3.85	0.36
3rd spiking	Amb <sub>3rd</sub>	3.45	2.58 – 4.59	0.20
	E <sub>3rd</sub>	2.58	2.28 – 2.92	0.15
	HW <sub>3rd</sub>	2.44	2.03 – 2.93	0.15

temperature treatments with DT<sub>50</sub>-values ranging from 2 to 5 days and TWA concentrations ranging from 0.15 to 0.4 µg/L. The highest DT<sub>50</sub>-values and TWA concentrations were noted for Amb and the lowest for HW with exception of the first spiking when concentrations were lower for E (Table 1). DT<sub>50</sub>-values were decreasing across all temperature treatments from one spiking event to the next with DT<sub>50</sub> Amb ≥ DT<sub>50</sub> E ≥ DT<sub>50</sub> HW. All imidacloprid concentrations in the sediment were below the limit of quantification (LOQ) of 1 ng/g.

### 3.3. Physico-chemical water quality variables

Significant effects of the temperature treatments, the chemical, and their interaction on the physico-chemical water quality variables measured in the morning and evening are presented in Table S1. Geometric mean DO concentrations and saturations (Fig. 3A,B,C,D) ranged from 7 to 14 mg/L and 85 to 163 % in the morning and 10 to 18 mg/L and 125 to 221 % in the evening, respectively. Significantly lower concentrations were measured during the first HW compared to Amb both in the morning and in the evening (Fig. 3A,B). Mean DO saturation in the morning (Fig. 3C) was significantly increased during the second HW and significantly decreased after the third HW. Similar to the morning measurements, significant temperature effects on DO saturation appeared in the evening measurements (Fig. 3D) with higher values after the first two HWs. Significantly lower values were found during the last HW when compared to E. Significant imidacloprid effects occurred once at a single timepoint with a lower DO saturation. Ecosystem productivity (Fig. 3K) presented a temperature-dependent pattern with lows and peaks in the absence and presence of a HW, respectively. Following the last HW, a significant interaction effect was noted.

Mean pH-values ranged from 8.5 to 10.5 in both the morning and evening with a similar trend across treatments over time (Fig. 3E,F). pH had significantly lower values during the first and third HW in the morning when comparing HW to E. Significant interaction effects on pH occurred early in the experiment, whereas no significant differences in pH were found in the evening.

Mean TDS- and EC-values ranged from 79 to 139 ppm and 156 to 238 µS/cm in the morning and 76 to 130 ppm and 176 to 255 µS/cm in the evening, respectively. Both variables were similarly affected by temperature with significantly higher values for E and HW, compared to Amb (Fig. 3G,H,I,J). After the third imidacloprid spiking, TDS was significantly higher in the morning. The second HW caused significantly higher TDS-values when compared to E in the morning. A significant interaction of both stressors was only observed at a single timepoint for TDS.

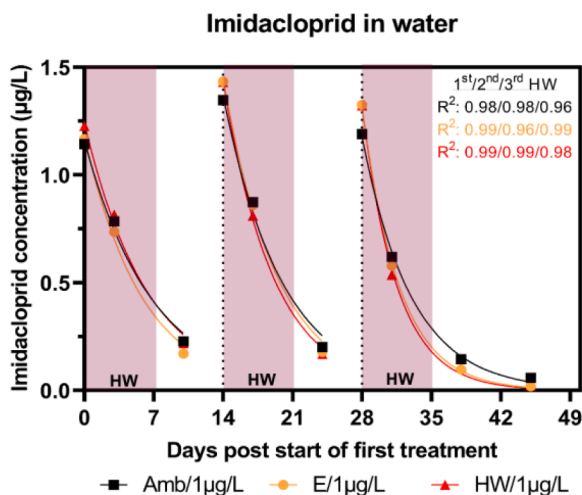
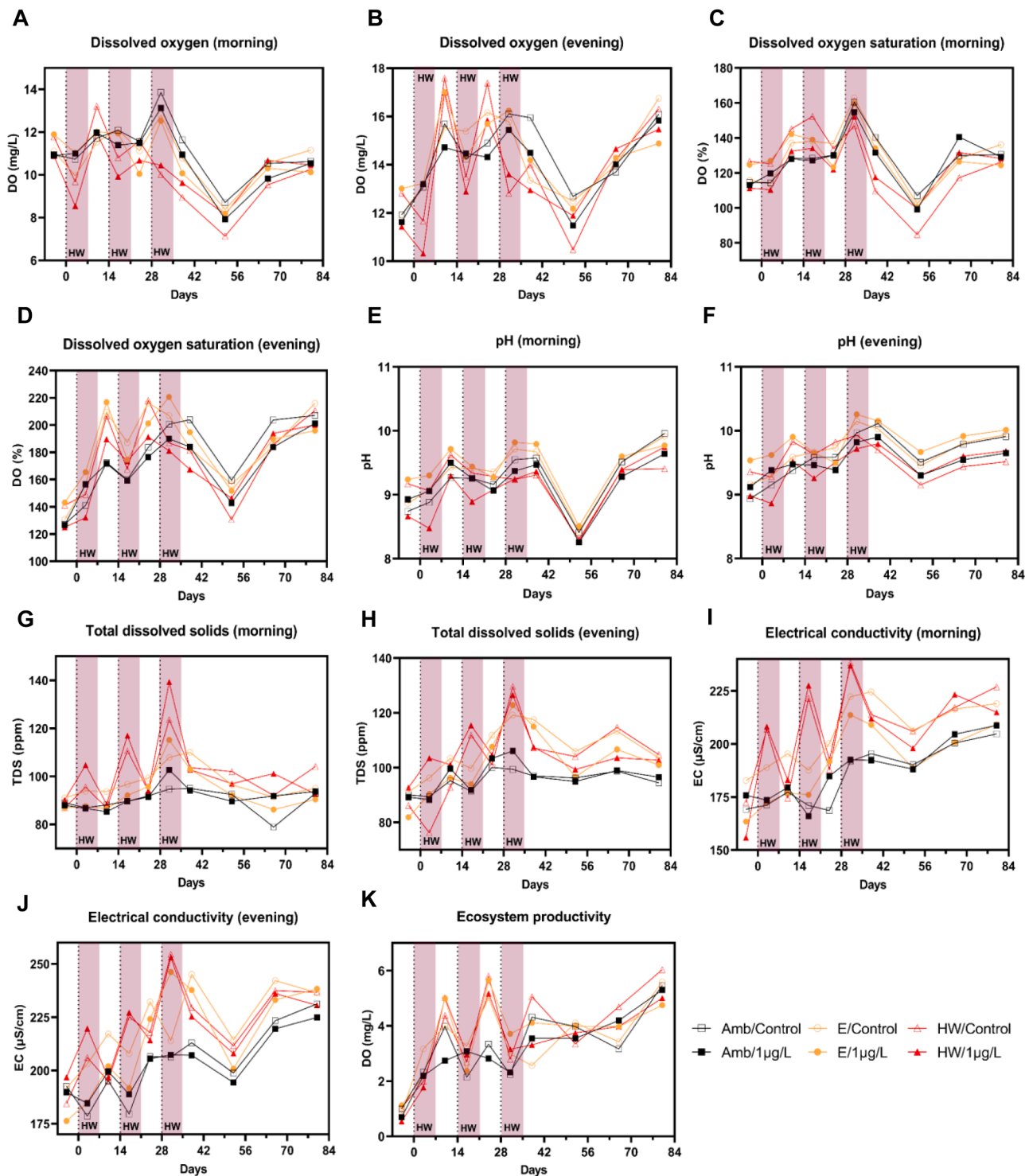


Fig. 2. Geometric mean ( $n = 4$ ) of imidacloprid concentrations (µg/L) in water under ambient (Amb), elevated (E), and heatwave (HW) temperatures over 45 days of the experiment. The dotted lines indicate the imidacloprid spiking and red blocks the timing of HWs.



**Fig. 3.** Geometric mean ( $n = 4$ ) of physico-chemical water parameters, including (A,B) dissolved oxygen, (C,D) dissolved oxygen saturation, (E,F) pH, (G,H) total dissolved solids, (I,J) electrical conductivity, and (K) ecosystem productivity. Except of (K), all parameters were measured in the morning and evening, respectively. The dotted lines indicate the imidacloprid spiking and the red blocks the timing of HWs. Confidence intervals were excluded to facilitate readability.

Temperature and imidacloprid effects on nutrients were only significant at single timepoints (Table S1, Figure S1A,B,C). Significantly lower nitrate (Figure S1A) and total phosphorus (Figure S1C) concentrations were noticeable for the imidacloprid treatment compared to chemical control during and after the third HW. Orthophosphate was significantly affected by both interacting stressors and was significantly higher for E (Figure S1B).

### 3.4. Zooplankton community dynamics

We identified 21 different zooplankton taxa, of which 10 were classified as macro- and 11 as micro-zooplankton taxa. Amongst the community, taxa that showed highest positive and negative species weights with the PRC diagram ( $b_k$ -values in Fig. 4) were two rotifers (*Polyartha*, Lecanidae), Cyclopoids (separated in nauplius stages and adults), and two Cladocera (*Daphnia longispina*, *Ceriodaphnia*). *Polyartha*,

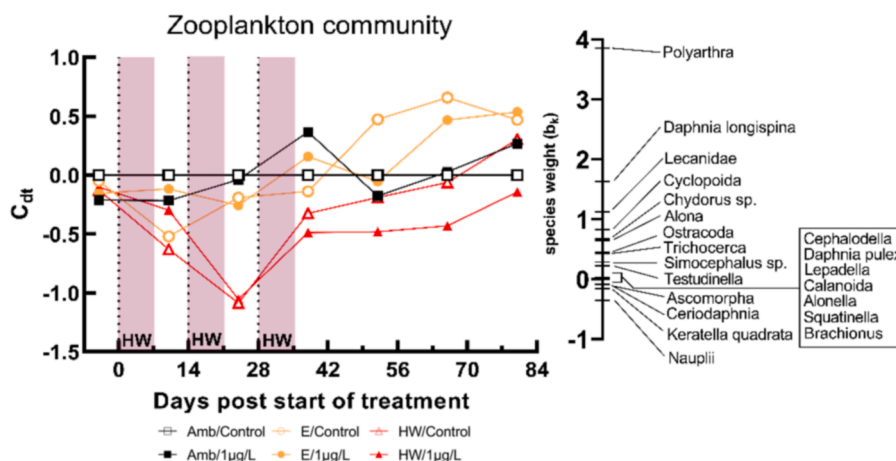


Fig. 4. Principal response curve (PRC) depicting combined temperature and imidacloprid effects on the zooplankton community over time. Sample weights ( $c_{dt}$ ) present differences between control and treatments on each sampling day. Taxon specific affinity of the community related to the response indicated by the PRC is presented by the species weights ( $b_k$ ). The dotted lines indicate the imidacloprid spiking and the red blocks the timing of HWs.

*D. longispina*, Lecanidae, and Cyclopoida had the highest positive species weights, indicating a decrease in abundance, whereas nauplius and *Ceriodaphnia* had minor negative  $b_k$ -values, indicating an increase in abundance. The first PRC of the zooplankton community displays a significant 35 % of the variation in species composition ( $p = 0.02$ ) due to the evaluated treatments (Fig. 4). Of the total variation present, 32 % was explained by factor time and 19 % by the evaluated treatments, whereas the remaining variance is due to differences among replicates. The PRC graph displayed a temperature-dependent separation of the treatments with HWs being the only significant one, causing significant deviations in community structure compared to the Amb control, whereas the effects of E and imidacloprid seemed modest (Fig. 4). Recovery dynamics of the community, indicated by a return in  $c_{dt}$ -values to 0, appeared late after the second HW in the presence and absence of imidacloprid and with a faster return to the reference community when no chemical stressor was involved. Monte Carlo permutations tests confirmed the displayed differences in the PRC and presented significant effects mainly for the heatwave treatment after the first and second heatwave for the chemical control treatment and after the second and third heatwave for the imidacloprid treatment (Table 2). Significant effects of E, imidacloprid and the interaction of temperature and chemical were absent. Monte Carlo permutation tests indicate that community effects were significantly larger for HWs when compared to E at single timepoints after the second HW for the chemical control treatment and towards the end of the experiment for the imidacloprid treatment. Furthermore, a consistent pattern of significant effects of HWs was noted when compared to E and Amb across non-chemical and

chemical treatment. The absence of significant effects on day -4 indicate homogeneity across all treatments (Table 2).

### 3.5. Zooplankton population dynamics

The dynamics of the six taxa showing highest positive and negative species weights are displayed in Fig. 5A-F, and show significant effects of the single and interacting stressors on abundance of the zooplankton populations per individual day. Amongst all taxa, imidacloprid effects were only noticeable for Cyclopoida with significantly lower population abundances during the recovery phase (Fig. 5E). Considering temperature effects, E showed significantly higher abundance values than Amb for copepod nauplii and *Ceriodaphnia* during the recovery phase (Fig. 5C,F). Following the second HW, population abundances were significantly lower for *Polyarthra* (Fig. 5A), and multiple HWs caused significant negative effects on two consecutive sampling days at the beginning of the recovery phase for Cyclopoida. Contrary to these negative HW effects, *Ceriodaphnia* abundances were significantly higher following the second HW. HWs caused significantly lower abundances for *Polyarthra* and Lecanidae (Fig. 5B), *D. longispina* (Fig. 5D), and Cyclopoida when compared to E, mainly during the recovery phase and for *Polyarthra* also during the treatment phase. Significant temperature  $\times$  chemical interaction effects were only noted for copepod nauplii and Cyclopoida during the recovery phase. Significant effects of the single and combined treatments were found at isolated time points for the other taxa in the community, except for *Daphnia pulex*, *Lepadella*, *Testudinella*, and *Brachionus*, which showed no significant effects.

Table 2

Significant results (p-values) from Monte Carlo permutation tests performed under the redundancy analyses (RDA) option testing individual temperature effects of elevated (E) and heatwave (HW) versus ambient (Amb), between HW and E, the chemical effects of imidacloprid (C1) versus control (C0), the treatments combined (C0C1; AEHW), and the interaction of temperature (T) and chemical (C) on the zooplankton community on each sampling day. Empty cells denote p-values > 0.05.

Day	Temperature									Chemical				Combination
	E vs. Amb			HW vs. Amb			HW vs. E			C1 vs. C0				Interaction
	C0	C1	C0C1	C0	C1	C0C1	C0	C1	C0C1	A	E	HW	AEHW	T $\times$ C
-4														
10				0.0238		0.0194							0.0491	
24				0.0238	0.0238	0.0006	0.0509						0.0026	
38						0.0072							0.0015	
52													0.0238	
66									0.0238				0.0186	
80														

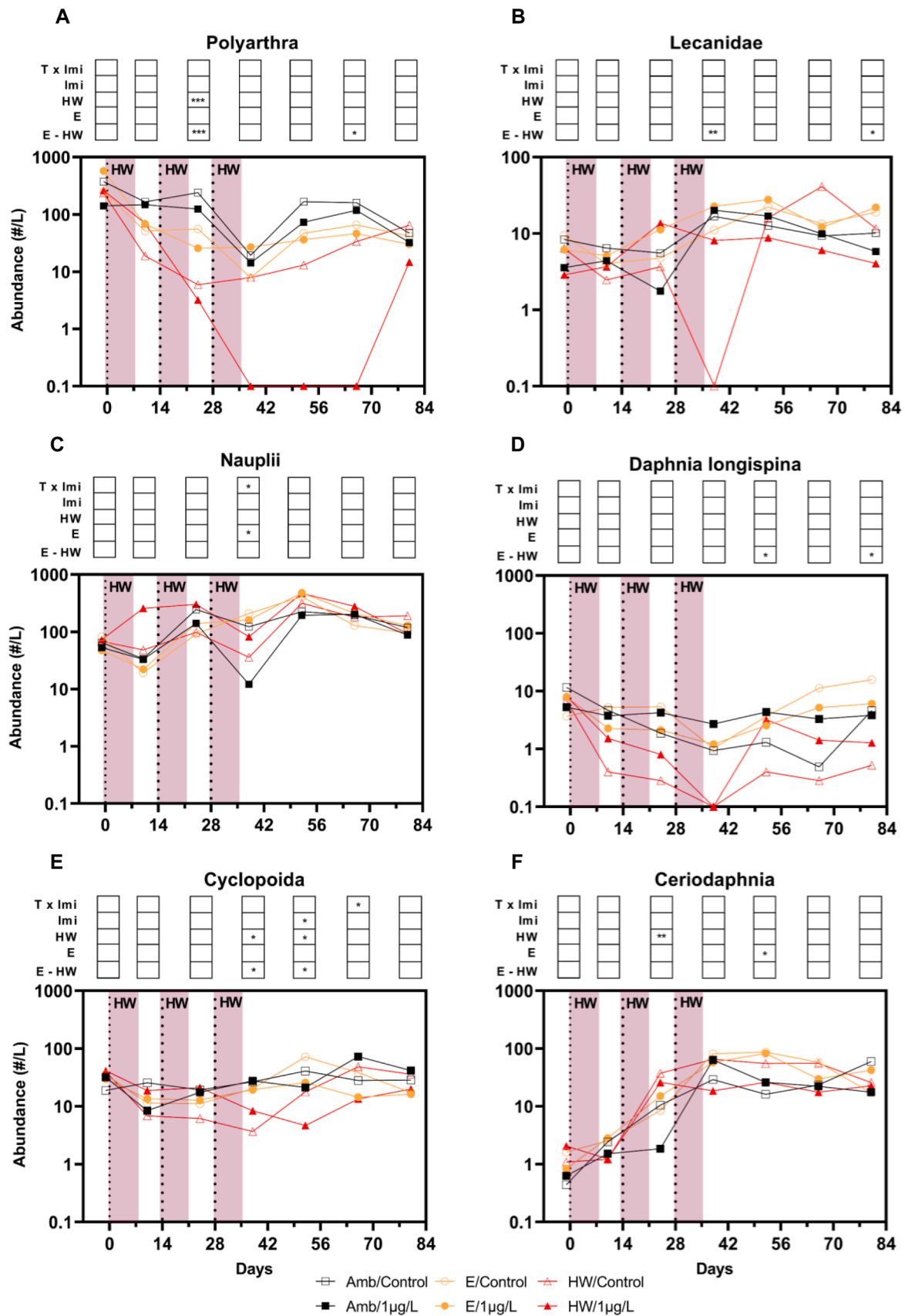


Fig. 5. Zooplankton taxa dynamics of the six most important species in the PRC community analyses. Figure A to F show the geometric mean ( $n = 4$ ) of the counted organisms per individual treatment per liter (#/L) of (A) Polyarthra, (B) Lecanidae, (C) Nauplii, (D) *D. longispina*, (E) Cyclopoida, (F) Ceriodaphnia. The absence of organisms is denoted by 0.1. Significant effects are indicated by \*0.01, \*\*0.001, and \*\*\* $\leq 0.001$ . The dotted lines indicate the imidacloprid spiking and the red blocks indicate the timing of HWs. Confidence intervals were excluded to facilitate readability.



### 3.6. Macroinvertebrate community and population dynamics

No significant treatment effects were displayed by the first PRC of the macroinvertebrate community over time (Figure S2A). Considering the individual sampling days, significant imidacloprid effects occurred on days 35, 49 and 77. Marginally significant HW effects occurred on day 21 and 49 when compared to Amb and E (Table S3A). The population dynamics of the taxa showing highest positive and negative species weights are shown in Figure S3A-F, including significant effects of the single and interacting stressors on the populations per individual day. For the other taxa in the community, significant effects of the single and interacting stressors were found at isolated time points, except for Planorbidae, Notonectidae, Zygoptera, *Erpobdella* sp., Elmidae (larvae), Ancyliidae, Trichoptera (stone/wood house), Sphaeriidae, Heptageniidae, and Hydrobiida, which showed no significant effects.

### 3.7. Phytoplankton, macroinvertebrate feeding and microbial leaf litter decomposition

Chlorophyll-a and phycoerythrin concentrations presented similar patterns across all treatments (Fig. 6A,B,C,D). This was regardless of time of day and with low fluctuations during treatment phase. Increasing concentrations appeared with time during recovery phase followed by a decrease at the end of the experiment. Significantly lower concentrations of both variables were found in both temperature treatments compared to Amb during the first and third HW, while this was

the case at single timepoints for E. Significant interactions between the temperature and chemical stressors were detected for phycoerythrin concentrations after the first HW and for both variables individually at the end of the study (Table S2). No significant treatment-related effects on the phytoplankton community were displayed by the first PRC (Figure S2B) with significant HW effects when compared to Amb and E only on day 38 and marginally significant imidacloprid effects under E only on day 10 (Table S3B). The phytoplankton community comprised 35 taxa in total with *Cryptomonas* sp., *Peridinium*, *Achnanthes* (< 5  $\mu\text{m}$ ), *Aphanocapsa*, and *Nostoc* showing highest positive species weights and *Eudorina*, *Merismopedia*, *Phacus* sp. (triqueter), *Chroococcus*, and *Epi-themia* showing highest negative species weights (Figure S2B).

Macroinvertebrate feeding (Table 3; Figure S4A) was significantly increased in the HW treatment compared to Amb after the second and third HW, as well as at the end of the experiment. HWs caused significantly higher feeding when compared to E after the second HW. Furthermore, a significantly increased feeding was observed for E compared to Amb at the end of the experiment. Considering chemical effects, feeding was significantly lower after the third imidacloprid spiking. A significant interaction effect of temperature and imidacloprid was noted at the end of the experiment.

MLLD was significantly and persistently higher for E compared to Amb during the recovery but not the treatment phase (Table 3; Figure S4B). HWs caused significantly higher MLLD when compared to Amb and E after the third HW and towards the end of the experiment. Imidacloprid induced a significant lower MLLD compared to the

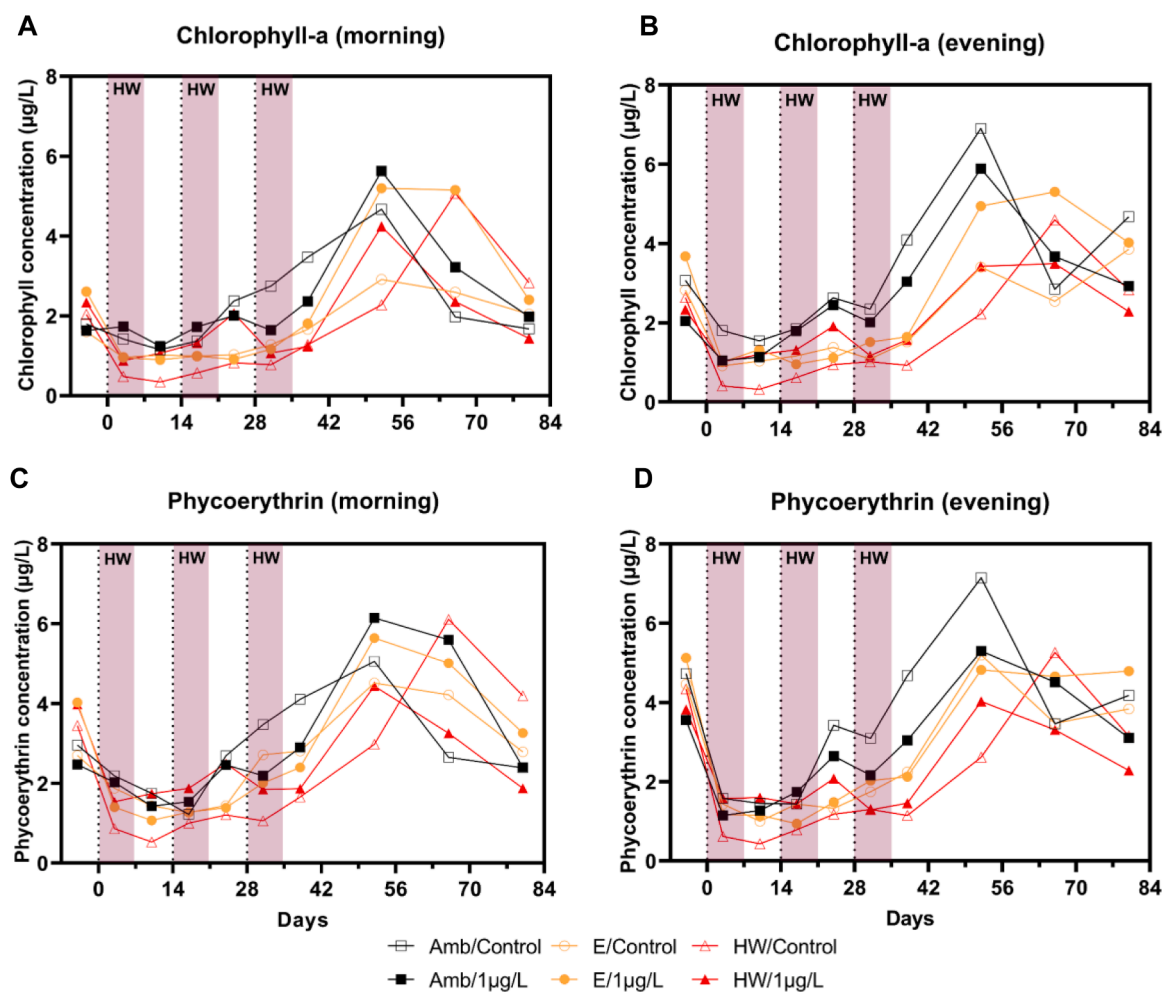


Fig. 6. Geometric means ( $n = 4$ ) of (A,B) chlorophyll-a and (B,C) phycoerythrin concentrations (measured in the morning, and evening, respectively). The dotted lines indicate the imidacloprid spiking and the red blocks the timing of heatwaves. Confidence intervals were excluded to facilitate readability.

**Table 3**

Significant (p-values) temperature effects of elevated (E) and heatwave (HW), between E and HW, the chemical effects of imidacloprid (C1), and the interaction of temperature (T) and chemical (C) on macroinvertebrate feeding and microbial leaf litter degradation on each sampling day. Standardized effect sizes (Cohen's d) are presented in brackets.

Endpoint	Day	Temperature			Chemical Imidacloprid	Interaction T × C
		Elevated (E)	Heatwave (HW)	E - HW		
Macroinvertebrate feeding	14					
	28		<0.001 (3.40)	0.007 (2.15)		
	42		0.02 (1.81)		0.01 (1.42)	
	56					
	84	0.048 (1.60)	<0.001 (2.58)			0.04 (0.45)
Microbial leaf litter decomposition (MLLD)	14				0.048 (0.97)	
	28					
	42		<0.001 (2.77)	0.007 (1.76)		
	56	0.007 (1.77)				
	70	0.002 (2.00)	<0.001 (2.95)			
	84	<0.001 (3.17)	<0.001 (2.87)			

chemical control after the first spiking at a single timepoint.

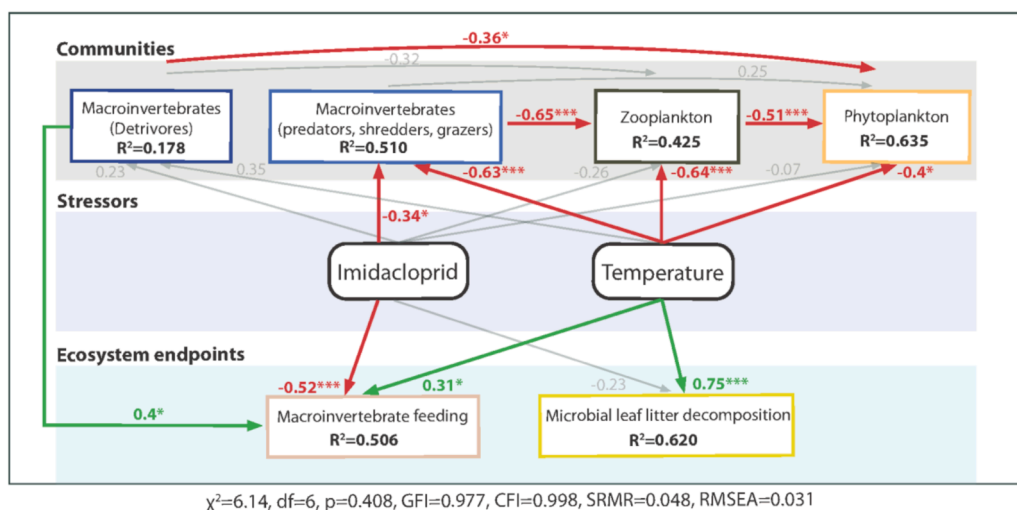
3.8. Structural equation modelling of food web effects

The SEM illustrated that temperature and imidacloprid had significant direct and indirect effects on the freshwater food web (Fig. 7) at the end of the treatment period (see Fig. 1). Significant standardized path coefficients explained negative and direct relationships of temperature with macroinvertebrates (-0.63; predators, shredders, grazers), zooplankton (-0.64), and phytoplankton (-0.4), and positive relationships with macroinvertebrate feeding (0.31) and MLLD (0.75). Significant negative and direct relationships of imidacloprid were only evident for macroinvertebrates (-0.34; predators, shredder, grazers) and for macroinvertebrate feeding (-0.52). Macroinvertebrate feeding was further significantly positive related to macroinvertebrate abundances (0.4). The SEM identified cascading food web effects by calculating significant negative relationships between phytoplankton and zooplankton (-0.51) and macroinvertebrate (predators, shredders, grazers) and zooplankton communities (-0.65), indicating a stronger negative relationship between macroinvertebrate and zooplankton community within the ecological effect chain. Non-adjacent ecological relationships, such as those between the phytoplankton and macroinvertebrate community were only significant for the detritivores (-0.36).

4. Discussion

4.1. Imidacloprid fate

Temperature is one of the dominating environmental factors amongst ultraviolet light, pH, and microbial activity determining imidacloprid dissipation from water (Anderson et al., 2015; Bonmatin et al., 2015). A previous cosm study conducted in the same time period (April to June) and research facility (Rico et al., 2018) reported a half-life dissipation time (DT50) of 13 days, which is three to six times slower than imidacloprid dissipation in our investigation. Similar to our observations, fast imidacloprid dissipation (DT50: ≤ 3 days) was observed in 2014 in a rice cosm study in Japan (Kobashi et al., 2017) and a notably slower dissipation (DT50: 9 days) in a follow-up experiment in 2015 (Hayasaka et al., 2019). One possible explanation for the slower imidacloprid dissipation in the cosm study by Rico et al. (2018) is limited photodegradation through shading effects caused by continuous growth of filamentous algae and macrophytes. In fact, aqueous photolysis was regarded as the main breakdown pathway in a cosm experiment in Germany, where a fast imidacloprid concentration decline was associated with high levels of sunlight (6 to 11 μW/cm<sup>2</sup>; Colombo et al., 2013). Apart from photodegradation, our study suggests that temperature can be another strong driver in the aqueous degradation pathway of imidacloprid (Table 1; Fig. 2). This is indicated by a low irradiance for



**Fig. 7.** Structural equation model (SEM) presenting the direct and indirect effects of imidacloprid and temperature on different trophic levels of the food web, comprising macroinvertebrate, zooplankton, and phytoplankton communities. Further relationships for macroinvertebrate feeding and leaf litter degradation are displayed. Arrows denote significantly positive (green) and negative (red) relationships, as well as non-significant relationships (grey). Standardized path coefficients on the arrows and R<sup>2</sup>-statistics describe explained variance. Goodness-of-fit statistics for the SEM are presented below.

HW-, followed by E- and Amb-conditions (9.6, 11.4, and 11.9  $\mu\text{W}/\text{cm}^2$ , respectively) and increasing  $\text{DT}_{50}$ -values under these different temperature scenarios. Temperatures of up to 30 °C for the Amb-conditions in our study compared to a temperature range of 16 to 25 °C in the cosm study by Rico et al. (2018) corroborate the expected increase in imidacloprid dissipation with increasing temperatures. Microbial degradation processes of imidacloprid (Pang et al., 2020), which were enhanced by temperature in our study (Table 3), can be one of the explanatory mechanisms for the faster imidacloprid dissipation while hydrolysis of imidacloprid is more pH than temperature dependent (Liu et al., 2006). Similarly, Sumon et al. (2018) concluded that higher temperatures (28.2  $\pm$  2 °C) lead to a faster imidacloprid dissipation in tropical conditions compared to a slower dissipation in temperate regions mainly through biological degradation processes and hydrolysis (Sánchez-Bayo and Hyne, 2011). Other factors potentially influencing imidacloprid fate, such as high primary productivity by algae, can be ruled out because chlorophyll-a and phycoerythrin concentrations were significantly lower in the temperature-manipulated cosms (Fig. 6A,B,C,D; Table S2).

#### 4.2. Imidacloprid effects

Although the zooplankton community response was significant towards both chemical and thermal stressor, we observed a lower imidacloprid sensitivity than in previous studies reported despite different temperature scenarios (Table 2). Earlier cosm studies in the (sub-)tropics reported adverse effects on the zooplankton community at considerably lower and marginally higher imidacloprid concentrations of 0.01 and 0.3  $\mu\text{g}/\text{L}$  (Merga and Van den Brink, 2021; Van de Perre et al., 2021) and of 2.3 and 3.0  $\mu\text{g}/\text{L}$ , respectively (Chará-Serna et al., 2019; Sumon et al., 2018). Other cosm studies found significantly adverse effects on zooplankton communities at concentrations 5 to 25 times higher compared to our study (Rico et al., 2018; Lewis et al., 2021). In this context, it was also surprising that the macroinvertebrate community showed significant negative effects only on day 35 and 49 (Table S3; Figure S2A), considering that it comprised highly sensitive taxa like Leptoceridae (caddisfly) and *Cloeon* sp. (mayfly) (Roessink et al., 2013; Macaulay et al., 2020). One of the possible reasons could be that imidacloprid exposure was likely too short and low (Fig. 2; Table 1), causing a low bioconcentration also due to the low imidacloprid uptake rate of *C. dipterum*. In essence, the chemical-biota interaction was too short for the internal concentration reaching lethal level (Huang et al., 2021). Cladocerans like *Daphnia magna* showed a very high tolerance towards imidacloprid with 24–96-h  $\text{LC}_{50}$  values exceeding 100  $\text{mg}/\text{L}$  (Morrissey et al., 2015), which corresponds to non-significant imidacloprid effects on *D. longispina* and *Ceriodaphnia* in the present study. Furthermore, the significantly imidacloprid-induced population decline of Cyclopoida (Fig. 5E) supports previous findings on high copepod sensitivity (Rico et al., 2018; Hébert et al., 2021; Macaulay et al., 2021a; Van de Perre et al., 2021). Yet, these observed adverse effects occurred late in the course of the experiment, which is in agreement with previous studies suggesting imidacloprid effects at low concentrations after chronic exposure (Lewis et al., 2021; Merga and Van den Brink, 2021; Van de Perre et al., 2021). This could be explained by the low elimination rate of the toxic biotransformation product, imidacloprid-olefin, as reported for *C. dipterum* and *Gammarus pulex* (Huang et al., 2021).

#### 4.3. Temperature effects

Temperature was the more pervasive stressor when compared to imidacloprid in the present study. This becomes evident with a series of significant heatwave (HW) effects on the zooplankton community (Table 2). Interestingly, significant effects occurred early and with large effects on the zooplankton community following the first HW in the absence of imidacloprid, whereas modest and non-significant effects of heatwaves were noted for the chemically stressed community (Fig. 4). Nonetheless, the second HW fostered the imidacloprid effect by

following the same negative trend in the community response for the heatwave-imidacloprid treatment which resulted in structural changes. These changes were similar to heatwave-control conditions where a significant difference from the Amb treatment was found (Table 2, Fig. 4). Such negative effects of heatwaves are consistent with previous studies assessing the impact of thermal extremes on the zooplankton community structure when exposed to a three days HW (+5.2 °C vs. average ambient of 21 °C and highest temperatures of 28.2 °C; Sun and Arnott, 2022). The zooplankton community in this study consisted of similar species as in our study, including cyclopoids (nauplii and adults), rotifers, and cladocerans. Furthermore, our investigations on taxon-specific population dynamics provide evidence that *Polyarthra*, Lecanidae, *D. longispina*, and Cyclopoida are significantly more vulnerable to heatwaves than elevated temperatures (Fig. 5A,B,D,E), which is in agreement with the reported negative warming effects on Cladocera and Copepoda in studies by Arenas-Sánchez et al. (2018) and Sun and Arnott (2022). However, while Arenas-Sánchez et al. (2018) reported increased rotifer densities in response to heat (28 °C), Sun and Arnott (2022) found in alignment with our study, reduced rotifer abundances when populations were exposed to heat. Particularly for *Polyarthra* and Cyclopoida, amongst the most sensitive taxa of the community in the present study, heatwaves caused significantly lower abundances which goes in line with the significant decrease in rotifer and cyclopoid abundances and extinction towards comparable temperature extremes as found by others (HWs of 29 and 39 °C in Seifert et al., 2015; 28 °C in Arenas-Sánchez et al., 2018; 27.5 °C in Castaño-Sánchez et al., 2020; +4, +8 °C above ambient in Zhang et al., 2018).

*D. longispina* and cyclopoids showed higher sensitivity towards heatwaves through lower abundances compared to *Ceriodaphnia* and copepod nauplii, which showed thermal tolerance through increasing abundances in both the HW and E treatments (Fig. 4, Fig. 5C,D,E,F). Our findings support recent observations from a study investigating interspecific differences in HW responses of three different *Daphnia* species (Vanvelk et al., 2021) and further suggest that *D. longispina* is more sensitive to temperature extremes than *Ceriodaphnia*. Similar to rotifers, interspecific variations in species sensitivity to heatwaves (26 °C) were noted amongst several rotifer taxa (*Notholca labis*, *Anuraeopsis fissa*, *Ascomorpha*, and *Monommata longiseta*) in another multiple stressor experiment (Hermann et al., 2023).

#### 4.4. Interactive effects of temperature and imidacloprid

Given the varying temperature tolerance ranges of species, evolutionary, physiological and behavioural adaptation may act as mitigating mechanisms to a temperature-altered environment (Dam and Baumann, 2017). Interestingly, significant negative heatwave effects occurred only at the end of the treatment phase for the rotifers *Polyarthra* and Lecanidae and cyclopoids, as well as, during the recovery phase for the rotifers, the cladoceran *D. longispina*, and cyclopoids (Fig. 5A,B,D,E). This suggests time-cumulative heatwaves effects as it also has been observed in a multiple-stressor experiment evaluating the interactive effects of heatwaves and imidacloprid (Macaulay et al., 2021b). The authors of the study reported that a synergistic imidacloprid-heatwave interaction impaired mayfly nymphs after 24 days of exposure with lasting synergistic interaction effects until day 30 of the experiment. Possible explanations for such postponed effects could be a shorter juvenile period in the naupliar stage (Frisch and Santer, 2004), earlier recruitment of copepodites from sediment (Adrian et al., 2006), and/or higher energy allocation in reproduction (Devreker et al., 2009). Furthermore, differences in generation times of focal species may explain the time delayed effects which may be a result of selection (Jackson et al., 2021). In contrast to these negative effects of warming, the elevated temperature treatment had a positive effect on copepod nauplii abundance (Fig. 5C). In this context and based on the low abundance of calanoids, a large number of nauplii were very likely cyclopoids, which would agree with the high temperature tolerance of freshwater copepods (Sasaki and

Dam, 2021). Another important finding of this study was the relatively short recovery period of the zooplankton community which resulted in less than 60 days to similar undisturbed, control conditions for all treatments (Fig. 4; Table 2). Vilas-Boas et al. (2021) and Arenas-Sánchez et al. (2018) also found a faster post-exposure recovery on zooplankton communities when exposed to higher temperatures. The characteristics of heating across space and time, such as intensity, duration, and frequency may play a crucial role in explaining the mechanisms which modulate post stress recovery (Walberg and Green, 2021). Recovery was faster and more favorable in the absence of imidacloprid which explains the significant interactive effects of temperature and imidacloprid on copepod nauplii and cyclopoid abundance during recovery phase (Fig. 5C,E). Hence, negative effects of imidacloprid may be positively related with temperature which would also account for the greater series of more significant negative effects of HWs than E.

Heatwaves caused significantly lower chlorophyll-a and phycoerythrin concentrations in our study (Fig. 6A,B,C,D; Table S2), which is in agreement with reported negative effects of thermal extremes on phytoplankton (Eggers et al., 2012; Remy et al., 2017). Other recent investigations support our observed heatwave phytoplankton decline which is significantly associated with species thermal tolerance range (Baker and Geider, 2021). This could further explain the onset of increasing chlorophyll-a and phycoerythrin concentrations during the recovery phase (Fig. 6A,B,C,D). Additionally, a release in grazing pressure is expected because of significantly lower abundance of zooplankters like cyclopoids in the HW treatment during the recovery phase. Apart from these changes in consumer-resources dynamics, zooplankton community structure may also have been reshaped by altered predator-prey dynamics and abiotic variables, such as that large zooplankton taxa like *Ceriodaphnia* and predatory cyclopoids may have further increased predation and resource competition on small rotifers like *Polyarthra* and Lecanidae (MacIsaac and Gilbert, 1989; Barnett et al., 2007; Polazzo et al., 2022a). Recent studies demonstrated that amongst abiotic variables, temperature best correlated with zooplankton abundance, followed by pH, phytoplankton biomass and nitrate concentration (Cremona et al., 2020). Thus, our significant effects on pH and nitrate (Fig. 3E,F; Table S1; Figure S1A) likely did not play a substantial role in the observed effects on the zooplankton community (Smith et al., 2018; Gao et al., 2022).

#### 4.5. Cascading food web effects

The SEM confirmed the previous analyses and demonstrated significant cascading food web effects from the macroinvertebrates via zooplankton to the phytoplankton community (Fig. 7). Interestingly, there is a stronger significant negative relationship ( $-0.65$ ) between the zooplankton and macroinvertebrate community than between the phytoplankton and zooplankton community ( $-0.51$ ). These findings suggest that warming and chemical effects may explain stronger relationships at higher than at lower trophic levels. Recent studies on trophic cascade strength in freshwater ecosystems confirm our assumption that warming strengthens trophic cascade effects and point out that top-down control increases as temperature increases (Ingram and Burns, 2018; Su et al., 2021). Hereby, our results align with the conclusions of a meta-analysis on SEM path coefficients of freshwater warming studies (Murphy et al., 2020), that warming may exert severe impacts particularly at the top of the food web indicated by stronger relationships (i.e., more negative standard path coefficients) than at lower trophic levels.

The SEM illustrated a significant positive and negative relationship between macroinvertebrate feeding and temperature and imidacloprid, respectively (Fig. 7). These findings are consistent with recent observations for a temperature-driven increased feeding at similar temperature scenarios (Hermann et al., 2023). Furthermore, significantly reduced feeding as a result of single or multiple imidacloprid pulses has also been observed in other studies (Kreutzweiser et al., 2007; Mohr

et al., 2012; Pestana et al., 2009), explaining the delayed and time-accumulative imidacloprid effects on feeding in our study (Table 3; Figure S4A). Imidacloprid effects on MLLD seem to be divergent and only few studies found, in alignment with our results, significantly lower MLLD (Kreutzweiser et al., 2008; Merga and Van den Brink, 2021). Our results confirm previously reported temperature-enhanced MLLD (Monroy et al., 2022) and support experimental evidence that litter breakdown rates may increase by 5 to 21 % with a 1 to 4 °C temperature increase (Follstad Shah et al., 2017). Overall, the SEM visualized that the temperature treatments induced both positive and negative effects while the imidacloprid treatment induced only negative effects on the different communities and ecosystem endpoints.

## 5. Conclusion

Our study provides empirical evidence that elevated temperatures and heatwaves induce severe stress on a Mediterranean freshwater ecosystem, and when combined with an insecticide, such as the neonicotinoid imidacloprid, they lead to significant effects on zooplankton community structure and population dynamics. In particular, we revealed how the presence or absence of imidacloprid modified post-exposure recovery dynamics for a community that experienced heatwaves. Additionally, we showed differences in species thermal sensitivities with some zooplankton species being significantly more affected by heatwaves than others when exposed to imidacloprid stress. A structural equation model summarizes significant effects and depicts cascading effects on the freshwater food web implying stronger negative effects at higher trophic levels. Overall, the study highlights the increased threat of heatwaves to imidacloprid-stressed freshwaters compared to elevated temperatures alone. Further research on temperature effects should focus on its interaction with other stressors to better predict the combined effects in the future.

### CRedit authorship contribution statement

**Markus Hermann:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesco Polazzo:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **Laura Cherta:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Melina Crettaz-Minaglia:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ariadna García-Astillero:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Edwin T.H.M. Peeters:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Andreu Rico:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Paul J. Van den Brink:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, 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

The supporting data of this data can be found online at: <https://doi.org/10.5281/zenodo.11520459>.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2024.121903](https://doi.org/10.1016/j.watres.2024.121903).

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