Multiple Stressors Simplify Freshwater Food Webs

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Authors: Peiyu Zhang[1,*], Huan Zhang[1], Shaopeng Wang[2], Guy Woodward[3], Eoin J. O'Gorman[4], Michelle C. Jackson[5], Lars-Anders Hansson[6], Sabine Hilt[7], Thijs Frenken[8], Huan Wang[1], Libin Zhou[2], Tao Wang[1], Min Zhang[9], Jun Xu[1,*]

[1]Key Laboratory of Lake and Watershed Science for Water Security, Key Laboratory of Breeding Biotechnology and Sustainable Aquaculture, Xiangxi River Ecosystem Research Station in the Three Gorges Reservoir Region, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China

[2]Institute of Ecology, Key Laboratory for Earth Surface Processes of the Ministry of Education, College of Urban and Environmental Sciences, Peking University, Beijing, China

[3]The Georgina Mace Centre for the Living Planet, Department of Life Sciences, Imperial College London, Ascot, United Kingdom

[4]School of Life Sciences, University of Essex, Colchester, United Kingdom

[5]Department of Biology, University of Oxford, Oxford, United Kingdom

[6]Department of Biology, Aquatic Ecology, Lund University, Lund, Sweden

[7]Department of Community and Ecosystem Ecology, Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany

[8]Cluster Nature & Society, HAS green academy, 's-Hertogenbosch, the Netherlands

[9]Hubei Provincial Engineering Laboratory for Pond Aquaculture, Engineering Research Center of Green Development for Conventional Aquatic Biological Industry in the Yangtze River Economic Belt, College of Fisheries, Huazhong Agricultural University, Wuhan, China

[*] Corresponding authors: Peiyu Zhang zhangpeiyu@ihb.ac.cn +86 15807110925

Jun Xu xujun@ihb.ac.cn +86 02768780195

Address: No. 7 Donghu South Road, Wuchang District, Wuhan, Hubei Province, China, 430072

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Abstract

Globally, freshwater ecosystems are threatened by multiple stressors, yet our knowledge on how they interact to affect food web structure remains scant. To address this knowledge gap, we conducted a large-scale mesocosm experiment to quantify the single and combined effects of three common anthropogenic stressors, warming, increased nutrient loading, and insecticide pollution, on the network structure of shallow lake food webs. We identified both antagonistic and synergistic interactive effects depending on whether the stressors affected negative or positive feedback loops, respectively. Overall, multiple stressors simplified the food web, elongated energy transfer pathways, and shifted biomass distribution from benthic to more pelagic pathways. This increased the risk of a regime shift from a clear-water state dominated by submerged macrophytes to a turbid state dominated by phytoplankton. Our novel results highlight how multiple anthropogenic stressors can interactively disrupt food webs, with implications for understanding and managing aquatic ecosystems in a changing world.

Keywords: climate change, food webs, interactive effects, anthropogenic stressors, regime shift, shallow lakes, global environmental change

Introduction

The world's freshwaters are under unprecedented pressure on multiple fronts, with impacts from both climate change and pollution, which are themselves consequences of human population growth and associated land-use changes (Reid *et al.* 2019; Birk *et al.* 2020). Despite both numbers and intensity of stressors are increasing (Reid *et al.* 2019; Almond *et al.* 2020), surprisingly little is known about their impacts beyond the lower ecological organisational levels of individuals or populations (Orr *et al.* 2024). Even less is known about how stressors interact to generate potentially interactive effects which can be antagonistic, where one stressor dampens the effects of another (Zhou & Wang 2023), or synergistic, where stressors amplify each other (Orr *et al.* 2020; He *et al.* 2023). This lack of predictive power reflects the difficulties in extrapolating from simple laboratory systems to the more complex reality in the wild, especially where multiple pathways through the food web and species interactions can rapidly swamp the far more subtle responses often seen in the laboratory (Beauchesne *et al.* 2021; van Moorsel *et al.* 2023). Hence, in order to meet and manage multiple, simultaneously acting anthropogenic threats to ecosystem function, studies addressing the interactions of such threats acting in concert are urgently needed.

Recent meta-analyses have shown that both antagonistic and synergistic interactions of multiple stressors can affect higher organisational levels, such as whole communities (Crain *et al.* 2008; Jackson *et al.* 2016; Morris *et al.* 2022). Experimental studies have also revealed some interactive effects of multiple anthropogenic stressors on different components of freshwater food webs (Allen *et al.* 2021; Vijayaraj *et al.* 2022). However, the interactive effects of multiple stressors on food web properties such as species and link richness, connectance, mean trophic level or the degree of omnivory (Bersier *et al.* 2002) have rarely been explored (Polazzo *et al.* 2022; Wang *et al.* 2023). In recent years, these traditional univariate or bivariate metrics have been augmented by trivariate approaches that consider not only network topologies, but also how these map onto mass-abundance scaling relationships, and this has generated new insights into individual stressor impacts, including both warming (O'Gorman *et al.* 2019) and pollution (Thompson *et al.* 2016). Specifically, these approaches plot averaged species populations on $\log_{10}$[body mass (M)] and $\log_{10}$[abundance (N)] axes as nodes that are connected via feeding links in trivariate food webs, which provides a potentially powerful method to assess how the size structure of trophic networks might change under multiple stressors. For instance, the allometric slope of the food webs represents the scaling coefficient obtained by regressing $\log_{10}$(N) on $\log_{10}$(M) for all trophically connected species within the web, so a steepening of the slope may indicate weakened top-down effects, as consumers become smaller and/or less abundant relative to their resources (Woodward *et al.* 2012). It can also indicate a less efficient transfer of energy through the food web, if, for instance the same abundance of resources supports fewer large consumers than under reference conditions (Yvon-Durocher *et al.* 2011). The lengths and angles of all pairwise consumer-resource links in $\log_{10}$[M] and $\log_{10}$[N] space, can also be used to assess the impact of these structural changes on energy transfer pathways within the food web (Cohen *et al.* 2009; O'Gorman *et al.* 2019).

In shallow aquatic ecosystems energy flow typically occur through both benthic and pelagic pathways (Lischke *et al.* 2017; Mehner *et al.* 2022), and the relative importance of them may indicate the presence of stable equilibria, such as a clear-water state dominated by submerged macrophytes (benthic pathway) or a turbid-state dominated by phytoplankton (pelagic pathway) (Phillips *et al.* 2016; Mehner *et al.* 2022). Environmental stressors can affect the critical threshold levels for shifts between these equilibria (Polst *et al.* 2022). However, how multiple stressors might affect the distribution of energy flow within these food webs, and hence their propensity for triggering ecosystem level shifts, has not been explored to date.

In order to advance our understanding on how multiple, simultaneously occurring anthropogenic threats may affect ecosystem function in freshwater ecosystems, we established food webs in a field array of 48 large (2500 L) mesocosms simulating shallow pond/lake ecosystems of a subtropical region, by seeding them with the same amount of sediment, common macrophytes, plankton, zoobenthos, and fish. We subsequently manipulated environmental conditions to simulate a range of human-induced stressor scenarios commonly affecting freshwater ecosystems globally: warming, nutrient loading, and insecticide addition in a full-factorial design (details can be found in Methods and Fig. S1, S2 & S3). The experiment spanned the entire growing season (10 months), and at the end, we harvested all major food web components in each mesocosm, including all primary producers, zooplankton, zoobenthos and the top-predators (Fig. 1). Using literature data on tropic interactions between species, we constructed food webs based on 14 taxonomic groups, which included all major components of a typical shallow freshwater food web (see Methods and Fig. S4, Tables S1 & S2). We then quantified food web structure with mass-abundance data integrated, using the trivariate approach (Cohen *et al.* 2009; O'Gorman *et al.* 2019).

The aim of this study was to provide urgently needed understanding on how multiple stressors affect food web structure in complex shallow freshwater ecosystems. Specifically, we tested the hypotheses that, (1) multiple stressors will interactively influence food web structure, with both antagonistic and synergistic effects depending on the type and combination of stressors; (2) an increased number of stressors will simplify the structure of the food web. We expected that, as different stressors affect various components of the ecosystem (Fig. 1), an increase in the number of stressors would result in a more pronounced disruption and damage to the overall structure of the food web.

Methods and Materials

Experiment design

The experiment was conducted at Huazhong Agricultural University, Wuhan (China) for 10 months from February to November 2021. Forty-eight cylindrical polyethylene mesocosms (volume: 2500 L, diameter: 1.5 m, depth: 1.4 m) were buried up to their rims to improve insulation from prevailing weather conditions and to simulate a shallow lake ecosystem (see Fig. S3). The mesocosms are situated near Lake Nanhu, an urban lake, with several experimental ponds also located in the vicinity. Three stressors were applied: a warming treatment (W) at 3.5 °C above ambient conditions, accompanied by multiple heatwaves imposed throughout the 10-month duration of the experiment; a nutrient loading treatment (N) involving the addition of dissolved nitrogen and phosphorus compounds; and an insecticide treatment (I) with imidacloprid (a neonicotinoid). The three stressors were randomly assigned to all mesocosms, using a fully factorial design that included each of three stressors and all their possible two- and three-way combinations, resulting in seven treatments with six replicates, and six control replicates (no treatments applied). W, N, I, W*N, W*I, N*I, W*N*I and C.

The frequency and magnitude of the heatwaves was based on model predictions from the historical meteorological data in the middle and lower reaches of Yangtze River Basin, China, predicted to be reached in this area by the end of this century given the ongoing climate warming (duration and magnitude of heatwaves can be found in Fig. S2) (IPCC 2014). Warming was achieved using a computer-controlled system with two temperature sensors (DS18B20) and a heating element (600 W) in each of the heated treatment mesocosm. The heating element was installed 30 cm below the water surface close to an aquarium pump to evenly distribute the heat throughout the water column. Water temperatures in the heated mesocosms were elevated based on the mean temperatures in the ambient mesocosms (Zhang *et al.* 2018; Zhang *et al.* 2022b). Nutrient loading and insecticide treatments were applied once per fortnight, with the doses changed according to the agricultural activities and precipitation intensities in this area (Zhou *et al.* 2019; Xu *et al.* 2020). This simulated more realistic scenarios whereby multiple stressors typically exhibit temporal shifts rather than being applied in (unrealistic) perfect synchrony (Jackson *et al.* 2021). Nitrogen and phosphorus were added to the nutrient loading treatment at an elemental mass ratio of 10:1, by dissolving $NaNO_3$ and KH_2PO_4 powder in de-mineralised water, respectively. Averaged nutrient loading doses were 0.90 mg L^{-1} (range from 0.25 to 1.6 mg L^{-1}) and 0.09 mg L^{-1} (range from 0.025 to 0.16 mg L^{-1}) for nitrogen and phsphorus, respectively (Fig. S1b). Insecticide treatments were applied by adding imidacloprid (70% active ingredients, PD20120072, Bayer, Germany) solution to the mesocosms, a common insecticide belonging to the neonicotinoids, one of the most commonly used agricultural pesticides worldwide (Egli *et al.*

2023), and neonicotinoids are toxic to many invertebrate species (including aquatic insects and crustaceans) (Morrissey *et al.* 2015; Dimitri *et al.* 2021; Sargent *et al.* 2023). The average insecticide loading of 32.7 μg L^{-1} (range from 10 to 50 μg L^{-1}) during the experiment (Fig. S1c). The loading doses were within concentrations found in natural water bodies in agricultural areas worldwide (Jeppesen *et al.* 2007; Morrissey *et al.* 2015).

Experiment set-up

In February, half area of the bottom of each mesocosm was filled with 10 cm of muddy sediment, which was collected from Lake Liangzi (N 30°11′3″, E114°37′59″). All sediment was homogenised and sieved through a 5*5 mm metal mesh to remove large debris, macrophyte seeds, and molluscs. Initially, the sediment contained 5.5 ± 0.4 mg total nitrogen (TN) g^{-1} dry weight and 0.42 ± 0.08 mg total phosphorus (TP) g^{-1} dry weight. *Potamogeton crispus* and *Hydrilla verticillata* are the dominant submerged macrophytes in this area (Zhang *et al.* 2016). *P. crispus* is an early season submerged macrophyte dominant in spring, while *H. verticillata* is a warm-adapted species that is typically dominant in summer (Zhang *et al.* 2016; Zhang *et al.* 2022a). Turions (50 g fresh weight per species) obtained from nearby Lake Honghu (29°51′N, 113°20′ E), were added to the sediment of each mesocosm.

The water level was gradually raised by tap water addition and natural rainfall to 1.2 m depth to allow the establishment of submerged macrophytes. We aimed at simulating a food web in the mesocosms with all major lake functional groups by seeding each with 14 individuals of the snail *Radix swinhoei* (1 to 2.5 cm) and 20 individuals of the snail *Bellamya aeruginosa* (around 2.5 cm) as periphyton grazers. Five freshwater shrimps *Macrobrachium nipponense* (length around 4 cm), four bitterling *Rhodeus sinensis* (around 3 cm), and four crucian carp *Carassius auratus auratus* (around 4 cm) were added as representative omnivores feeding on zooplankton, macroinvertebrates, detritus, periphyton and phytoplankton (diet composition can be found in Table S2). The fishes and shrimp were commercially obtained. The organisms we selected are all common species coexisting in water bodies in this region, and the densities and biomasses were within the range occurring in nature (Ye *et al.* 2007; Gong *et al.* 2009; Zhi *et al.* 2020; Mao *et al.* 2021; Yu *et al.* 2021). In nearby lakes, the density of small fish can reach up to 8.71 individuals per m² (Ye *et al.* 2007). Our mesocosms, each with an area of 1.77 m², previously housed eight fish per unit, and all fish grew well (Zhang *et al.* 2022a). In addition, aerial aquatic insects can freely colonize these mesocosms. Ten litres of lake water were added from nearby Lake Nanhu to each mesocosm to inoculate with plankton and other microbes (N 30°28′57″, E14°22′34″). Deionised water was added to the heated mesocosms to compensate for evaporation. The submerged macrophyte *Ceratophyllum demersum* and floating macrophyte *Lemna minor* emerged in a few mesocosms and were removed as soon as they were observed.

Regular sampling

The experiment was set up in early February of 2021, during winter, when most organisms were relatively dormant (Zuo & Li 2011). The mesocosms were acclimated for two months. All treatments were applied when the macrophytes had established and the water was clear in all mesocosms on the 8th of April 2021. Water quality was measured once per fortnight, including conductivity, dissolved oxygen concentrations (DO), pH, turbidity, nutrient concentrations and phytoplankton chlorophyll a concentrations. Conductivity, DO and pH were measured using a HACH HQD portable meter (HQ60d, HACH, USA). Turbidity was measured by a portable WGZ-2B turbidity meter (Xinrui, Shanghai, China). Depth-integrated water samples were collected using a transparent plexiglas tube (diameter 70 mm, length 1 m) to analyse nutrient and phytoplankton chlorophyll *a* concentrations. Total nitrogen (TN) and total phosphorus (TP) concentrations were determined spectrophotometrically (Chinese National Standards 1996) after digestion with potassium peroxodisulfate. Concentrations of nitrate (NO_3^-), ammonia (NH_4^+) and phosphate (PO_4^{3-}) were also measured spectrophotometrically after filtering the water through GF/C filters (Chinese National Standards 1996). Phytoplankton chlorophyll *a* concentration was determined by filtering water on Whatman GF/C filters and spectrophotometric analysis after acetone extraction (HJ 897-2017) (Chinese National Standards 1996).

Periphyton chlorophyll *a* content and plant volume infested (PVI) were also quantified. Periphyton chlorophyll *a* content (indicating the biomass of periphytic algae) was measured by hanging a polyethylene board (rough surface, 10*10 cm) vertically attached to the wall in the middle of each mesocosm (60 cm below the water surface). The board was taken out every two weeks and the periphyton was brushed off and extracted in acetone for 24 h. The solution was centrifuged, and analysed spectrophotometrically. After brushing, the board was replaced in the mesocosms until the next sampling. Plant volume infested (PVI) was determined separately for each macrophyte species in each mesocosm. The percentage cover of the plant was estimated, and the average height and depth were measured to the nearest centimetre with a ruler. PVI was calculated as: PVI = plant coverage × averaged plant height / depth of water (Sayer et al. 2010). Dead fish were recorded and removed during the experiment, and higher mortality was observed in the warmed than the ambient treatments, and the highest mortality occurred during the heatwave period with the highest temperatures (Fig. S2).

Food web sampling

The experiment ended in early November 2021, whereby the water from each mesocosm was drained, and plants and animals were harvested. All fish, shrimp, and large snails were collected, carefully cleaned, blotted dry, counted, and weighted.

(1) Primary producers: All macrophytes were harvested and the dominant macrophyte was generally *H. verticillata*, whereas only a few mesocosms had *P. crispus*, and then at low abundances. Water drops attached to macrophytes were removed by putting the plants in a tuck net and manually centrifuging them, and then fresh weight was determined to the nearest gram. A Plexiglas tube (diameter 70 mm, length 1 m) was used to randomly collect multiple integrated samples of the whole water column and pooled in a bucket (> 10 L) for phytoplankton sampling. Phytoplankton was preserved by fixing 1000 ml of homogenised water sample with Lugol´s solution. Periphyton was collected by brushing off three areas of periphyton (3*3 cm) from the wall of each mesocosm (10 cm below the water surface, 10 cm above the sediment and in the middle between these), fixed with Lugol´s solution. Algae was identified to the lowest possible taxonomic level and counted and size was measured (> 500 cells per sample) under the microscope (OLYMPUS CX31) with 400X magnifications to determine their abundance and

biomass. We estimated the mean cellolume based on the geometric shape that most closely resembled the actual cell shape, following Hillebrand *et al.* (1999) and Hu and Wei (2006). Total periphyton biomass of each mesocosm was then estimated by adding the total wall periphyton biomass and the periphyton biomass on the leaves of macrophytes. The leaf surface of the macrophyte was calculated from their biomass following the equation of Fisher and Kelso (2007) for *H. verticillata*: Surface Area = 2390.3618 * Weight$^{0.8362}$.

(2) Zooplankton: Ten litres of depth-integrated water were filtered through a 112 μm net to collect crustacean zooplankton (Copepoda and Cladocera). All zooplankton samples were fixed with Lugol´s solution. Rotifers were quantified using the same samples as phytoplankton. Subsequently, these were identified to the lowest possible taxonomic level and counted under the microscope (OLYMPUS BX53) at 40X of magnification using a 5 mL plankton counting chamber (Zhang & Huang 1991). The biomasses of the zooplankton were estimated by their abundance multiplied by averaged individual wet body mass, according to Zhao (2015).

(3) Benthos: species of larger size gastropods such as the snail *Radix swinhoei* and *Bellamya aeruginosa* were all collected from the mesocosm walls and from the sediment surface, and a second search was performed after 24 hours to make sure large individuals were all collected. Other benthos were quantified according to Brock *et al.* (1992). A metal basket (L*W*H, 15 cm*15 cm*10 cm) with pebbles (1-6 cm sized) was placed on the sediment in each mesocosm at the beginning of the experiment. The baskets were taken out once a month, all pebbles were rinsed to collect all benthos, and then put back to the baskets. All species were identified to the highest taxonomic resolution. We then grouped them into three taxon groups: insects, oligochaeta and other tiny snails (mainly *Bithynia fuchsiana* and *Hippeutis umbilicalis*) in the analysis.

(4) Secondary consumers: All fish and shrimp were caught, counted, and weighted after draining the water from each mesocosm.

Food web analyses

To construct a food web for each mesocosm, we used the data generated from the end of the experiment. Taxa were assembled into 14 functional groups, based on their known trophic position (Table S1), and their biomasses were calculated and expressed as per gram fresh weight per mesocosm. The trophic links in the food webs were established based on dietary data collated from previous studies (Table S2). Food webs were visualized and their properties were calculated using the cheddar package in R (Hudson *et al.* 2013), including mean trophic level, connectance, omnivory, allometric slope (NvM slope), mean link length and mean link angle. Trivariate food webs were visualized using the PlotMvN functions. Mean trophic level was determined using the ShortWeightedTrophicLevel function, which represents the average shortest trophic level of a consumer plus one, and the mean trophic level of all its trophic resources. This metric closely approximates flow-based trophic level, where each link is weighted according to its relative energetic contribution to the diet of a consumer (Williams & Martinez 2004). Connectance was calculated using the DirectedConnectance function, which measures the proportion of realized links in a food web relative to the total possible links. It provides an indication of community sensitivity to perturbations (Dunne *et al.* 2002). Omnivory was quantified using the Omnivory function, which assesses the proportion of species that feed at different trophic levels. This metric provides insights into the trophic flexibility of an ecosystem (Gellner & McCann 2012). The allometric slope (NvM slope) was computed as the scaling coefficient of $\log_{10}(N)$ regressed on $\log_{10}(M)$ for all trophically connected species. A steeper slope may suggest a reduction in top-down effects, as consumers decrease in size and/or abundance relative to their available resources (Woodward *et al.* 2012). Mean link length and mean link angle were calculated using the links data frame obtained from the NvMTriTrophicStatistics function. Link lengths describe the mass-abundance space distance between each consumer and its resources in the food web, while link angles represent the body mass of each consumer relative to its resources.

Statistical analyses

To determine the effects of warming, nutrient loading, insecticide, and their interactions on response variables (biomass of different organisms, food web properties, see Table 1 and Table S3) at the end of the experiment, we employed multiple generalized linear models (glm function in lme4 package) (Bates *et al.* 2015) with Gaussian distributions. Additionally, we utilized multiple linear mixed-effect models (lmer function in lme4 package) (Bates *et al.* 2015) to analyse the main and interactive effects of treatments on water quality parameters (TN, TP, NO_3^-, NH_4^+, PO_4^{3-}, DO, pH, conductivity, and turbidity) and biological parameters (macrophyte PVI, phytoplankton and periphyton chlorophyll a concentrations) during the experiments (Table S4), with sampling date and mesocosm ID included as random factors.

To characterize the type of interactions, we employed a simple classification scheme based on the main effects of both stressors and their interaction, as well as their standardised effect sizes (see Fig. S5 for classification of stressor interaction). The classification of stressor interactions was adapted from previous studies (Crain *et al.* 2008; Piggott *et al.* 2015; Jackson *et al.* 2016; Birk *et al.* 2020; Jackson *et al.* 2020). Initially, a global model was constructed, encompassing all factors and their interactions. The response variables were log or sqrt transformed if normality and homoscedasticity assumptions were not met, as indicated in Table 1, Table S3 and S4. We then conducted stepwise model selection based on the Akaike information criterion (corrected for small sample sizes; AICc), using the MuMIm package in R (Bartoń 2023), selecting the model with the lowest AICc value. The fixed effects (the main effects of both stressors and their interactions) of the final model were evaluated from the standardised partial regression coefficients and their significance (t-test), in the following, referred to as standardised effect sizes (SES, please note that it is not hedge's d). Interactive effects of multiple drivers were considered if the selected model had significant interaction terms. The classification can be summarized as four scenarios, specifically:

(i) If the SES of both main stressors was neutral (not different from zero), the interactive effect was consistently synergistic.

(ii) If the SES of one stressor was positive and the other stressor's SES was either positive or neutral, we determined the interaction effect to be synergistic if the interaction term (I_e) was positive; conversely, if I_e was negative, the interaction effect was regarded as antagonistic.

(iii) If the SES of one stressor was negative and the other stressor's SES was either negative or neutral, we classified the interaction effect as synergistic if I_e was negative, and as antagonistic if I_e was positive.

(iv) For interactions where the two stressors exhibited opposite signs, if the SES of the interaction fell below $-A_e$ or exceeded $-B_e$ (where A_e represents the positive SES of stressor A and B_e represents the negative SES of stressor B), we designated the effect as synergistic; otherwise, it was considered antagonistic.

To better evaluate the biological relevance of our findings, we set the significance level (α) for all tests at 0.1, and the corresponding *p*-values are presented in Table 1 and Tables S3 & S4. Null hypothesis significance testing is widely used in biology despite its often-overlooked issues. Biologists should focus on the biological significance of effect sizes rather than statistical significance (Nakagawa & Cuthill 2007). A 0.1 significance level is suggested for model selection (Nakagawa & Cuthill 2007) and has been applied in interaction classification (Macaulay *et al.* 2021). Given the absence of higher-order interactions (involving three stressors) in our study, we did not specify their types in our analysis. Additionally, to indicate the explanatory contribution of each term in the final model, we calculated partial η^2 values using the effectsize package (Ben-Shachar *et al.* 2020). To test the robustness of our methods, we have also classified the type of interactions using different null models (additive, multiplicative and dominance) (Morris *et al.* 2022) (Fig. S6 & S7). Results showed that most of the interaction types based on our model selection can be captured by at least one of the null models. We thus believe our method can provide strong evidence that multiple stressors can produce synergistic and antagonistic effects on properties of freshwater food webs.

Simple linear models (lm) were used to examine the relationships between food web properties with the number of stressors. To assess model validity of all the linear models, we evaluated residuals for correlations with fitted values and tested for deviations from normality using the Shapiro–Wilk test. If the assumption was not met, data were transformed (log or sqrt) accordingly. Structural equation models were conducted with the lavaan (version 0.6–5) package (Rosseel 2012). All statistical analyses were conducted using R software version 4.3.0 (R Core Team 2023). All data is provided in Dryad (Zhang *et al.* 2024).

Results

Each individual stressor induced significant effects within and across organisational levels, from individual species to food web or ecosystem properties. However, the identity of the sensitive response variables and the magnitude of the effect varied, and interactive effects were commonly detected for multiple stressors. The individual and interactive effects of stressors on food webs at the end of the experiment were all analysed by generalized linear models after model selection.

Specifically, the biomasses of individual taxa (Fig. 2 and Table S3), community (Fig. 3 and Table S3) and food web structure (Fig. 4 and Table 1) were all altered by the three stressors, and both antagonistic and synergistic effects were observed, contingent upon the specific combination of stressors.

Individual effects of warming, nutrient loading, and insecticides

In our experiment, higher trophic level consumers, such as the omnivorous fish and shrimps, were extremely sensitive to warming, with significant reductions in both population biomass (Fig. 2,3, and Table S3) and diversity (Fig. S8 and Table S3) at the top of the food web and community biomass of zoobenthos (Fig. 3 and Table S3). Consequently, warming alone led to a significant decrease in the traditional descriptors of food web complexity: species richness, trophic links, mean trophic level and omnivory (Fig. 4c, Fig. S9, and Table 1). In addition, total biomasses of shrimps, insects, and copepods were all negatively affected by the neonicotinoid insecticide in our study (Fig. 2 and Table S3). Accordingly, we observed a reduced mean trophic level and omivory with the addition of this stressor (Fig. 4c, Fig. S9, and Table 1). Both warming and insecticide exposure alone also resulted in reduced network connectivity within the food webs (Fig. 4d and Table 1). Both nutrient loading and insecticide addition independently led to increased phytoplankton biomass at the base of the food web (Fig. 2 and Table S3). Steeper allometric slopes were also observed under both nutrient loading and insecticide addition (Fig. 4e and Table 1). In addition, the mean link length of the food web became longer under each stressor (Fig. S9 and Table 1), and both warming and insecticide application independently led to an increase in mean link angles (Fig. 4f and Table 1).

Interactive effects of the stressors

Significant interactions between stressors in the food webs were identified, with the type of these interactions depending on the specific combination of stressors. Specifically, warming and insecticide application exhibited antagonistic interactions on mean trophic level, connectance, and mean link angle (Fig. 4c,d,f and Table 1). In contrast to this antagonistic interaction, nutrient loading and insecticide exhibited synergistic effects on the biomasses of phytoplankton (Fig. S10 and Table S4), zoobenthos (Fig. 3 and Table S3), and the ratio of zoobenthos to zooplankton biomass (Fig. 3 and Table S3), as well as on food web allometric slopes (Fig. 4e and Table 1). As the number of stressors increased, various food web attributes displayed directional changes. Specifically, the total biomass of zoobenthos, the biomass ratio of zoobenthos to zooplankton, food web nodes, links, connectance, trophic level, omnivory, allometric slopes, and mean link angles all decreased, while the mean link length increased, indicating a simplification of the food web structure (Fig. 5 & Fig. S11, F and p values of linear models can be found in the figures).

Discussion

Individual effects of warming, nutrient loading, and insecticides

In our experiment, warming reduced both the population biomass and diversity of higher trophic level consumers. This may reflect an inability to meet the increased metabolic demand at warmer conditions (Brown *et al.* 2004) and/or to handle heatwaves exceeding their thermal optima (Peralta-Maraver & Rezende 2020). Consequently, warming alone led to a significant decrease in species richness, trophic links, mean trophic level and omnivory, with herbivorous interactions becoming more dominant, in line with some recent natural experimental studies in geothermal streams (O'Gorman *et al.* 2019; O'Gorman *et al.* 2023). Some macroinvertebrate species (e.g., shrimp, insect, and copepod) are sensitive to the insecticide imidacloprid, and this was reflected in reduced mean trophic level and omnivory with the addition of this stressor. Both warming and insecticide addition, when acting alone, also resulted in reduced network connectance within the food webs, indicating elevated susceptibility to secondary extinctions (Dunne *et al.* 2002; Gilbert 2009) and a dominance of more specialised and stronger links that are often associated with reduced food web stability (Binzer *et al.* 2016).

Nutrient loading and insecticide addition both independently increased phytoplankton biomass at the base of the food web, but via different processes. Excessive nutrient loading (nitrogen and phosphorus) generally induces phytoplankton blooms which can outcompete submerged macrophytes by shading (Phillips *et al.* 2016), whereas insecticide addition led to higher phytoplankton biomass, likely reflecting reduced top-down control by copepods (Dimitri *et al.* 2021) (also shown in Fig. S12 & S13), and a reduction in total zoobenthos biomass (mainly insects, Oligochaeta and Bellamya snail) might release more nutrients to the water column. Similar blooms in benthic algae have been attributed to the suppression of herbivores by pesticides in large riverine food webs (Thompson *et al.* 2016) and are an emerging threat for clear lakes (Vadeboncoeur *et al.* 2021). Steeper allometric slopes under both nutrient loading and insecticide addition due to increases in abundances of smaller organisms were consequently associated wth increased bottom-up or weaker top-down control, respectively (Woodward *et al.* 2012). In addition, the mean link length of the food web increased under each stressor, and both warming and insecticide application independently led to higher mean link angles. These findings suggest a reduction in feeding pressure, as the abundance of resources increased relative to the abundance of their consumers (Fig. S14) (O'Gorman *et al.* 2019). Consequently, these stressors may alter the typical inverted biomass pyramids commonly seen in these freshwater ecosystems, resulting in a more pyramidal shape with a truncated bottom structure, reduced consumer biomasses and lower energy transfer efficiency (McCauley *et al.* 2018; Woodson *et al.* 2018; Barneche *et al.* 2021).

Interactive effects of the stressors

Both synergistic and antagonistic interactions were identified in the food webs, with the type of these interactions depending of the specific combination of stressors. These findings support our first hypothesis. Specifically, warming and insecticide addition exhibited antagonistic interactions on mean trophic level, connectance, and mean link angle. This implies that the presence of the insecticide alleviated the negative effects of warming on these food web properties, or vice versa, which may be attributed to higher temperatures accelerating the decomposition of the insecticide, thus reducing its realised toxicity in the field (Macaulay *et al.* 2021). Furthermore, in our study, warming reduced the population biomass of secondary consumers (carp, bitterling and shrimp, Fig. 2,3 and Table S3), which can all directly graze on macrophytes (Table S2). Similarly, the insecticide led to a reduction in shrimp population biomass, which stimulated the growth of submerged macrophytes (Fig. 2 and Fig. S10 and Table S4). The growth of submerged macrophytes enhanced the habitat quality for aquatic animals, by providing higher oxygen levels (Fig. S15 and Table S4) and structurally complex habitats reducing predation pressure on lower trophic levels (Phillips *et al.* 2016; Thomaz 2023). These might partially alleviate the otherwise negative effects of warming and insecticide on the animals. Combined, warming and insecticide triggered a negative feedback loop via regulating consumer-macrophyte interaction and thus exhibited antagonistic effects on food web structure (Fig. 6a).

In contrast to this nutrients × warming antagonistic interaction, nutrients × insecticide generated a positive feedback loop (Fig. 6b) and thus exhibited synergistic effects on the biomasses of phytoplankton, and zoobenthos, as well as on the whole food web allometric slopes. Specifically, high phytoplankton biomass may have affected the benthic habitat through shading or toxicity (Huisman *et al.* 2018), and elevated mortality of benthic organisms would release more nutrients to the water column (Fig. S16), thereby further boosting the growth of phytoplankton (Scheffer *et al.* 2001; Phillips *et al.* 2016; Mehner *et al.* 2022). Both nutrient loading and insecticide addition promoted the growth of phytoplankton, making the water turbid (Fig. S15), limiting light penetration through the water column, and suppressing the growth of benthic primary producers (Hansson 1988, 1992). Additionally, the presence of insecticides reduced the total biomass of zoobenthos (Fig. 3) and thereby further shifted nutrient and energy flow from benthic to pelagic pathways.

Our results thus support mechanistic explanations for antagonistic or synergistic effects of stressor combinations on food web properties. If two stressors trigger a negative feedback loop in a food web, they are likely to exhibit antagonistic interactions. Conversely, if the two stressors trigger a positive feedback loop, there appears to be a synergistic interaction (Fig. 6). This highlights the danger of simply extrapolating the impacts of individual stressors on single model organisms in the laboratory to the more complex ecosystems in the field, where multiple drivers and responses have a huge potential to attenuate or even reverse such relationships.

In addition to detecting a range of interactions among stressors on food webs, we observed a general simplification of food web structure with an increasing number of stressors. This was evident in the transition from a relatively diffuse, reticulate network to one characterised by fewer and shorter chains (Fig. 5), in line with our second hypothesis. Hence, our findings suggest that under field conditions food webs may be prone to significant simplification in response to an increasing number of stressors, as multiple stressors are generally expected to drive directional changes in ecosystems (Rillig *et al.* 2019; Rillig *et al.* 2023). In addition, we observed a shift in energy transfer from the benthic to pelagic pathway with an increasing number of stressors. This could also have further implications at the whole-ecosystem level, as detrital pathways are mostly benthic and donor-controlled, whereas pelagic systems are dominated more by classical consumer-resource dynamics and hence stronger interactions that tend to reduce food web stability (Rooney & McCann 2012).

Caveats and implications

Nearly all ecosystems are subject to multiple stressors due to human activities, and their interactions will depend on their combination, intensity, timing, and overall impact (Jackson *et al.* 2021; Ostrowski *et al.* 2022). The three stressors we selected represent a small fraction of those operating in nature, and different stressors, applied in different modes, intensities, or locations, may yield different outcomes. Nonetheless, vanishingly few studies to date have even considered more than two stressors across multiple organisational levels (Orr *et al.* 2024). As such, our findings have broad implications, including those independent of the specific stressors involved here. For instance, the discovery that combined impacts on negative or positive feedback loops in food webs could trigger antagonistic or synergistic effects, respectively, is an important new insight since these particular modes and mechanisms of stressor modulation have been effectively overlooked, but could be commonplace in nature. Our experiment focused on larger organisms and their interactions within the food webs, which meant that bacterial, picoplankton, microzooplankton and other organisms were not included directly in our analysis. The challenges involved in quantifying the microbial component are non-trivial and these groups are still largely treated as unresolved "black boxes" in most food web research to date. Future monitoring techniques overcoming these challenges would provide a more comprehensive understanding on the full scope of response of aquatic system to multiple stressors.

For the construction and analysis of the food web, species feeding interactions were based on observational data from published sources rather than direct measurements in our particular system. While we acknowledge that this approach may not be the most ideal, it is *de rigeur* in the vast majority of trait-based and food web research and was the most practical given the scale of the experiment and the considerable time and labour required for maintenance, sampling, and analysis. Relying on observed trophic interactions reported in the literature, combined with directly observed organism biomass and abundance data, is a recommended method in most modern food web studies, especially given the time-intensive nature of sampling and risk of under sampling in diet analysis (Woodward *et al.* 2010; Borrelli *et al.* 2023). Future studies should aim to optimize experimental design and incorporate direct measurements where feasible, whilst recognising that complete direct observation of entire individual food webs *in situ* will rarely, if ever, be feasible in large scale multispecies field experiments and literature-based network characterisations may continue to provide useful complementary information.

Shallow lakes and ponds represent the most abundant freshwater ecosystems on Earth (Verpoorter *et al.* 2014), yet anthropogenic activities are currently degrading both their biodiversity and key ecosystem functions at an alarming rate (Hilt *et al.* 2017; Janssen *et al.* 2021). We show here that multiple anthropogenic stressors, acting in concert, jointly simplify food web structure and reduce energy transfer from lower to higher trophic levels (Nagelkerken *et al.* 2020). Furthermore, multiple stressors induce a shift in energy flow from benthic to pelagic pathways, thereby potentially destabilising food webs through shifting energy transfer to "greener" pathways (Rooney & McCann 2012), and also increasing the risk of shifting from submerged macrophyte-dominated clear-water states to phytoplankton-dominated turbid conditions (Mehner *et al.* 2022).

The conclusions from our study are specifically important since they provide a more realistic and common scenario, including several human-induced stressors acting simultaneously. Our results demonstrate that different pairs of stressors could mediate both negative and positive feedback loops within food webs more widely, leading to antagonistic and synergistic interactions, respectively. Such interactions between stressors, if they are indeed the rule rather than the exception in nature, complicate our (still limited) ability to predict ecosystem responses to multiple stressors (Birk *et al.* 2020). To predict ecosystem responses accurately in face of multiple global change factors, we will need to embrace and grapple with this potential reality by considering both the scope for masking effects of different stressors and signal attenuation among the response variables as complexity increases. This raises particular challenges for regulation, conservation or restoration efforts, as removing one stressor might not yield the desired ecological recovery that might be expected, due to the huge potential, but largely overlooked, range of redundancy and compensatory mechanisms that may be embedded within natural food webs.

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Table 1 Effects of treatments on food web properties at the end of the experiment. The best generalized linear models are presented after model selection based on AICc values. The signs denote the direction of the significant standardised partial regression coefficients (SES), with "+" representing a positive effect and "-" representing a negative effect. Significant SES are emphasised with p-values in bold. The values in brackets represent partial η^2, indicating the contribution of each variable to the explained variance in the final model. For interactive terms, "A" indicates antagonistic effects, while "S" indicates synergistic effects.

Category	**Food web properties**	**W**	**N**	**I**	**N*I**
Trivariate	NvM slope		- **0.011** (0.28)	0.128 (0.43)	- **0.056** (0.08)(S)
	Mean link length	+ **<0.001** (0.36)	+ **0.016** (0.12)	+ **0.011** (0.14)	
	Mean trophic angle	- **<0.001** (0.34)		- **0.004** (0.05)	
Topology	Nodes	- **0.005** (0.16)			
	log(Links)	- **<0.001** (0.33)			
	Connectance	- **<0.001** (0.45)		- **0.005** (0.07)	
	log(Trophic level)	- **<0.001** (0.30)		- **<0.001** (0.15)	
	log(Omnivory+0.1)	- **<0.001** (0.31)		- **0.005** (0.17)	

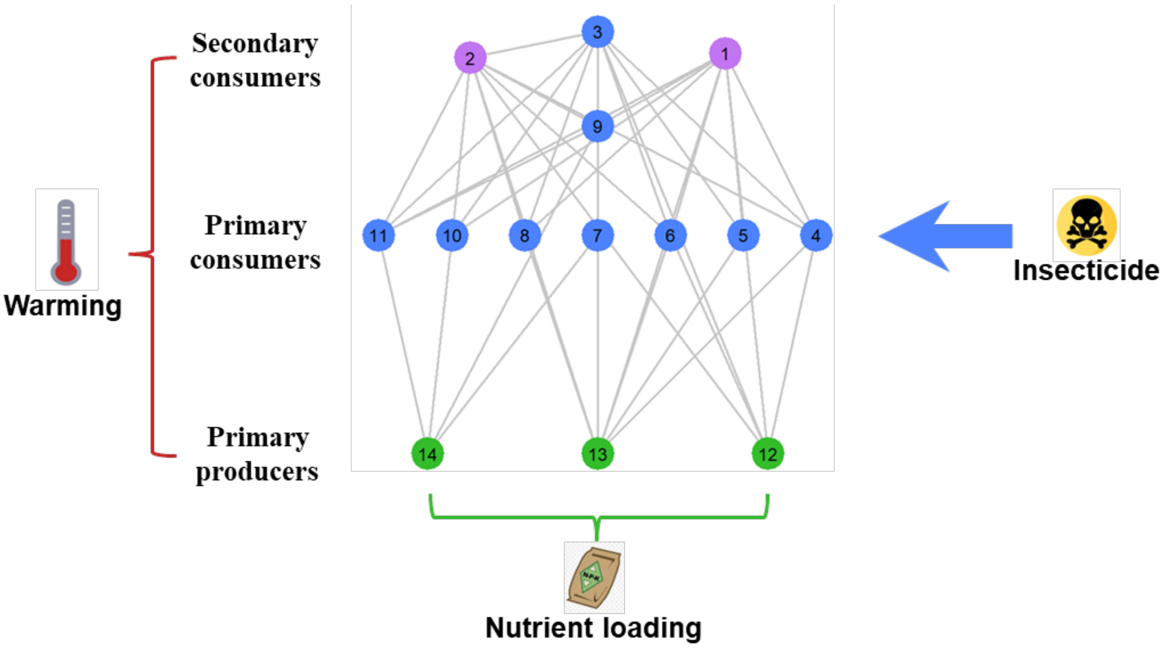

Figure 1 Expected impacts of multiple stressors on the different components in the food web. We expected that warming would affect metabolic rates of all organisms in the food web, nutrient loading would directly affect the composition of primary producers, and insecticide would negatively affect some invertebrate consumers. Organisms are labelled as follows: 1. Crucian carp, 2. Bitterling, 3. Shrimp, 4. Insecta, 5. Oligochaeta, 6. Snail Radix, 7. Snail Bellamya, 8. Other tiny snails, 9. Copepoda, 10. Cladocera, 11. Rotifers, 12. Macrophytes, 13. Periphyton, 14. Phytoplankton.

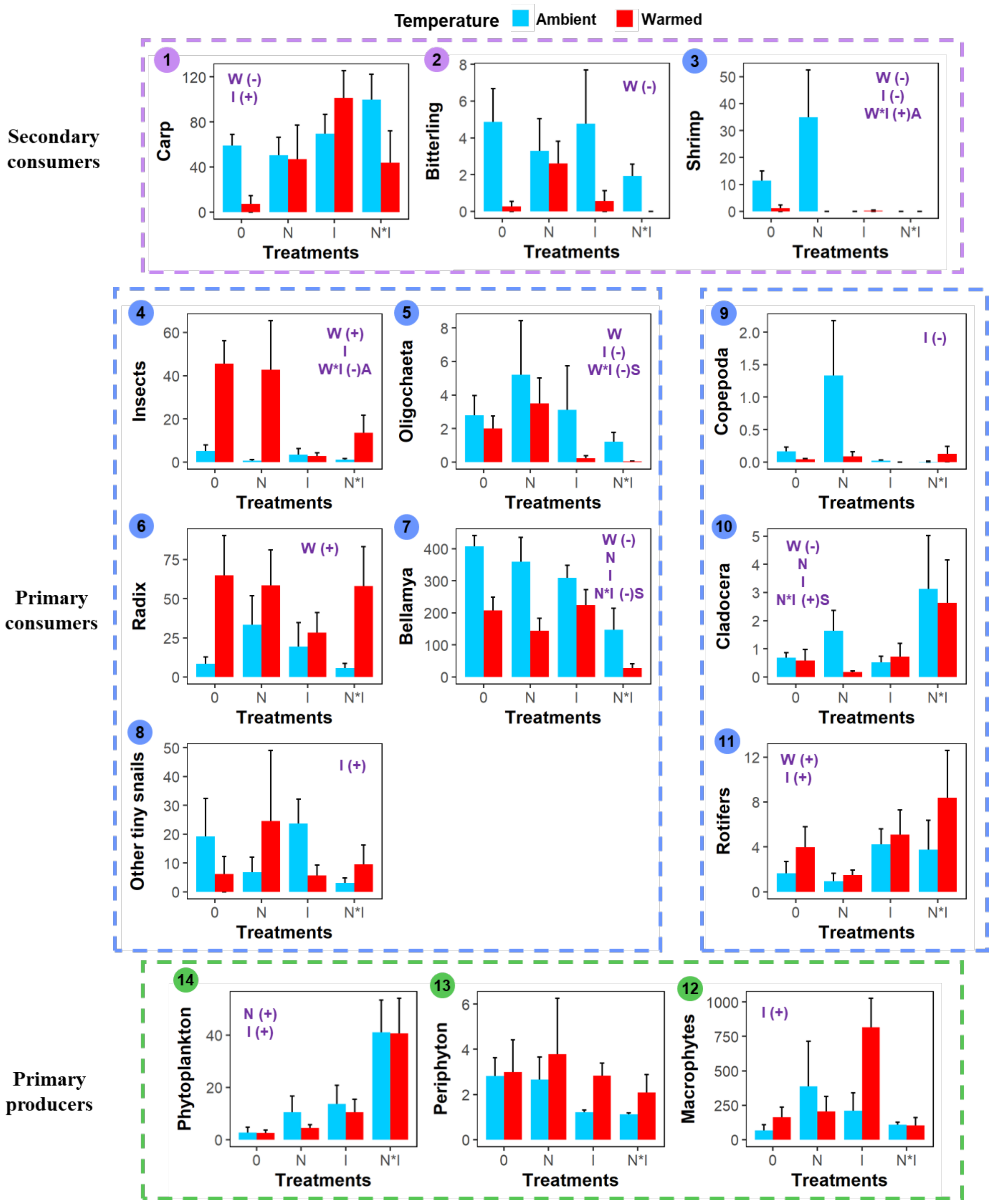

Figure 2 Detected impacts of multiple stressors on the biomass of different components in the food web. At the bottom are primary producers, at the middle-left are zoobenthos, at the middle-right are zooplankton, and on the top are secondary consumers. The unit is the same for all the response variables, gram per mesocosm. The labelled numbers are the same with Figure 1. Treatments are indicated as follows: 0 (control), W (warming), N (nutrient loading), and I (insecticide). Bars indicate means ± standard errors for each treatment. The final selected variables after model selection are displayed in each subplot, with significant directions indicated by "+" (positive) or "-" (negative). For interactive terms, "A" indicates antagonistic effects, while "S" indicates synergistic effects. Detailed statistical results can be found in Table S3.

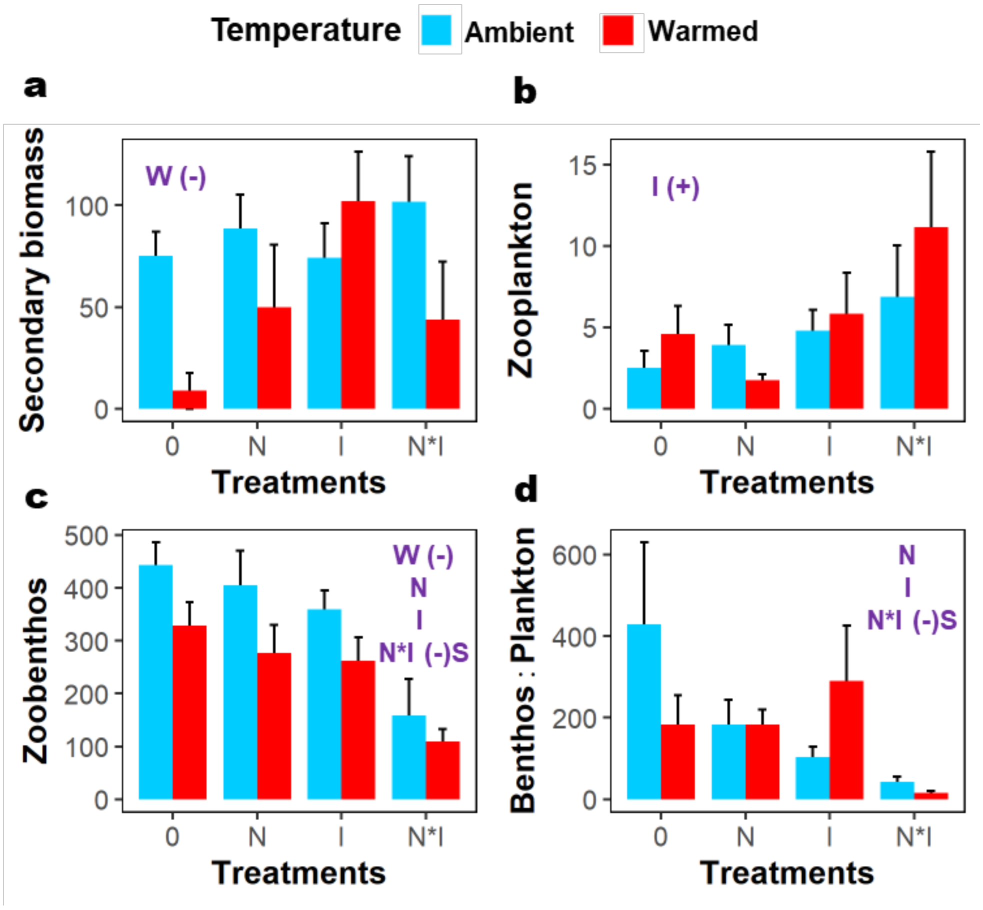

Figure 3 Secondary species biomass (fish and shrimp), total zooplankton biomass, total zoobenthos biomass, and ratio of zoobenthos to zooplankton in different treatments. 0 (control), W (warming), N (nutrient loading), and I (insecticide). The unit is the same for all the biomasses, gram per mesocosm. Bars represent means ± standard errors for each treatment. The final selected variables after model selection are displayed in each subplot, with their significant directions indicated by "+" (positive) or "-" (negative). For interactive terms, "A" indicates antagonistic effects, while "S" indicates synergistic effects.

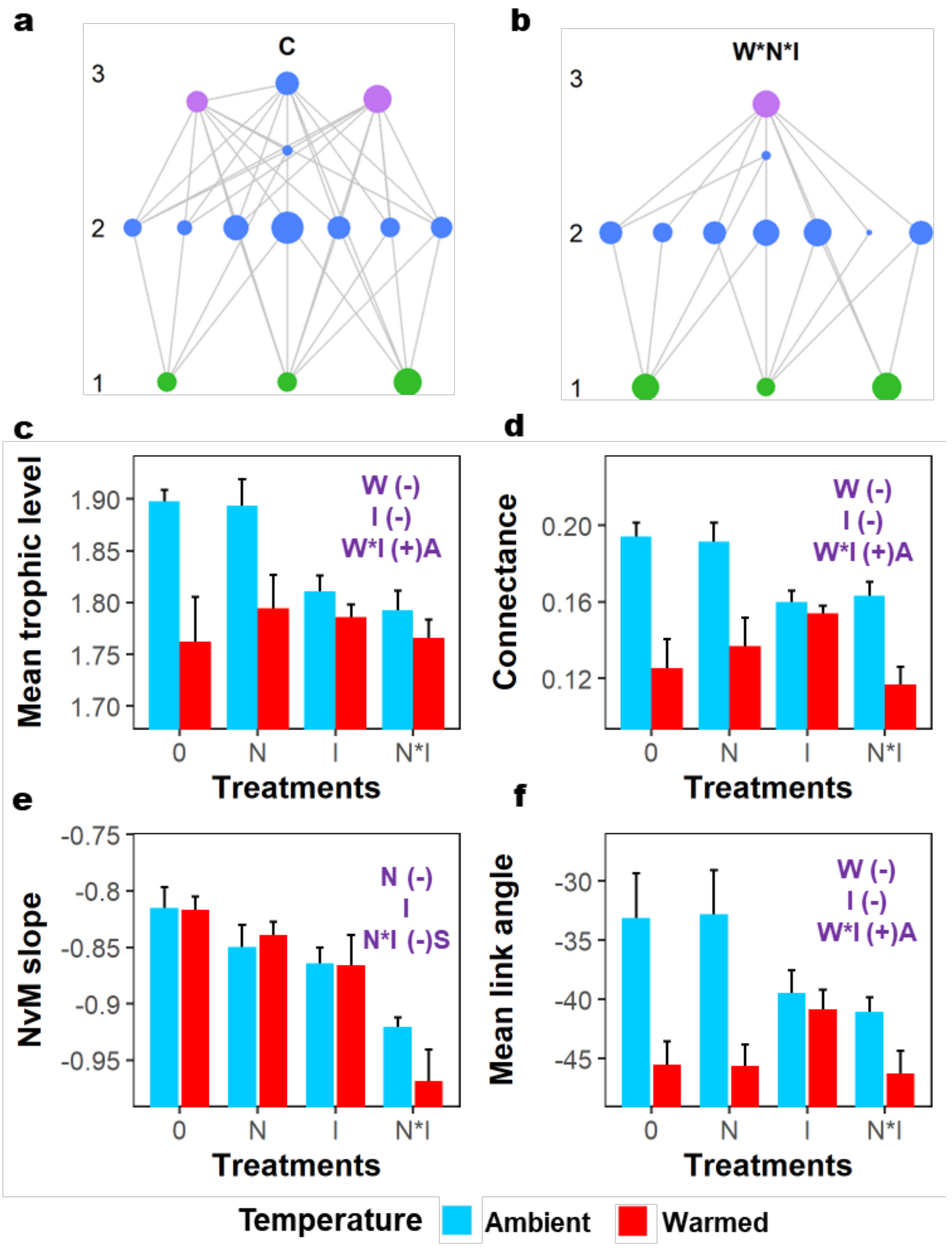

Figure 4 Effects of single stressors and their combinations on food web properties. Trivariate food webs for the control (a) and with all stressors applied (b), based on the summed web data from six replicate mesocosms for each treatment. Area of the circle size is proportional to biomass of the taxonomic group, and grey lines depict feeding interactions. Primary producers are denoted by green circles, invertebrates by blue circles, and fish by purple circles. Treatments are represented as follows: C (control), W (warming), N (nutrient loading), and I (insecticide). Effects of treatment on food web mean trophic level (c), connectance (d), allometric (NvM) slope (e), and mean link angle (f). Bars indicate means ± standard errors for each treatment. The final selected variables after model selection are displayed in each subplot, with significant directions indicated by "+" (positive) or "-" (negative). For interactive terms, "A" indicates antagonistic effects, while "S" indicates synergistic effects. Detailed statistical results can be found in Table 1.

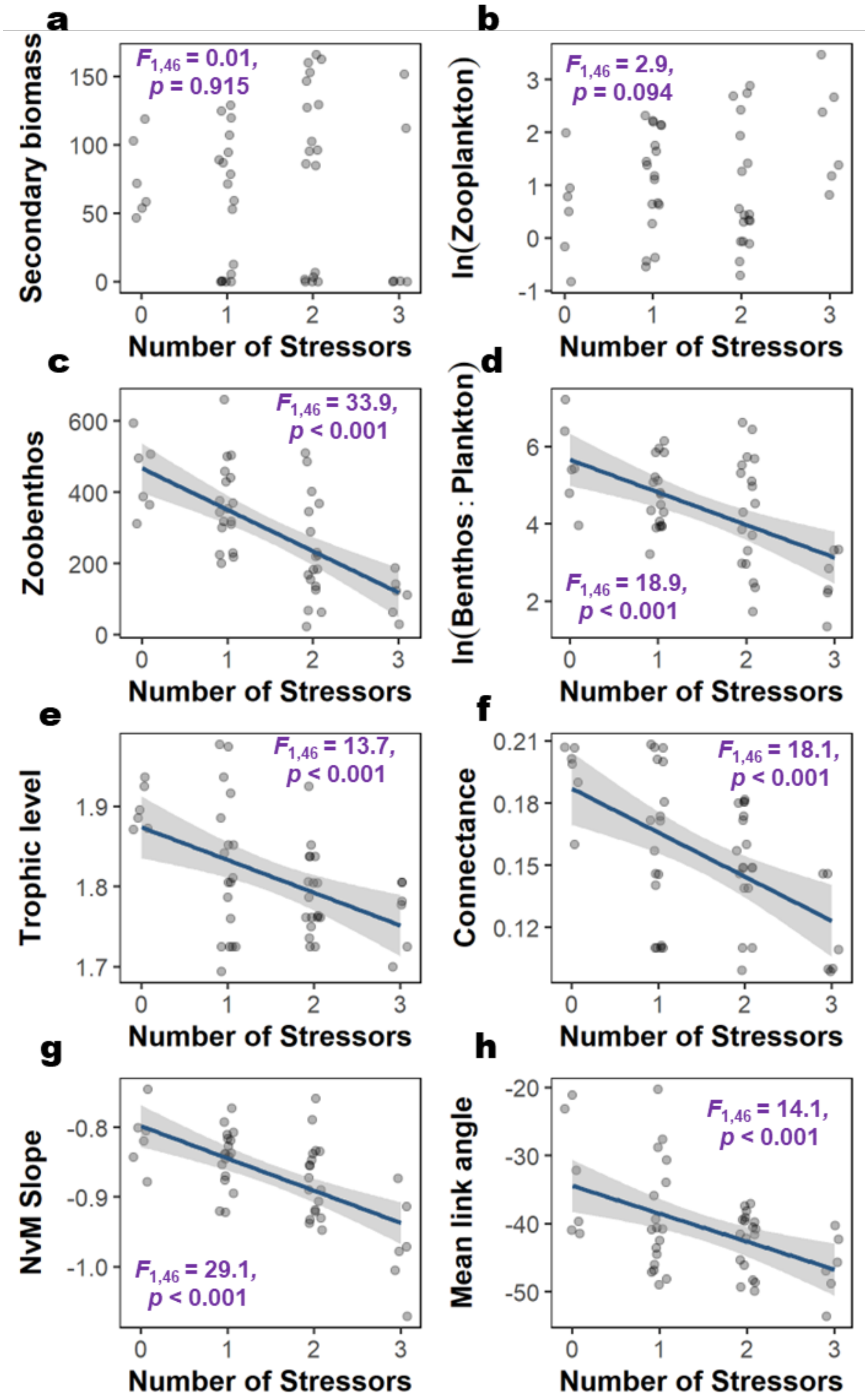

Figure 5 Relationships between food web properties and the number of stressors. Significant regressions are represented by bold lines, and corresponding *F* and *p*-values are indicated in each subplot.

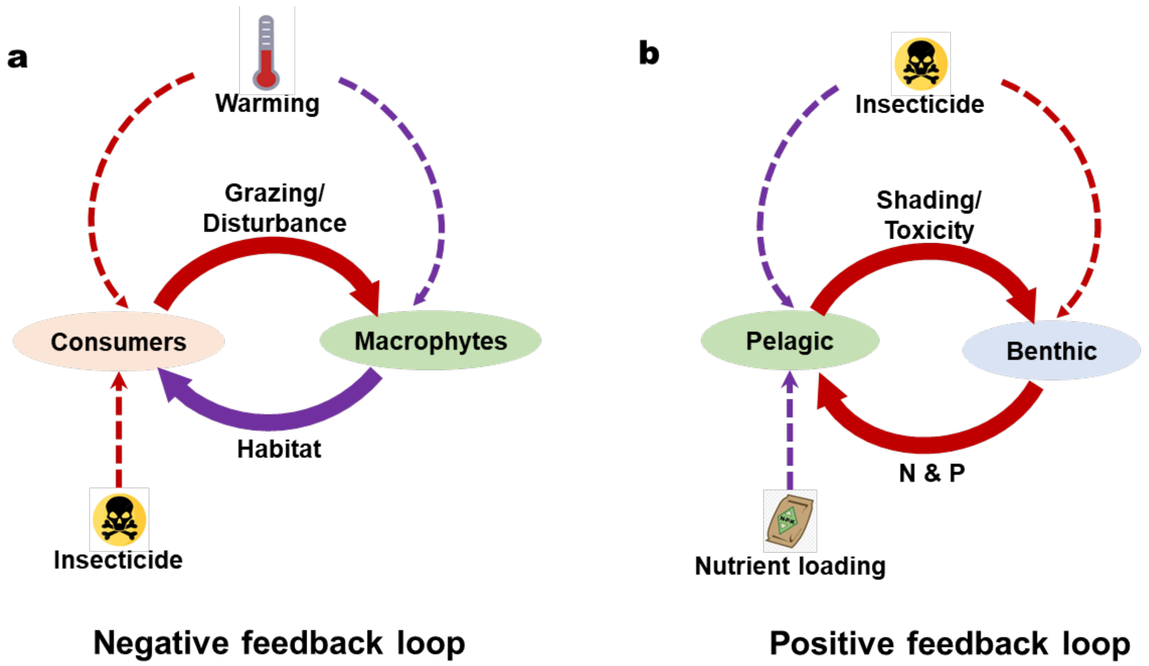

Figure 6 Stressors interactively generate antagonism and synergism in the food web through negative (a) and positive (b) feedback loops (bold arrows), respectively. Dashed arrows indicate the impacts of stressors (red: negative, purple: positive) on the components in the loops. **Negative feedback loop**: some consumers negatively affect submerged macrophytes through direct grazing or indirect disturbance resulting in the loss of their habitat, thus alleviating their negative impacts on macrophytes. **Positive feedback loop**: excessive growth of phytoplankton in the pelagic habitat, supported by nutrient loading and insecticides inhibiting zooplankton, inhibit the development of benthic habitat through shading or toxicity on benthic organisms, the loss of benthic organisms will result in more nutrients release to water columns, thus further boosting the growth of phytoplankton.