Combined effects of climate warming and environmentally relevant concentrations of pharmaceutical active compounds on a freshwater community

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Abstract

Predicting the combined effects of stressors on ecosystems is a pressing concern due to increasing anthropogenic pressures on the Earth's biota. Climate warming and chemical pollution are two major stressors affecting freshwater biota. The latter is becoming more common due to the widespread use of mixtures of chemical compounds and the low removal efficiency of water treatment plants, especially in the case of pharmaceutical active compounds (PhACs).

To evaluate the effects of warming and PhACs, we conducted a full factorial experiment (with/without PhAC mixture and with/without warming) in heated outdoor mesocosms with a common community of macroinvertebrates and plankton taxa from different trophic levels. The experiment was conducted twice, in winter and in summer. We repeatedly measured PhAC concentrations, environmental parameters, zooplankton density, and aquatic insect emergence. The summer experiment showed much stronger effects of stressors, dominated by temperature effects, on community structure and temporal dynamics of individual taxa. Both warming and PhACs altered invertebrate community composition in the summer experiment, with contrasting effects observed between insect emergence and phytoplankton and zooplankton responses. Our results suggest that PhACs at environmentally relevant concentrations can alter the effects of climate warming on freshwater biota, with individual-level responses such as delayed or accelerated development and altered phenology of key taxa.

Keywords: warming, pharmaceutical active compound mixture, freshwater invertebrates, community-levels, trophic interactions

Introduction

Freshwater ecosystems are severely impacted by numerous stressors, leading to strong declines in species abundance and biomass (Sánchez-Bayo and Wyckhuys, 2019) (Hallmann et al., 2017). The main causes of species declines are habitat loss due to intensive agriculture and urbanisation, pollution, increases in pathogens and invasive species, and climate change (Sánchez-Bayo and Wyckhuys, 2019). Climate warming and chemical pollution are two ubiquitous stressors (Loos et al., 2013; Parmesan, 2006) that lead to rapid environmental changes and pose a major threat to freshwater biodiversity and ecosystem functioning (Vörösmarty et al., 2010).

Pharmaceutically active compounds (PhACs) represent an emerging class of chemical pollutants. Their importance has increased due to the growing human population and recent advances in pharmaceutical research (Fekadu et al., 2019). Due to their widespread use and low removal efficiency in wastewater treatment plants, PhACs are found in aquatic systems worldwide (Brodin et al., 2014; Koba et al., 2018; Loos et al., 2013; Wilkinson et al., 2022). PhACs bioaccumulate in fish (Du et al., 2014) and benthic macroinvertebrates (Grabicova et al., 2015). At environmentally relevant concentrations ($ng.L^{-1}$ to low $\mu g.L^{-1}$), PhACs can affect the behaviour of aquatic ectotherms (Brodin et al., 2014; Saaristo et al., 2019), alter feeding rates (Bláha et al., 2019), and disrupt reproduction (Fursdon et al., 2019). These responses directly affect individual fitness (Fursdon et al., 2019) and can trigger indirect effects such as altered trophic interactions (Bláha et al., 2019). Altered species phenologies and trophic interactions can also redirect the flow of PhACs in food webs through bioaccumulation (Lagesson et al., 2016) and biomagnification (Zenker et al., 2014), increasing their potential toxicity to higher trophic levels including fish and humans.

PhACs are usually detected as complex mixtures in the environment (Grabicova et al., 2015; Koba et al., 2018; Loos et al., 2013). However, their effects on aquatic ecosystems are primarily understood through single-species laboratory bioassays, often conducted at concentrations higher than those commonly found in the environment (Richards et al., 2004). More empirical data are therefore needed to understand the ecotoxicology of PhAC mixtures under environmentally realistic conditions and the quantitative consequences of PhACs on ecological interactions and whole communities (Backhaus, 2014).

Average temperatures are predicted to increase by +1.4–4.4°C by 2100 due to a global warming (Masson-Delmotte et al., 2021). Freshwater ecosystems dominated by ectotherms are expected to be particularly impacted by warming (Parmesan and Yohe, 2003; Woodward et al., 2010). These organisms have a limited dispersal ability and therefore have to deal directly with changing environments (Woodward et al., 2010). These changes often result in reduced body sizes, altered species interactions, shifts in species distribution ranges and phenology, and cascading impacts on community dynamics and ecosystem functioning (Boukal et al., 2019; Post, 2013). Due to the strong correlation between water surface and air temperatures, climate warming will strongly affect communities in shallow freshwaters. Thermal stress often prompts organisms to seek refuge in colder waters at the bottom of water bodies (Balogová and Gvoždík, 2015), but even these microhabitats may be close to the upper thermal tolerance limits of many species during heat waves (Stoks et al., 2017).

Despite the widespread distribution of PhACs in freshwater ecosystems and the reality of climate warming, we currently lack evidence of the combined effects of PhACs and warming at the population and community level (Heye et al., 2019). Typically, studies have only examined the combined effects of temperature and single toxicants on individual organisms and have not considered how PhACs may interact with other stressors at the community level. The effects of multiple stressors are complex and can be synergistic (greater than the sum of

96 the individual effects), antagonistic (due to opposing effects of two or more stressors) or
97 additive (sum of the individual effects; Sala et al., 2000), making predictions about the effects
98 of multiple stressors difficult (Spaak et al., 2017). However, recent advances in predicting the
99 interactions between warming and other stressors have shown that impacts on freshwater
100 ecosystems are usually best explained by the effect of the stronger stressor alone (dominance
101 null model; Morris et al., 2022).

102 Warming can modulate the effects of pollutants on freshwater ecosystems, both directly
103 and indirectly. While many pollutants become more toxic at higher temperatures, warming may
104 also accelerate their degradation, thereby reducing the exposure of biota to PhACs (Noyes et
105 al., 2009; Op de Beeck et al., 2017). Population-level responses to the combined stressors can
106 vary dramatically due to differences in environmental sensitivity (Venâncio et al., 2016),
107 tolerance of individuals (Baert et al., 2016), ecological trade-offs and local adaptation patterns
108 (Bennett and Lenski, 2007; Kneitel and Chase, 2004). Furthermore, the fate of species
109 embedded in communities depends on direct and indirect changes in intraspecific and
110 interspecific interactions (Post, 2013; Relyea, 2009). These include effects mediated by indirect
111 traits and population densities and include top-down and bottom-up effects (Abrams, 1996;
112 Brogan and Relyea, 2015; Guedes et al., 2016; Rodrigues et al., 2018). Persistent pollutants
113 can also alter community structure and affect or enhance various aspects of ecosystem
114 functioning (Duchet et al., 2018; Sánchez-Bayo, 2021).

115 To address these knowledge gaps, we conducted a full-factorial experiment to
116 investigate the combined effects of warming and a mixture of PhACs on freshwater
117 communities in outdoor mesocosms. Our experimental community consisted of common
118 macroinvertebrate and planktonic taxa from different trophic levels and feeding guilds,
119 including predators, filter feeders, grazers, detritivores, and primary producers. We exposed
120 the community to a single-pulse cocktail of 15 PhACs from major drug categories, including

121 cardiovascular, psychoactive, antihistaminics, and antibiotics, at concentrations commonly
122 found in surface waters (Fedorova et al., 2022; Grabicova et al., 2015; Koba et al., 2018;
123 Švecová et al., 2021), in combination with warming. We predicted an interactive effect between
124 the PhAC cocktail and warming on community composition and abundance, resulting in
125 reduced grazing pressure and an increase in phytoplankton biomass cascading through the food
126 web. Specifically, we predicted that high temperatures above therma optima (25°C for most
127 taxa in the community) in summer would have a strong and negative effect on the community,
128 whereas in winter, warming would have a mild but positive effect. The combined effects of
129 PhACs and warming on predators may indirectly affect filter feeder biomass by top-down
130 control, and thus indirectly increase phytoplankton biomass due to reduced grazing pressure
131 (Belden et al., 2007; Polazzo et al., 2022).

132

133 **2. Material and Methods**

134 *2.1. Mesocosms*

135 The mesocosm complex is located at the University of South Bohemia (České Budějovice,
136 Czech Republic; 48°58'36.6"N 14°26'41.8"E, 381 m above sea level). It consists of 32 circular
137 outdoor mesocosms (inner diameter 120 cm, water volume 1.13 m^3 each) set into the ground
138 and equipped with a custom-built, remote-controlled heating system. The 28 mesocosms used
139 in this study were filled 6 weeks before the start of the experiment with tap water filtered
140 through a granulated activated carbon filter and enriched with 100 g of dry leaves of common
141 alder (*Alnus glutinosa*) and 8 g of fish food (Pondstick granules, Apetit®) to add nutrients. The
142 water in each mesocosm was gently mixed with an air stone to avoid thermal stratification,
143 except in winter when mixing was stopped in all treatments between 12 January 2021 and 03
144 February 2021 to allow the water surface in the unheated mesocosms to freeze naturally.

The experimental community included common macroinvertebrate and plankton taxa from different trophic levels and feeding guilds (predators, filter feeders and grazers, detritivores, and primary producers; Table S1.1). During the maturation period, the mesocosms were populated with phytoplankton and zooplankton from a nearby fishpond one month before the start of the experiment. The water with phytoplankton and zooplankton was thoroughly mixed in a barrel before a 2.5-litre aliquot was added to each mesocosm. Two weeks later, the mesocosms were gradually inoculated with macroinvertebrates from nearby fishponds, ditches and sand pits: water column predators (*Notonecta glauca* backswimmers, 9 per mesocosm and *Aeshna* or *Anax* dragonfly larvae, 6 individuals per mesocosm; hereafter *Aeshna* and *Anax*), benthic predators (libellulid dragonfly larvae, 3 per mesocosm), benthic detritivores (*Asellus aquaticus,* ca. 100 per mesocosm), grazers (*Cloeon* cf. *dipterum*, mayfly larvae, ca. 200 per mesocosm), omnivorous scrapers (*Planorbarius corneus* pond snails, 9 individuals per mesocosm), omnivorous piercers (*Sigara falleni*, water boatmen, 50 individuals per mesocosm), and phytophilous predators occupying artificial vegetation and mesocosm walls (damselfly larvae, ca. 50 per mesocosm). The predators were added to the mesocosms only one week before the start of the experiment to allow the community to settle in.

Three artificial submerged macrophytes made of strips of green plastic mesh (5×100 cm) attached to a granite stone were added to each mesocosm to provide additional microhabitat. Two plastic strips (6 cm wide, 50 cm long) hanging vertically in each mesocosm were used to incubate periphyton that had been pre-cultured for four weeks under natural conditions in one of the fishponds where the macroinvertebrates were also collected. Finally, a coarse (20x20 cm; 20 mm mesh) and a fine (20x20 cm; 0.5 mm mesh) litter bag containing 9.5 g of dried alder (*Alnus glutinosa*) leaves were added in each mesocosm to distinguish the contribution of detritivores and microbial decomposers, respectively, to the decomposition process (Fig. 1).

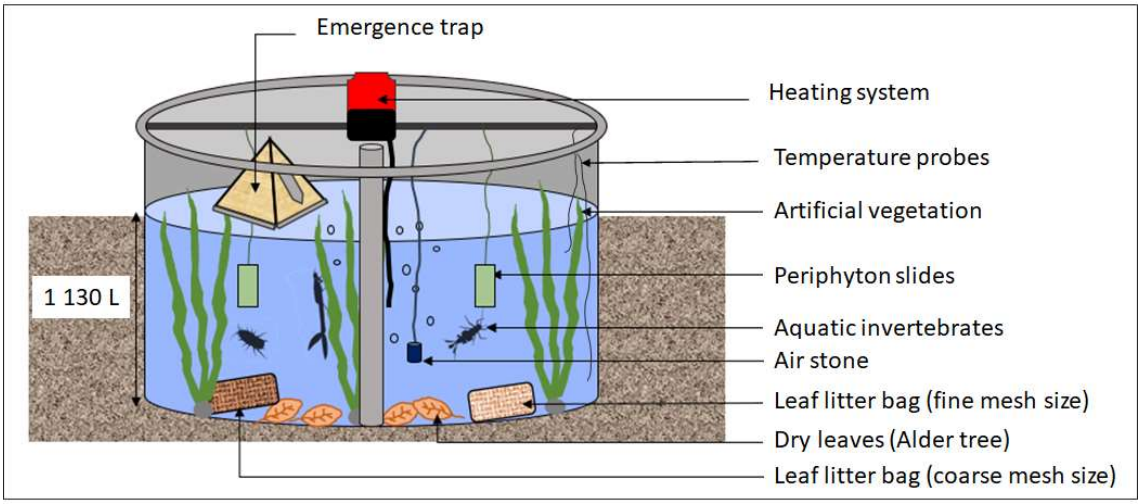

Figure 1. Schematic view of the mesocosm set-up

2.1. Experimental design

Seven mesocosms were each assigned to one of four treatments: (1) not heated, without PhACs, (2) not heated, treated with PhACs, (3) heated, treated with PhACs, and (4) heated, with PhACs. Each mesocosm was subjected to the same treatment in both experiments. Heating was maintained at +4°C above ambient throughout the experiment (see temperature profiles for each experiment in S2). The PhAC mixture was a cocktail of 15 compounds from the major drug categories (cardiovascular: telmisartan, valsartan, metoprolol, atenolol; psychoactive: carbamazepine, lamotrigine, venlafaxine, citalopram, tramadol; antihistaminic: cetirizine, fexofenadine; antibiotic: sulfamethoxazole, trimethoprim, clarithromycin, clindamycin; Table S3.1), at concentrations commonly detected in surface waters in the Czech Republic (Fedorova et al., 2022; Grabicova et al., 2015; Švecová et al., 2021). The total added PhACs were 20 $ng.L^{-1}$ for citalopram and fexofenadine, 50 $ng.L^{-1}$ for atenolol and clindamycin, 100 $ng.L^{-1}$ for clarithromycin, sulfamethoxazole, trimethoprim and venlafaxine, 150 $ng.L^{-1}$ for valsartan, 200 $ng.L^{-1}$ for cetirizine, 250 $ng.L^{-1}$ for carbamazepine and metoprolol and 500 $ng.L^{-1}$ for

186 lamotrigine, telmisartan and tramadol (total concentration: 2890 ng.L^{-1}). The PhAC mixture
187 was administered as a single pulse exposure.

188 We conducted the experiment twice, from 24 September 2020 to 12 March 2021
189 (hereafter 'winter experiment') and from 2 June to 2 August 2021 (hereafter "summer
190 experiment'; Figures S4.1 and S4.2). We refer to the start of each experiment (just before the
191 PhACs were added and the warming started) as Day 0. The first three samplings for all the
192 above parameters were done on Day 0 before the PhAC mixture was added on Day 2 and on
193 Day 7. From then on, samples were taken monthly in winter and fortnightly in summer, until
194 the end of the experiment.

195

196 *2.3. Sampling*

197 In both experiments, we repeatedly measured PhAC concentrations, environmental parameters
198 (dissolved O_2, conductivity, pH, turbidity, and temperature with a YSI 556 MPS probe (®YSI,
199 USA)), chlorophyll-a concentration, zooplankton density and aquatic insect emergence (the
200 latter occurred only in summer; traps were emptied three times per week).

201 Zooplankton was sampled with a tube sampler (length 1.2 m, inner diameter 7 cm)
202 equipped with a one-way valve at the bottom. Water column samples were taken from three
203 regularly spaced locations within each mesocosm to reduce the effects of plankton patchiness
204 (Arnold et al., 1991; Stephenson et al., 1984). The resulting bulk sample (3 × 3L, depending
205 on the water level in the mesocosms: min = 2.5L, max = 3.2L in summer; min = 2.8L, max =
206 3.5L in winter) was collected in a 20L bucket. One litre of the 9L of the bulk sample was
207 filtered through a plankton net with 80-µm mesh size. The retained organisms (zooplankton
208 and some other pelagic invertebrates) were transferred to a 120 mL plastic vial and preserved
209 with formaldehyde (4%). Fixed specimens were counted under a stereomicroscope (Olympus

SZX2-ILLT stereomicroscope; Olympus, Tokyo, Japan) and identified to the lowest feasible taxonomic level using identification keys (Amoros, 1984; Johannsen, 1937). Biomass of each taxon was estimated from the literature (Table S5) or from our own measurements on the macroinvertebrates.

On each sampling day, water samples (6 mL) were collected in each mesocosm, filtered through a 0.2-µm filter and stored at -20°C until the PhAC compound concentrations were analysed with an in-line solid-phase extraction liquid chromatography with tandem mass spectrometry (in-line SPE/LC-MS/MS, triple quadrupole mass spectrometer Quantiva, Thermo Fisher Scientific, USA; see Text S3 for details).

Dissolved organic carbon (DOC) and a measure of nitrogen and phosphorus concentration were measured at Day 0 and at the end of each experiment. For technical reasons, total nitrogen (TN) and total phosphorus (TP) were measured during the winter experiment, while dissolved nitrogen (DN) and soluble reactive phosphorus (SRP) were measured during the winter experiment.

Phytoplankton samples were collected from the surface water layer of 0-1 m in 500 ml PET bottles and fixed with Lugol solution immediately after sampling. After complete sedimentation in the laboratory, the water was decanted, and the sample transferred to a Bürker counting chamber. The algal cells were counted using an optical microscope (OLYMPUS CX23) at 400× magnification. The results are given in cell number.mL^{-1}. Taxa were identified to species or genus level (where possible) according to (Kaštovský et al., 2018a, 2018b). We used the currently accepted phytoplankton nomenclature based on the AlgaeBase (Guiry and Guiry, 2023).

During the summer experiment, a pyramid-shaped floating emergence trap (35 × 35 cm base dimensions) with a 250 mL collection bottle of soapy water (Cadmus et al., 2016) was

installed in each mesocosm on Day 0. Emerging insects were collected every 2-3 days and preserved in 70% ethanol (mayflies, chironomids) or frozen (odonates) for later identification and analysis of PhAC content.

At the end of the experiment, macroinvertebrates were destructively sampled and periphyton stripes were collected and analysed to quantify algal growth. The contents of the litter bags were dried at 60°C for 48 hours before being weighed to quantify the litter decomposition process by invertebrate detritivores and microorganisms.

We focus here only on the temporal dynamics of the pelagic community during the experiment and include in it only the predatory insects feeding on zooplankton. The whole community data collected at the end of each experiment are subject to a separate study (see summary of response variables collected during the experiments, Table S6).

2.3. Statistical analyses

Temporal changes in environmental parameters (pH, dissolved O_2, conductivity, turbidity and water level) and chlorophyll-a concentrations during the experiment were tested by a redundancy analysis (RDA on centred data) separately for the summer and the winter experiment. Treatment was used as a categorical explanatory variable and environmental variables were used as a response. Day of the experiment was used as a covariate. Statistical significance of the RDAs was tested by Monte Carlo permutation tests (999 unrestricted permutations under the reduced model in split-plot hierarchical design).

Differences in community composition of algal groups and species between treatments were visualised by principal component analyses (PCAs) with centred and log-transformed data. Only species found in more than three samples were analysed.

Changes in the planktonic community structure were analysed using the principal response curve (PRC) method (Van den Brink and Braak, 1999) using the most practical level of taxonomic identification for each group. Interaction of treatment and day was used as the explanatory variable and community composition as a response. Day of the experiment was used as a covariate. Before analysis, the biomass of each taxon was log-transformed. Monte Carlo permutation tests were performed on each sampling date to identify the dates on which the treatments differed significantly. In all analyses, the permutations were set in a hierarchical split-plot design and thus permuted for each mesocosm individually. All multivariate and PRC analyses were conducted using CANOCO 5 (Braak and Smilauer, 2012).

We ran generalised linear mixed models (GLMMs; implemented in packages *glmmTMB* and *sjplot*) with the appropriate distribution and link functions. A log-link Gamma GLMM was used for the analyses of the treatment effects on nutrients (dissolved organic carbon, nitrogen and phosphorus concentrations), while binomial GLMMs were used for the analyses of treatment effects on cumulative emergence of predators in summer and on predator survival in winter, respectively. These analyses used only data measured at the end of each experiment. Probability of successful emergence of odonates in the summer experiment was defined as the ratio of the estimated number of individuals emerged by Day 56 to the size of the inoculum. Similarly, survival probability of odonates and *Notonecta* in the winter experiment was defined as the ratio of the estimated number of individuals collected on Day 169 to the size of the inoculum. Model selection approach with the corrected Akaike information criterion was used to identify the most parsimonious model for each univariate response.

Finally, to disentangle the direct and indirect effects of the PhAC mixture and warming on interactions within the aquatic community, we tested simple networks of cause-effect relationships describing a trophic cascade and predator-prey relationships using confirmatory path analysis (piecewise SEM; (Shipley and Douma, 2020), implemented in *piecewiseSEM*

package (Lefcheck, 2016)). This approach can identify mechanistic pathways underlying the effects of different stressors on aquatic ecosystems (Schmidt et al., 2022). Using *a priori* knowledge, we built a complete model of cause-effect relationships based on hypothetical pathways supported by literature (Table S1.1). PhACs and warming were assumed to directly reduce the biomass of filter feeders (= cladocerans in our mesocosms) and thus indirectly increase phytoplankton biomass due to reduced grazing pressure (Belden et al., 2007). We also assessed the effects of PhACs and warming on predators (odonates in summer, and odonates and *Notonecta* in winter), which indirectly affect filter feeder biomass by top-down control. PhACs and warming were used as predictors. Phytoplankton (chlorophyll-*a* concentration), filter feeder biomass and predator biomass were used as response variables. For predator biomass, we used cumulative emergence of odonates in summer and the survival of *Notonecta* and odonate larvae in winter, as there were very few odonate larvae left at the end of the summer experiment and no adults emerged during the winter experiment. We used Fisher's C statistics to assess the overall fit of each SEM model, with $p > 0.05$ defined as indicating a good fit for each model. All these analyses were done in R version 4.1.2 (RStudio, 2020; R Core Team, 2018).

3. Results

3.1. PhAC concentrations

Concentrations of the PhAC mixture gradually decreased over time to ~25% of the initial concentration in the heated mesocosms and to ~30% in the non-heated mesocosms in the summer experiment (Fig. 2A). Metabolite concentrations increased slightly in the first month and then remained stable until the end of the experiment (Fig. 2B). In the winter experiment, the PhACs were also degraded during the course of the experiment (Fig. 2C). In contrast to the

summer experiment, however, the metabolite concentrations increased greatly during the first two months of the experiment, reaching 50% of the initial concentration of the parent compounds, and then remained stable (Fig. 2D). Concentrations of all PhACs and their metabolites in both experiments were below detection limits in all samples taken from the non-treated mesocosms.

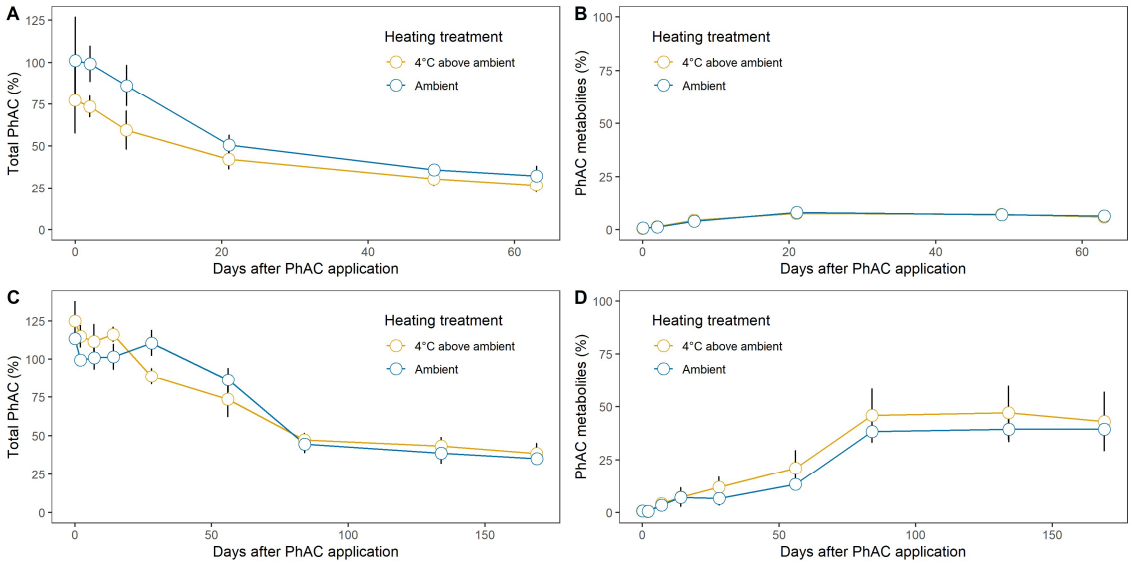

Figure 2. Total concentrations of PhAC main compounds (A, C) and their metabolites (B, D) in the winter (A, B) and summer (C, D) experiments in heated (orange) and non-heated (blue) mesocosms. Symbols with error bars = treatment-specific mean values ± SD (n = 7); mean values connected by colour lines. Data include only treated mesocosms.

3.2. Environmental conditions

Environmental conditions in the summer experiment differed significantly between treatments in the summer (RDA: pseudo-F = 4.9, p = 0.002, explained variation = 7.4%) and winter (RDA: pseudo-F = 53.7, p = 0.001, explained variation = 43.1%) experiments. In the summer experiment, non-heated mesocosms were characterised by higher water levels, higher turbidity,

lower conductivity and lower chlorophyll-*a* concentrations (the latter mainly in the non-treated treatment) compared to heated mesocosms (Fig. 3A, Table S7.1). In the winter experiment, the treatments did not differ in chlorophyll-*a* concentration, dissolved oxygen, turbidity and pH, but non-heated mesocosms were characterised by higher water levels and lower conductivity compared to the heated ones (Fig. 3B, Table S7.2).

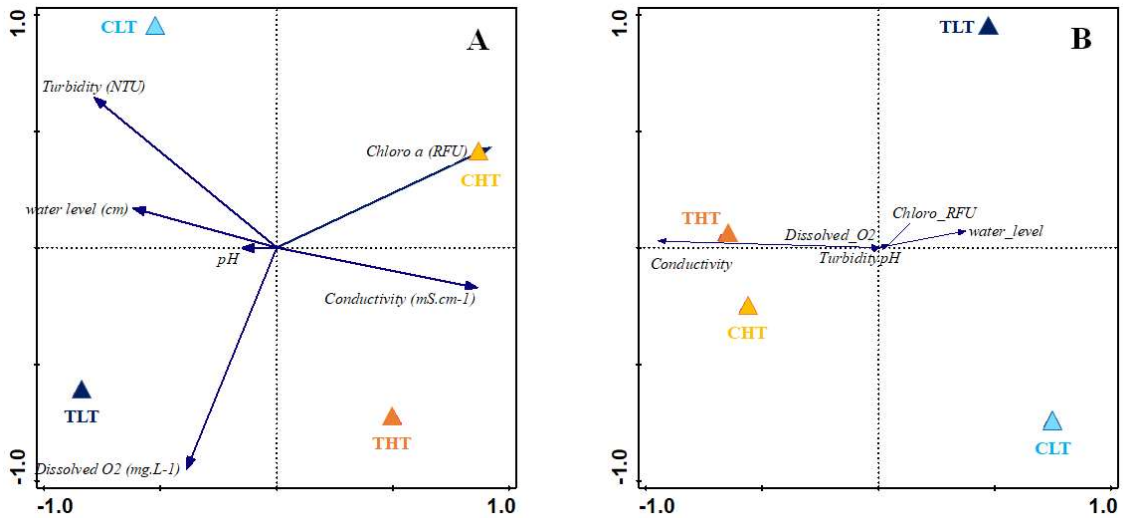

Figure 3. Ordination diagram (RDA) showing differences between the treatments in environmental parameters and chlorophyll-*a* concentration during the summer (A) and winter (B) experiment. Treatments: CLT = non-heated, non-treated; CHT = heated, non-treated; TLT = non-heated, treated; THT = heated and treated.

3.3. Effect of the stressors on phytoplankton and nutrients

Community composition showed no significant differences in the major algal groups between treatments in both the summer and winter experiments (PCA, Fig. S8.1). Species composition was more homogeneous and PCA showed no specific patterns in the summer experiment (Fig. S8.1AB). However, during the winter experiment, Chrysophyta seemed to be more abundant in the non-heated controls, while Streptophyta and Cryptophyta were comparatively more abundant in the non-heated, treated treatment compared to other treatments. Nevertheless, there

was considerable overlap in the samples (Fig. S8.1C). In terms of species composition, temperature was the main driver of differences in the winter experiment (second axis in Fig. S8.1D). The Bacillariophyta *Achnanthes* sp. and the Chlorophyta *Oocystis* spp. and *Scenedesmus* sp. tended to be more abundant in the heated controls, while Chrysophyta tended to be more abundant in the non-heated controls. The Bacillariophyta *Synedra* sp., *Gomphonema* spp., *Navicula* sp. and *Ulnaria* were more abundant in the non-heated, treated treatment, while none of the species were more abundant in the heated treatment with added PhACs than in the other treatments (Fig. S8.1D).

The most parsimonious models identified partly disparate effects of the stressors on nutrients in the summer and winter experiments. In the summer experiment, the final concentrations of DOC, DN and SRP were lower in the heated mesocosms than in the non-heated ones and the added PhAC mixture had a small negative effect on DOC and a significant negative effect on DN and SRP (Fig. S8.2ACE). In the winter experiment, the final concentrations of DOC, TN and TP were also lower in the heated mesocosms than in the non-heated ones, while no effects of PhACs on nutrients could be detected (Fig. S8.2BDF). In both experiments, we did not detect any synergistic effects of the two stressors on nutrients (Tables S8.1 and S8.2).

3.3. Effect of the stressors on zooplankton community dynamics

The dynamics of the zooplankton community and its responses to the stressors also differed markedly between the summer and winter experiments. In the summer experiment, the biomass of the main zooplankton groups differed significantly between treatments (PRC: $F = 0.7$, $p = 0.003$; Fig. 4A). More specifically, differences between treatments appeared immediately after Day 0 and continued to increase until Day 35, with differences primarily caused by warming rather than PhACs and mainly affecting ostracods and *Daphnia* (Fig. 4A). Ostracod biomass

increased while *Daphnia* biomass decreased in the heated mesocosms compared to the non-heated mesocosms, while the other groups did not contribute to the differences between treatments (Fig. 4A). That is, *Daphnia* biomass decreased in all treatments, but the decline was steeper in the heated mesocosms (Fig. S9.1). On the other hand, copepod biomass tended to increase towards the end of the experiment in all treatments, with an intermediate peak in biomass around Day 35, which appeared to be higher in the treatments without PhAC addition (Fig. S9.2). In contrast, zooplankton composition did not differ significantly between treatments in the winter experiment (F = 0.3, p = 0.41; Fig. 4B).

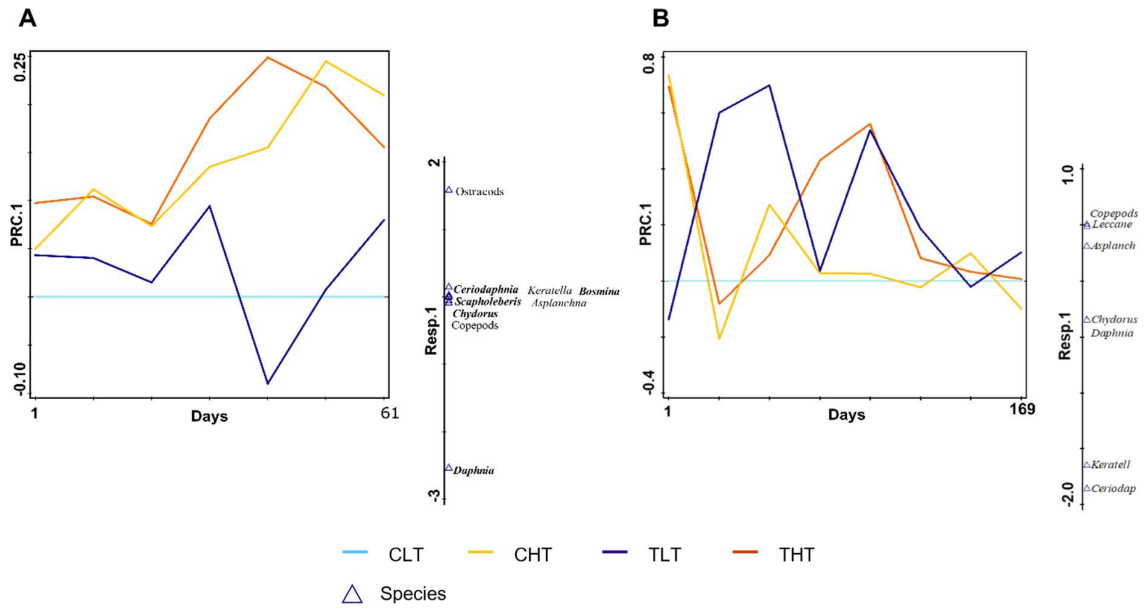

Figure 4. Principal response curves (PRC) with species weights (vertical bars) indicating treatment-specific effects on zooplankton during the summer (A) and winter (B) experiment. Treatments: non-heated, without added PhACs (light blue); yellow line: heated, non-heated, without added PhACs (yellow); non-heated, with added PhACs (dark blue); heated, with added PhACs (orange). Days = days after exposure.

385 *3.3. Effect of the stressors on insect predator survival and emergence*

386 The effects of the two stressors on predatory insects differed between taxa and experiments
387 (Fig. 5).

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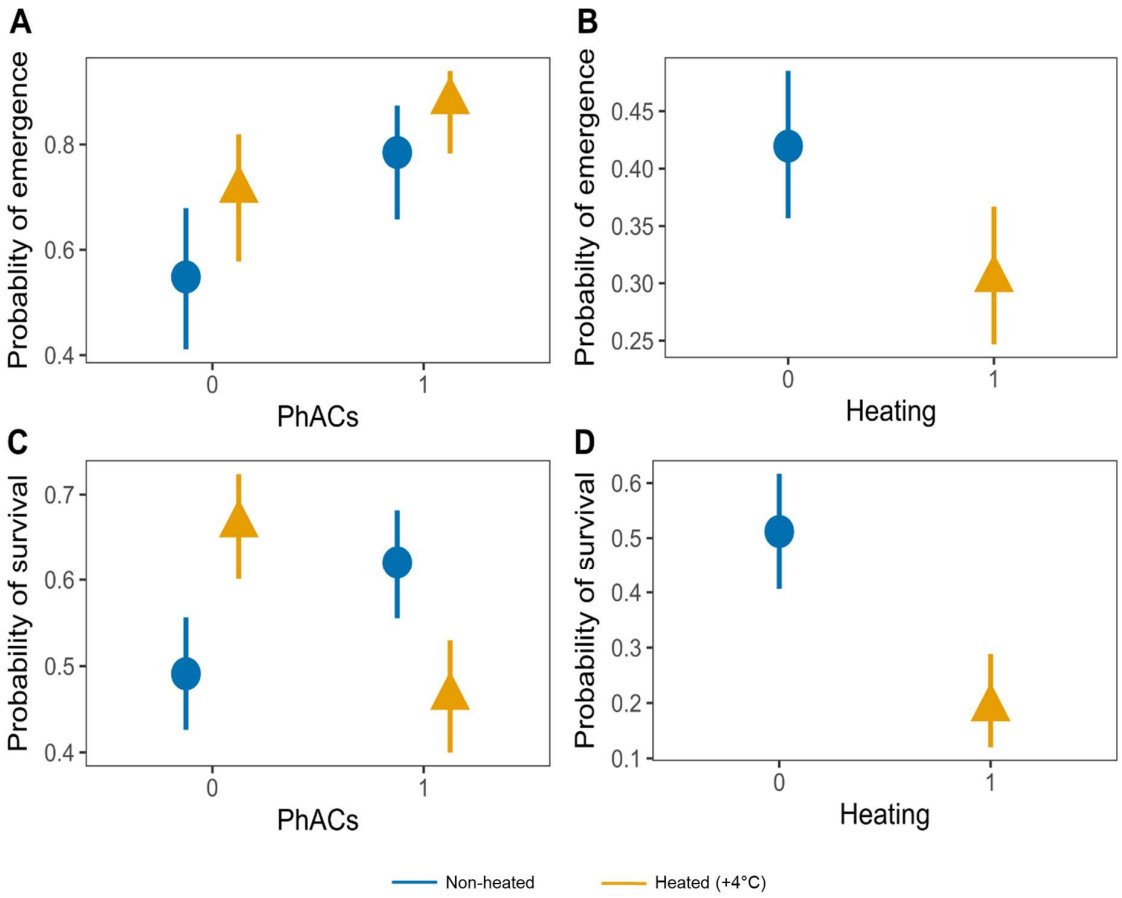

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391 **Figure 5.** Estimates of treatment-specific probability of emergence of insect predators in the summer
392 experiment (A: anisopteran larvae (*Aeshna*), B: zygopteran larvae) and probability of survival of insect
393 predators in the winter experiment (C: zygopteran larvae, D: *Notonecta* backswimmers). Model
394 estimates of the most parsimonious models, shown as mean values with 95% confidence intervals based
395 on fixed effects.

396

397 The effects of warming and PhACs on the cumulative probability of emergence in the summer
398 experiment and on the survival probability in the winter experiment differed between taxa

399 (Tables S10.1 and S10.2). In the summer experiment, both warming and PhACs increased the
400 cumulative number of anisopteran (*Aeshna*) larvae that emerged, with an additive effect on the
401 predictor scale (Fig. 5A). In contrast, warming had a negative effect on damselfly emergence
402 in the summer experiment (Fig. 5B). In the winter experiment, survival of anisopteran (*Anax*)
403 larvae was similar in all treatments, while damselfly larvae survived better when exposed to
404 PhACs only or heating only, with an antagonistic effect of both stressors resulting in similar
405 survival of the larvae in the treatments without both stressors and with both stressors(Fig. 5C).
406 Finally, *Notonecta* survived less when exposed to warming, but PhACs had no effect on their
407 survival in the winter experiment (Fig. 5D).
408

409 *3.3. Effect of the stressors on the pelagic food web*

410 The effects of the two stressors on relationships in the pelagic food web differed markedly
411 between the summer and winter experiments (Fig. 6 and Tables S11.1 and 11.2). In the summer
412 experiment, chlorophyll-*a* concentration as a proxy of phytoplankton biomass was directly and
413 negatively affected by PhACs (standardised path coefficient = -0.0001; Fig. 6A and Table
414 S11.1) and directly and positively affected by warming (standardised path coefficient = +0.08).
415 Both stressors had a direct and negative effect on cladocerans as the main filter-feeding group
416 (standardised path coefficients = -0.0001 for PhAC and -0.14 for warming). However, the
417 relationship between primary producers and filter feeders was positive and not significant
418 (standardised path coefficient = -0.09). These results do not support the hypothesis that a
419 decrease in filter-feeding biomass due to the stressors leads to a trophic cascade and thus to
420 higher algal biomass. The effects of stressors on the predators were positive but not significant
421 (direct effect, standardised path coefficients = +0.0001 for PhAC and +0.02 for warming);
422 however, predators caused a direct decrease in filter feeder biomass (standardised path
423 coefficient = -0.21; Fig. 6A).

In contrast, the PhAC mixture had no direct or indirect significant effect on any component of the simplified pelagic food web in the winter experiment, while only the phytoplankton biomass was directly and positively affected by warming during winter (standardized path coefficient = 0.28; Fig. 6B and Table S11.2).

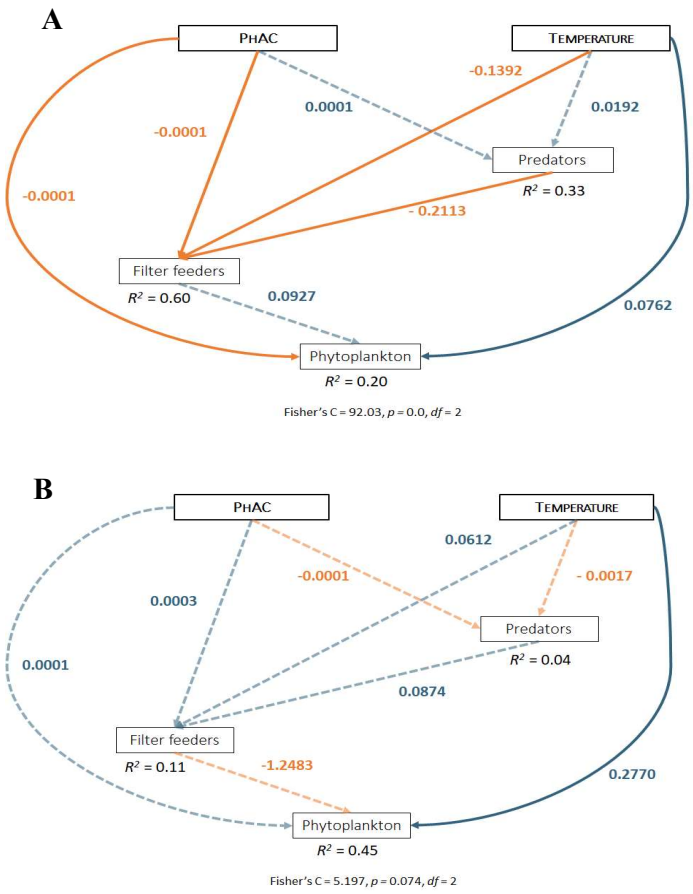

Figure 6. Path diagram of the selected structural equation model based on average data collected during the summer experiment, and last sampling data during the winter experiment. Orange, blue, and dashed arrows respectively indicate significantly ($p < 0.05$) negative, significantly positive, and non-significant ($p > 0.05$) relationships between variables. Proportions of variation explained by the model for each response variable given by R^2 values. See Tables S5 and S6 for details.

4. Discussion

To the best of our knowledge, this is the first study to examine the combined toxic effects of a mixture of PhACs at environmentally relevant concentrations and warming on freshwater communities. In the winter experiment, we observed very limited effects, while in the summer experiment, warming acted as the stronger stressor and amplified the effects of PhACs on the freshwater community. This led to a PhAC- and temperature-mediated trophic cascade in the planktonic food web, with warming negatively affecting zooplankton and promoting phytoplankton development. Although no effects of PhACs and warming on secondary consumers were observed, our results suggest that low and environmentally realistic concentrations of PhACs may be detrimental to the aquatic community at higher temperatures as a resulting of climate warming. This was evidenced by a significant negative effect of the combined stressors on filter feeder biomass, which even reversed the expected result based on the effects of the individual stressors.

4.1. Effects of warming on the freshwater community

Temperature was the stronger stress factor for the freshwater community in our study, especially in summer. During the summer experiment, the highest temperatures in the heated mesocosms reached up to 29.5°C (compared to 25.5°C in the non-heated mesocosms) and remained above 25°C for almost three weeks in July.

The effects of global warming on freshwater ecosystems have been well studied recently and are expected to have significant impacts on the pelagic communities. For example, warming can stimulate phytoplankton growth in temperate and shallow lakes (Zhang et al., 2019), leading to altered primary productivity, nutrient recycling, and higher trophic productivity (Mooij et al., 2008). However, warming may also promote the development of bacteria that can increase competition for resources with phytoplankton (Joint et al., 2002). The

463 increased stratification caused by warming may alter plankton structure and dynamics,
464 favouring small-bodied and buoyancy-regulating species, ultimately leading to changes in
465 primary productivity, nutrient cycling, and higher trophic levels (Winder and Hunter, 2008).
466 Warming is not expected to have a significant effect on the total phytoplankton biomass, but
467 the associated phenomenon of brownification can significantly reduce local phytoplankton
468 biodiversity due to the dominance of mixotrophic algae that displace other phytoplankton taxa
469 (Urrutia-Cordero et al., 2017). In our study, we observed an increase in phytoplankton biomass
470 (measured by chlorophyll-*a* concentration) under warming in both the summer and winter
471 experiments. However, by the end of the summer experiment, the phytoplankton community
472 in the heated mesocosms shifted towards a dominance of cyanobacteria and diatoms, while
473 green algae continued to dominate the non-heated mesocosms.

474 Moreover, the effects of warming on phytoplankton are context-specific and may be
475 offset by the grazing effects of filter feeders such as *Daphnia* and other zooplankton taxa
476 (Kazanjian et al., 2018; Velthuis et al., 2017). Invasive species may also colonize lakes in
477 response to warming, but the long-term consequences of this disturbance may differ from short-
478 term observations (Dziuba et al., 2020). In our summer experiment, the zooplankton
479 community was initially dominated by *Daphnia* and other large cladocerans and gradually
480 shifted to the dominance by smaller zooplankton species in response to warmer temperatures.

481 Finally, the presence of top predators may also exacerbate the effects of climate warming
482 on phytoplankton in shallow aquatic ecosystems by reducing grazing pressure on zooplankton
483 (Hansson et al. 2013; Šorf et al., 2015; Velthuis et al., 2017; Yvon-Durocher et al., 2011). Our
484 observations corroborate these studies, as we found that phytoplankton biomass was
485 significantly higher in the warmer mesocosms, and we attribute this pattern to a lower
486 zooplankton grazing pressure caused by both predation by odonate larvae and higher
487 temperatures. Overall, our results reiterate that the complex and non-linear responses of

488 freshwater communities to environmental stressors should be considered when predicting
489 future biodiversity patterns (Polazzo et al., 2022).
490
491 *4.2. Effects of PhACs on the freshwater community*
492 PhACs are emerging contaminants that can have toxic effects on freshwater communities (Fent
493 et al., 2006). The toxicity of PhACs can be even more pronounced when present in mixtures,
494 causing significant structural and functional changes in microbial and algal communities and
495 affecting the survival and behaviour of organisms from zooplankton to macroinvertebrates to
496 fish (Brodin et al., 2014). We observed no significant effects of PhACs in the winter
497 experiment. However, in the summer experiment, the PhAC mixture alone had a negative effect
498 on phytoplankton biomass (chlorophyll-*a* concentration) as well as on nutrients (DN, SRP).
499 The PhAC mixture also negatively affected predator survival, which may have attenuated the
500 indirect effect of lower phytoplankton biomass on cladoceran herbivores.
501 Several previous studies have reported negative effects of PhAC mixtures on freshwater
502 organisms, but these effects were typically observed at concentrations much higher than those
503 used in our study. A laboratory study showed that a mixture of 10 PhACs (antidepressants,
504 antihistaminics, antibiotics, anticonvulsants and analgesics) can be toxic to algae
505 (*Pseudokirchneriella subcapitata*), daphniids (*Ceriodaphnia dubia*) and zebrafish (*Danio*
506 *rerio*) at concentrations much higher (up to 15,000 times higher for the zebrafish) than the
507 environmental concentrations of the individual compounds (Watanabe et al., 2016). In outdoor
508 microcosms, high concentrations of a PhAC mixture (analgesics, antidepressants and
509 antibiotics; >60 $mg.L^{-1}$ for each compound) resulted in elevated fish mortality and a decline in
510 zooplankton and phytoplankton diversity (Richards et al., 2004). However, David et al. (2020)
511 did not observe strong effects of a mixture of environmentally relevant concentrations of five

512 PhACs (antiepileptics, analgesics, and antihypertensives) on three-spined stickleback
513 populations in mesocosms.

514 In the field, an antibiotic mixture increased mortality of biofilm and decreased
515 extracellular leucine-aminopeptidase and alkaline phosphatase, resulting in significant
516 structural and functional changes in microbial attached communities (Proia et al., 2013), while
517 algal biomass and biofilm respiration were suppressed by 18% and 40%, respectively, and
518 photosynthesis by 88% in a mixed PhAC treatment (Rosi-Marshall et al., 2013). The effects on
519 phytoplankton observed in the summer experiment corroborate the results of these two studies.
520

521 *4.3. Interactions between warming and PhACs*

522 The interactions between temperature and contaminants pose a major challenge for ecological
523 risk assessment, especially with global warming (Zhang et al., 2019). The effects of warming
524 on contaminant toxicity have been extensively studied. However, the complex impacts of
525 warming and chemical stressors on shallow freshwater ecosystems can be influenced by
526 additional factors such as the type of contaminants and community composition, including the
527 number of trophic levels and their feeding behaviour. For example, heat waves in combination
528 with pesticide exposure can have antagonistic or synergistic effects, depending on the type of
529 pesticide used (Polazzo et al., 2022).

530 The combined effects of the PhAC mixture and warming in our study could not be
531 predicted from the individual effects of each stressor, especially in the summer experiment.
532 The PhAC mixture combined with warming strongly reduced phytoplankton biomass and its
533 control by primary consumers (zooplankton filter feeders) directly and indirectly due to
534 increased predation pressure from higher trophic levels. This trophic cascade also led to a
535 change in phytoplankton composition.
536

5. Conclusions and perspective

Although several studies of simplified food webs and communities have included both warming and chemical stressors (Knillmann et al., 2013; Wijewardene et al., 2021), current understanding of the joint effects of complex mixtures of PhACs and warming on freshwater communities is very limited. This hinders our ability to distinguish direct and indirect effects of each stressor and identify emergent phenomena that cannot be inferred from observations based on single stressors. Indeed, the current bias towards single species and population-level experiments has resulted in a knowledge gap for relevant community and ecosystem-level endpoints that prevents the exploration of important indirect effects of interacting multiple stressors that can compromise food web stability (Polazzo et al., 2022).

Our results contribute to filling these gaps and have implications for decision making to mitigate the effects of stressors on freshwater ecosystems. In our study, the PhAC mixture combined with warming strongly reduced phytoplankton biomass and its control by zooplankton. PhACs alone caused little or no impact at the population or community level, but when they were present in combination, they altered the aquatic community in the summer experiment, most likely when high temperatures increased close to or above the upper limits of the thermal niche of many taxa from phytoplankton to the insect predators. Moreover, we found that the effects of warming and PhACs differed strongly between seasons. Future research can extend our study by considering larger spatio-temporal scales and increasing the resolution and scope of the food web interactions. This would help us predict long-term effects of the current levels of anthropogenic stressors on freshwater communities and ecosystem functioning.

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