Doctoral program in Hydrology and Water Resource Management

MULTIPLE STRESSORS EFFECTS ON COMMUNITY STABILIY

Francesco Polazzo
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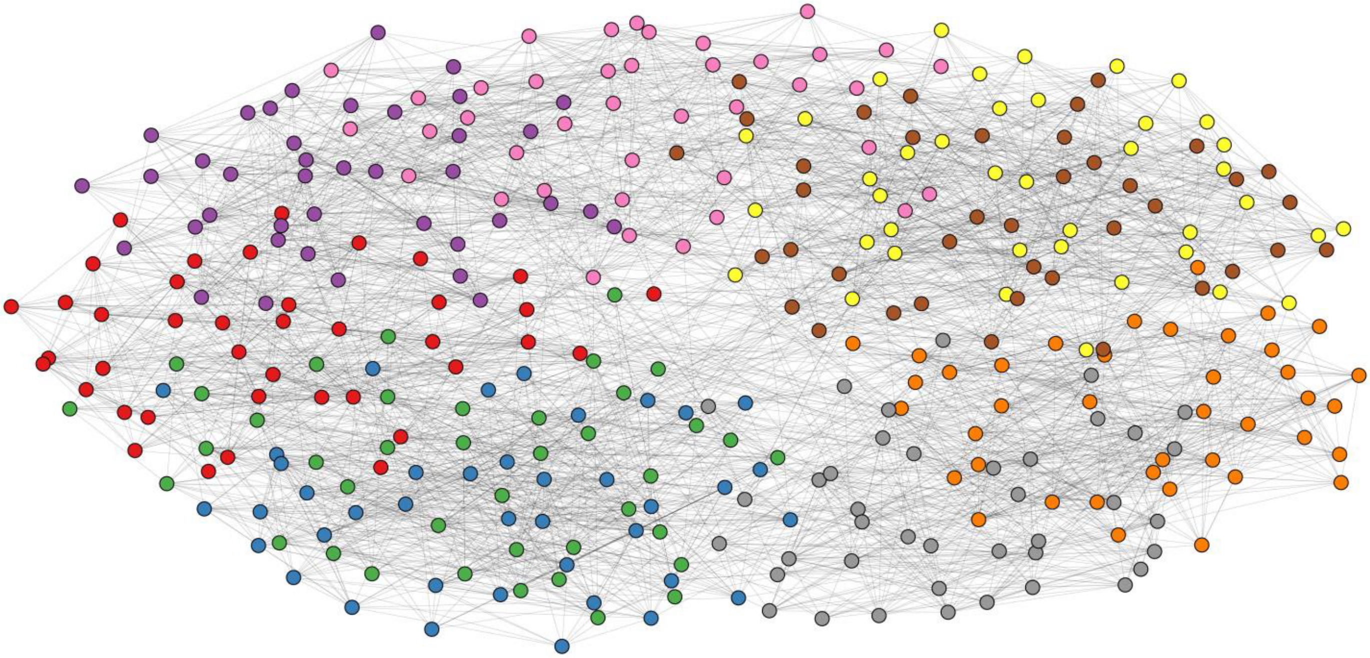

Universidad de Alcalá

Hydrology and Water Resource Management

Multiple stressors effects on community stability

Doctoral Thesis presented by

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To Elisa and to my family

“Not all those who wander are lost.”

J.R.R. Tolkien, *The Fellowship of the Ring*

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Contents

Summary

Humans are posing increasing pressure upon natural ecosystems. Given the variety of disturbances that anthropogenic activities generate (e.g., climate change, chemical contamination, invasive species, land use changes), it is unrealistic to think that only one stress factor will affect the environment at a given time. Contrasting with this simple assumption, most of the ecological research has traditionally studied the effects of one stressor at the time on different response variables. Only in the last decade the investigation of multiple stress factors impairing simultaneously the environment has become more common. Yet, most of the studies assessing the combined effects of multiple stressor have only measured the responses of single species in isolation or even isolated individuals. Additionally, multiple stressors research has focused on measuring the effects of interacting stressors at a single time point. By doing so, such studies disregarded two fundamental ecological processes. First, stressors' interactions are temporal scale dependent, meaning that non-additivity between stressor might appear only at a late stage, and thus measuring only one time-point reduces the chances of detecting non-additive effects. Second, describing the response of a biological system to a disturbance through only one observation does not provide information on how the response changes over time, on how the system responds after the application of the stress (its resistance), and on whether and how it recovers. Yet, describing the response dynamics of complex ecological systems exposed to multiple stressors is the first step in understanding and identifying general response patterns. Knowing and identifying general response patterns to multiple stressor will allow the formulation of accurate predictions, and, thus, of efficient protection measures.

The aim of this thesis is to explore, assess, and implement new approaches to mechanistically understand the individual and combined effects of anthropogenic stressors on the stability of freshwater communities and ecosystems.

This thesis begins with a literature review of the existing knowledge of the combined effects of extreme climatic events (e.g., heatwaves) and micropollutants on freshwater ecosystems (Chapter 2). This Chapter also reviews common approaches to study individual and combined effects of multiple stressors, highlights knowledge gaps and provides indications for a better integration of extreme climatic events into multiple stressors research. Generally, the literature review found only few studies that investigated the biological effects of HWs in combination with chemical pollution. The reported interactive effects of HWs and chemicals varied largely not only within the different trophic levels but also depending on the studied endpoints for populations or individuals. Hence, owing also to the little number of studies available, no consistent interactive effects could be highlighted at any level of biological organization. Moreover, it found an imbalance towards single species and population experiments, with only few studies using a multitrophic approach. This results in a knowledge gap for relevant community and ecosystem level endpoints, which prevents the exploration of important indirect effects that can compromise food web stability.

Chapter 3 evaluates the single and combined effects of two pesticides of widespread global use (chlorpyrifos and diuron) under different nutrient regimes (oligotrophic and eutrophic) on community structure and ecosystem functions in replicated pond mesocosms. The individual application of nutrients and pesticides affected community composition and species richness. Ecosystem functioning was generally less sensitive to chemical stress than community structure, while eutrophication fostered the dominance of species that are more resilient to pesticides. Stressor interactions were significant at different time points, with late stressor interactions affecting the recovery of community composition. Additionally, the correlation between biodiversity and relevant ecosystem functions, such as primary productivity and total ecosystem respiration, can be shifted from positive to negative under particular stress conditions. This Chapter demonstrates that nutrients enrichment is a key factor influencing the resilience of freshwater ecosystems to multiple stressors and that functional redundancy allows maintaining constant levels of functioning even under high toxic stress pressure.

Chapter 4 evaluates the effects of the agricultural stressors studied in Chapter 3 on four dimensions of community stability (resistance, resilience, recovery, and invariability) and on the overall dimensionality of stability (DS). Functional recovery and resilience to pesticides were enhanced in nutrient-enriched systems, whereas compositional recovery was generally not achieved. Pesticides did not affect compositional DS, whereas functional DS was significantly increased by the insecticide only in non-enriched systems. Stressor interactions acted non-additively on single stability dimensions as well as on functional DS. Moreover, Chapter 4 demonstrates that pesticides can modify the correlation between functional and compositional aspects of stability. This study shows that different disturbance types, and their interactions, require specific management actions to promote ecosystem stability.

Chapter 5 uses again the dataset described in Chapter 3 to assess the capacity of ecological network analyses to elucidate the mechanisms driving long-term community-composition dissimilarity and late-stage disturbance interactions at the community level. It was found that changes in interactions' strength are strongly related to compositional changes, and post disturbance interaction strength rewiring has been identified as responsible for most of the observed compositional changes. Additionally, pesticides interactions were found to be significant in the long term only when both interaction strength and food-web architecture were reshaped by the disturbances. Overall, this work suggests that quantitative network analysis has the potential to unveil ecological processes that prevent long-term community recovery.

Chapter 6 uses the results of another outdoor pond mesocosm experiment to study the effects of extreme events (heatwaves) on the complexity of realistic freshwater ecosystems using topological and quantitative trophic network metrics. Next, changes in network complexity are linked with the investigation of four stability components (temporal stability, resistance, resilience, and recovery) of community's functional, compositional, and energy fluxes stability. Reduction in topological network complexity was found to be correlated with reduction of functional and compositional resistance. However, temperature-driven increase in link-weighted network complexity increased functional and energy fluxes recovery and resilience, but at the cost of increased compositional instability. Overall, Chapter 6 proposes an overarching approach to eviscerate the effects of climate change on multidimensional stability through the lens of network complexity, providing helpful insights for preserving ecosystems stability under climate change.

Finally, in Chapter 7 the overall results of the various studies that compose this thesis are discussed, and avenues for future research are indicated.

Resumen

Los seres humanos ejercen una presión cada vez mayor sobre los ecosistemas naturales. Estas presiones incluyen, por ejemplo, el cambio climático, la contaminación química, la introducción de especies invasoras, o los cambios en el uso del suelo. Dada la gran variedad de presiones que ejercemos sobre los ecosistemas, no es realista pensar que los impactos sobre la biodiversidad se deban únicamente a un factor de estrés. Sin embargo, la mayoría de los estudios que han evaluado la respuesta de los ecosistemas naturales a los impactos antrópicos se han centrado en un factor de estrés particular. Solo en la última década se ha vuelto más común la investigación de las perturbaciones humanas sobre el medio ambiente evaluando un gran número de factores de estrés de forma simultánea. Dichos trabajos, se han centrado en evaluar el impacto de estos factores sobre una especie o un grupo de individuos de forma aislada, en un momento concreto, ignorando por tanto algunos aspectos fundamentales del ecosistema. En primer lugar, no tienen en cuenta que las interacciones de los factores de estrés múltiple dependen de la escala temporal, y que podríamos apreciar respuestas no aditivas si muestreamos los ecosistemas en diferentes momentos. En segundo lugar, al basarse en una única observación, no entran a valorar cómo responde el sistema después de la aplicación del estrés (su resistencia), y si y cómo el ecosistema se recupera. La evaluación de la respuesta de los sistemas ecológicos formados por varias especies a los factores de estrés múltiple a lo largo del tiempo es el primer paso para comprender e identificar patrones generales de respuesta. Además, el conocer e identificar dichos patrones permitirá ampliar nuestra capacidad de predecir las respuestas biológicas y nos ayudará a establecer medidas de protección medioambiental eficientes.

Esta tesis se centró en evaluar el conocimiento existente y proponer nuevos métodos para comprender la estabilidad de las comunidades y ecosistemas acuáticos a los factores de estrés antropogénicos de manera individual y combinada.

La tesis comienza con una revisión bibliográfica sobre los efectos combinados de los eventos climáticos extremos (p. ej., olas de calor) y los microcontaminantes sobre los ecosistemas de agua dulce (Capítulo 2). Este Capítulo destaca las lagunas del conocimiento actual y proporciona indicaciones para una mejor la integración de los eventos extremos en la investigación sobre los factores de estrés múltiple. Además, esta revisión demuestra que, en general, existen solo unos pocos estudios que investigaron los efectos biológicos de las olas de calor en combinación con la contaminación química. Los trabajos

disponibles muestran que los efectos interactivos entre las olas de calor y los contaminantes variaron en gran medida, no solo dentro de los diferentes niveles tróficos, sino también en individuos y poblaciones. Por tanto, no se pudieron identificar patrones claros de respuesta para ningún nivel de organización biológica. Además, se encontró que el número de estudios que evaluar la respuesta en varios niveles tróficos son muy escasos. Esto ha impedido la exploración de efectos indirectos importantes que puedan comprometer la estabilidad de las redes tróficas.

El Capítulo 3 evalúa los efectos de dos pesticidas (clorpirifos y diurón) y las condiciones de eutrofización del sistema (mesotróficos y eutróficos) sobre la estructura de la comunidad acuática, y sobre las funciones del ecosistema usando mesocosmos. La aplicación individual de nutrientes y pesticidas afectó a la composición de la comunidad y a la riqueza de especies. El funcionamiento del ecosistema fue generalmente menos sensible al estrés químico que la estructura de la comunidad, mientras que la eutrofización fomentó el predominio de especies que son más resistentes a los pesticidas. Las interacciones entre los factores evaluados fueron significativas en diferentes momentos del experimento, y se observó que, en el largo plazo, la interacción entre los factores de estrés impidió la recuperación de la composición de la comunidad. Además, el signo de la correlación entre la biodiversidad y algunas funciones clave del ecosistema, como la productividad primaria y la respiración total, se vio alterado bajo algunas condiciones de estrés específicas. Este capítulo demuestra que el enriquecimiento de nutrientes es un factor clave que influye en la resiliencia de los ecosistemas de agua dulce ante múltiples factores de estrés y que la redundancia funcional permite mantener niveles constantes de funcionamiento, incluso bajo una alta presión de estrés por la contaminación.

En el Capítulo 4 se utilizaron los datos obtenidos en el experimento anteriormente descrito para evaluar el impacto de los factores de estrés estudiados sobre los cuatro parámetros que definen la estabilidad del ecosistema (resistencia, resiliencia, recuperación e invariabilidad), así como la dimensionalidad de dichos factores. Se observo como la recuperación funcional y la resiliencia a los pesticidas mejoraron en los sistemas enriquecidos con nutrientes, mientras que la recuperación de la composición generalmente no se logró. Los pesticidas no afectaron a la dimensionalidad de la estabilidad del sistema en lo que se refiere a composición de la comunidad acuática, mientras que la dimensionalidad de los parámetros funcionales aumentó significativamente con el insecticida en los sistemas no enriquecidos con nutrientes. La interacción entre los factores

de estrés fueron no aditivas respecto a los parámetros que definen la estabilidad del ecosistema, así como la dimensionalidad de la estabilidad funcional. Finalmente, este capítulo demuestra que los plaguicidas pueden modificar la correlación existente entre los parámetros que definen la estabilidad funcional y composicional. Este estudio concluye que los diferentes tipos de perturbaciones y sus interacciones requieren acciones de manejo específicas para promover la estabilidad del ecosistema.

El Capítulo 5 también utiliza los datos obtenidos en el experimento de mesocosmos descrito anteriormente para tratar de dilucidar los mecanismos que impulsan la disimilitud entre la composición de las comunidades acuáticas en el largo plazo usando redes tróficas. En este capítulo se demostró que los cambios en la intensidad de las interacciones entre las especies están relacionados con los cambios en la composición de la comunidad y que los factores de estrés evaluados influyen sobre la reorganización de estas interacciones. Además, se muestra como las interacciones entre los pesticidas son significativas en el largo plazo solo cuando se ha modificado la fuerza de las interacciones y la arquitectura de la red trófica. En general, el Capítulo 5 concluye que el análisis cuantitativo de redes tróficas tiene un gran potencial para revelar los procesos ecológicos que impiden la recuperación de las comunidades acuáticas en el largo plazo.

El Capítulo 6 usa los resultados de otro experimento de mesocosmos para analizar los efectos de los eventos extremos (olas de calor) sobre la estructura de la comunidad acuática utilizando redes tróficas basadas en métricas topológicas y cuantitativas. Además, los cambios en la estructura de la red se asocian a los cuatro componentes principales de la estabilidad del ecosistema (estabilidad temporal, resistencia, resiliencia y recuperación), calculados en base a datos funcionales, estructurales y de flujos de energía para la comunidad. Se encontró que la reducción en la complejidad de la red topológica está intrínsecamente correlacionada con la reducción de la resistencia funcional y composicional. Sin embargo, el aumento de complejidad estructural de la red impulsado por la temperatura aumentó la recuperación y la resiliencia de los flujos de energía y funcionales, a costa de una mayor inestabilidad de la composición estructural de la comunidad. En general, el Capítulo 6 propone un nuevo enfoque para evaluar los efectos del cambio climático en la estabilidad del ecosistema en base al análisis de redes tróficas, brindando información útil para la preservación de estos.

Finalmente, en el Capítulo 7 se discuten los resultados generales de los diversos estudios que componen esta tesis y se hacen propuestas para futuras investigaciones.

Riassunto

Le attività antropiche esercitano una pressione sempre maggiore sugli ecosistemi naturali. Data la varietà di fattori impattanti generati dall’uomo, come il cambiamento climatico, la contaminazione chimica, le specie invasive, il cambio dell’uso del suolo, è irrealistico pensare che solo un fattore di stress stia compromettendo l’ambiente in uno specifico momento. In contrasto con questa semplice constatazione, la maggior parte della ricerca ecologica ha tradizionalmente studiato gli effetti di un fattore di stress alla volta su diverse variabili di risposta. Solo nell'ultimo decennio lo studio di molteplici fattori di stress che danneggiano simultaneamente l'ambiente è diventato più comune. Tuttavia, la maggior parte degli studi che valutano gli effetti combinati di molteplici fattori di stress hanno misurato solo le risposte di singole specie o di singoli individui isolati. Inoltre, la maggior parte della ricerca sui fattori di stress multipli ha misurato solo gli effetti di multipli fattori di stress in un singolo momento. Tali studi, infatti, hanno ignorato due processi ecologici fondamentali. In primo luogo, le interazioni dei fattori di stress cambiano con il tempo, il che significa che la non additività tra i fattori di stress potrebbe apparire solo in una fase avanzata, e quindi misurare un solo punto temporale riduce le possibilità di rilevare effetti non additivi. In secondo luogo, descrivere la risposta di un sistema biologico a un fattore di disturbo attraverso una sola osservazione non fornisce informazioni di come la risposta cambi nel tempo, di come il sistema risponda dopo l'applicazione dello stress (la sua resistenza) e di se e come esso recuperi. Tuttavia, descrivere le dinamiche di risposta di complessi sistemi ecologici esposti a molteplici fattori di stress è il primo passo per comprendere e identificare i pattern di risposta generali. Conoscere e identificare i pattern di risposta generali a molteplici fattori di stress consentirà la formulazione di previsioni accurate e, quindi, di misure di protezione efficienti. Lo scopo di questa tesi è di esplorare, valutare e implementare nuovi approcci per comprendere meccanicamente gli effetti individuali e combinati dei fattori di stress antropogenici sulla stabilità delle comunità e degli ecosistemi d’acqua dolce.

Questa tesi inizia con una revisione della letteratura sulle conoscenze esistenti degli effetti combinati di eventi climatici estremi (es. ondate di calore) e microinquinanti sugli ecosistemi di acqua dolce (Capitolo 2). Questo capitolo esamina anche gli approcci più comuni per studiare gli effetti individuali e combinati di più fattori di stress, evidenzia le lacune di conoscenza e fornisce indicazioni per una migliore integrazione di eventi climatici estremi nella ricerca degli impatti antropici. In generale, la revisione della letteratura ha

trovato solo pochi studi che hanno investigato gli effetti biologici delle ondate di calore in combinazione con l'inquinamento chimico. Gli effetti interattivi riportati tra onde di calore e sostanze chimiche variavano ampiamente non solo all'interno dei diversi livelli trofici, ma anche a seconda delle variabili di risposta studiate per popolazioni o individui. Quindi, anche a causa del numero limitato di studi disponibili, non è stato possibile evidenziare effetti interattivi coerenti a qualsivoglia livello dell'organizzazione biologica. Inoltre, è stato riscontrato uno squilibrio verso studi focalizzati su singole specie o popolazioni, con solo pochi studi che hanno incluso lo studio di più livelli trofici. Ciò si traduce in delle lacune di conoscenza per gli endpoint pertinenti a livello di comunità e ecosistema, che impedisce l'esplorazione di importanti effetti indiretti che possono compromettere la stabilità della rete alimentare.

Il capitolo 3 valuta gli effetti singoli e combinati di due pesticidi di uso diffuso (chlorpyrifos e diuron) in diversi regimi di nutrienti (mesotrofici ed eutrofici) sulla struttura della comunità e sulle funzioni ecosistemiche usando mesocosmi come sistemi sperimentali. L'applicazione individuale di nutrienti e pesticidi ha influito sulla composizione della comunità e sulla ricchezza delle specie. Il funzionamento dell'ecosistema è risultato generalmente meno sensibile allo stress chimico rispetto alla struttura della comunità, mentre l'eutrofizzazione ha favorito il predominio di specie più resistenti ai pesticidi. Le interazioni dei fattori di stress sono risultate statisticamente significative in momenti diversi. In particolare, interazioni tardive tra i pesticidi hanno rallentato il recupero della struttura della comunità. Inoltre, la correlazione tra la biodiversità e le funzioni ecosistemiche, come la produttività primaria e la respirazione totale dell'ecosistema, può essere spostata da positiva a negativa in particolari condizioni di stress. Questo capitolo dimostra che l’aumento della concentrazione dei nutrienti è un fattore chiave che influenza la resilienza degli ecosistemi di acqua dolce a molteplici fattori di stress e che la ridondanza funzionale consente di mantenere livelli di funzionamento ecosistemico costanti anche in condizioni di elevata pressione di stress tossico.

Il Capitolo 4 valuta gli effetti dei fattori di stress agricoli studiati nel Capitolo 3 su quattro dimensioni della stabilità ecologica (resistenza, resilienza, recupero e invariabilità) e sulla dimensionalità complessiva della stabilità (DS). Il recupero funzionale e la resilienza ai pesticidi sono stati favoriti dall’alta concertazione di nutrienti nei sistemi eutrofici, mentre il recupero della composizione generalmente non è stato raggiunto. I pesticidi non hanno influenzato il DS in termini di composizione della comunità, mentre il DS funzionale è stato significativamente aumentato dall'insetticida solo nei sistemi oligotrofici. Le interazioni dei fattori di stress hanno agito in modo non additivo sulle singole dimensioni di stabilità e sul DS funzionale. Inoltre, il Capitolo 4 dimostra che i pesticidi possono modificare la correlazione tra aspetti funzionali e compositivi della stabilità. Questo studio mostra che diversi tipi di disturbo e le loro interazioni richiedono azioni di gestione specifiche per promuovere la stabilità dell'ecosistema.

Il Capitolo 5 utilizza nuovamente il set di dati descritto nel Capitolo 3 per valutare la capacità delle analisi della rete ecologica di chiarire i meccanismi che guidano la dissimilarità a lungo termine nella composizione della comunità e le interazioni tardive tra fattori di stress a livello di comunità. È stato riscontrato che i cambiamenti nella intensità delle interazioni sono fortemente correlati ai cambiamenti della composizione e la ridistribuzione della forza delle interazioni tra specie dopo l'applicazione dei fattori di stress è stato identificato come responsabile della maggior parte dei cambiamenti compositivi osservati. Inoltre, le interazioni dei pesticidi sono risultate significative a lungo termine solo quando sia la forza delle interazioni che l'architettura della rete alimentare vengono rimodellate dai fattori di stress. Nel complesso, questo lavoro suggerisce che l'analisi quantitativa della rete trofica ha il potenziale di spiegare i processi ecologici che impediscono il recupero della comunità a lungo termine.

Il capitolo 6 utilizza i risultati di un altro esperimento di mesocosmi per studiare gli effetti di eventi estremi (onde di calore) sulla complessità di ecosistemi d'acqua dolce utilizzando metriche di rete trofica topologiche e quantitative. Successivamente, i cambiamenti nella complessità della rete sono collegati all'indagine di quattro componenti di stabilità (stabilità temporale, resistenza, resilienza e recupero) della stabilità funzionale, compositiva e dei flussi energetici della comunità. La riduzione della complessità topologica della rete è risultata correlata alla riduzione della resistenza funzionale e compositiva. Tuttavia, l'aumento della complessità della rete ponderato in base alla temperatura ha aumentato il recupero e la resilienza dei flussi di energia e funzionali, ma a scapito di una maggiore instabilità compositiva. Nel complesso, il Capitolo 6 propone un nuovo approccio per sviscerare gli effetti del cambiamento climatico sulla stabilità ecologica attraverso la lente della complessità della rete trofica, fornendo utili informazioni per preservare la stabilità degli ecosistemi durante il cambiamento climatico.

Chapter 1

General introduction

1. Multiple stressors

Anthropogenic activities are increasingly threatening biodiversity and ecosystems globally. Human- induced impacts, such as climate change, pollution, habitat loss and degradation, are progressively putting pressure on the natural environment (Millennium Ecosystem Assessment 2005). Additionally, all these anthropogenic pressures are becoming more intense as well as more widespread worldwide (Vörösmarty *et al.* 2010). Traditionally, ecologists and environmental scientists have tended to study the effects of each of these human-related stressors in isolation, where “stressors” are here defined as all the various biotic or abiotic factors that can disrupt the structure of an ecosystem, community, or population, and change resource availability or the physical environment (White & Pickett 1985). Yet, in nature, multiple stressors acting in concert are the norm, rather than the exception. Consistently, in the last decade, an increasing amount of research has been focusing on trying to understand the effects of multiple stressors across different levels of biological organisation (i.e., from individuals to ecosystems). Studying, but especially predicting, the effects of multiple stressors acting simultaneously has proven to be particularly challenging. This difficulty mainly derives from the fact that stressors’ interactions may result in effects larger than (synergism) or smaller than (antagonism) those one may expect from the sum of the effects of the single stressors (addition). Meta-analysis summarising available research on multiple stressors impacts have highlighted that non-additive effects (i.e., different from the sum of their individual impacts) are common (Crain *et al.* 2008; Jackson *et al.* 2016; Birk *et al.* 2020), and have shown specific cases in which synergistic interactions may occur. However, antagonism has been identified as the most common type of interaction between two stressors by several meta-analyses (Jackson *et al.* 2016; Tekin *et al.* 2020).

Given our limited understanding of stressors interactions, investigating the mechanisms driving non-additive effects of multiple stressors is a priority in ecological research. Indeed, expanding our knowledge on multiple stressors interactions at different levels of biological organization and at multiple spatial scales will eventually enable us to produce meaningful expectations on the overall pressure exerted on ecosystems, and to set efficient protection measures.

Freshwater ecosystems are extremely vulnerable to environmental stressors, among other factors, because they are embedded within a largely terrestrial landscape, that contributes to their fragmentation. Freshwaters are biodiversity hotspots, and play a crucial role in providing essential ecosystem services, involving drinking water, food, flood prevention, and recreational opportunities (Maasri *et al.* 2022). Therefore, multiple interacting stressors represent pressing threats to both natural systems and human societies (Mantyka-Pringle *et al.* 2014; Maasri *et al.* 2022). Freshwater ecosystems are exposed to a large variety of stressors, ranging from land-use change to alteration of hydrological properties, to chemical pollution. Generally, stressors can be divided into two main categories: pulse and press stressors. Pulse stressors are characterized by a sudden onset and are limited in time. Typical examples of pulse stressors are heatwaves, hurricanes, and chemical spills. Contrary, press stressors exert a continuous pressure on the environment. Classical press stressors are global warming, eutrophication, and ocean acidification. Among the several stressors affecting freshwater ecosystems, eutrophication has been recently highlighted as an urgent problem, being responsible for the alteration of the ecological status of almost 35% of European freshwaters (Lemm *et al.* 2020), and has been recognised as a key threat to freshwater biodiversity (Donohue *et al.* 2009). Alongside with eutrophication, chemical contamination has also been found to limit the overall status of European rivers (Posthuma *et al.* 2020). Despite the increasing annual application of pesticides (Food and Agriculture Organization of the United Nations – FASOSTAT, 2020), and the rising amount of pharmaceuticals and personal care products traced in freshwater ecosystems (Wilkinson *et al.* 2022), chemical contamination is still only partially considered as a primary stressor driving global change.

Although being particularly vulnerable to environmental stressors and extremely valuable for humanity, our knowledge on the combined effects of multiple stressors on freshwater ecosystems is still limited, with current understanding mostly restricted to a few model organisms and a little number of stressor combinations (Jackson *et al.* 2016). Particularly, the combined effects of multiple stressors on multi-species assemblages are poorly characterised, despite being the prevalent scenario in natural systems (Jackson *et al.* 2018). The reasons why we lack mechanistic understanding of multiple stressors effects at the community and ecosystem level are several, and range from the logistic difficulties of running large-scale experiments, to the absence of methods enabling mechanistic investigations of stressors' effects in multi-species assemblages. Additionally, understanding the effects of one single stressors across a wide range of species (horizontal extrapolation), as well as upscaling effects from one single endpoint (single species/population) to higher levels of biological organization (vertical extrapolation), are challenges that impairs our comprehension of stressors' interactions.

2. Extreme Events

Global climate change is one of the most pervasive anthropogenic disturbances to our planet (IPCC 2021). In the last century, the global surface temperature has risen by roughly 1°C, and projections indicate that it might increase an additional 1.5 – 4.5°C by the end of the century (IPCC 2021). Following the need to understand all possible implications of climate change for ecological systems, ecological research has been focusing for the last two decades almost exclusively on the effects of increased mean temperature (Jentsch *et al.* 2007; Thompson *et al.* 2013). Climate warming has been shown to have profound implications for the great majority of biological mechanisms and ecological processes, and is expected to be one of the main forces shaping future ecosystems (Woodward *et al.* 2010).

Yet, several other phenomena compose climate change. Among the multiple aspects of climate change, extreme weather events, such as heatwaves, droughts, floods, and hurricanes are receiving growing attention. An extreme climatic or weather event is defined as the occurrence of a "value of a weather or climate variable above (or below) a threshold value near the upper (or lower) ends ('tails') of the range of observed values of the variable" (Seneviratne *et al.* 2012). Extreme events are rising concerns as they have been predicted to become more frequent as well as more severe in the nearby future (Woolway *et al.* 2021).

Extreme climatic events have been found to have severe detrimental effects on freshwater ecosystems. For example, a summer heatwave in 2003 caused a drastic mortality, and consequent abrupt reduction in species richness of benthic macroinvertebrates in a French river (Mouthon & Daufresne 2006). Heatwaves have also been shown to slow down fundamental river functions, such as litter break down and benthonic microalgae biomass production (Correa-Araneda *et al.* 2020; Ross *et al.* 2021). As much as extreme climatic events, also extreme hydrological events have the potential to alter both the structure and the functioning of freshwater ecosystems. Intense drought events have been revealed to cause alteration in the composition, body size and trophic structure of experimental rivers' invertebrate assemblage (Aspin *et al.* 2019). On the other side of the spectrum, floods are also known to cause notable changes in river's macroinvertebrates assemblages, which might require years to recover (Woodward *et al.* 2015).

Despite the accumulating evidence reporting profound detrimental effects of extreme events on freshwater ecosystems, extreme events have only recently started to be considered by ecological research. Information and understanding of extreme events' effects on ecological systems is still scarce. Moreover, extreme weather events have not yet been incorporated in multiple stressors studies. The few studies that have tested the combined effects of extreme events in combination with additional stressors are extremely limited, and have only investigated low levels of biological organisation (i.e., individuals or populations) (Dinh *et al.* 2016; Verheyen & Stoks 2020). The projected increase in frequency and intensity of extreme events in the future, together with the increasing pressure exerted by human activities on the environment, call for urgent investigation of the mechanisms driving the combined impacts of extreme events in a multiple stressors' context. Only by disentangling the single and joint effects of extreme events and additional stressors we may be able to develop and employ efficient protection measures.

3. Ecological network approach to study community-level effects under stress

Most ecological studies assessing the effects of single or multiple stressors measure direct impacts of disturbance on meaningful biological endpoints, such as abundance, biomass, composition, or diversity of target organisms. This has contributed to the provision of invaluable information for the management and restoration of disturbed ecosystems. However, the vast majority of these studies, by adopting this approach, completely overlook some fundamental characteristics of communities and ecosystems. This is, organisms in communities are interlinked by biotic interactions, forming complex ecological networks. Biotic interactions are relevant for ecosystems undergoing disturbance because they mediate the indirect effects of a stressor at the community level. The intensity and the organisation of biotic interactions within an ecological networks are the result of abundance, biomass, and identity of interacting species, which may all be impacted by stressors. Hence, stress-driven alterations in biomass or abundance in one trophic level or in a population might influence the structure and the interaction strength of the whole network (Paine 1980; Bartley *et al.* 2019). Single and multiple stressors can, thus, alter the structural properties of the network, such as the number and type of interactions, as well as the interaction's strength (O’Gorman *et al.* 2012; Kortsch *et al.* 2021). Consequently, disturbances may cause the “topological rewiring” of an ecological network, a concept describing the changes in network’s topology by removal or addition of nodes (i.e., species) and links (i.e., species interactions). Yet, stressors might also determine “interaction-strength rewiring”, a notion describing the changes in the interactions’ strength of interconnected species resulting from density or biomass variation in populations composing the network (Blanchard 2015; Kortsch *et al.* 2015; Bartley *et al.* 2019).

At present, there has been no experimental attempt trying to investigate the effects of multiple stressors on realistic species assemblages using unweighted (considering only species presence/absence) and weighted (also considering interactions’ strength) network properties. Nevertheless, studying stressors interactions through the lens of ecological network analysis may shed light on community-level processes that risk being otherwise overlooked, limiting our understanding of how stressors jointly impact communities and ecosystems. Particularly, quantitative network properties (that is, weighted properties) have the potential to mechanistically explain the processes driving the combined effects of multiple stressors at high levels of biological organisation. Indeed, weighted metrics are sensitive to changes in biomass of the species forming the network, and the consequent changes in interactions strength. Profound alterations in interactions strength, in turn, cause significant changes in quantitative network properties which can be translated into shifts in community composition or ecosystem functioning. Overall, studying how disturbance(s) modify the distribution and intensity of the interaction’s strength in the network, as well as the structure of the network, may notably advance our understanding of disturbance effects on community processes and ecosystem functioning.

4. Ecological stability

Understanding the factors determining the stability of ecological communities and ecosystems has been a major point of discussion among ecologists for decades (MacArthur 1955; May 1972; Allesina & Tang 2012). Ecological stability is of paramount importance, as it underpins the ability of communities and ecosystems to absorb, withstand, and recover multiple types of environmental change. Understanding ecological stability is key to develop knowledge about how ecosystems respond to stress. Since understanding is the first step in the way of making predictions, expanding our comprehension of how ecosystems’ stability is impacted by multiple disturbances underlies our ability to protect them. Indeed, enhancing stability of populations, communities and ecosystems has been highlighted as one of the main goals of environmental managers and policy makers (Donohue *et al.* 2016).

One of the main challenges that has hampered the advancement of our understanding of how stability is influenced by disturbance is that stability may be defined in different ways (Pimm 1984). Ecological stability can be defined as asymptotic stability, resilience, resistance, recovery, persistence, and variability (Donohue *et al.* 2013). The existence of such a rich variety of definitions has made comparison between studies rather difficult. Additionally, a gap exists between theoretical and empirical studies. The firsts usually focus on asymptotic stability (May 1972; Allesina & Tang 2012), a mathematical concept based on a binary assumption where communities are either stable or unstable respect to an equilibrium point. Conversely, empiricists typically measure stability as the temporal variability of some aggregate metric of populations or communities (e.g., coefficient of variation of community biomass).

Donohue *et al.* (2013), in a seminal paper, overcame the challenge posed by the multiple possible definition of stability proposing a framework able to consider simultaneously all the different aspects of stability. They suggested that stability is composed of several aspects, or “dimensions”, and that only considering all of them, scientists may be able to eviscerate the mechanisms determining ecological stability. The “multidimensional” conception of stability contrasts profoundly with the historically one-dimensional approach that has been dominating ecological studies for more than fifty years. Indeed, a literature review revealed that almost 90% of the studies investigating stability in ecological systems only evaluated the response of one stability’s dimension, usually in a species poor setting (< than 10 species) (Kéfi *et al.* 2019). Ever since this overarching approach was proposed, some studies have started to assess how multiple stability dimensions respond to disturbances of different type (Hillebrand *et al.* 2018b; Radchuk *et al.* 2019; White *et al.* 2020). Overall, these studies have evidenced that multiple stability metrics are indeed necessary to depict and understand all possible response aspects of stability. Finally, the framework proposed by Donohue *et al.* (2013) explores the correlation between the different dimensions of stability. The study shows that when two stability components correlate strongly and positively, they are basically one-dimensional. Oppositely, if the two stability dimensions do not correlate, they are essentially independent, and describe different response aspects. If two stability components correlate negatively, though, it means that a trade-off exists between the two components. This is, one stability component cannot be enhanced without reducing the other. Obviously, this has profound implication for management and conservation, as it forces regulators to privilege one stability component over another. Therefore, protecting communities and ecosystems requires the investigation of the mechanisms driving the response aspects of several stability components in face of multiple stressors, stressors type, and intensity. Only in this way, we might be able to unveil the processes determining the response of the different stability dimensions and how they relate on each other.

5. Research objectives and scope

This thesis aims to improve our understanding on how single and multiple anthropogenic stressors related to global change affect the composition and the functioning of freshwater communities, their network organisation, and multiple aspects of their stability. Moreover, it provides novel insights on how to mechanistically investigate the effects of disturbances on ecological stability through a quantitative ecological network approach.

The specific research objectives of this thesis are:

1. To critically evaluate the state of the knowledge regarding the combined effects of key extreme events (i.e., heat waves) and micropollutants on freshwater ecosystems, with a particular emphasis on identifying knowledge gaps.
2. To evaluate the effects of multiple agricultural stressors on freshwater ecosystems. Specifically, investigating the role of community structure, trophic status, and biodiversity-functioning on ecosystem responses.
3. To assess whether the dimensionality of ecological stability, and the different components that form it, is affected by the interaction among multiple stressors.
4. To assess the potential of ecological network approaches (i.e., unweighted and weighted) to explain long-term compositional dissimilarity after pulse perturbations and late stressor interactions on freshwater communities.
5. To understand how extreme climatic events can influence trophic network complexity and community multidimensional stability of freshwater ecosystems, and the relationship among these two.

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The ECORISK2050 project brings together a world leading and interdisciplinary consortium of universities, research institutes, industry, and regulatory and governmental authorities to deliver a cohort of early stage researchers (or PhDs) that become experts on assessing global change impacts on freshwater ecosystems. The research objectives of the project are: (a) to assess how the inputs of chemicals from agriculture and urban environments and their fate and transport are affected by different environmental conditions, including those of specific EU regions, and how this will change under global change scenarios in order to assess the likely increase in chemical risks to human and ecosystem health, (b) to identify potential adaptation and mitigation strategies that can be implemented in the short and medium term, to abate unacceptable changes in risks, and use the global change scenarios to propose robust implementation pathways, and (c) to develop a set of tools for use by industry and policy makers, that allow the impacts of a range of global change related drivers on chemicals risks being assessed and managed.

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Chapter 2

Combined effects of heatwaves and micropollutants on freshwater ecosystems: towards an integrated assessment of extreme events in multiple stressors research

Francesco Polazzo, Sabrina K. Roth, Markus Hermann, Annika Mangold-Döring, Andreu Rico, Anna Sobek, Paul J. Van den Brink, Michelle C. Jackson

Abstract

Freshwater ecosystems are strongly influenced by weather extremes such as heatwaves (HWs), which are predicted to increase in frequency and magnitude in the future. In addition to these climate extremes, the freshwater realm is impacted by the exposure to various classes of chemicals emitted by anthropogenic activities. Currently, there is limited knowledge on how the combined exposure to HWs and chemicals affects the structure and functioning of freshwater ecosystems. Here, we review the available literature describing the single and combined effects of HWs and chemicals on different levels of biological organization, to obtain a holistic view of their potential interactive effects. We only found a few studies (13 out of the 61 studies included in this review) that investigated the biological effects of HWs in combination with chemical pollution. The reported interactive effects of HWs and chemicals varied largely not only within the different trophic levels but also depending on the studied endpoints for populations or individuals. Hence, owing also to the little number of studies available, no consistent interactive effects could be highlighted at any level of biological organization. Moreover, we found an imbalance towards single species and population experiments, with only five studies using a multitrophic approach. This results in a knowledge gap for relevant community and ecosystem level endpoints, which prevents the exploration of important indirect effects that can compromise food web stability. Moreover, this knowledge gap impairs the validity of chemical risk assessments and our ability to protect ecosystems. Finally, we highlight the urgency of integrating extreme events into multiple stressors studies and provide specific recommendations to guide further experimental research in this regard.

1. Introduction

Multiple anthropogenic disturbances drive the so-called global change (Schlesinger 2006), which is leading to various, and still unpredictable, changes in biodiversity, species interactions and ecosystem functioning (Cardinale *et al.* 2012). Among the several drivers of global change, Climate change is one of the key and most concerning ones (Parry *et al.* 2001). Climate change is composed of different processes and phenomena (IPCC 2013), including extreme weather events such as heavy precipitations or heatwaves (HWs; Jentsch *et al.* 2007). HWs are characterized by a short-term, rapid increase in temperature, which can impact all trophic levels, from microorganisms (Szymczak *et al.* 2020) to large predators (de Mira-Mendes *et al.* 2019), across all ecosystem types (Stillman 2019). HWs are of particular concern because their magnitude and frequency are predicted to increase in the future (Meehl & Tebaldi 2004; Woolway *et al.* 2021). A growing amount of literature has described the effects of HWs on freshwater ecosystems (Maazouzi *et al.* 2008; Huber *et al.* 2010; Ledger & Milner 2015; Bartosiewicz *et al.* 2016; Weisse *et al.* 2016; Woodward *et al.* 2016) and showed that they can lead to high mortality rates (Mouthon & Daufresne 2006; Strydom *et al.* 2020). This is particularly relevant for ectotherm animals (Brown *et al.* 2004; Cereja 2020), which represent the vast majority (>95%) of species in aquatic ecosystems (Willmer et al., 2000). Temperature directly controls the metabolic rate of cells and their size (Gillooly *et al.* 2001). It also affects carbon allocation (García-Carreras *et al.* 2018), population growth (Savage *et al.* 2004), carrying capacity (Fussmann *et al.* 2014) and ecosystem respiration (Yvon-Durocher *et al.* 2010). Generally, ectotherm plants and animals show increased metabolism but reduced size at elevated temperature (Brown *et al.* 2004; Yvon-Durocher *et al.* 2011; Zohary *et al.* 2021).

Extreme weather events are not the only disturbances affecting aquatic ecosystems. Among the large number of stressors affecting the freshwater realm (Birk *et al.* 2020), a serious, yet somewhat overlooked, dimension of global change is chemical pollution (Rockström *et al.* 2009; Steffen *et al.* 2015; Mazor *et al.* 2018). A review by Bernhardt *et al.* (2017) showed that chemicals are only partially considered as drivers of global change. Yet, the global annual application of pesticides grows constantly (Food and Agriculture Organization of the United Nations—FAOSTAT, 2020), and pharmaceuticals and personal care products are increasingly traced in freshwater ecosystems worldwide (Ebele *et al.* 2017; Danner *et al.* 2019). Micropollutants reaching water bodies act as selective stressors targeting different organisms based on their physicochemical properties and toxicological mode of action, leading to non-random effects on communities (De Laender *et al.* 2016). Synthetic chemicals are known to reduce diversity (Bray *et al.* 2019) cause trophic interaction shifts (Schrama *et al.* 2017), affect ecosystem functioning (Spaak *et al.* 2017), and have been found to limit the overall ecological status of European rivers (Posthuma *et al.* 2020). Heatwaves are predicted to increase more in their frequency, duration, and intensity particularly in spring and summer (Woolway *et al.* 2021). Spring and summer are the seasons when higher amounts of pesticides are generally applied (Phillips & Bode 2004; Scheyer *et al.* 2007), whereas the emission of other micropollutants (e.g., pharmaceuticals and metals) is less time-bound (Ebele *et al.* 2017; Danner *et al.* 2019). Surface water contamination by micropollutants has already been reported worldwide (Hughes *et al.* 2013; Sharma *et al.* 2019). Additionally, pesticide application (Kattwinkel *et al.* 2011) and pharmaceutical consumption is predicted to rise (Hughes *et al.* 2013). Shallow aquatic systems, such as shallow lakes, ponds, ditches, intermittent rivers and streams are expected to be the most impacted from the combined exposure of HWs and chemicals. In larger freshwater ecosystems, the concentration of chemicals may be lower due to dilution, whereas they are expected to experience less intense HWs due to their large thermal inertia (Woolway *et al.* 2021).

Multiple stressors research has received increasing attention in recent years (Orr *et al.* 2020), following the need for a more realistic and comprehensive assessment of the multiple drivers of global change across the different levels of biological organization. However, most research on climate change in a multiple stressor context focuses on warming under constant elevated temperature regimes (Piggott *et al.* 2015a, c; Arenas-Sánchez *et al.* 2019a). The significance of increasing temperature on freshwater biota has been recognized long ago (Schindler 1997), and the effects of elevated and constant temperatures have been largely studied in isolation (Döll *et al.* 2018) but occasionally also in combination with chemical stressors (Heugens *et al.* 2001; Holmstrup *et al.* 2010). Similarly, the relevance of extreme weather events as drivers of detrimental effects on biological systems was recognized more than a decade ago (Jentsch *et al.* 2007). Since then, scientists have made calls to drive the attention on extreme weather events, rather than on trends (Thompson *et al.* 2013; Woodward *et al.* 2016), and extreme temperature fluctuations have been shown to pose a greater risk to alter species performance than elevated mean temperature (Vasseur *et al.* 2014). Yet, we still lack a comprehensive understanding of the combined effects of extreme weather events and other stressors, such as chemical pollution, as well as of the mechanisms underpinning those effects in environmentally realistic species assemblages.

Understanding the combined effects of HWs and chemicals is pressing not only because they can interact resulting in effects larger then (synergism) or smaller than (antagonism) additive effects but also because their interactions are known to be dependent on the application order (Dinh *et al.* 2016). The toxicity of many micropollutants may increase for organisms previously exposed to warming, following the “climate change induced toxicant sensitivity” (CITS) concept (Hooper *et al.* 2013; Moe *et al.* 2013). Conversely, micropollutants can reduce the heat tolerance of organisms, according to the “toxicant induced climate change sensitivity” (TICS) concept (Hooper *et al.* 2013; Moe *et al.* 2013). Consequently, disentangling the processes causing these effects requires systematic testing of different exposure orders at different levels of organization.

The aim of this work was to critically evaluate the state of knowledge on the combined effects of HWs and chemicals with different physicochemical properties and toxicological modes of action, highlighting knowledge gaps. Here, we first develop predictions on how HWs and micropollutants may affect and interact on multiple endpoints spanning different organism groups and levels of organization (Figure 1). Then, we review laboratory, semi-field and field studies assessing the effects of HWs alone and in combination with micropollutants on different trophic levels of freshwater ecosystems and used a food web approach to identify possible indirect effects that may be propagated across the different trophic levels. Finally, we provide recommendations for a better integration of HWs into multiple stressor's research and chemical risk assessment.

2. Materials and methods

We performed a scoping review (Munn *et al.* 2018) and discussed the available information regarding the impact of HWs alone and in combination with micropollutants on different trophic levels. This allowed us to focus on the combined effects of HWs and the class of micropollutants that is expected to have the largest direct impact on the trophic level under investigation. This included, antibiotics for bacteria, herbicides for primary producers (phytoplankton, cyanobacteria and macrophytes), and insecticides for arthropods (micro- and macro-crustacea and insects) and all of the above for vertebrate predators. We defined HW following The World Meteorological Association as “five or more consecutive days of prolonged heat in which the daily maximum temperature is higher than the average maximum temperature by 5°C (9°F) or more”. We also include a maximum based on

Woolway *et al.* (2021), who projected maximum HWs of 27 days in the future (IPCC scenario RCP 2.6). These criteria were used to select studies, and we report HW's characteristics in parentheses in the main text (i.e., duration, C: temperature of controls [reported only for laboratory studies], H: treatment temperature). Many of the studies we included aimed to investigate the effects of climate change but, because they used sudden increases of temperature larger than 5°C, they fit our criteria of a HW (rather than the more gradual temperature increases associated with Climate Change; IPCC, 2013). However, we excluded all experiments that included long (>1 week) acclimatization periods at high temperatures before chemical exposure and not testing the CITS or TICS phenomena, as they may have allowed adaptation and species recombination prior to the temperature increase. The complete report of the databases used for the literature search, the key words used for each trophic level as well as the complete list of the papers reviewed, are reported in Supporting Information (SI1, Table S1 and SI2, Table S1).

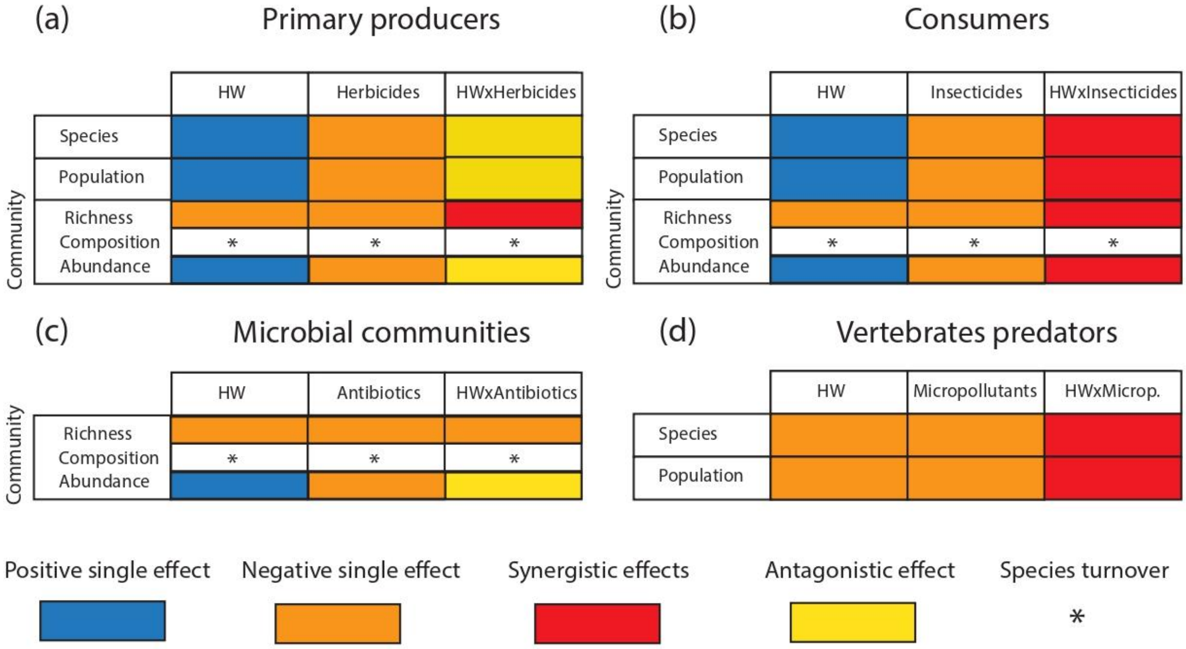

Figure 1. Predicted single and combined effects of heatwaves (HWs) and micropollutants on the different trophic levels (a, primary producers; b, consumers; c, microbial communities, and d vertebrate predators) based on available literature (61 articles). Within each trophic level, the rows represent different types of endpoints. Columns show the effect of HWs and the chemical stressor that is projected to have the largest negative effects on the organism groups under consideration. Note that because micropollutants are expected to have mainly a negative direct effect on the different endpoints, we classified the possible synergism only as negative. The individual positive effects of HWs are related to the individual temperature-stimulated increase in metabolism, which may result in increased population growth.

3. Results

Our literature search resulted in 61 studies fitting our HW definition. Of these studies, 13 assessed the combined effects of HWs and chemicals. Only 21 studies explicitly tested the effects of HWs, whereas the others were conceived as conventional temperature raise studies, but their features suited our HW definition.

Studies assessing the combined effects of HW and chemical pollution included only one trophic level and were unequally spread between trophic levels: microbial communities (n = 0), primary producers (n = 3), primary consumers (n = 6) and predators (n = 4; Figure 2). No study assessed the combined effects of HWs and micropollutants on multitrophic systems.

In the following subsections, we report the results of the literature review divided by trophic levels. Each subsection deals with one single trophic level. We first discuss the direct effects of HWs on each level and continue with the combined effects of HWs and micropollutants.

3.1 Microbes

3.1.1 Direct effects of HWs on microbial communities

Microbial communities comprise bacteria, fungi, protozoa, viruses and nematodes (Margulis *et al.* 1986). Aligning with the species-sorting model (Leibold *et al.* 2004), microbial communities are shaped by the local environmental conditions (Logue & Lindström 2010), such as temperature (Ziegler *et al.* 2019), salinity (Herlemann *et al.* 2011), resource availability (Pradeep Ram *et al.* 2020) or environmental degradation processes (Mykrä *et al.* 2017). Although some soil microbial communities are relatively tolerant (e.g., no significant changes in biomass or respiration) to HWs (de Oliveira *et al.* 2020; Pérez-Guzmán *et al.* 2020), microbial communities in freshwater ecosystems have been found to have a higher sensitivity towards warmer temperature or HWs. This is due to faster conduction of temperature through water than terrestrial media (van Rooyen & Winterkorn 1957; Singh & Devid 2000). HWs may have an effect on the metabolism, biomass, composition, and the stoichiometry of freshwater bacteria, as shown in experiments (10–30° C) where elevated temperature increased nutrient cycling on suspended bacteria from oligotrophic lakes (Phillips *et al.* 2017; Figure 3). The changes in stoichiometry are more pronounced when access to nutrients, particularly phosphorus, is lacking, thus resulting in larger effects of

HWs in oligotrophic ecosystems compared with more eutrophic ones (Phillips *et al.* 2017). Further studies demonstrated that elevated temperatures in freshwater systems enhance both the growth rate and the leaf litter decomposition by aquatic fungi (Duarte *et al.* 2013), which promotes the organic nitrogen concentration in leaf litter (Kaushik & Hynes 1971), and, in turn, stimulates microbial respiration rates (Stelzer *et al.* 2003; Figure 4). The biomass of freshwater microbial communities increases with temperature, which leads to an increased rate of leaf litter decomposition (Donnelly *et al.* 1990; Stelzer *et al.* 2003; Fernandes *et al.* 2012). This can enhance nutrient cycling, at least during short-term periods. Some microbial communities prosper with temperature increases, for example, functional groups such as ammonia-oxidizing (Zeng *et al.* 2014) or biocalcifying (Zamarreňo *et al.* 2009) bacteria. Consequently, bacteria growing faster under HW conditions may outcompete slower-growing bacteria, leading to changes in community structure (Fetzer *et al.* 2015).

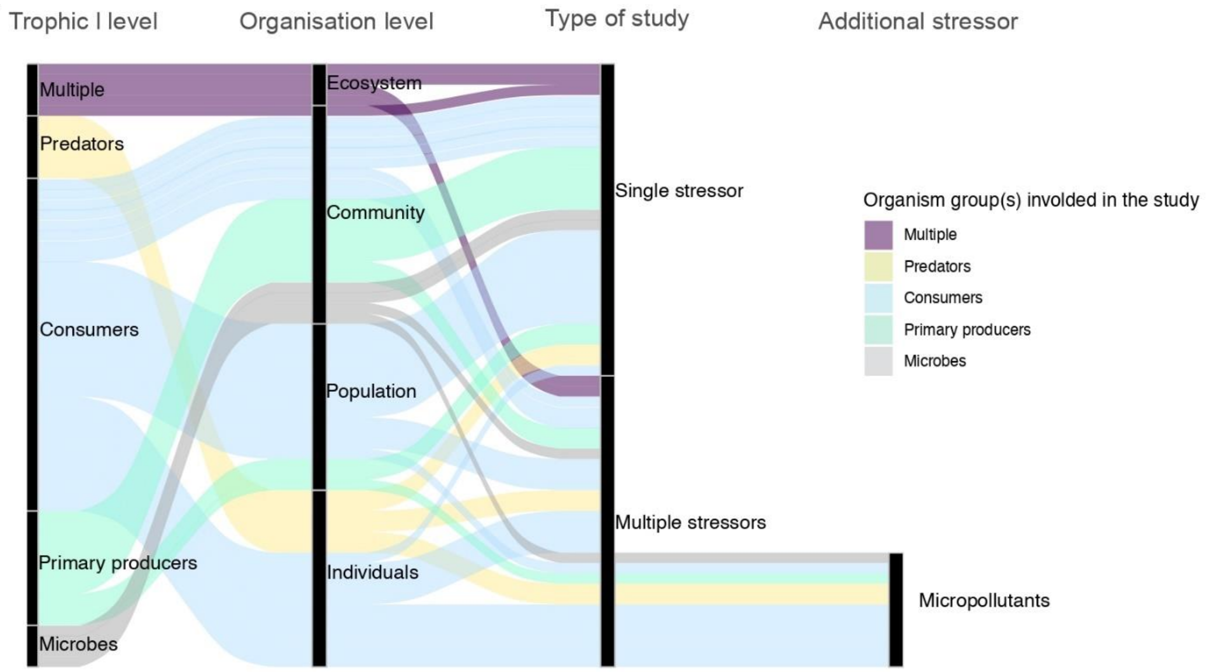

Figure 2. Alluvial plot showing the studies available dealing with heatwaves (HWs) only and HWs in combination with other stressors and the level of biological organization and trophic levels evaluated. The band width is proportional to the number of studies evaluated (total number of studies included = 61).

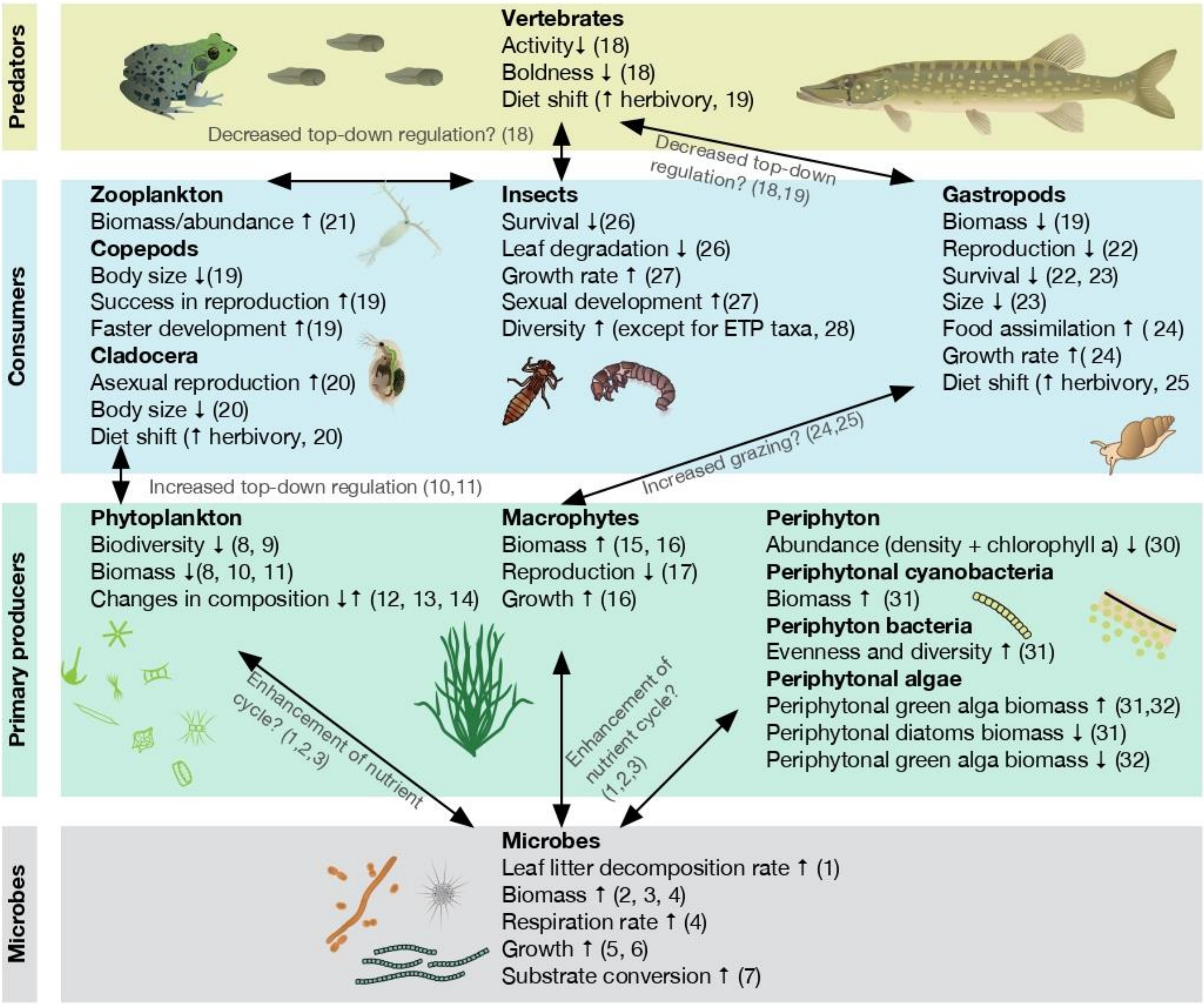

Figure 3. Graphical representation synthesizing the results found in the literature on the effects of HWs on aquatic food-webs. Arrows connecting different organism groups indicate trophic and/or indirect effects. Upward arrows indicate an increase/raise and downward arrows indicate a decrease of the evaluated ecological parameter. Numbers in brackets refer to references: (1) Duarte et al 2013; (2) Donnelly et al 1990; (3) Fernandes *et al.* 2012; (4) Stelzer et al 2003; (5) Zeng *et al.* 2014; (6) Zamarreňo *et al.* 2009; (7) Höfle 1979; (8) Remy *et al.* 2017; (9) Egger *et al.* 2011; (10) Velthuis *et al.* 2017; (11) O'Connor *et al.* 2009; (12) Maazouzi *et al.* 2008; (13) Bergkemper & Weisse 2017; (14) Weisse *et al.* 2016; (15) Bertani *et al.* 2016; (16) Hansson *et al.* 2020; (17) Li *et al.* 2017; (18) Mammeri *et al.* 2020; (19) Carraira *et al.* 2016; (20) Nguyen *et al.* 2020; (21) Johnsen *et al.* 2020; (22) Cremona *et al.* 2020; (23) DeWhatley & Alexander, 2018; (24) Leicht & Seppälä, 2019; (25) Carreira *et al.* 2020; (26) Zhang *et al.* 2020; (27) Vander Vorste *et al.* 2017; (28) Prato *et al.* 2008; (29) Fornaroli *et al.* 2020; (30) Hao *et al.* 2020; (31) Piggott *et al.* 2015; (32) Bondar-Kunze *et al.* 2021.

The assembly of microbial community changes due to changing environmental conditions (Langenheder & Lindström 2019). This can alter biogeological fluxes and the availability of key elements for the ecosystem (Zhao *et al.* 2017; Figure 4). For instance, increased bacterial growth promoted by higher temperatures may lead to a phosphorus depletion for phytoplankton (Currie 1990) and to food scarcity for herbivores. Conversely, a substantial increase in the bacterial abundance can, depending on the microbial community, facilitate the remineralization of nitrogen (Berthelot *et al.* 2019; Hayes *et al.* 2019) and phosphorus (Klausmeier *et al.* 2004), which precedes an increase in phytoplankton biomass that would, otherwise, function as a self-limited nutrient sink (Pomeroy & Wiebe 1988).

The ability of microbial communities to resist and recover from extreme climate events is still understudied, but needed to help the characterization of ecosystem response to important ecological functions after extreme events (Bardgett & Caruso 2020).

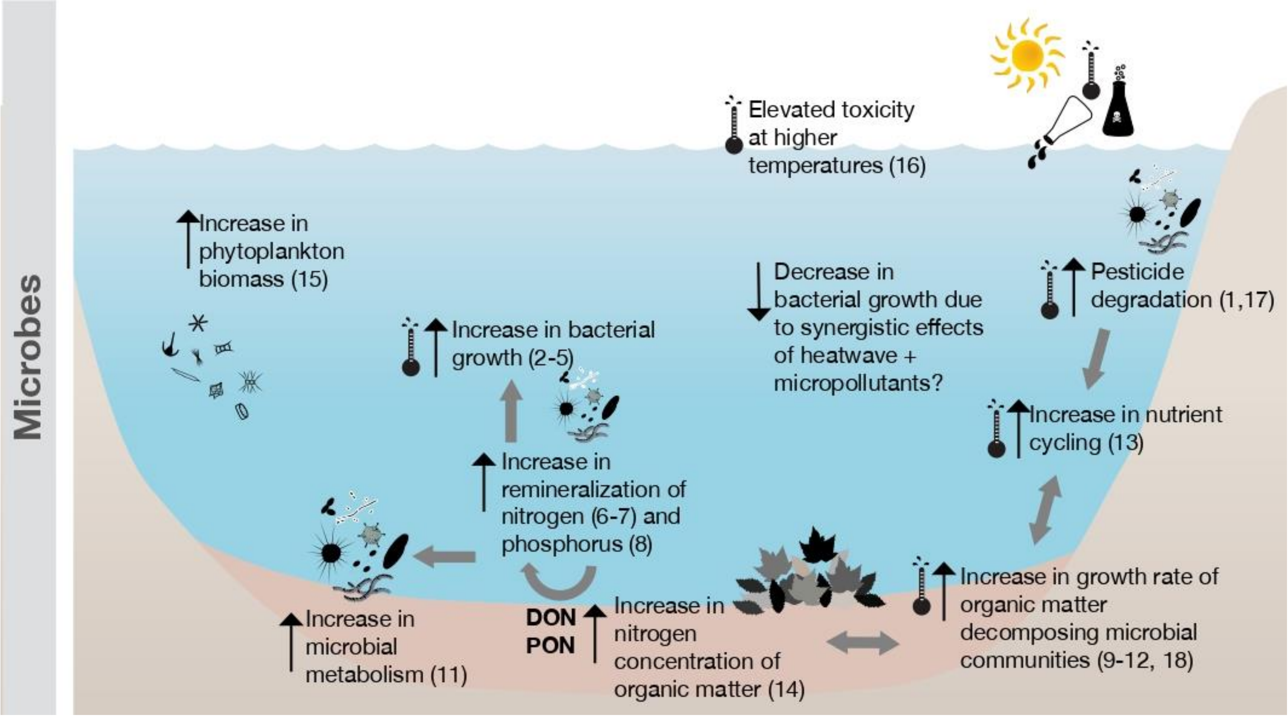

Figure 4. Conceptual overview of the potential combined effects of HWs and chemicals on microbial communities. Black upward or downward arrows indicate an increase or decrease of the respective processes. Grey arrows indicate direct and indirect effects on other processes. The thermometer symbol indicates an effect related to temperature only, while the symbols of the thermometer and of the chemical together indicate a combined effect of both. (1) Bighiu & Goedkoop 2021); (2) Zeng *et al.* 2014; (3) Zamarreño *et al.* 2009; (4) Höfle 1979; (5) White *et al.* 1991; (6) Berthelot *et al.* 2019; (7) Hayes *et al.* 2019; (8) Klausmeier *et al.* 2004; (9) Duarte *et al.* 2013; (10) Donnelly *et al.* 1990; (11) Stelzer *et al.* 2003; (12) Fernandes *et al.* 2012; (13) Phillips *et al.* 2017; (14) Kaushik & Hynes 1971; (15) Pomeroy & Wiebe 1988; (16) Delnat *et al.* 2021; (17) Wickham *et al.* 2020; (18) Arias Font *et al.* 2021.

3.1.2 Combined effects of HWs and chemicals on microbial communities

Microbes living in freshwater habitats are exposed to micropollutants that form complex mixtures (Escher *et al.* 2020). Yet, it remains unknown how microbial communities might take up, degrade, or metabolize micropollutants when simultaneously exposed to HWs and chronic chemical stress, as no studies were found investigating this (Figure 2).

Although lake microbial communities have been shown to be highly resilient to long-term warming (i.e. composition was recovered within 1 week after the warming was interrupted; Shade *et al.* 2012), we do not know whether a simultaneous exposure to micropollutants may impair the recovery capacity. Similarly, it is unknown whether the shift in community composition resulting from HW (albeit short-term) may increase the sensitivity towards chemicals.

Freshwater microbial organisms are capable of degrading pesticides and other synthetic chemicals (Mishra *et al.* 2020). Microbial degradation of micropollutants, thus, directly impact the exposure of other organism groups, which may benefit from an increased microbial degradation of toxic substances. Microbial communities repeatedly exposed to chemicals may enhance their biodegradative activity and reduce micropollutant persistence (Barra Caracciolo *et al.* 2015). However, this is only true when microbial populations able to degrade specific micropollutants are present in the environment (Barra Caracciolo *et al.* 2015; Coll *et al.* 2020). If such populations are removed by a disturbance (e.g., HWs), the biotransformation and degradation of chemicals may be impaired.

3.2 Primary producers

3.2.1 Direct effects of HWs on primary producers

Although metabolic theory (López-Urrutia *et al.* 2006; O'Connor *et al.* 2011) and empirical data (O'Connor *et al.* 2009) indicate that respiration-limited (heterotrophic) metabolism is more sensitive to changing temperature than photosynthesis-limited (autotrophic) metabolism and production, negative effects of HWs have also been reported for primary producers (Eggers *et al.* 2012; Weisse *et al.* 2016). HWs have been shown to promote a shift in community composition, both in experimental systems and field studies (Blenckner *et al.* 2007; Maazouzi *et al.* 2008; Weisse *et al.* 2016; Bergkemper & Weisse 2017). Generally, those compositional changes are linked to a reduction in phytoplankton diversity in microcosm experiments (Eggers *et al.* 2012; Weisse *et al.* 2016; Remy *et al.* 2017) as well as in field studies (Maazouzi *et al.* 2008; Bergkemper & Weisse 2017; Figure 3). The reduction in species number is commonly driven by a decline of thermal sensitive species (Urrutia-Cordero *et al.* 2017), which in turn allows more tolerant species to thrive and to increase their abundance (Eggers *et al.* 2012). Although a decline in richness is often reported as a consequence of a HW, some studies do not highlight a net decrease in species number, but rather a species turnover driven by the different species' thermal sensitivities (Li *et al.* 2017; Hansson *et al.* 2020). Several studies show that compositional changes caused by HWs often promote cyanobacteria dominance, even in nutrient-and light-deficient aquatic environments (Calderó-Pascual *et al.* 2020). HWs can also promote cyanobacteria blooms by inducing a seasonal advancement and increasing their recruitment rates from sediments (Urrutia-Cordero *et al.* 2020; Richardson *et al.* 2019; Bergkemper & Weisse 2017). A common consequence of these blooms is the alteration of ecosystem functions, such as biomass production and chlorophyll-a concentration, as well as toxicity through the release of cyanotoxins (Eggers *et al.* 2012; Remy *et al.* 2017).

Macrophytes have been shown to be sensitive to HWs, too. As for phytoplankton, their response to heat stress is species-specific. Li *et al.* 2017 showed that *Carya tomentosa* exposed to extreme temperature fluctuations (+8°C, different durations) increased the mortality rate up to 60%. Conversely, HWs tolerant species, such as *Myriophyllum spicatum*, exhibit opposite responses, by increasing their total abundance and biomass under HW (+8°C, different durations, Hansson *et al.* 2020). Nevertheless, when exposed to HWs, *M. spicatum* showed impaired sexual reproduction expressed as severe reduction in the number of flowers produced during the vegetative season, suggesting that the balance between vegetative and generative reproduction under HWs is shifted towards more asexual reproduction (Li *et al.* 2017).

The majority of the experimental set-ups investigating the effects of HWs on primary producers have not included other trophic levels. The only experiment testing HW (+8°C, 7 days) effects on a freshwater food web (phytoplankton and zooplankton) reported reduced primary producer biomass as a result of an increased grazing activity from zooplankton (Velthuis *et al.* 2017). The same effects have been found by a similar study investigating a marine bi-trophic community (+6°C, 8 days, O'Connor *et al.* 2009). Other studies have shown that HWs can influence the competition between algae and macrophytes. For instance, Bertani *et al.* (2016) found that after a natural HW, a shallow lake shifted from a phytoplankton-dominating stage to a macrophyte-dominating one. Such bottom-up interactions triggered cascading effects at higher trophic levels, resulting in a decline in planktonic herbivores (Bertani *et al.* 2016).

Temperature can directly affect plant palatability. Zhang *et al.* (2019) showed that some aquatic macrophytes were less palatable to consumers (e.g., the aquatic gastropod *Lymnaea stagnalis*) when grown under constant elevated temperature. A reduction in plant palatability directly leads to decreased biomass, abundance and lower fitness of consumers (Zhang *et al.* 2019). Potentially, this could lead to a general decreased top-down control also under HW stress, which may lead to unforeseen effects on ecosystem functioning.

3.2.2 Combined effects of HWs and micropollutants on primary producers

All the studies (n=3) available on the combined effects of herbicides and HW on primary producers are single-species laboratory tests (Delorenzo *et al.* 2013; Tasmin *et al.* 2014; Wilkinson *et al.* 2017). These studies show consistent trends for some endpoints and opposite trends for others. When the freshwater algae *Pseudokirchneriella subcapitata* was simultaneously exposed to high temperature and the herbicide diuron for six consecutive days, the acute toxicity of the herbicide decreased (6 days, C:20°C, H: 30°C; Tasmin *et al.* 2014). The species' growth was less reduced by diuron at high temperature compared to colder temperatures, and the photosynthetic activity was less impaired by the herbicide when undergoing the HW treatment (Fig. 5, Tasmin *et al.* 2014). Delorenzo *et al.* (2013) studied the effects of increasing temperature and salinity on the toxicity of herbicides (irgarol, diuron, atrazine, and ametryn, tested individually) to the phytoplankton species *Dunaliella tertiolecta*. The authors found that a HW exposure (5 days, C: 25°C, H: 35°C) generally decreased the negative effects of the herbicides on the chlorophyll-a concentration, lipid content, and starch content (Fig. 5). However, opposite to Tasmin *et al.* (2014), under simultaneous exposure to HW and herbicides, growth rate, and cell density were generally decreased compared to the treatment containing only herbicides (Fig. 5) (Delorenzo *et al.* 2013).

The lower sensitivity of primary producers to herbicides, when simultaneously exposed to rapid temperature increase, is a frequently observed response across ecosystems (Gomes & Juneau 2017). Wilkinson *et al.* (2017) observed that the decrease in photosynthetic activity in the seagrass *Halophila ovalis* exposed to diuron was reduced when exposed to extreme temperatures (3 days, C:25°C, HW:35°C).

The available studies highlight that herbicides and HWs have mostly an antagonistic (i.e., less than additive) effect on the photosynthetic efficiency and algal growth of primary producers (Delorenzo *et al.* 2013; Tasmin *et al.* 2014; Gomes & Juneau 2017). The same trend has also been found by a recent review summarizing the combined effects of elevated temperature and herbicides on primary producers (Gomes & Juneau 2017). Nevertheless, the sensitivity range of photosynthetic aquatic organisms to extreme temperature fluctuations and herbicides is wide, and further investigations are needed on the combined effects of HWs and herbicides on realistic species assemblages and on higher trophic levels. Potentially, the reduction in primary producers' biomass caused by herbicides coupled with the shift to cyanobacteria dominance driven by HWs could reduce the availability of food source for consumers. Cyanobacteria are relatively tolerant to some classes of herbicides, particularly to the globally most applied phosphonate herbicide glyphosate (Forlani *et al.* 2008; Annett *et al.* 2014). The combined effects of HWs and herbicides could promote cyanobacteria blooms and reduce the portion of palatable phytoplankton and macrophytes for consumers. Such an effect could reduce consumers' ability to carry out detoxification processes from cyanotoxins, leading to an overall biomass decline across the food web (Fig. 3).

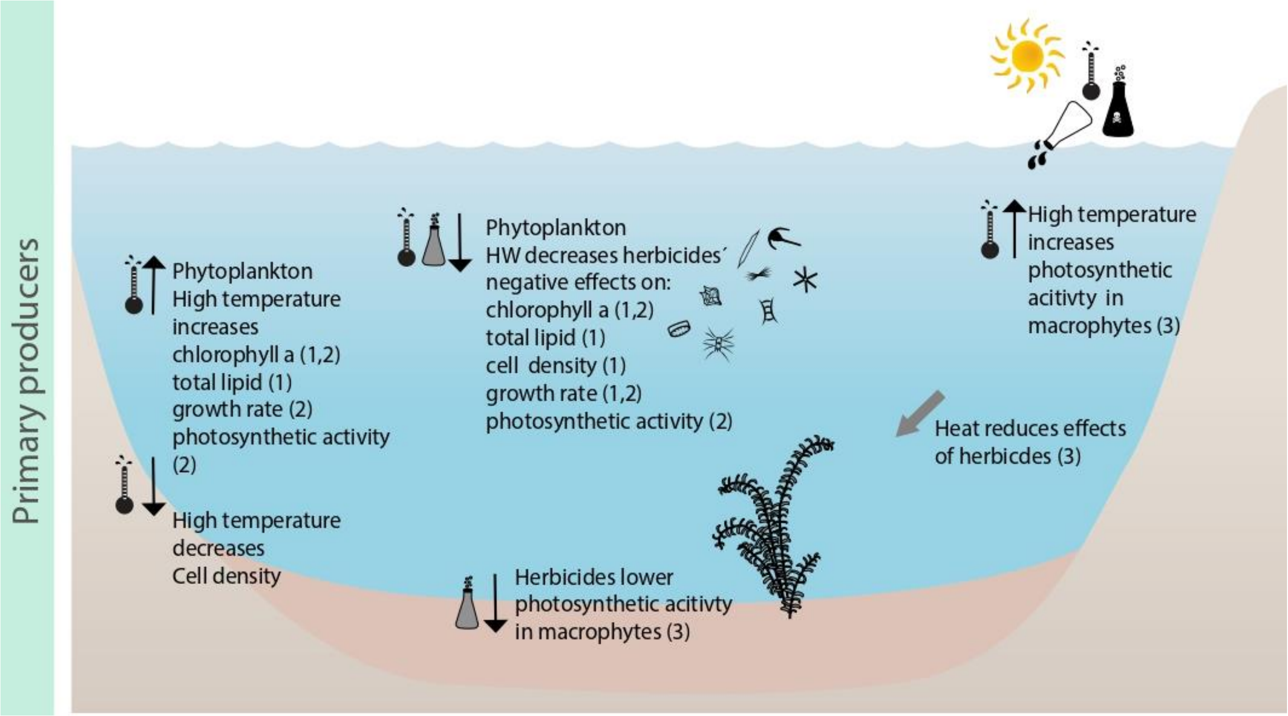

Figure 5. Conceptual overview of the single and combined effects of heatwaves and herbicide(s) on primary producers. Black upward or downward arrows indicate an increase or decrease of the respective processes. Grey arrows indicate direct and indirect effects on other processes. The thermometer symbol indicates an effect related to temperature only, while the symbols of the thermometer and of the chemical together indicate a combined effect. (1) Delorenzo *et al.* 2013. (2) Tasmin *et al.* 2014. (3) Wilkinson *et al.* 2017.

3.3 Primary consumers

3.3.1 Direct effects of HWs

Temperature plays a key role in ectotherms' physiology, as their body temperature changes with the temperature of the environment. Thus, for ectotherms, changes in temperature result in alterations of the rate of all physiological and metabolic reactions (Brown *et al.* 2004). As a consequence, temperature drives the selection of specific individual traits as well as behavioural alterations. A recent mesocosm study on zooplankton community dynamics showed that under warming (sampling over 3 years, C: 11,7 °C, HW: 19,7 °C) smaller body size and asexual (parthenogenetic) reproduction dominated (Johnsen *et al.* 2020). Responses of tropical zooplankton to a HW (12 days, C: 26 °C, HW: 34 °C) revealed a faster development but a reduced body size, clutch size, hatching success, and nauplii production for copepods (Nguyen *et al.* 2020). Similar warming-driven selections were predicted under future climate models for shallow, eutrophic lakes in which copepod's biomass and abundance are projected to increase (Cremona *et al.* 2020). Under the same projections, however, cladoceras are expected to reduce their biomass, but not their abundance (Cremona *et al.* 2020).

The features of a HW, including duration and the speed of temperature increase over time, determines the magnitude of the adverse impact as investigated on the filtration capacity of *Daphnia magna* (Müller *et al.* 2018). Individuals showed an immediate negative response with rapid temperature changes (up to 20 days, T-range: 11 °C to 29 °C). More severe responses were noticed at higher temperatures and faster temperature changes, although quasi-acclimatization with higher filtration capacities was reported (ca. 7 days, T-range: 11 °C to 25 °C) (Müller *et al.* 2018).

Freshwater gastropods exposed to a HW showed thermal sensitivity through significantly higher mortality rates at high temperatures (10 days, C: 20 °C, HW: 35 °C and 30 °C) (DeWhatley & Alexander 2018). Similar observations were reported after a HW in 2003, when the snail populations of two European rivers strongly declined (Mouthon & Daufresne 2008). HW effects were noticed also in the following years (2004, 2005), with the snail populations showing smaller cohorts (Mouthon & Daufresne 2008).

HW effects can differ depending on the life-history trait under investigation. For instance, hatching success of laid eggs increased with high temperature (7 days, C: 15 °C, HW: 25 °C), but also caused reduction in the size of eggs, egg survival rate, and the number of hatched juveniles. However, the surviving offspring showed an increased developmental rate and growth (Leicht & Seppälä 2019). The duration and speed of temperature increase of these extreme events appear to be crucial for the response direction of the organism's trait. Supporting this, two experimental HWs (1 and 7 weeks, C: 20 °C , HW: 25 °C) with identical magnitude but different duration have been found to increase snails' food assimilation and growth rate, with only minor negative effects on reproduction (Carreira *et al.* 2020). Yet, responses on these endpoints were dependent on the HW duration, as the short HW caused a relatively stronger response (Carreira *et al.* 2020). Similar time-dependent effects were reported for prolonged HWs (7, 9, or 11 days, C: 15 °C, HW: 25 °C) when compared to short ones (1, 3, 5 days) with increased growth and reproduction only in the first week of observation and reduced levels of immune function only under prolonged HWs (Leicht *et al.* 2013).

To maintain higher metabolic rates at elevated temperatures, omnivorous ectotherms seem to change their diet by increasing herbivory instead of carnivory, which is also observed in other aquatic taxa, e.g., zooplankton and fish. These temperature-induced diet shifts may cascade through the aquatic food web, increasing top-down pressures on primary producers (Zhang *et al.* 2020) (Fig. 3).

HW effects on benthic organisms and insects are poorly documented in freshwater research and only a few publications studied their sensitivity towards such extreme events. Under laboratory conditions (15 days, C: 15 °C, HW: 25 °C), the amphipod *Gammarus pulex* showed behavioural alterations, consisting in vertical immigration to potential refuge areas (i.e., hyporheic zone) (Vander Vorste *et al.* 2017). These tries of avoiding heat stress, came along with decreases in survival, leaf consumption, and energy recourses. HWs may also favour the dominance of more resistant invasive species. In an experimental study, the allochthonous gammarid *Dikerogammarus villosus* was found to be less sensitive to HWs compared to the native *Gammarus pulex* (Truhlar *et al.* 2014). This lower thermal sensitivity of the invasive species led to a rapid shift in macroinvertebrates' community structure, which was linked to changes in nutrients dynamics (Maazouzi *et al.* 2011; Truhlar *et al.* 2014). Such changes in community structure after HWs have also been reported consistently in field studies (Daufresne *et al.* 2007), including the loss of the more sensitive *Ephemeroptera*, *Plecoptera,* and *Trichoptera* taxa (Fornaroli *et al.* 2020).

Such individual- and community- level changes seem to be linked to the temperature effects on the overall energy budget of the organisms, including energetic investments in homeostatic maintenance, growth, development, and reproduction (Verberk *et al.* 2020). In accordance with these general findings, low energy reserves of the caddisfly larvae

Stenopsyche marmorata were associated with high temperature (9 days, C: 10 °C, HW: 25 °C) (Suzuki *et al.* 2018). Dinh *et al.* (2016) described that heat stress effects (6 days, C: 22 °C, HW: 30 °C) caused a reduction in the immune function (activity of phenoloxidase) and metabolic rate (activity of the electron transport system). Additionally, the authors pointed out the importance of delayed HW effects in shaping the overall stressor impact on the organism when other stressors are subsequently applied (TICS concept).

Although *Odonata* are intermediary consumers/predators in aquatic food webs, we report here the effects of HWs on this organism group, as the following subsection only deals with vertebrate predators. Experimental warming studies on dragonfly larvae (*Odonata*) present strong thermal effects at environmentally relevant temperatures (ca. 35 days, C: ambient, HW: const. +5°C to ambient) with lower survival rates and premature emergence (Mccauley *et al.* 2015). Although no effects of increased temperature on the overall body size of adults were found, there was a significant interaction effect of temperature and sex with the trend for larger size with higher temperature being stronger in females than males.

3.3.2 Combined effects of HWs and chemicals on primary consumers

Interaction effect studies on HWs and chemical stressors towards aquatic primary and intermediate consumers are generally scarce (n=6), and all of them were published within the last ten years (Fig. 6). In a microcosm experiment, negative synergistic effects were observed initially (4 and 14 days, C: 20 °C, HW: 28 °C) in several zooplankton taxa (*Daphnia* sp., Cyclopoida, and Copepoda nauplii) exposed to increased temperature and the insecticide lufenuron (Arenas-Sánchez *et al.* 2019a). Observed positive effects of increased temperature alone (16 days, C: 20 °C, HW: 30 °C) may be nullified through interactions with chemicals. This was noted for a cladoceran species (*Moina micrura*), which exposed to a HW only (16 days, C: 20 °C, HW: 30 °C) showed increased population size, but when exposed to a HW and the fungicide carbendazim simultaneously showed reduction in population size and net decrease of reproductive rate (Miracle *et al.* 2011). Another multiple stressor study with the freshwater gastropod *Bellamya bengalensis* reported detrimental effects on molecular endpoints caused by HWs in combination with the pesticide chlorpyrifos (60 days, C: 25 °C, HW: 30 °C, 35 °C) (Baag *et al.* 2021). In a multiple stressor experiment, Macaulay *et al.* (2021) exposed mayfly nymphs (*Deleatidium* spp) to two subsequent HWs (both 6 days, C: 12 °C, HW: 20 °C). The first HW was combined with a non-chemical stressor (e.g., starvation) and the second with the insecticide imidacloprid. The mayfly nymphs revealed a delayed negative synergistic interaction between a HW and imidacloprid (0.4

ug/L) (Macaulay *et al.* 2021). In the same study, the second HW resulted in time-dependent stressor interactions and time-cumulative toxicity of imidacloprid affecting mayfly mobility. Lethal effects of imidacloprid were only observed when applied as single stressor, suggesting that previous exposure to the first HW might have increased the tolerance of the organisms to the combined effects of a HW and insecticide (Macaulay *et al.* 2021). Further, there are reported synergistic HW-imidacloprid interactions in *Deleatidium* spp*.* and *Coloburiscus humeralis* on their moulting, immobility, and mortality, suggesting temperature-enhanced toxicity of the neonicotinoid insecticide (Macaulay *et al.* 2020). In another study with imidacloprid, Camp and Buchwalter (2016) showed that insecticide uptake in *Isonychia bicolor* as well as oxygen consumption rates increased significantly with increasing temperature (5 days, T-range: 15, 18, 21, and 24 °C). Additional uptake tests with several aquatic invertebrates (*I. bicolor*, *Neocloeon triangulifer*, *Macaffertium modestum*, *Pteronarcys proteus*, *Acroneuria carolinensis*, and *Pleuroceridae* sp.) indicated that all the species tested had significantly increased imidacloprid uptake with increasing temperatures (Camp & Buchwalter 2016).

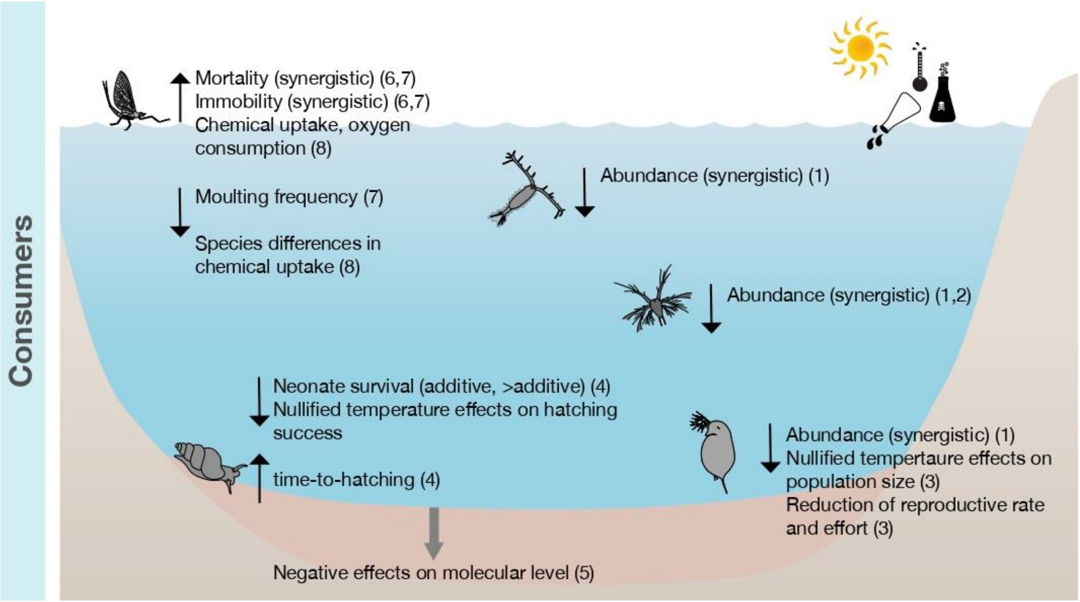

Figure 2.6. Conceptual overview of the combined effects of HWs and chemicals on primary consumers. Black upward or downward arrows indicate an increase or decrease of the respective ecological parameter. Grey arrows indicate direct and indirect effects on other processes. (1) Arenas-Sánchez *et al.* 2019, (2) Van de Perre *et al.* 2018, (3) Miracle *et al.* 2011, (4) Kimberly & Salice 2013, (5) Baag *et al.* 2021, (6) Macaulay *et al.* 2021, (7) Macaulay *et al.* 2020, (8) Camp & Buchwalter (2016).

3.4 Vertebrate predators

3.4.1 Direct effects of HWs onvertebrate predators

The focus in this section lays on larger vertebrate predators such as fish and amphibians, excluding macroinvertebrates and zooplankton. Increased water temperatures can affect the reproduction of fish by altering their spawning activity and impacting embryo development (Ashton *et al.* 2019; Warriner *et al.* 2020). Fish are known to respond to seasonal water temperatures by changes in movement behaviour, i.e., the timing of migration or changes in microhabitat, seeking temperature refugia (Coutant 2001). Consequently, HWs are likely to influence similar behavioural responses. In a mesocosm experiment, the swimming behaviour of the Iberian barbel (*Luciobarbus bocagei*), a Mediterranean freshwater fish, was impacted under HW conditions (6 days, C: 24.5 °C, H: 29.7 °C) resulting in decreased activity and boldness (Mameri *et al.* 2020). Next to direct consequences on individual performance, these temperature-dependent behavioural changes also influence predator-prey interactions (Öhlund *et al.* 2014). Altered predator-prey interactions may have indirect effects on other trophic levels resulting from a decreased predation pressure on preys, i.e., benthic invertebrates and other fish (Fig. 3).

A similar indirect effect across trophic levels has been observed for tadpoles under HW conditions. Diet shifts of tadpoles occurred in HW treatments (various HW scenarios tested, see Carreira *et al.* 2016) (Fig. 3) with a general trend to increased herbivory in response to a higher temperature (Carreira *et al.* 2016). Consequently, the direct effect of the HW on the predator (i.e., its diet shift) has limited consequences on other predators but rather affects other trophic levels indirectly (i.e., increased consumption of macrophytes and decreased predation on insect larvae). Conversely, HWs can also have indirect effects on predators via a direct effect on their prey or lower trophic levels through bottom-up mechanisms (Fig. 3). Additionally, indirect effects within the same trophic level, e.g., intensified interaction of predators through competition of food resources or microhabitats, may occur at least temporally.

3.4.2 Combined effects of heatwaves and micropollutants on vertebrate predators

The studies investigating the combined effects of HWs and micropollutants on predators (n = 7) include exposure experiments with metals, herbicides, and antidepressants (Fig. 7). The response categories of these studies comprise i) effects only present in the combined exposure to HWs and micropollutants, ii) effects dominated by the HW, or iii) effects in all treatments. It is worth noticing that this section reports mainly results of molecular endpoints. Extrapolation of effects in terms of fitness and reproduction are not easy because those are not typically measured.

For the following examples, the sensitivity of the organisms was only evident when exposed to the combination of both stressors, while each single-stressor application did not reveal significant effects. Tadpole metamorphosis was accelerated under HW conditions (7 days, C: 28 °C, H: 34 °C, diuron and its metabolite 3,4-DCA) only when combined with an herbicide (Freitas *et al.* 2016). In another study exposing tadpoles to an herbicide under HW conditions (8 days, C: 28 °C, H: 36 °C, clomazone), the activity of the biotransformation enzyme glutathione-S-transferase (GST), increased with increasing dose. A GST rise indicates an increased oxidative stress, which may lead to a reduced fitness of amphibians (Gripp *et al.* 2017). Such an increase was not observed at the same herbicide concentrations at lower temperature (Freitas *et al.* 2017). The HW treatment alone indicated no significant difference in the activity of this phase II detoxification enzyme.

Similarly, the survival rate of zebrafish embryos after a HW treatment (7 days, C: 26 °C, H: 34 °C, cadmium) was not affected in the HW only treatment but showed a dose-dependent reduction under joint cadmium exposure (Park *et al.* 2020). Furthermore, lower heart rates, increased cell death, upregulated genes for antioxidants, and genes involved in apoptotic responses as well as DNA methylation were observed for the combined exposure. Another study with zebrafish revealed that glucose levels and routine metabolic rates, indicating stress and potentially reduced fitness, were increased in the combined exposure of a HW and an antidepressant (21 days, C: 27 °C, H: 32 °C, venlafaxine), again with no significant effects for the respective single stressor treatments (Mehdi *et al.* 2019).

The two following studies showed single stressor effects of the HW treatment and the combined exposure, but no effect on the micropollutant single stressor application. The pyruvate kinase activity in *Danio rerio* increased (21 days, C: 27 °C, H: 32 °C, venlafaxine), indicating an increase in metabolic costs for the organism (Mehdi *et al.* 2019). For the Japanese medaka, the production of intracellular reactive oxygen species (ROS) increased under HW condition (7 and 14 days, C: 25 °C, H: 30 °C, nickel chloride), and increased even more when combined with the exposure to a metal, which itself did not induce this effect (Prophete *et al.* 2006). An increase of ROS is associated to oxidative stress in fish, which can influence responses to environmental change and life history strategies (Birnie-Gauvin *et al.* 2017).

The remaining two studies investigating the effect of HWs and micropollutants in predators did not show a combined effect mechanism of the tested stressors. No significant effect of combined exposures (15 days, C: 16 °C, H: 21 °C, cadmium) were detected and only the HW treatment decreased the hepatosomatic index in males and increased plasmatic vitellogenin content in female zebrafish (Hani *et al.* 2019). This result highlights the importance of considering different endpoints in multiple stressor research, as cadmium did show an interactive effect with HWs in zebrafish in the study mentioned earlier, investigating a different endpoint (Park *et al.* 2020). Conversely, the exposure to venlafaxine and a HW caused micro-RNA to decrease in their single exposures in the same amount as for their combined exposure (21 days, C: 27 °C, H: 32 °C, venlafaxine), indicating no significant interaction effect in zebrafish (Ikert & Craig 2020).

Though limited in their representation for the variety of top predators in freshwater ecosystems, these seven studies reveal a variety of combined effects of HWs and micropollutants on predators. Additionally, these studies highlight the importance of multiple stressor research, as only the combination of HWs and micropollutant revealed the adverse effects to predators. Chemical risk assessments based on exposure studies with standard temperature conditions are thus limited to detect the described effect occurring in combination with HWs.

Though the results of almost all mentioned studies indicate stronger effects of micropollutants on predators when simultaneously exposed to HWs, they also indicate that there are different response categories with different stressor interactions. To unravel the underlying mechanisms, more research is needed investigating how HWs and micropollutants interact in affecting predators’ responses.

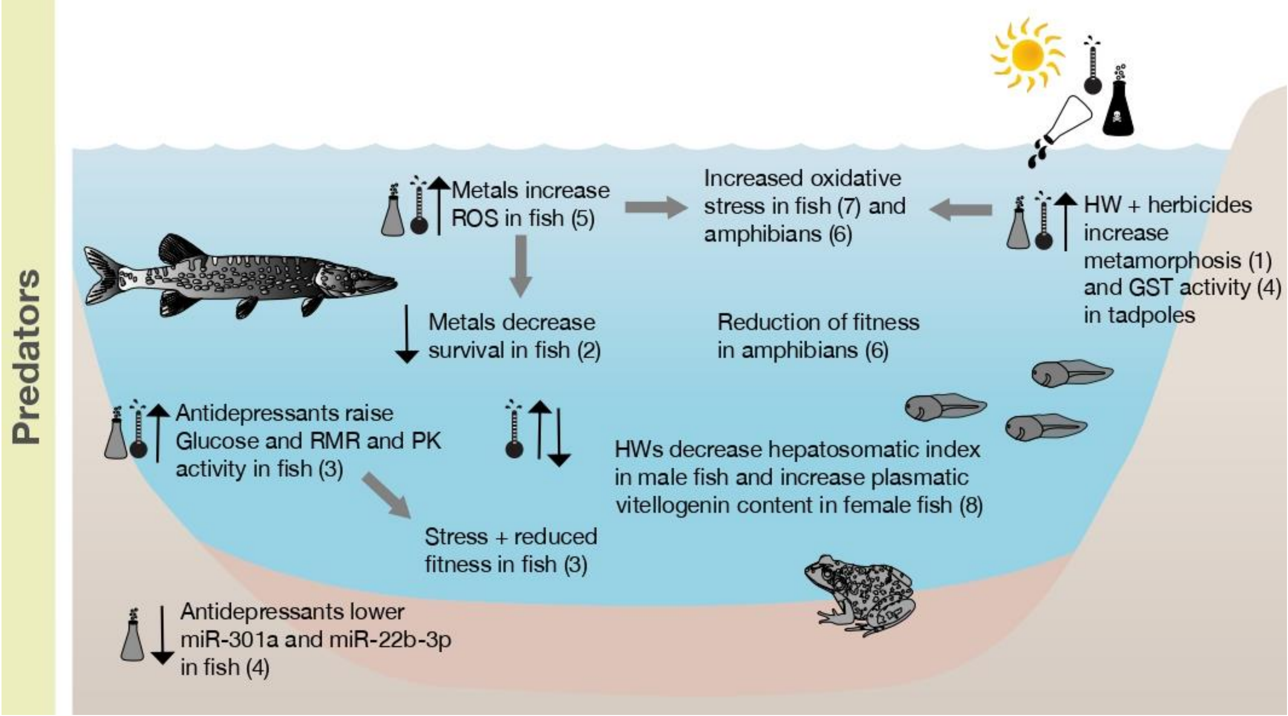

Figure 2.7. Conceptual overview of the single and combined effects of HWs and chemicals on predators. Black upward or downward arrows indicate an increase or decrease of the respective ecological parameter. Grey arrows indicate direct and indirect effects on other processes. The thermometer symbol indicates an effect related to temperature only, while the symbols of the thermometer and of the chemical together indicate a combined effect. (1) Freitas *et al.* 2016; (2) Park *et al.* 2020; (3) Mehdi *et al.* 2019; (4) Freitas *et al.* 2017; (5) Prophete *et al.* 2006; (6) Gripp *et al.*. 2017; (7) Birnie-Gauvin *et al.* 2017; (8) Hani *et al.* 2019.

4. Concluding remarks and recommendations

This literature review has revealed that only 5 out of the 61 included studies investigated the effects of HWs using a multitrophic approach. Those studies only focus on the effects of HWs in isolation. HWs have been assessed mainly through single species and/or population experiments containing one trophic level. Although these approaches are essential to reveal physiological responses, they are limited in ecological realism, excluding the investigation of the effects of trophic and non-trophic species interactions (Kéfi *et al.* 2015; Seibold *et al.* 2018). The lack of testing in complex species assemblages appears particularly problematic since ecological realism, and more community/ecosystem-oriented studies have recently been recognized as essential for the advancement of multiple stressors research (Orr *et al.* 2020).

Generally, HWs are reported to cause compositional changes in all trophic levels analysed individually, although the mechanisms driving those changes may be different. Changes in community composition resulting from HW exposure could result in altered sensitivity towards chemical stressors. Yet, the scarcity of available studies does not allow to draw general conclusions on how HW-driven compositional changes may affect community sensitivity towards different chemical classes. Furthermore, although a framework to mechanistically decompose the effects of multiple stressors on the different trophic levels has been recently propose (Bracewell *et al.* 2019; Van den Brink *et al.* 2019), at present, a mechanistic understanding of the processes underpinning the effects of HWs across organisation levels in a multiple stressor context is missing. Mainly, this is linked to the insufficient availability of theoretical and empirical work trying to unravel the processes behind the observed effects. Nevertheless, this review has provided some insights on the mechanisms driving the combined effects of HWs and micropollutants. In the next sub-sections we provide recommendations for further research.

4.1 Focus shift towards higher levels of biological organisation

We suggest that the assessment of the effects of extreme events combined with other stressors should involve investigations at the community and ecosystem level, which are primary focus of water managers, risk assessors, and policymakers interested in multiple stressor effects (Orr *et al.* 2020). Investigations at high levels of organization have the potential to unravel the combined effects of extreme events and other stressors on functional processes, species interactions and how impacts can cascade through the food web. This is particularly important since temperature effects mediated by species interactions may be larger than direct effects caused by extreme events (Higashi & Patten 1989; Montoya *et al.* 2009). Ecosystem response projections from single-species tests are challenging, as species performances differ depending on whether they are in monoculture or interacting in a community (Tabi *et al.* 2020). Moreover, it has been shown that food-web length and the number of species at each trophic level can modify the overall effects of perturbations, including chemicals, on species assemblages (Zhao *et al.* 2019), highlighting the need to test multiple stressors on multitrophic and species rich communities (Seibold *et al.* 2018). An improved understanding of how high levels of organization processes respond under combined disturbances, based on empirical data, is needed, and represents the major knowledge-gap identified in this review. In this context, micro- and mesocosm experiments represent one of the best ways to test the combined effects of micropollutants and extreme events at high levels of biological organization. Alongside with the experimental effort, the development of models able to predict combined effects of HWs and pollutants is needed to help ecosystems protection. Since testing all the possible stressors combination for all aquatic ecosystem types is unrealistic, we need modelling tools enabling us to project combined stressors' effects on different organisation levels.

4.2 Gradient testing

Experimental designs aiming to study effects of extreme events need to consider nonlinear responses. Non-linear responses seem to be the norm in biological systems exposed to climatic variability, as a consequence of Jensen's inequality (Ruel & Ayres 1999; Kreyling *et al.* 2018). Especially when effects of temperature are accounted for in freshwater systems, non-linear responses have been reported across all levels of biological organization, from individuals (Bernhardt *et al.* 2018), to communities (Baranov *et al.* 2020), and ecosystem processes (Lv *et al.* 2020). Testing non-linear responses requires the exploration of a wide gradient of the environmental driver(s) under investigation. Most experimental designs use only two levels of the tested drivers (i.e., applied/not applied) (Kreyling *et al.* 2018), which can limit our ability to derive hypotheses on the mechanisms behind the observed effects. Adopting gradient designs, therefore, may allow researchers to perform multiple stressor experiments investigating more combinations of stressors and a larger gradient of those stressors. Further, gradient approaches would consent to different realistic disturbance intensities (i.e., multiple temperature regimes or chemical concentrations) and HW durations to be tested. Experimental exploration along a large gradient of drivers' intensities and durations may also help in determining stress levels that have long-lasting legacies on communities' biomass and composition (Jacquet & Altermatt 2020). Moreover, more complex experimental designs allow studying different patterns of occurrence (i.e., reoccurring HWs, different timing of stressor application) and under different environmental scenarios. Finally, it has been pointed out that gradient designs allow more null models to be tested, which could improve our understanding of stressors' interactions (Schäfer & Piggott 2018).

4.3 Temporal dependency of stressor interactions and temporal dynamics of multiple stressors

The effects of multiple stressors (and their interactions) are temporal-scale dependent (Garnier *et al.* 2017). That is, interactions between stressors can appear right after disturbance's application, as well as in the recovery phase. Yet, in multiple stressors studies, performing only a single sampling point after the stressors' application is the rule (Halstead *et al.* 2014; Piggott *et al.* 2015b, a; Beermann *et al.* 2018). Accordingly, we did not find any study assessing the combined effects of HWs and chemicals over time, which is required to describe potential adaptation and/or recovery. This is particularly relevant for the combination of HWs and micropollutant, as HWs, by definition, are temporary phenomena. Conversely, some micropollutants can persist in the aquatic environment for months or years (Arp *et al.* 2017), and many chemicals have a continuous discharge to the aquatic environment, maintaining exposure levels also for less persistent substances. Therefore, following the response of the study system(s) is important to detect interactions between stressors happening at different time points. Particularly, late stressors' interactions may prevent the recovery of both compositional and functional endpoints. Furthermore, HWs are predicted to become more frequent (Meehl & Tebaldi 2004) and may appear multiple times in a season (Woolway *et al.* 2021). The re-occurrence of HWs in ecosystems stressed by persistent micropollutants (or receiving short-lived micropollutants multiple times) makes it necessary to follow the system's response over time in order to identify late-stage interactions and to take adequate protection measures.

Since perfect synchrony between stressors is rarely found in nature, designing experiments involving different order of stressor applications and measuring the organisms and ecosystem's responses over multiple time points is crucial (Jackson *et al.* 2021). Stressors' sequence has consequences for the so-called ecological memory, which is the ability of previous stressors to influence future responses (Hughes *et al.* 2019; Jackson *et al.* 2021). Among the literature we reviewed, only a single study explicitly investigated whether different orders of stressors application may result in different effects (Dinh *et al.* 2016). All the other studies reviewed here only tested simultaneous exposure to combined stressors. Testing different stressors' sequence may be challenging as it requires large experimental designs. Yet, it appears to be a central research aspect, as the abovementioned CITS and TICS phenomena might produce unexpected effects across all levels of biological organisation.

Some studies have explicitly assessed the effects of CITS and TICS, but this has been done prevalently at the individual and population level (Delnat *et al.* 2019; Verheyen & Stoks 2019, 2020; Verheyen *et al.* 2019). Thus, consequences of these phenomena for higher levels of biological organization are still unexplored. Restricting the investigations of temporal dynamics of multiple stressors to the individual or population level will preclude the understanding of the role played by ecological memory in driving the responses at higher levels of biological organisation.

Finally, the sampling of multiple time points can be translated in the measurement of different ecological metrics, such as resistance, recovery, resilience, and invariability (Donohue *et al.* 2013; Donohue *et al.* 2016; Hillebrand *et al.* 2018). Those ecological metrics have been shown to compose the different “dimensions” of ecological stability (Pimm 1984; Donohue *et al.* 2013), which can be altered by different stressor combinations (Polazzo & Rico 2021). Maintaining stable ecosystems is fundamental, as only stable systems can deliver functions and services consistently. Therefore, studying how HWs and micropollutants affect different stability indices, and how the order of stressors application plays in this context, may help to solve current managing conflicts, protect biodiversity in the long-term, and guarantee the delivery of ecosystem functions and services.

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Chapter 3

Effect of multiple agricultural stressors on freshwater ecosystems: the role of community structure, trophic status, and biodiversity-functioning relationships on ecosystem responses

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Abstract

The toxicity and widespread use of agricultural pesticides threaten freshwater biodiversity, but their long-term effects under different nutrient concentrations are poorly understood. We evaluated the single or combined effects of two pesticides (chlorpyrifos and diuron) under different nutrient regimes (mesotrophic and eutrophic) on community structure and ecosystem functions in replicated pond mesocosms. The individual application of nutrients and pesticides affected community composition and species richness. Ecosystem functioning was generally less sensitive to chemical stress than community structure, while eutrophication fostered the dominance of species that are more resilient to pesticides. Stressor interactions were significant at different time points, with late stressor interactions affecting the recovery of community composition. We also found that the correlation between biodiversity and relevant ecosystem functions, such as primary productivity and total ecosystem respiration, can be shifted from positive to negative under particular stress conditions. Our study demonstrates that nutrients enrichment is a key factor influencing the resilience of freshwater ecosystems to multiple stressors and that functional redundancy allows maintaining constant levels of functioning even under high toxic stress pressure.

1. Introduction

Freshwater ecosystems are threatened by a wide range of anthropogenic stressors (Millennium Ecosystem Assessment, 2005). One of the most important stressors affecting freshwater biodiversity is pesticide pollution (Bernhardt *et al.* 2017; Posthuma *et al.* 2020). Pesticides can reach freshwater ecosystems through spray-drift, runoff and leaching, and may form complex mixtures (Rohr *et al.* 2006). Although they have a significant influence on freshwater biodiversity conservation (Côté *et al.* 2016), recent meta-analysis have shown that studies addressing the interactions between pesticide mixtures and other freshwater stressors are scarce (Jackson *et al.* 2016; Birk *et al.* 2020).

Pesticides reaching aquatic habitats act as selective stressors targeting different organisms based on their physicochemical properties and their toxicological mode of action. Consequently, pesticide contamination may lead to non-random effects on populations and communities (De Laender *et al.* 2016). Increasing our understanding of the link between toxicological properties of pesticides and their implications for community composition, species diversity, and ecosystem functioning is crucial to predict the net effects of chemical mixtures on aquatic ecosystems, which is the ultimate goal of ecological risk assessment (Liess *et al.* 2016).

Most studies evaluating the effects of multiple stressors and their interactions have focused on measuring effects on a single time-point after disturbance (Halstead *et al.* 2014; Barmentlo *et al.* 2018; Bray *et al.* 2019). However, multiple stressor effects can vary on a temporal scale (Garnier *et al.* 2017). This means that measuring stressor interactions shortly after disturbance (i.e., resistance) may disregard delayed, non-additive effects caused by the depletion of energy reserves of individuals or changes in species interactions (Jackson *et al.* 2021; Orr *et al.* 2021). Thus, to set effective management actions, it is important to understand how multiple stressors affect the sensitivity of populations and communities in the short-term, as well as their long-term responses, which also influence their capacity to adapt and recover.

Among the list of stressors affecting freshwater ecosystems, nutrient contamination has been recognized as one of the most threatening together with pesticides (Birk *et al.* 2020). Nutrient enrichment has been found to be responsible for almost 35% of the variance in the ecological status of European waters, and frequently interacts with toxic substances (Lemm *et al.* 2020). As described in some studies, nutrient enrichment reduces the magnitude of the effects caused by pesticides (Halstead *et al.* 2014) or fine sediment inputs coming from agriculture (Piggott *et al.* 2015a, b). This is because nutrient enrichment, and the consequent eutrophication, generally reduce biodiversity and homogenize communities at the local and regional scale (Donohue *et al.* 2009), selecting for tolerant species (Roessink *et al.* 2005; Belando *et al.* 2017). However, studies describing this phenomenon are limited and have been performed mostly under temperate or cold conditions. The studies considering nutrient addition under warm conditions, such as the Mediterranean region, show notable differences in terms of species dominance and ecosystem dynamics as compared to the temperate ones (Romo *et al.* 2004), which may in turn influence ecosystem resilience to pesticide stress.

The relationship between biodiversity and ecosystem functioning (B-EF) has been a central and long-standing debate among ecologists for decades (Yachi & Loreau 1999; Loreau *et al.* 2001; Hooper *et al.* 2005). Although there is a consensus on the positive effects of biodiversity on ecosystem functioning (Hooper *et al.* 2012), we do not have sufficient information on how different stressors affect this relationship (De Laender *et al.* 2016). While there is some evidence suggesting that the positive correlation between B-EF holds true also under environmentally stressful conditions (Baert *et al.* 2016a, 2018), these conclusions have been derived by applying random manipulations on relatively simple species assemblages, often containing just one trophic level (Tilman *et al.* 2014). Biodiversity manipulations are essential to unveil underpinning mechanisms driving the B-EF relationship. However, these approaches are biased in several ways. First, under natural conditions, biodiversity changes are non-random (Mcmahon *et al.* 2012; Gerhard *et al.* 2020), but rather depend on the type of anthropogenic impact (e.g., species introductions, climate change, chemical pollution). Second, the consequences of biodiversity changes do not stop at one single trophic level, but propagate across the whole food web (Rumschlag *et al.* 2021). And, finally, the impacts of stressors on both biodiversity and ecosystem functioning are not constant over time, which can lead to different responses and recovery trajectories depending on stressor interactions (Garnier *et al.* 2017). Therefore, studying the effects of multiple agrochemicals on community structure and ecosystem functioning may help not only to define thresholds for ecosystem management, but also to improve our understanding on the B-EF relationship under environmentally relevant sources and levels of stress.

In this study we addressed some of the most pressing issues related to freshwater contamination by agrochemicals, which include the temporality of the multiple stressor effects, the influence of community composition related to nutrient enrichment on the observed responses, and the relationship between biodiversity and ecosystem functioning under stress conditions. Explicitly, we tested the following hypotheses:

H1: Multiple stressor effects on community structure may be delayed, as a result of stress-driven changes in population and community dynamics;

H2: Nutrient enrichment modifies community composition, leading to changes in their resilience to subsequent pesticide contamination;

H3: The generally accepted positive correlation between biodiversity and ecosystem functioning is maintained under stressful conditions.

These hypotheses were tested by performing a mesocosm experiment in which we evaluated the single and combined effects of two pesticides (i.e., the herbicide diuron and the insecticide chlorpyrifos), in presence and absence of nutrient enrichment. The long-term effects of these stressors were assessed on the structure and diversity of phytoplankton, zooplankton, and benthonic macroinvertebrates communities, as well as on ecosystem functional parameters such as primary productivity (using chlorophyll-a as a proxy of phytoplankton biomass) and total ecosystem respiration.

2. Materials and methods

2.1 Experimental design

An outdoor experiment was performed between May and July of 2019 at the mesocosm facilities of the IMDEA Water Institute, which is located in Alcalá de Henares (Spain), and subjected to a semi-arid Mediterranean climate. Each mesocosm consisted of a PVC tank (diameter: 120 cm; water depth: 75 cm) initially filled with approximately 40 cm of silty-sand sediments and 850 L of water from an artificial lagoon. A total of 24 mesocosms were stocked with aquatic macrophytes (*Myriophyllum sp.* and *Elodea sp.*) and invertebrates collected from unpolluted water bodies in the vicinity of Alcalá de Henares. The biological community in the mesocosms was allowed to establish, and homogenized among experimental units, for two months prior to the start of the experiment.

A full factorial design was used with eight different treatments applied in a randomized fashion and three replicates per treatment. The treatments included: the insecticide chlorpyrifos (present and absent), the herbicide diuron (present and absent), nutrients (added, not added), and their binary and tertiary interactions. Diuron is a phenylurea herbicide that affects the photosystem-II and reduces the growth of a wide range of primary producers, while chlorpyrifos is an organophosphate insecticide that inhibits the acetylcholinesterase enzymatic activity and is highly toxic to aquatic crustaceans and insects. The two selected pesticides have been widely used in agriculture (Knauer & Hommen 2012; Huang *et al.* 2020) and are routinely monitored as part of the European Water Framework Directive (EU 2013). Moreover, they have been found to co-occur in freshwater ecosystems and to be major drivers of ecotoxicological risks (Arenas-Sánchez *et al.* 2019b). In this study, chlorpyrifos and diuron were applied at the concentration of 1 µg/L and 18 µg/L, respectively, which represent peak exposure concentrations measured in the edge of agricultural field surface waters (Knauert *et al.* 2008; Thompson *et al.* 2016). Nutrients (P and N) were applied twice per week as a solution containing 1.820 g of NH_4NO_3 and 0.208 g of KH_2PO_4, which resulted in a nutrient addition of 750 µg/L of N and 75 µg/L of P, respectively. These nutrient levels correspond to a eutrophic/hypereutrophic ecological scenario. Nutrient additions started 3 weeks prior to the pesticide's application. Moreover, before the first nutrient addition, macrophytes were initially removed in order to create a phytoplankton dominated system, typically occurring in hypereutrophic shallow waters. The mesocosms that did not receive nutrients were considered oligo-mesotrophic based on nutrient analyses. In these systems, macrophytes were allowed to grow naturally. Details on chemical applications are provided in the Supporting Information (SI), while the analytical methods and the results of their analysis are provided in Oliveira dos Anjos et al. (2021)

2.2 Water quality parameters

Water samples (1 L) were collected on days -5, 15, 30, and 50 relative to the application of the pesticides in order to analyse the concentrations of ammonia, nitrate, ortho-phosphate, and total phosphorous. The total inorganic nitrogen concentration in the mesocosm water was calculated as the sum of the concentrations of ammonia and nitrate, assuming nitrite as negligible. Analysis of nutrients concentrations were performed by colorimetry following the methods described in APHA (2005). Temperature, dissolved oxygen, pH, conductivity, and turbidity were measured by a multimeter (HANNA HI0194) in the morning (8 a.m.) and evening (7 p.m.) on the days -5, 7, 15, 30, and 50 relative to the application of the pesticides. Additional water samples (0.5 L) were taken on days -5, 15, 30, and 50 relative to the pesticides application to assess the concentration of chlorophyll-a. Chlorophyll-a was measured following the protocol described in APHA (2005) and was used as a proxy of primary productivity, as it measures the biomass of the suspended microalgae community. Ecosystem respiration was calculated as the difference between dissolved oxygen at dawn and at dusk of the same day.

2.3 Phytoplankton

Phytoplankton were sampled on days -5, 7, 15, 50 relative to the pesticides' application. Depth- integrated water samples were taken with a PVC tube (six sub-samples per mesocosm mixed in a bucket). Next, 250 mL of this sample was introduced into glass amber bottles and 10% Lugol's iodine was added for preservation. Counting and identification were done to the lowest taxonomic resolution possible with an inverted microscope (Motic AE31) coupled to a Motic Plan x 100/1.25 Oil ∞/0.17 objective.

2.4 Zooplankton

Zooplankton samples were taken on days -5, 7, 14, 30, and 50 relative to the application of pesticides. Six depth-integrated samples of approximately 1 L were collected from each mesocosm using a PVC tube and mixed together. Next, 5 L of the composed water sample were passed through a zooplankton net (55 µm) and concentrated to an approximate volume of 100 mL. The concentrated samples were fixed with Lugol's iodine solution and stored in dark conditions at room temperature until identification and counting. Cladocera, Ostracoda and Copepoda were identified and counted in the entire zooplankton sample using an Olympus SZx2-TR30 stereomicroscope with a magnification of 20×. As for Rotifera and Copepoda nauplii, a subsample of 1 mL was taken and analysed using a binocular Olympus UCTR30-2 microscope with a magnification of 100×. Cladocera and Rotifera were identified up to the genus or species level. For Cyclopoida, a distinction was made between the adult stages (as the sum of true adults and copepodite stages) and the nauplius stages during counting and for further statistical analyses. Ostracoda appeared in relatively low abundance and were not further identified.

2.5 Macroinvertebrates

Macroinvertebrates were sampled on days -32, 15, 30, and 50 relative to the application of the pesticides. In order to collect pelagic and benthic individuals, three sampling methods were used. First, a net (mesh size: 0.5 mm) was passed twice through the side of the mesocosms (in both directions) to catch the animals that were swimming or resting on the mesocosm's wall. Second, two pebble baskets positioned over the sediment surface were collected, and third, two traps filled with macrophyte shots (*Elodea sp.*), *Populus sp.* leaves and stones were collected from the sediment's surface using a net. The invertebrates sampled from each mesocosm with the three sampling methods were pooled together, identified, and counted alive. Afterwards, the invertebrates were returned to their original mesocosms together with the colonizing pebble baskets and traps. The macroinvertebrate taxonomic identification was performed to the lowest practical resolution level making use of freshwater biology guides (e.g. Tachet *et al.* 2000).

2.6 Statistical analyses

We examined the effects of diuron, chlorpyrifos, nutrients and their interactions on the single communities (phytoplankton, zooplankton, macroinvertebrates) in isolation, using a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations (Anderson 2001), based on Bray-Curtis distances between each treatment and the control obtained from log-transformed abundance data. Homogeneity of data dispersion across the different treatments was tested using a distance-based dispersion test, followed by a permutation-based test of multivariate homogeneity of group dispersions (variances). The resulting PERMANOVA model was: Chlorpyrifos + Diuron + Nutrients + Chlorpyrifos x Diuron + Chlorpyrifos x Nutrients + Diuron x Nutrients + Chlorpyrifos x Diuron x Nutrients. To further explore the effects of the treatments on the experimental communities, we analysed single taxon abundance response for some relevant taxa using a three-way ANOVA. We tested normal distribution and homogeneity of variances of residuals using the Shapiro-Wilk test combined with Quantile-Quantile plots and Levene's test respectively. When the assumptions were not met, the data were either log or square root transformed. Three-way ANOVA was used to study the effects of the treatments on water quality parameters as well. Furthermore, we applied some diversity indexes to investigate the effects of single and combined stressors on communities. We calculated species richness, Shannon's index and Berger-Parker dominance index using the R packages “BiodiversityR” (Kindt & Coe 2005) and “lawstat” (Hui *et al.* 2008). Differences among indexes' values and ecosystem functioning parameters caused by treatments were analysed by means of a three-way ANOVA. The three-way ANOVA model was the same used for the PERMANOVA. To visualize the influence of the treatments on the B-EF correlation, we calculated the Spearman correlation coefficient between the total richness of phytoplankton taxa and the two ecosystem functions we measured (i.e., primary productivity and total ecosystem respiration). We considered statistically significant effects when the calculated p-values were <0.05. All statistical analyses and figures were created using R (version 3.5.1) in Rstudio (2020)

3. Results

3.1 Phytoplankton responses

The PERMANOVA analysis showed that the application of nutrients significantly modified the phytoplankton community composition during the whole experimental period (Table 1, Figure 1). Mesocosms treated with nutrients were generally characterized by high total abundance, and lower species richness (Figure 2 and 3, Table S1). Chlorpyrifos significantly affected phytoplankton community composition, starting from day 15 onwards (Table 1, Figure 1). In the mesocosms treated with chlorpyrifos, abundance was significantly increased. Particularly, Chlorophyta (the most abundant taxonomic group) significantly increased in abundance on days 7 and 50 (Figure S1, S2, Table S1, and S2). Diuron modified community composition in every sampling day after its application (Table 1, Figure 1). Total abundance was not affected, but species richness strongly decreased after the herbicide application (Figure 2, Table S1). Diatoms, Cryptophyta, and Cyanobacteria were the most affected taxonomic groups (Table S2). Only on day 50 a significant interaction between the herbicide and nutrients was found by the PERMANOVA analysis.

3.2 Zooplankton responses

Nutrients addition caused significant changes in the zooplankton community structure on every sampling day (Table 1, Figure 1). High nutrient concentrations resulted in some cases in increased abundances (Figure 3, Table S3), but also a reduction of species richness and increased values of species dominance (Figure 2, Table S3). Particularly, some Rotifera species (*Testudinella sp.* and *Polyarthra sp.*) were severely impaired by the nutrients treatment (Table S4). Chlorpyrifos also shifted the community composition in each sampling day after its application (Table 1, Figure 1). In the mesocosms treated with chlorpyrifos, zooplankton abundance was low until day 15 (Figure 3), as were species richness (Figure 2) and the Shannon diversity index, while taxa dominance was higher (Table S3). From day 30 to the end of the experiment, abundance levels were equal or higher as compared to the controls, and richness, diversity, and dominance were still dissimilar to the control mesocosms (Table S3). Chlorpyrifos caused a drastic reduction in Cladocera abundance (Figure S3 and S4), while Copepoda increased in abundance. The herbicide treatment significantly modified the zooplankton community composition on day 30 (Table 1). Consistently, zooplankton species richness was significantly reduced on day 30 (Table S3). The most impaired taxa were *Daphnia sp*. and *Simocephalus sp*. (Table S4).

The chlorpyrifos and diuron were found to interact significantly by the PERMANOVA, both on days 15 and 50 (Table 1). On the same day, chlorpyrifos and diuron interacted synergistically decreasing species richness (Figure 2, Table S3) and abundance was generally decreased (Figure 3). The most affected taxa were Copepoda (nauplius stage) on day 15, and *Daphnia sp*. and *Ceriodaphnia sp*. on day 50. On day 7, a significant interaction between the insecticide and the nutrients treatment on community composition was found (Table 1).

	N	I	H	IxN	HxN	IxH	IxHxN
Phytoplankton							
D-5	**0.023 ***	0.517	0.904	0.48	0.431	0.36	0.68
D7	**0.048 ***	0.213	**<0.001 *****	0.65	0.783	0.112	0.501
D15	**0.007 ****	**<0.001 *****	**<0.001 *****	0.421	0.199	0.712	0.461
D50	**0.003 ****	**0.007 ****	**0.006 ****	0.566	**0.002 ****	0.566	0.293
Zooplankton							
D-5	0.178	0.941	0.483	0.632	0.162	0.578	0.647
D7	**0.011***	**0.001*****	0.649	**0.037***	0.169	**0.037***	0.183
D15	**<0.001*****	**0.001*****	0.232	0.129	0.05	0.846	0.516
D30	**0.025***	**0.001*****	**0.011***	0.975	0.222	0.284	0.981
D50	**0.014***	**0.002****	0.265	0.514	0.514	**0.044***	0.265
Macroinvertebrates							
D-32	0.883	0.3	0.442	0.01	0.952	0.57	0.273
D15	**0.041 ***	**<0.001 *****	**0.003****	0.349	0.071	0.079	0.407
D30	0.121	0.054	**<0.001*****	0.405	0.642	0.263	0.076
D50	**0.005****	0.628	0.203	0.709	0.865	0.071	0.087

Table 1. Summary of p-values resulting from the Permutational Analysis of Variance (PERMANOVA) based on Bray-Curtis dissimilarities using abundance data for the phytoplankton, zooplankton and macroinvertebrate communities for the period pre (Day -5 and -32) and post-pesticide application (Days 7, 15, 30 and 50). Significant effects ($p < 0.05$) of the treatments are shown in bold. '***' p<0.001, '**' p<0.01, '*'p< 0.05. N: nutrients; I: insecticide/chlorpyrifos; H: herbicide/diuron.

3.3 Macroinvertebrate responses

The addition of nutrients caused a shift in macroinvertebrates community structure on days 15 and 50 (Table 1, Figure 1). The eutrophic conditions were generally associated with higher total abundance (Figure 3). However, nutrients enrichment led to a decrease in diversity and to an increase in dominance (Figure 2, Table S5). Chlorpyrifos had significant effects on the macroinvertebrate's community structure in the first half of the experiment (Table 1, Figure 1). The community impaired by the insecticide showed reduced total abundance (Figure 3), and species richness (Figure 2, Table S5). Some sensitive taxa were temporally eradicated (Ephemeroptera, Figure S5 and S6) and generally most of the insect taxa were significantly reduced (*Chironomini*, *Cloeon sp*, *Leptoceridae*, Table S6). However, more tolerant taxa with a simple nervous system (Annelida), increased their abundance (Figure S5). On day 30, chlorpyrifos did not have a significant effect on community structure (Table 1), nor any deviation from the controls was noticed regarding total abundance or diversity indices (Table S5). The macroinvertebrate community treated with chlorpyrifos no longer deviated from controls at the end of the experiment for any compositional or diversity matrix. The herbicide treatment significantly shifted community composition on day 15 and 30 (Table 1). The most affected taxa were *Chironomini*, *Cloeon sp*, *Caenidae* and *Leptoceridae*, which significantly decreased in abundance (Table S6).

No significant interaction between stressors was found by the PERMANOVA analysis (Table 1), but there were significant interactions among stressors at the population level (Table S6). The Diptera taxa Chironomini and Orthocladiinae were negatively impaired by the interaction between diuron and chlorpyrifos, whereas *Dugesia sp.* increased in abundance almost every sampling day (Table S6).

3.4 Effects on primary productivity, ecosystem functioning and physicochemical parameters

Physicochemical parameters responded consistently to the treatments (Table S7 and S8). As expected, nutrient concentrations were significantly higher in the mesocosms undergoing nutrients addition. In those systems, chlorophyll-a, oxygen concentration and pH were also significantly higher. Diuron application, significantly decreased the parameters related to photosynthetic activity: oxygen concentration and pH, with the exception of chlorophyll-a concentration (Figure 4, Tables S7 and S8). Primary productivity (measured as chlorophyll-a concentration) was generally increased by nutrient enrichment (Figure 4). Diuron increased primary productivity on day 7, but did not have other significant effect during the experimental period on chlorophyll-a concentration (Table S8). Diuron and nutrients significantly interacted on day 7 (negative synergism), causing a decrease in primary productivity. Conversely, systems treated with diuron and nutrients simultaneously increased primary productivity, even though differences were not statistically significant from day 15 onwards. The other treatments did not have any significant effect on primary productivity.

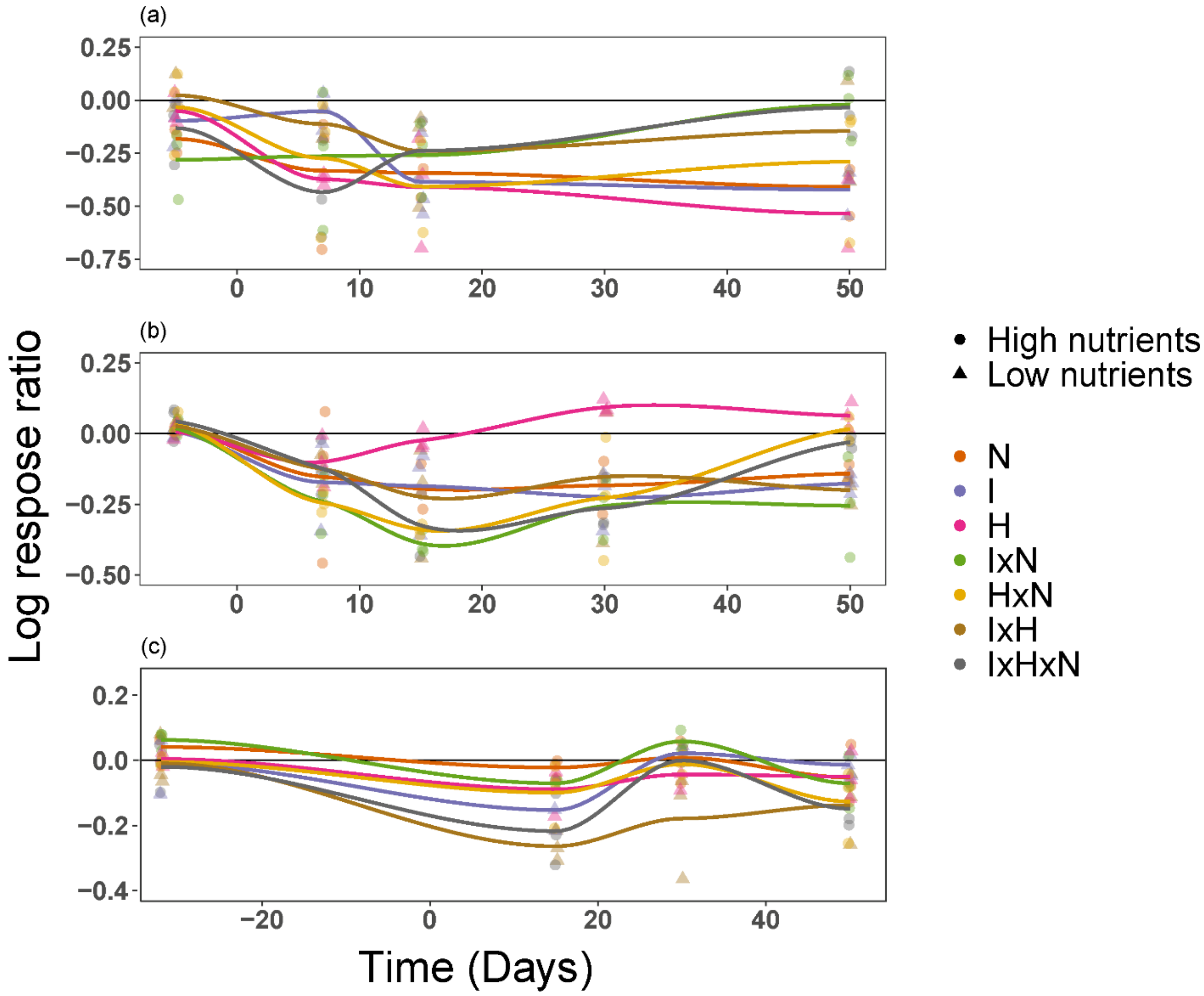

Figure 1. Log response ratio (calculated as: $LRR = ln\ (\frac{BC_t}{BC_c})$, where BC_t is the Bray-Curtis distance of the treatment and BC_c is the Bray-Curtis distance of the control) of community composition across the different treatments for phytoplankton (a), zooplankton (b) and macroinvertebrates (c). Note: in (a), (b) and (b), the solid line on zero represents the control. Thus, the closer a treatment line is to zero, the more similar it is to the control. N: nutrients; **I**: insecticide/chlorpyrifos; H: herbicide/diuron.

Total ecosystem respiration was generally increased in systems treated with nutrients, whereas diuron decreased total respiration (Figure 4, Table S8). The mixture of chlorpyrifos and diuron interacted significantly at the end of the experiment (day 50), reducing total respiration (negative synergism). The mixture of pesticides also interacted significantly under eutrophic conditions on day 30 (three-way interaction).

The sign and strength of the correlation between biodiversity of primary producers and ecosystem functioning changed depending on the function measured (productivity or respiration) and on the stressor(s) applied (Figure 5). The B-EF were positively correlated in the control (significantly for primary productivity). Generally, a clear trend could be identified for primary productivity, where the B-EF correlation was always positive when a pesticide was applied together with nutrients, whereas it was negative when pesticides or nutrients were applied individually. No clear trends were found for ecosystem respiration (Figure 5).

4. Discussion

4.1 Single stressor effects

The insecticide disturbance caused the decline of the most sensitive taxa (Cladocera), allowing the more tolerant taxa (Copepoda) to thrive. The post-disturbance disbalance in relative densities determined an alternative dominance state (*sensu* Fukami & Nakajima, 2011), in which Copepoda became the dominant species, likely through competitive exclusion and niche pre-emption after Cladocera's decline (Fukami 2015). Supporting this argument, the mesocosms treated with chlorpyrifos (as a single stressor or as a mixture with diuron) showed the lowest abundance of Cladocera across all sampling days (Figure S4), even when the insecticide concentration was negligible (day 50) and not expected to cause any harm to aquatic invertebrates (Huang et al., 2020).

Chlorpyrifos was not expected to have a direct effect on primary producers at the tested concentration (Huang *et al.* 2020). Yet, phytoplankton community composition resulted altered during a large part of the experimental period in the insecticide treatment. Different zooplankton taxa are known to have different selective grazing behaviors (Fulton & Paerl, 1988; Hambright et al., 2007), which can shape primary producers' community composition (Hillebrand et al., 2007; Müren et al., 2005; O'Connor et al., 2009). In our systems, the reduction in Cladocera abundance, and the inverse response of Copepoda, changed the selective grazing pressure on phytoplankton. Thus, we show that a change in the nature of the top-down regulation can actively modify the community structure of primary producers. These findings suggest that selective stressors such as pesticides, which have a very specific target related to their toxic mode of action, can result in indirect effects that propagate through the food web, determining different community dynamics and recovery trajectories in taxonomic groups not directly impaired by the stressor.

Diuron shifted the primary producer's community structure and contributed to a decrease of palatable phytoplankton species, reducing energy resources for zooplankton grazers (Chen *et al.* 2004). The bottom-up effect was reflected in the macroinvertebrate community, which showed compositional changes in response to the herbicide treatment. The most impacted macroinvertebrates taxa (particularly chironomid larvae and

Ephemeroptera, all herbivore taxa) have been previously shown to be indirectly affected by herbicides as a result of decreased abundance of food resources (primary producers) (Relyea 2005; Rohr & Crumrine 2005).

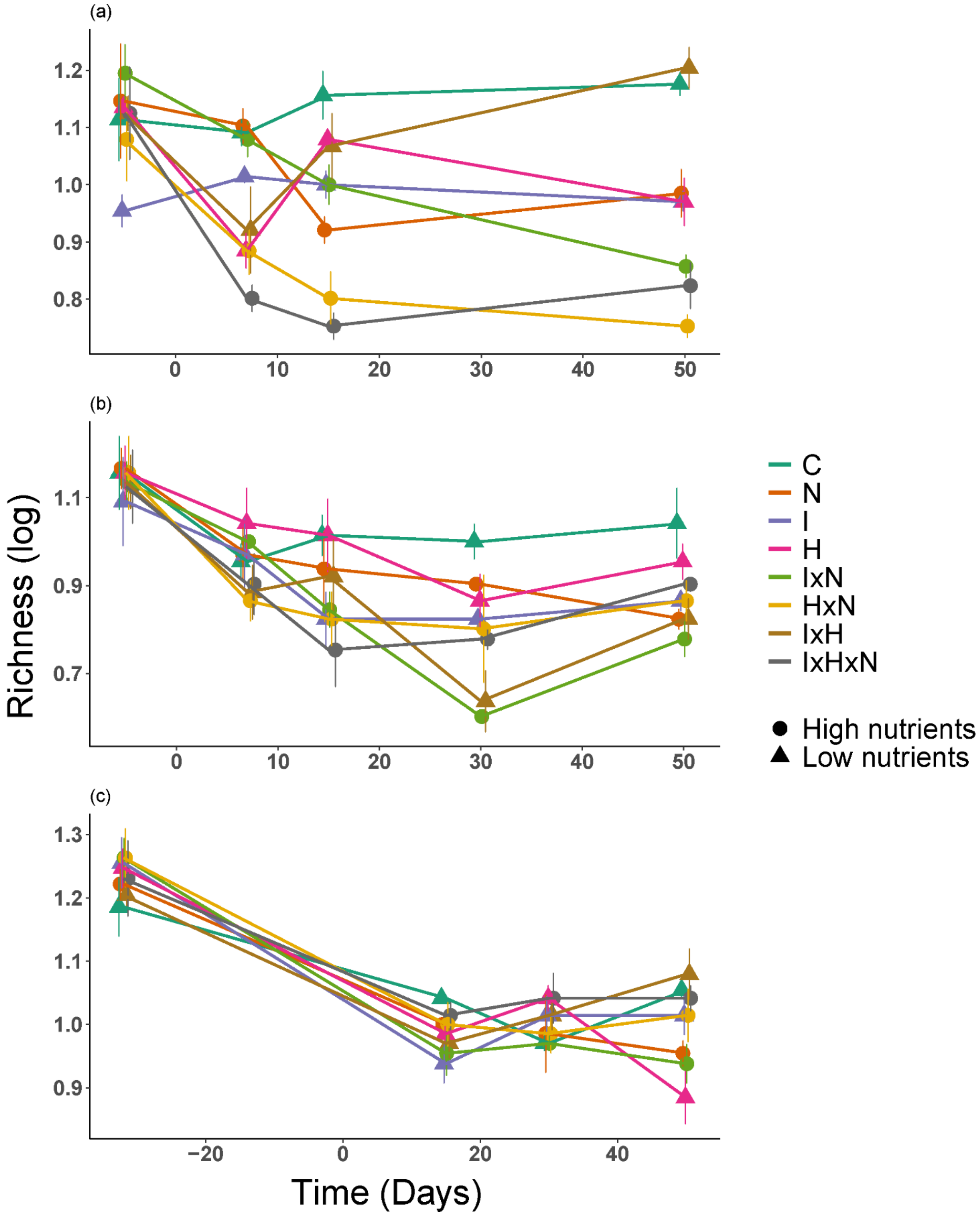

Figure 2. Time series of taxa richness in the different treatments over the course of the experiment for (a) phytoplankton, (b) zooplankton and (c) macroinvertebrates. Symbols and colours indicate nutrient and pesticide treatments, respectively. The error bar represents the standard deviation. Please note that a small offset on x-axis values was introduced to better distinguish symbols and error bars. C: control; N: nutrients; I: insecticide/chlorpyrifos; H: herbicide/diuron.

4.2 Temporality of multiple stressor effects

Significant stressor interactions appeared in different moments of the experiment and significantly affected communities' structure by the end of the experimental period (supporting H1). Particularly, the significant interaction between chlorpyrifos and diuron on the zooplankton composition appeared only on day 50 (Figure 1). Several zooplankton sensitive species were still significantly reduced in abundance on day 50 (*Daphnia sp*. and *Cerodaphnia sp*., Table S4), indicating a missing recovery. Additionally, the insecticide and the herbicide interacted significantly on zooplankton's species richness on day 15 and 50. The direct negative effects of the insecticide and the reduction of food source caused by the herbicide resulted in a negative synergistic interaction in both the medium and the long-term. Chlorpyrifos and diuron interacted significantly on day 50 impairing several individual taxa of other organism groups (Tables S2, S4, S6). Direct effects of both pesticides contributed to impair sensitive species of different organism groups, leading to indirect top-down and bottom-up effects that are subtle and not easy to unravel. These findings indicate that to utterly assess the effects of multiple stressors, it is not sufficient to limit experimental research to one short-term temporal scale (i.e., resistance), but that also recovery and resilience should be evaluated during prolonged periods, as late stressors interactions may occur and preclude recovery.

On aggregate ecosystem properties (productivity and respiration) interactions between stressors showed opposite temporal dynamics. For productivity, nutrients and the herbicide interacted on day 7 showing a negative synergism. The press disturbance created by the application of nutrients reduced the overall phytoplankton diversity and the combined exposure to the herbicide decreased the photosynthetic activity. The initial reduction in richness caused by the nutrients limited the number of species able to maintain high photosynthetic activity under herbicide exposure (that is, reduction of the insurance effect, see Yachi & Loreau, 1999 for details), which resulted in a negative synergistic interaction.

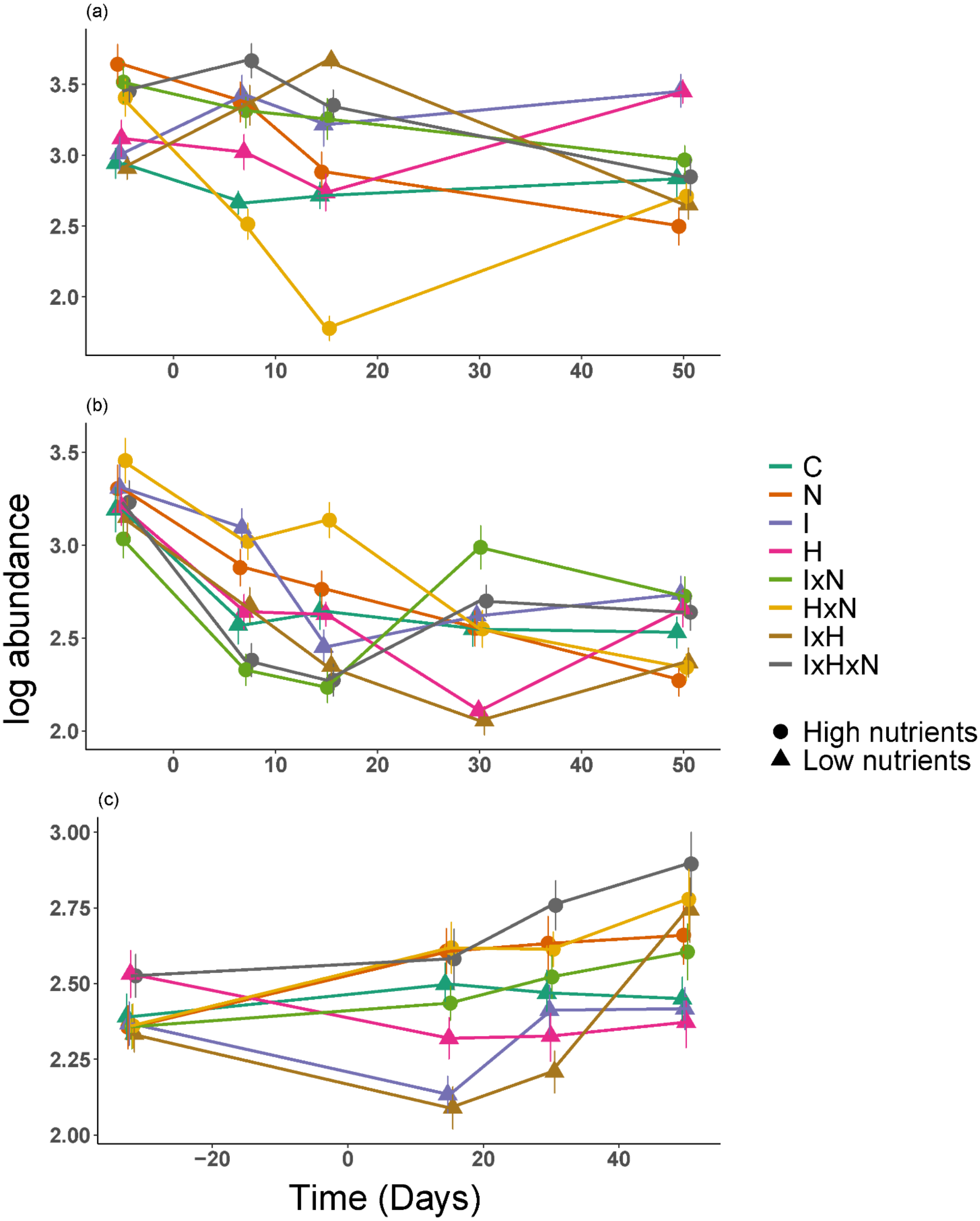

Figure 3. Time series of log-transformed abundance data in the different treatments over the course of the experiment for (a) phytoplankton (individuals/mL), (b) zooplankton (individuals/L) and (c) macroinvertebrates (individuals/sample). Symbols and colours indicate nutrient and pesticide treatments, respectively. The error bar represents the standard deviation. Please note that a small offset on x-axis values was introduced to better distinguish symbols and error bars. C: control; N: nutrients; I: insecticide/chlorpyrifos; H: herbicide/diuron. On the x-axis, Time (days) is relative to pesticides' application.

Chlorpyrifos and diuron showed a negative synergism on ecosystem respiration only on day 50. The long-term change in phytoplankton and zooplankton community structures promoted by the two stressors (Table 1), might have induced a simpler community with overall reduced respiration.

4.3 Effects of nutrient enrichment on pesticide responses

In eutrophic systems, the biotic response was rather consistent across different organism groups. Despite a general increase in total abundance and a faster recovery of tolerant species from pesticide stress, nutrients caused a decrease in species richness and an increase in species dominance (supporting H2). At the beginning of the experiment (day -5) phytoplankton abundance was higher in all treatments undergoing nutrients enrichment. Later, the initial increase in primary producers' abundance was followed by an increased density of grazers, which limited phytoplankton proliferation. Consistently with our results, high nutrient concentrations have been shown to alter the heterotrophs/autotrophs ratio in the long term, by increasing consumer biomass relative to producer biomass, and reducing the total biomass of the food web despite increases in primary productivity (O'Connor *et al.* 2009). The increase in primary productivity was reflected in our study by the phytoplankton biomass (measured as chlorophyll-a concentration) as well as by the water's physicochemical parameters (pH and oxygen saturation), which were significantly higher in the mesocosms with nutrients' enrichment.

Nutrient enrichment is often considered as an antagonistic factor against detrimental effects of other disturbance types such as pesticides in temperate regions (Barmentlo et al., 2019; Halstead et al., 2014; Matthaei et al., 2010; Piggott et al., 2015a, b). Consistently, in our experiment, nutrients often reduced the negative effects of pesticides on total abundance, especially at the population level (Tables S2, S4, S6). This antagonism between nutrients and pesticides is mainly related to three factors. First, high nutrient concentrations reduced bioavailability and accelerated the dissipation of the added pesticides in the water column, reducing the overall toxic pressure (Tables S9 and S10). Second, nutrients provided larger resource availability for primary producers, which was translated into higher phytoplankton productivity even under herbicide exposure (Figure 4). Higher phytoplankton biomass fueled growth and reproduction of invertebrates, which recovered faster from both insecticide and herbicide exposure under eutrophic conditions. Third, eutrophic conditions modified the habitat and contributed to the selection of species better adapted to daily oxygen fluctuations and higher pH, which are also more resilient to additional pesticide stressors (Belando *et al.* 2017).

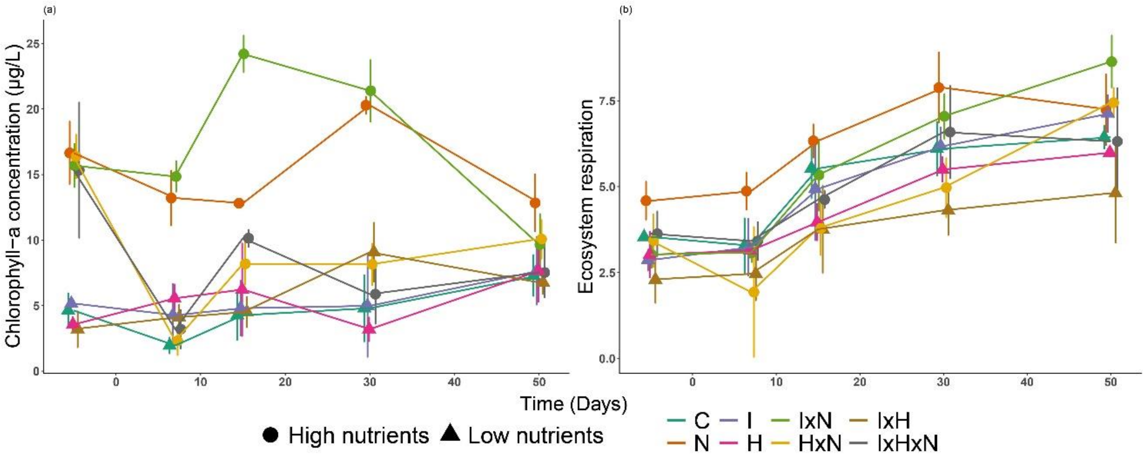

Figure 4. (a) Time series of chlorophyll a concentration (a proxy for phytoplankton biomass/primary productivity) in all treatments over the course of the experiment. (b) Time series of total ecosystem respiration (a proxy for ecosystem functioning) in all treatments over the course of the experiment. Symbols and colours indicate nutrient and pesticide treatments, respectively. C: control; N: nutrients; I: insecticide/chlorpyrifos; H: herbicide/diuron.

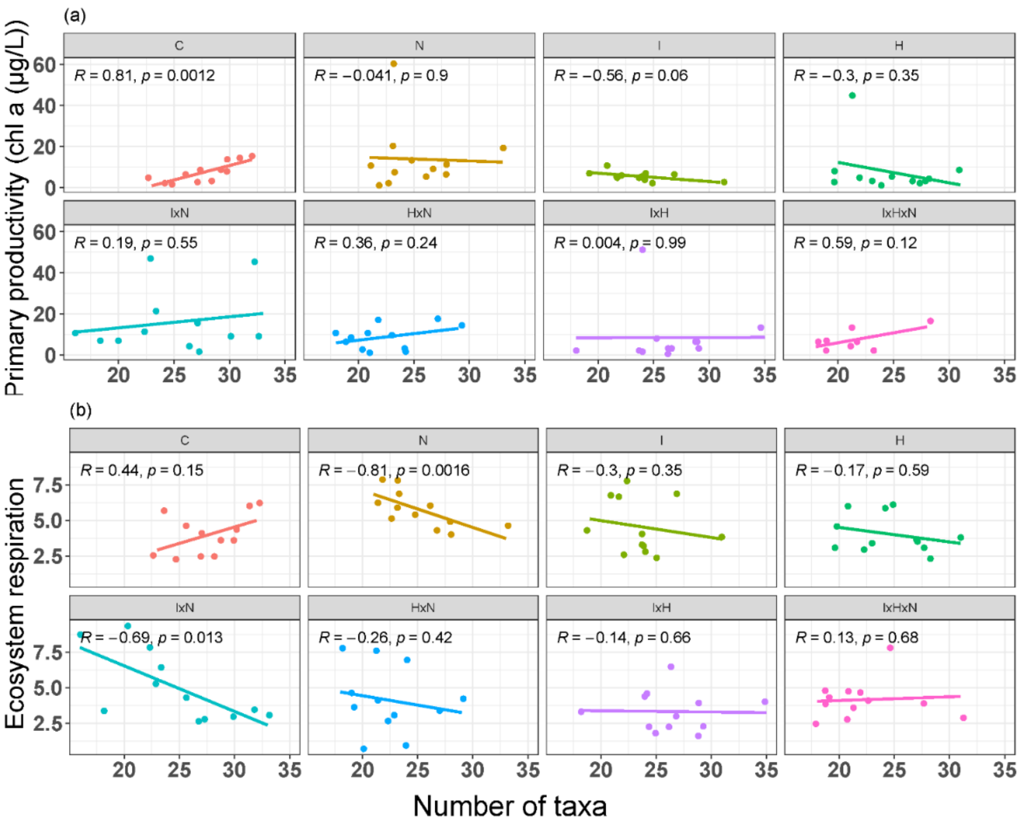

Figure 5. (a) Correlation between number of phytoplankton taxa and primary productivity. (b) Correlation between number of phytoplankton taxa and total ecosystem respiration. R represents the Spearman correlation coefficient, and p represents the p-value of the correlation. C: control; N: nutrients; I: insecticide/chlorpyrifos; H: herbicide/diuron.

4.4 Relationship between biodiversity and ecosystem functioning

Nutrients enrichment generally increased primary productivity (chlorophyll-a concentration) and ecosystem respiration. Especially in the insecticide-nutrients treatment, primary productivity and ecosystem respiration were higher compared to the other treatments. Likely, primary producers benefited from the release from the grazing pressure caused by the insecticide, which allowed phytoplankton biomass to increase. However, high nutrients availability promoted a faster zooplankton recovery from the insecticide disturbance, which reduced the primary productivity at the end of the experiment because of increased grazing activity. Diuron application did not significantly affect primary productivity, but did significantly decrease ecosystem respiration during the whole experimental period. Herbicide exposure also led to a significant decrease in pH, which might reflect the reduction in photosynthetic activity (Rumschlag *et al.* 2021). As for community composition, significant stressor interactions on ecosystem functioning were found only in the last part of the experiment. This might be a consequence of stress-driven changes in community composition and dynamics, which may have determined an overall shift in ecosystem respiration rate.

In line with previous investigations (Hooper *et al.* 2005, 2012), in the control systems we found a significant positive correlation between primary producers species richness and ecosystem functioning, both for primary productivity and respiration. However, in different treatments, the positive effect of biodiversity on ecosystem functioning was reversed. For primary producers' productivity, consistent trends could be highlighted. The slope of the B-EF correlation was always positive when a pesticide was applied together with nutrients, whereas it was negative when pesticides or nutrients were applied individually (against H3, Figure 5). This might be related to the general positive effect of nutrients enrichment on the detrimental perturbation exerted by the pesticides, as the positive effect of biodiversity on ecosystem functioning has been shown to decrease along a gradient of increasing stress (Steudel *et al.* 2012). Thus, nutrients enrichment may have alleviated the environmental stress caused by the pesticides application, changing the overall sign of the B-EF relationship. Yet, such a clear trend was not detected for ecosystem respiration. For ecosystem respiration, the B-EF relationship was negative for all applied stressors and stressor combinations, except for the mixture of pesticides applied with nutrients. This indicates that the hypothetical stress threshold reversing the positive effect of biodiversity on ecosystem functioning might differ for distinct functions (Hiddink *et al.* 2009). Future research should focus on determining such thresholds in complex communities under environmentally relevant gradients of stress using a gradient experimental approach.

Despite the generally accepted positive correlation between diversity and ecosystem functioning, it is not the first time that deviations from this relationship are reported (Caldeira *et al.* 2005; Downing & Leibold 2010). Yet, our experiment is one of the first studies to provide evidence supporting this concept in a multi-trophic community, where disturbance affected several trophic levels, causing changes in the whole community structure. Our results also show that, in a species-rich assemblage, the chances to have tolerant species are high (Yachi & Loreau 1999). Stress tolerant species can benefit from the removal of competitors and/or grazers and increase their abundance. When the tolerant species have high per capita contribution to function, the overall functional level may increase (Baert *et al.* 2016a). In our experiment, changes in fitness through interspecific differences in stress tolerance might have triggered a dominance effect in favor of highly functional species, which thrived and boosted functional parameters, despite a general decrease in diversity (Schäfer *et al.* 2012).

5. Conclusions

Our study shows that agrochemicals, as single and as multiple stressors, can result in long-term effects on the structure and functioning of freshwater ecosystems. Studying community and ecosystems responses measuring only one time point after stressors application may underestimate non-additive effects on freshwater systems occurring after prolonged time periods. Moreover, toxic mode of action and consequent changes in species interactions are important drivers of community structure and ecosystem functioning, which in turn, influence post-exposure recovery patterns and resilience of freshwater ecosystems. We found that nutrients enrichment and trophic status is a key driver in modifying community structure and influencing the sensitivity and recovery of ecosystems to additional agricultural stressors such as pesticides, and therefore should be considered in pesticide risk assessment. Finally, we demonstrated that freshwater ecosystems show a high functional redundancy to selective stressors such as pesticides, and that significant losses of biodiversity are not always directly translated into a loss of important ecological functions such as primary productivity or ecosystem respiration. However, as we showed here, some agrochemical stressor combinations can decouple the otherwise positive relationship between biodiversity and ecosystem functioning.

Acknowledgements

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Chapter 4

Effects of multiple stressors on the dimensionality of ecological stability

Francesco Polazzo and Andreu Rico

Abstract

Ecological stability is a multidimensional construct. Investigating multiple stability dimensions is key to understand how ecosystems respond to disturbance. Here, we evaluated the single and combined effects of common agricultural stressors (insecticide, herbicide, and nutrients) on four dimensions of stability (resistance, resilience, recovery and invariability) and on the overall dimensionality of stability (DS) using the results of a freshwater mesocosm experiment. Functional recovery and resilience to pesticides were enhanced in nutrient-enriched systems, whereas compositional recovery was generally not achieved. Pesticides did not affect compositional DS, whereas functional DS was significantly increased by the insecticide only in nutrient-poor systems. Stressor interactions acted non-additively on single stability dimensions as well as on functional DS. Moreover, we demonstrate that pesticides can modify the correlation between functional and compositional aspects of stability. Our study shows that different disturbance types, and their interactions, require specific management actions to promote ecosystem stability.

1. Introduction

Ecosystems are exposed to several co-occurring disturbances (Millennium Ecosystem Assessment 2005). Human-mediated disturbances differ greatly in nature (climate change, species invasion, chemical pollution), temporal extension (press, pulse) and intensity (Turner 2010; Jacquet & Altermatt 2020). Understanding the impact of anthropogenic disturbances across levels of biological organization is critical to improve our capacity to manage and restore natural systems (De Laender *et al.* 2016). Recently, the overall understanding of ecosystems stability in relation to disturbance has improved greatly, starting with the work by Donohue *et al.* (2013), which unravelled the multidimensional nature of stability. According to their work, the dimensionality of stability (DS) requires the assessment of several ecological properties, including resistance, resilience, recovery and invariability (Donohue *et al.* 2013, 2016).

Different stability properties can be strongly or poorly correlated among each other (Donohue *et al.* 2013; Hillebrand *et al.* 2018). When such properties are strongly correlated, the overall DS is reduced, resembling a one-dimensional concept, and all the correlating metrics give similar information (Domínguez-García *et al.* 2019). Conversely, when stability properties are not correlated, the dimensionality increases, meaning that every single metric provides a unique information on the system's stability. Consequently, DS has important policy and ecosystem management implications. When DS is high, managers could decide to maximize one stability property (e.g., resistance) without influencing other stability's aspects. On the other hand, when DS is low, no action on a single stability property can be taken without affecting the others and, therefore, management actions cannot specifically address one stability property in isolation. Moreover, when DS is low, the sign of these correlations becomes pivotal. If two stability properties correlate negatively, a trade-off exists, which prevents maximising both stability aspects. In this way, one stability property can be promoted only at the expenses of the other. When the correlation is positive, such trade-off does not exist, allowing to tackle both stability properties simultaneously.

The measurement of several components of stability is not yet a common practice in ecology. Despite the large number of metrics existing to quantify stability, most of the available studies (> 90%) used a one-dimensional approach (Donohue *et al.* 2016), therefore failing to describe its multidimensional nature. Furthermore, Kéfi *et al.* (2019) pointed out that there is a disbalance towards the investigation of stability in species-poor systems. Among empirical studies, almost 30% involved systems with < 10 species, and a negligible number of experiments used a species assemblage large enough to resemble a natural ecosystem (Kéfi *et al.* 2019).

The majority of studies addressing ecological stability have investigated the response of ecosystems to one single stressor (Kéfi *et al.* 2019). However, ecosystems are exposed to multiple disturbances at a time (Birk *et al.* 2020; Lemm *et al.* 2020). Multiple stressors research has accordingly grown in recent years (Orr *et al.* 2020), providing invaluable information on stressors interactions and unravelling mechanisms behind the observed responses. Meta-analyses have tried to summarize common stressors interactions across ecosystems (Crain *et al.* 2008; Jackson *et al.* 2016; Birk *et al.* 2020) and new methods have been proposed to understand and classify non-additive interactions (Piggott *et al.* 2015c; Thompson *et al.* 2018a; Tekin *et al.* 2020). However, the multidimensional conception of stability has never been applied in multiple stressors' research. Disentangling the multidimensionality of stability is key to unlock our understanding of - and our ability to predict - how biological systems respond to multiple disturbances. The urge of understanding how multiple stressors affect the different stability components has become pressing since interactions between stressors have been shown to be dependent on temporal scales (Garnier *et al.* 2017). That is, non-additive interactions between stressors can modify several stability dimensions, from early response to pulse disturbance (e.g., resistance) and recovery (e.g., resilience and recovery), to long-term responses to press disturbances (e.g., temporal invariability).

Here, we investigate the effects of multiple stressors on the dimensionality of stability in freshwater communities, by using data from a species-rich mesocosm experiment. We quantified four stability properties: resistance, recovery, resilience, and invariability, and analysed how stability is influenced by single and multiple disturbances in both, functional and compositional aspects, using a multitrophic aquatic community. Additionally, we assessed the correlation between stability properties related to different disturbance types and between compositional and functional stability properties. As stressors, we selected two pesticides with different toxicological properties. We used the herbicide (diuron) to selectively reduce primary producers' diversity and abundance, and an insecticide (chlorpyrifos) to reduce consumers' diversity and abundance. Both pesticides were applied at a dose that is expected to affect 50% of the primary producers and consumers, respectively. Also, we assessed whether nutrients enrichment (as a press stressor) can influence the DS to the pesticides. For this, we treated half of the mesocosms with a press (continuous) addition of nutrients (N and P) starting one month before the application of the pesticides, while the other half were not treated with nutrients. Nutrients enrichment is known to reduce diversity and to homogenise species assemblages at different scales (Donohue *et al.* 2009; Woodward 2012; Isbell *et al.* 2013). Ecosystem stability has been shown to change non-monotonically with diversity (Pennekamp *et al.* 2018). That is, diversity can increase as well as decrease stability. However, the underpinning mechanism driving the occasionally reported negative correlation between increasing diversity and stability remains poorly understood (Loreau & de Mazancourt 2013; Pennekamp *et al.* 2018).

Through this study we addressed the following hypotheses:

H1: Single and multiple disturbances influence individual stability properties as well as the DS.

The metrics we used to quantify DS can be affected non-additively by multiple stressors (Halstead *et al* 2014; Garnier *et al.* 2017). Consequently, we may expect to find significant stressors interactions on single stability dimensions as well as on the overall DS. Since DS depends on the correlations between stability properties, we expect those correlations to be modified by disturbance.

The recent advancement in theoretical studies may help to formulate predictions on how DS behave under multiple stress situations. The work by Radchuk *et al.* (2019) does not only show that disturbance type determines DS, but also indicates that disturbances resembling pesticide applications generally increase functional DS, whereas compositional DS was not affected by disturbance.

Pesticides cause non-random effects according to their toxicological mode of action (De Laender *et al.* 2016). By using an equivalent dose that affects the same percentage of species of the most sensitive taxonomic group, we expect the herbicide to produce a smaller impact on the individual stability dimensions since primary producers (phytoplankton) usually show a higher adaptation capacity as compared to consumers, in part, due to their fast population growth (Hillebrand *et al.* 2018). Similarly, we expect the herbicide to result in a lower effect on DS as compared to the insecticide.

H2: Compositional and functional stability properties of nutrient-enriched systems are less affected by pesticides than those of non-enriched ones.

Species identity and richness determines communities' responses to disturbance as well as the speed at which communities recover from perturbations (Loreau & de Mazancourt 2013). That is, species identities influence different stability properties (Pennekamp *et al.* 2018; White *et al.* 2020). Accordingly, we expect nutrient enrichment to modify the species identity and richness (composition) of the community, promoting a less diverse assemblage dominated by few tolerant species and an overall increase of biomass (Donohue *et al.* 2009). A more homogeneous community dominated by tolerant species is expected to be less affected by disturbance, both functionally and compositionally, and to display differences in stability properties (Belando *et al.* 2017).

H3: Disturbance type influences the correlation between compositional and functional stability properties.

Diverse species assemblages are expected to have higher functional stability over time, as they consent faster changes in composition via asynchronous fluctuations in response to disturbance (Yachi & Loreau 1999; Hautier *et al.* 2014). In such cases, perturbations may compromise compositional stability, but functional stability can be maintained (Allan *et al.* 2011), thus resulting in a negative correlation between the two stability realms. However, studies have found generally positive correlations between functional and compositional stability properties (Baert *et al.* 2016b; Hillebrand *et al.* 2018b), and a recent meta-analysis has shown that correlations between functional and compositional stability are generally positive across ecosystems exposed to pulse disturbances (Hillebrand & Kunze 2020). Thus, we expect to find mainly positive correlations between compositional and functional stability properties in our study. Yet, whether different disturbance types (i.e., press *vs* pulse, or pesticides affecting different taxonomic groups) influence this general relationship has not been explicitly tested.

2. Materials and methods

2.1 Mesocosm experiment

We performed an outdoor mesocosm experiment at the IMDEA Water Institute (Alcalá de Henares, Madrid, Spain) between April and July of 2019. Each mesocosm was filled with approximately 40 cm of sediments and 850 L of water from an artificial lagoon. The community, composed of macrophytes (*Myriophyllum sp*. and *Elodea sp.*) and invertebrates collected from unpolluted water bodies in the vicinity of Alcalá de Henares, was allowed to establish and homogenize among experimental units for two months prior to the start of the experiment.

A full factorial design (n=3), including chlorpyrifos (two levels: 1 µg/L and absent), diuron (two levels: 18 µg/L and absent) and nutrients (added, not added) was used in a randomized fashion. Nutrients (P and N) were applied twice per week as a solution containing 1.820 g of NH_4NO_3 and 0.208 g of KH_2PO_4, which resulted in a nutrient addition of 750 µg/L of N and 75 µg/L of P, respectively. These nutrients levels correspond to a eutrophic system, whereas mesocosms not treated with nutrients resembled oligo-mesotrophic systems. Nutrient additions started 4 weeks before the pesticide application. Details on chemical and nutrient applications, sampling and analysis are provided in Polazzo *et al.* (2020).

Phytoplankton, zooplankton and macroinvertebrates communities were sampled one time before pesticides application and several times afterwards. For a complete list of the sampling days see Table S1 in the Supplementary Information (SI). In total we identified 128 taxa (68 phytoplankton taxa, 22 zooplankton taxa and 38 macroinvertebrates taxa). Details on sampling techniques are provided in Polazzo *et al.* (2020).

2.2 Criteria for organism groups selection

We calculated four stability properties at the community level for three different organism groups: phytoplankton, zooplankton and macroinvertebrates. Each group was composed of several species which are differently impaired by the treatments, reflecting the range of sensitivity typical of natural species assemblages. Phytoplankton was heavily impaired by the herbicide, which reduced algae diversity. Zooplankton and macroinvertebrates were mostly impaired by the insecticide, which reduced abundance and diversity in both groups. However, indirect effects, resulting from stress-driven changes in species density, propagated throughout the food-web, changing the structure of non-target groups composition. Stability was calculated also for some populations. Methods and results of the populations' analysis are reported in the SI.

2.3 Stability properties

We quantified four stability properties: resistance, resistance, resilience, and invariability. We calculated the four stability properties for both composition and function. Compositional stability was calculated using the Bray – Curtis similarity as a state variable (Donohue *et al.* 2013; Radchuk *et al.* 2019), whereas functional stability was based on total abundance (Hillebrand & Kunze 2020). Details on the measurement, calculation and interpretation of the stability properties are provided in Table 1. ANOVA was used to test for effects of treatments on invariability, resistance, resilience and recovery, separately for the functional and compositional stability components (White *et al.* 2020).

Table 1. Stability properties studied. The table shows definitions, how they were measured and interpretation of the different metrics. Measurement of stability properties were based on Hillebrand *et al.* (2018).

Property: definition	**Type of endpoint**	**How was it measured?**	**Formula**	**Limits and interpretation**
Resistance: ability of a system to withstand stress (Hillebrand *et al* 2018)	Functional	log response ratio of the abundance in a treatment compared to the control at the first sampling after treatment (day 15)	$Res_{func} = ln\ (\frac{Ab_t}{Ab_c})$	Res_{func} of 0 reflects maximum resistance (abundance in treatment and control are the same Resistance < of 0 means low resistance. Resistance > of 0 overperformance (increase in abundance).
	Compositional	Bray-Curtis similarity of the community composition between treatment and control at the first sampling after treatment (day 15)	$Res_{com} = BC\left(\frac{Comp_t}{Comp_c}\right)$	Res_{com} ranges between 0 and 1: 0 = low resistance, 1 = maximum resistance.
Recovery: capacity of a system to return to undisturbed state following a disturbance (Ingrisch & Bahn 2018)	Functional	log response ratio of the abundance in a treatment compared to the control at the final sampling (day 50)	$Res_{func} = \ln\ (\frac{Ab_t}{Ab_c})$	Res_{func} of 0 reflects maximum (complete) recovery (abundance in treatment and control are the same); Res_{func} < of 0 means low resistance. Recovery > of 0 means overperformance (increase in abundance).
	Compositional	Bray-Curtis similarity of the community composition between treatment and control at the final sampling (day 50)	$Rec_{com} = BC\left(\frac{Comp_t}{Comp_c}\right)$	$0 \leq Rec_{com} \leq 1$; Rec_{com} of 1 reflects maximum (complete) recovery (composition in treatment and control are the same); the closer Rec_{com} to 0 the more incomplete is the recovery.
Resilience: engineering resilience; the speed of recovery after	Functional	Slope of log response ratio of the abundance in a treatment compared to control over time	$i + (Rsln_{func}) * t = \ln\ (\frac{Ab_t}{Ab_c})$ where $i = intercept, t = time$	$Rsln_{func}$ = 0 means no recovery. $Rslm_{func} >$ 0 means recovery. $Rsln_{func} < 0$, means

perturbation (Pimm 1984)				further deviation from the control.
	Compositional	Slope of the regression of similarity (Bray-Curtis) over time between treatment and control	$i + Rsln_{com} * t = BC\left(\frac{Comp_t}{Comp_c}\right)$ where $i = intercept, t = time$	$Rsln_{com}$ = 0 means no recovery. $Rsln_{com} > 0$ means recovery. $Rsln_{com} < 0$, means further deviation from the control.
<u>Invariability (temporal)</u>: inverse of temporal variability of a state variable (Wang *et al.* 2017)	Functional	Inverse standard deviation of residuals from the linear model regressing log response ratio of the abundance (treatment vs control) on time	$Inv_{func} = \frac{1}{sd(resid(Rsln_{func}))}$	The larger Inv_{func} the higher is invariability, i.e. the less fluctuating is log response ratio around the linear temporal trend.
	Compositional	Inverse standard deviation of residuals from the linear model regressing Bray-Curtis similarity on time	$Inv_{com} = \frac{1}{sd(resid(Rsln_{com}))}$	The larger Inv_{com} the higher is invariability, i.e. the less fluctuating is Bray-Curtis similarity around the linear temporal trend.

2.4 Dimensionality of stability

We measured DS as multidimensional ellipsoids constructed from the matrix of pairwise covariances between all stability properties (Donohue *et al.* 2013). Disturbance can affect both volume and shape of the ellipsoid (Donohue *et al.* 2013), thus we considered both, using semi-axis length to characterize ellipsoids' shape. The semi-axis length was measured as $\lambda_i^{0.5}$, where λ_i is the i^{th} eigenvalue of the covariance matrix for a given treatment for each organism group. Ellipsoid volume (*V*) was calculated as:

$$V = \frac{\pi^{n/2}}{\Gamma\left(\frac{n}{2}+1\right)} \prod_{i=1}^{n} \left(\lambda_i^{0.5}\right) \qquad \text{Eq. 1}$$

Where n is the dimensionality of the covariance matrix. All measurements of stability were standardized prior to the volume calculation by subtracting the mean and then dividing by the standard deviation (Donohue *et al.* 2013). Then, for each set of semi-axis, we divided them by the maximum length within a set, thus the maximum standardized length was equal to 1. By doing so, the largest theoretically possible volume equalled 1 (i.e. all the semi-axis lengths are 1), which represents a spheroidal shape and, consequently, high DS (Figure 1, Radchuk *et al.* 2019). Dividing the calculated volume by the theoretical volume, we obtained a proportional volume which varies between 1 (high DS, spheroidal shape) and 0 (low DS, “cigar”-like shape of the ellipsoid).

To assess the effects of the treatments on DS (H1), we fitted generalized linear mixed-effects models (GLMMs, Gamma distribution) with either ellipsoid volume or semi-axis length as a response. Stressors and their interactions were used as fixed effect predictors, whereas organism group was used as random effect. To test for significance, we used likelihood – ratio tests (LRT).

2.5 Pairwise correlations between stability properties

To test whether the treatments affect the correlations between different stability properties, we first calculated the Pearson correlation coefficient for each pair of stability properties within each treatment. We did that for each group (phytoplankton, zooplankton, and macroinvertebrates), both for function and composition. Then, we fitted GLMMs (Gaussian distribution) using the correlation coefficient as the response variable. As fixed effects we included stressors and their interactions, while organism group was used as random effect. Again, LRT was used to test for significance.

2.6 Correlations between compositional and functional stability properties

To test whether there are some predisposed correlations between functional and compositional aspects of stability, and whether disturbance can modify these correlations (H3), first we calculated the pair-wise correlation coefficient (Pearson rho) between the same stability properties for function and composition. We first calculated the correlations across all treatments to look for the general correlation between functional and compositional stability. Then, we calculated the correlation coefficients for the single treatments. Eventually, we fitted GLMMs (Gaussian distribution) using the correlation coefficient as the response variable, the stressors and their interactions as fixed effects, and the organism group as random effect. LRT was used to test for significance.

All statistical analyses were conducted using R (version 3.5.1) in Rstudio (2020). GLMMs were run using the package lme4 (Bates *et al.* 2015). For all fitted models, the model diagnostics included tests of the normality of residuals. When testing for significance, we always considered p-values < 0.05 as significant.

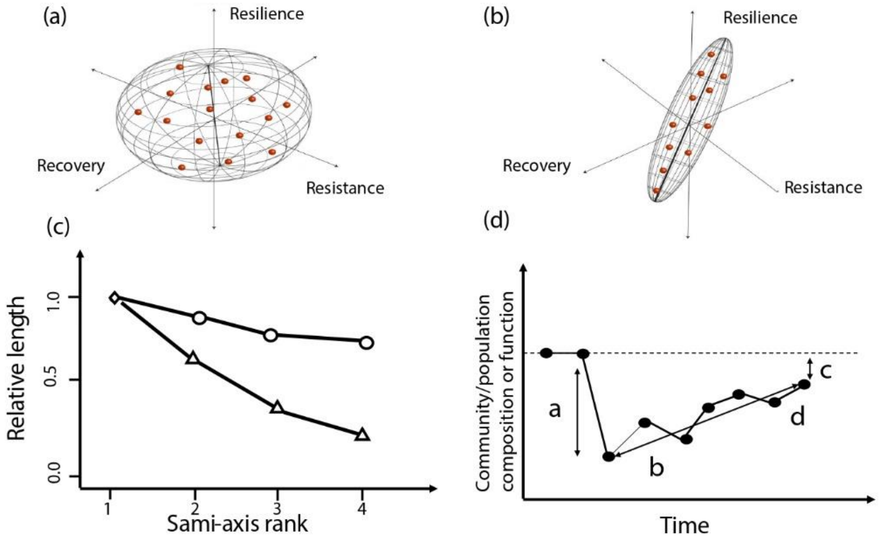

Figure 1. Graphical representation of the dimensionality of stability (a, b and c) and schematic representation of how the four stability aspects were measured (d) after Hillebrand *et al* 2018. The multidimensional ellipsoids represent a system with high dimensionality – spheroid shape (a) and a systems with low dimensionality – “cigar” shape (b) in a 3-dimensional space (thus, representing only 3 stability properties). Their relative axis lengths are shown in (c), where dots represent the semi-axis length of the system with high dimensionality (spheroid), and triangles represent the semi-axis length of the system with low dimensionality (“cigar”). In (d), a = resistance, b = resilience, c = recovery and (d) = residuals used to calculate temporal invariability.

3. Results

3.1 Effects of disturbance on individual stability properties and on the dimensionality of stability

3.1.1 Non-enriched systems

Phytoplankton functional resistance and invariability were both impaired by the herbicide, whereas the mixture of the insecticide and the herbicide improved phytoplankton resistance (Figure 2, Table S2). Zooplankton functional recovery and resistance were impaired by the pesticide mixture (Figure 2, Table S2). Macroinvertebrates functional resistance was impaired by the insecticide, whereas resilience was significantly dampened by the herbicide treatment. Phytoplankton compositional resistance and recovery were reduced by the herbicide treatment, as well as by the pesticide mixture. Zooplankton compositional resistance and recovery were significantly impaired by the insecticide treatment. Macroinvertebrates compositional resistance was reduced by the insecticide treatment, whereas the herbicide impaired compositional recovery and resilience. Overall, compositional resilience of macroinvertebrates was higher in systems treated with the pesticide mixture.

Disturbance type did not significantly affect the ellipsoid volume for what concerns composition in non-enriched systems (Figure 3, Table S4, p-values always >0.05). On the other hand, the dimensionality was influenced by pesticides for functional stability in non-enriched systems. The insecticide significantly increased the ellipsoid volume ($\chi2$ =13.0, p<0.001), whereas the herbicide decreased it ($\chi2$ =6.63, p=0.01). The pesticide mixture behaved non-additively ($\chi2$ =11.07, p<0.001).

Two out of the six pair-wise correlations were affected by disturbance in non-enriched systems for compositional stability (Table S8). For functional stability, only the correlation between resistance and invariability was modified by the pesticide mixture.

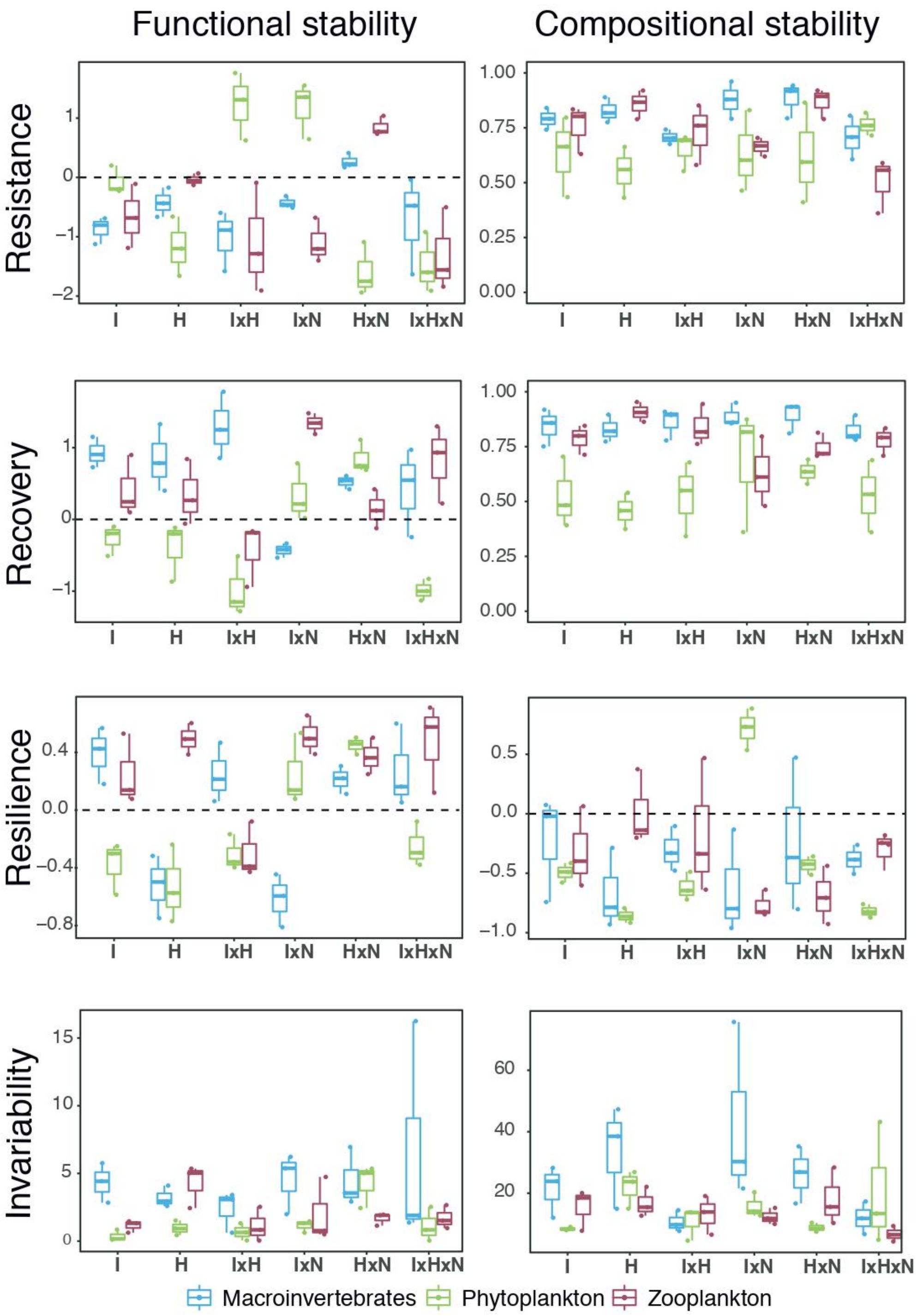

Figure 2. Box and whisker plots showing how the four dimensions of stability (resistance, recovery, resilience and invariability) responded to disturbance. Functional responses were based on abundance data, whereas compositional responses were based on community composition (Bray – Curtis similarity). Please note that functional resistance and recovery use a different scale than compositional resistance and recovery (see Table 1). I: chlorpyrifos; H: herbicide; IxH: mixture insecticide – herbicide; N: nutrients enrichment.

3.1.2 Nutrient-enriched systems

In enriched systems, phytoplankton functional resistance was significantly decreased by the herbicide treatment, while recovery and resilience were decreased only by the mixture of pesticides. Functional invariability was increased under the herbicide treatment, whereas the insecticide significantly decreased it. Zooplankton functional resistance was impaired by the insecticide, whereas functional recovery and resilience were significantly higher in systems treated with the insecticide or the pesticide mixture. Macroinvertebrates functional resistance and recovery were decreased by the insecticide treatment, whereas systems treated with the herbicide showed higher resilience. Phytoplankton compositional recovery was impaired by the herbicide. Zooplankton compositional resistance was significantly lower under the insecticide treatment, whereas it was significantly increased by the herbicide. The insecticide also significantly impaired the compositional recovery of zooplankton, whereas the mixture of the pesticides boosted both compositional recovery and resilience. Zooplankton compositional invariability was reduced by the insecticide, but increased by the herbicide. Macroinvertebrates compositional resistance was increased by the herbicide treatment, but reduced by the pesticide mixture. Macroinvertebrates compositional recovery was increased significantly by the herbicide treatment.

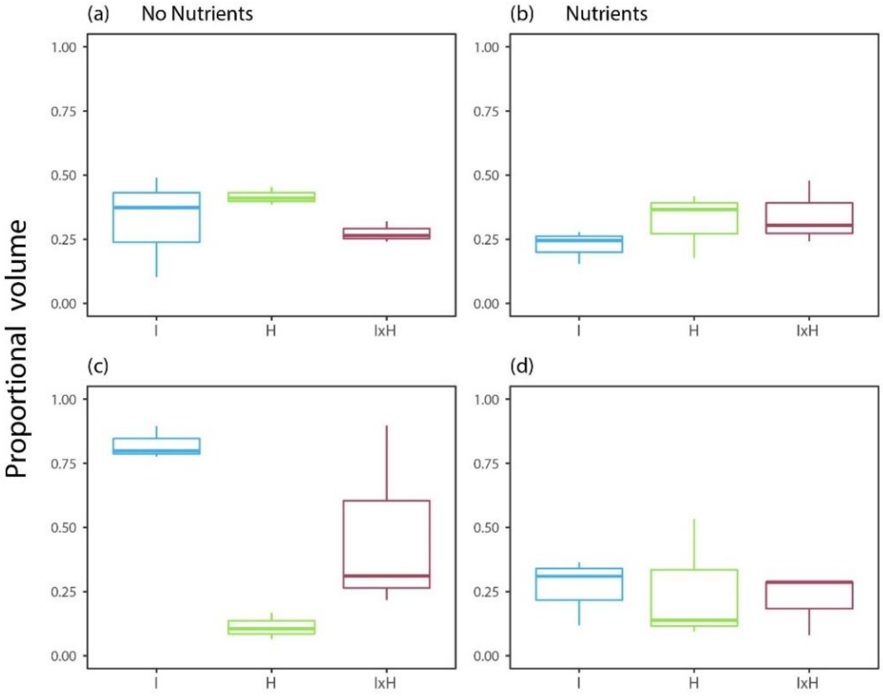

Figure 3. Proportional ellipsoid volumes under different disturbances at the community level. Plots (a) and (b) show the ellipsoid volumes calculated for compositional stability, (c) and (d) show the ellipsoid volumes calculated for functional stability. Plots on the left side show the results of the analysis for non-enriched systems, whereas those on the right side show the results of the enriched ones. **I**: chlorpyrifos; H: herbicide; IxH: mixture insecticide - herbicide.

Conversely to non-enriched systems, we did not find any difference in the ellipsoid volumes of functional stability in nutrient-enriched systems. No differences were found for compositional stability either.

For composition stability, four out of the six stability parameter correlations were influenced by disturbance in the nutrient-enriched systems (Table S8). As for functional stability, the correlation between resistance and invariability was modified by the insecticide, which shifted the correlation from generally positive to negative.

Overall, we found consistent trends in the correlation between different aspects of stability across enriched and non-enriched systems (Figure 4). Resilience and resistance were generally positively correlated, and negative values were often related to significant effects of the treatments. Recovery and resilience showed a positive consistent correlation across treatments, deviating only with a significant disturbance effect. Accordingly, resistance and recovery were positively correlated in most of the cases. Resistance and invariability were also generally positively correlated, while resilience and invariability were mostly negatively correlated. Recovery and invariability were generally positively correlated.

To sum up, differences in the effects of the disturbance between enriched and non-enriched systems on single stability properties emerged for functional recovery and resilience, which were improved in enriched systems. No clear differences between enriched and non-enriched systems appeared for single compositional stability properties, nor for functional resistance and invariability. When looking at the effects of disturbance on functional DS, differences were clear. Functional DS of non-enriched systems was modified by single and combined disturbances, whereas functional DS of enriched systems was never affected by disturbance. No effect of disturbance was detected on compositional DS regardless of the nutrients status.

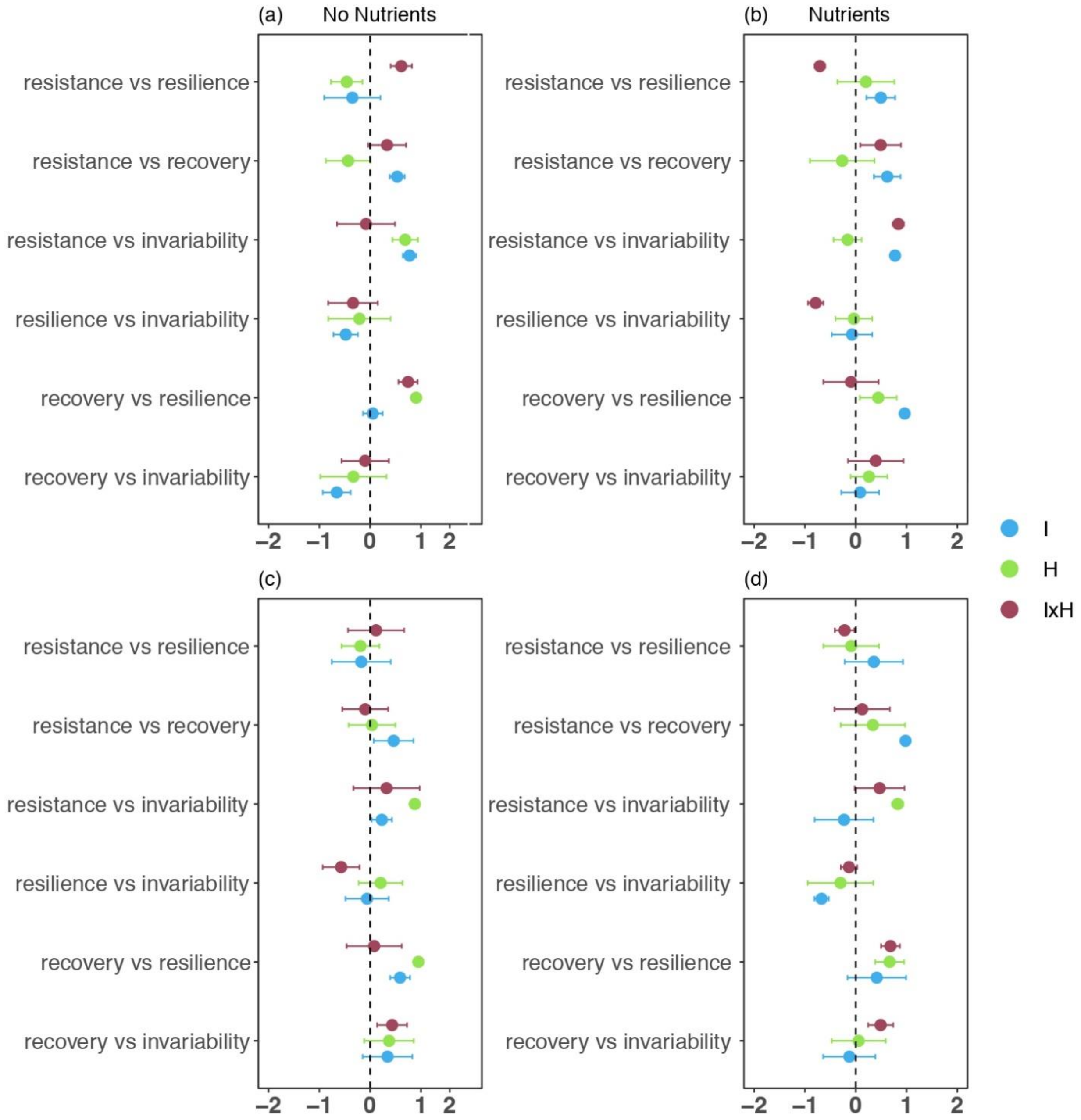

Figure 4. Correlation between aspects of stability at the community level for composition (a, b) and function (c, d). Plots on the left (a, c) show correlations for non-enriched systems. Plots on the right (b, d) show correlations for nutrient-enriched systems. **I**: insecticide; H: herbicide; **I**xH: mixture insecticide – herbicide.

3.2 Effects of disturbance on the correlation between compositional and functional stability

Overall, we found all correlation between composition and functional stability to be positive, with the exception of resilience, which was non-significantly negatively correlated (R= - 0.18, *p*= 0.19). Deviations from this general trend were associated to a significant effect of disturbance. In non-enriched systems, only one correlation between the compositional and functional invariability was affected by the herbicide (χ^2=5.89, p = 0.01) and the mixture of the pesticides (χ^2 =5.24, p = 0.02, Figure 5, Table S12). In enriched systems, the correlation between functional and compositional resistance was modified by the insecticide (χ^2 =27.69, $p < 0.001$), and invariability was affected by the herbicide (χ^2 =5.32, p = 0.02) and the mixture of the pesticides (χ^2 =5.04, p = 0.03, Figure 5, Table S12)

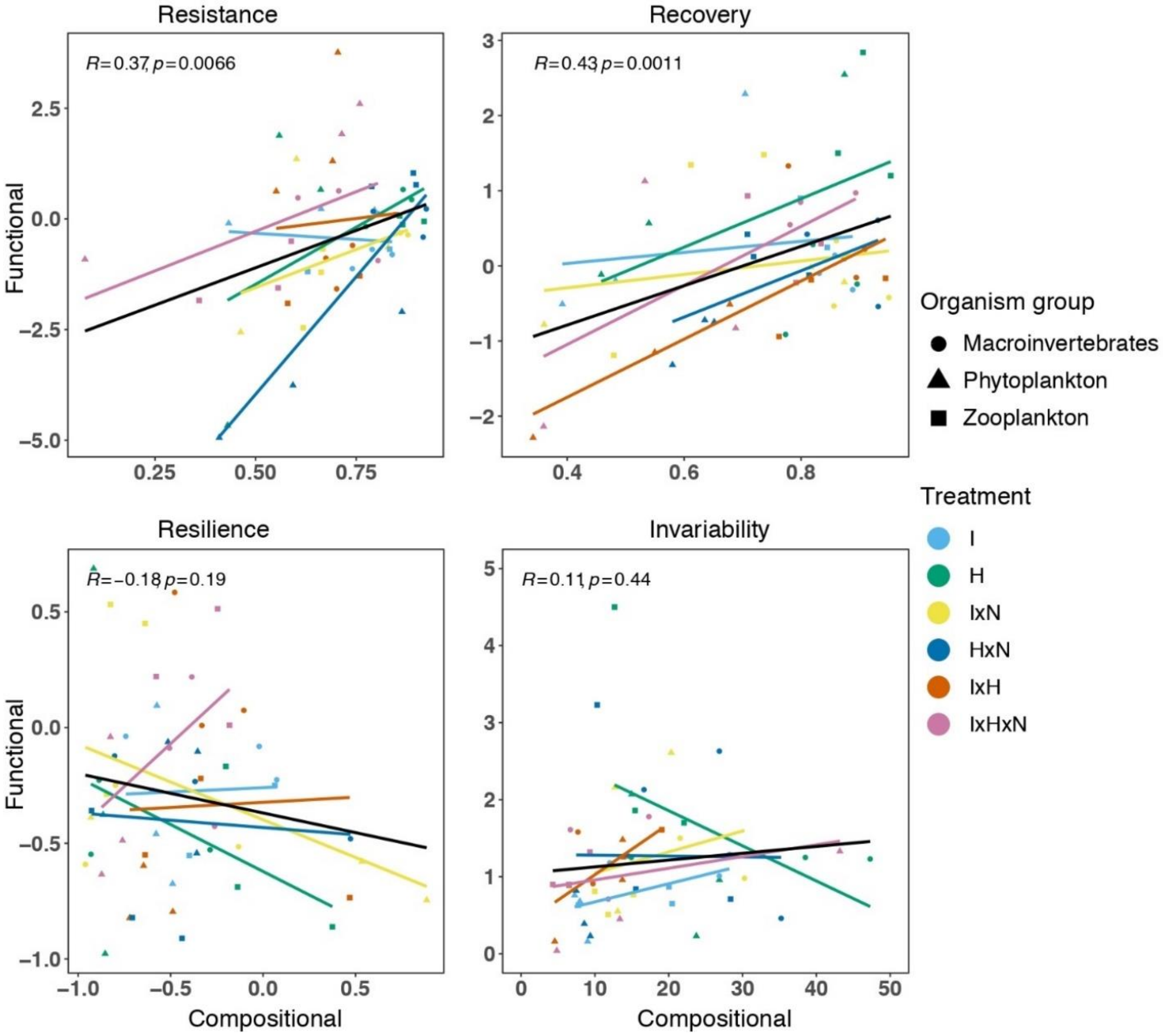

Figure 5. Correlations between the same aspects of compositional and functional stability. Each dot represents one sample. Coloured lines represent the correlation within a single treatment, whereas the black line represents the correlation between compositional and functional aspects of stability across all treatments. Each plot shows the calculated Spearman correlation coefficient (R) across the treatments and the corresponding *p*-value. I: insecticide; H: herbicide; IxH: mixture insecticide – herbicide; N: nutrients enrichment.

4. Discussion

4.1 Multiple stressors effects on the dimensionality of stability

We investigated whether and how pesticides affecting different trophic levels modify individual stability properties and the overall DS. We found that the pesticides affected the single stability properties of the different organism groups (H1) according to their expected sensitivity. That is, phytoplankton's stability properties were mainly affected by the herbicide application, whereas macroinvertebrates and zooplankton were significantly affected by the insecticide. Non-additive effects of the pesticides' mixture were found for all stability properties for functional and compositional stability. Indirect effects of the herbicide were also found to be significant on several stability properties of zooplankton and macroinvertebrates. Likewise, the insecticide significantly impaired some stability properties of phytoplankton. These significant indirect effects suggest that bottom-up and top-down effects play a large role in shaping both functional and compositional stability at the community level (White *et al.* 2020). Moreover, we found consistent differences between enriched and non-enriched systems for functional stability properties across organism groups. Generally, functional recovery and resilience were higher in nutrient-enriched systems compared to the non-enriched ones, while no clear differences were noted for compositional stability properties between the two nutrient availability scenarios.

Our study shows that neither the pesticides applied individually nor as a mixture influenced the dimensionality of compositional stability, in line with theoretical predictions (Radchuk *et al.* 2019). Inversely, pesticides did influence DS of functional stability (confirming H1). The insecticide significantly increased functional DS, and the joint application of the insecticide and the herbicide resulted in a higher functional DS compared to the herbicide alone. The herbicide treatment showed the smallest functional DS across all the measurements.

DS has been suggested to be driven by two mechanisms: changes in species interactions (Radchuk *et al.* 2019) and stochasticity (Yang *et al.* 2019). DS is commonly lower in systems with strong competitive interactions, as competitive interactions are usually more deterministic (Radchuk *et al.* 2019). Conversely, more stochastic species interactions likely result in weaker pair-wise correlations among stability properties, thus leading to higher DS (Radchuk *et al.* 2019; Yang *et al.* 2019). In our experiment, the herbicide treatment reduced the abundance of phytoplankton (Figure 2), which may have increased the strength of competition among consumers, leading to lower DS. Simultaneously, the herbicide reduced phytoplankton richness and increased dominance (Table S15). Communities sharing a limited number of traits (high dominance) have low resource use efficiency (Hodapp *et al.* 2019). Hence, the increase of dominance led to an increase in competitive interactions, contributing to the overall lower DS. Conversely, the insecticide and the mixture of pesticides may have reduced the strength of the interactions by reducing the abundance of sensitive consumer species, leading to an increase of DS. Sustaining this hypothesis, the phytoplankton abundance was not increased by the insecticide treatment. However, phytoplankton richness increased in the insecticide treatment, leading to an increased resources use efficiency and, thus, to a lower competition and DS.

We found consistent trends in the correlations between stability properties (see Results section), which are in line with those reported in other experimental and theoretical studies (Donohue *et al.* 2013; Radchuk *et al.* 2019). Most deviations from those trends were associated to a significant effect of the pesticides on the correlation between different stability properties. All stressors (single or multiple) were able to modify those correlations differently. This is, single pesticide or pesticide mixtures can modify the nature of the correlations between stability properties, which has important ecological implications (Donohue *et al.* 2013). For instance, resistance and recovery were always positively correlated across the treatments, both for compositional and functional stability, as by definition, the smaller the initial change, the higher is the likelihood of recovery (Hillebrand *et al.* 2018; Hillebrand & Kunze 2020). Yet, herbicide disturbance decoupled such positive correlation for compositional stability, regardless of nutrient status (Figure 4). This suggests that the features promoting the resistance of community composition to disturbance may, sometimes, be negatively related to the characteristics promoting recovery from perturbation and vice versa. This is in line with previous studies, in which features promoting both compositional and functional resistance under disturbance where found to be negatively correlated with recovery (Isbell *et al.* 2015; Baert *et al.* 2016b)

4.2 Nutrients enrichment and multidimensional stability

Interestingly, functional DS was modified by the treatments only in non-enriched systems, while in nutrient-enriched systems, no significant differences in DS among treatments were detected (H2). The lack of pesticide effects in the DS in nutrient-enriched systems might be explained by the fact that their effect on abundance was lower compared to the non-enriched ones (Table S15-S17). Nutrients are known to reduce the negative effects of a wide range of disturbances on biomass and abundance, from fine sediment addition (Matthaei *et al.*

2010; Piggott *et al.* 2015b, a) to chemicals (Halstead *et al.* 2014), as a result of enhanced primary productivity and increased energy fluxes. On the other hand, nutrients enrichment acted as an environmental filter, reducing diversity and promoting the dominance of tolerant species, less sensitive to additional disturbances, such as pesticides (Roessink *et al.* 2005). Based on our results we conclude that, in eutrophic systems, pesticide disturbances may not cause a significant change in functional stability, allowing the use of a restricted number of metrics (e.g., one or two) to quantify systems' stability. Conversely, the conservation of mesotrophic or oligotrophic ecosystems impacted by pesticides may need to consider specific actions targeting different stability properties (i.e., resistance, recovery, resilience, invariability) independently form each other.

Since our measurement of functional stability is based on abundance as state variable, the lower effect of the pesticides on abundance in nutrient-enriched systems may have kept DS lower and similar between treatments, despite a general reduction in species richness. Although these results seem to suggest a negative correlation between diversity and stability, we claim that, in this specific case, the higher functional stability observed in less diverse systems was not related with the total number of species. Actually, the change in species identity and the consequent dominance effect (see Fox 2005) caused by the nutrients promoted a community which is functionally more stable (Baert *et al.* 2016a). Therefore, our study suggests that the turnover rate in species identities and the dominance effect, rather than differences in species diversity, may drive the observed negative correlation between stability and diversity. Moreover, stochasticity may have contributed to determine the difference in DS found between nutrient-enriched and non-enriched systems (Yang *et al.* 2019). Indeed, more diverse communities are expected to have more unpredictable dynamics, and thus be characterized by higher stochasticity compared to species poor communities, whose dynamics are largely regulated by the dynamics of the dominant species (Reuman *et al.* 2006; Yang *et al.* 2019). A previous study has shown that communities with lower stochasticity have lower DS under disturbance (Radchuk *et al.* 2019). Consistently, our results show that nutrient-enriched systems (lower diversity and, thus, lower stochasticity) had generally lower functional DS.

4.3 Effects of disturbance type on the correlation between compositional and functional stability

In line with other studies, we found mostly positive correlation between compositional and functional aspects of stability (Hillebrand *et al.* 2018b; Hillebrand & Kunze 2020). Resistance and recovery showed a significant positive correlation across the treatments, suggesting that if compositional stability stays constant after disturbance application, high levels of functional stability will be maintained. Similarly, our results show that communities recovering their composition, will likely recover their function as well. Conversely, resilience and invariability did not show significant correlations between functional and compositional aspects of stability. Compositional resilience had negative values in most cases, indicating that community's composition further departed from the control after disturbance application. In contrast, functional resilience was often positive, suggesting that abundance can be recovered rapidly by communities that progressively diverged compositionally. Studies on ecological restoration as well as experiments on pulse disturbances have provided similar examples showing that functional recovery can be achieved even in absence of compositional recovery (Borja *et al.* 2010; Hillebrand *et al.* 2018). This generally faster functional recovery compared to compositional recovery forces us to consider the time-dependency of stability responses to disturbance, across which our predictive capacity still requires further research (Garnier *et al.* 2017; Yang *et al.* 2019; White *et al.* 2020).

Our study shows that the correlation between functional and compositional resistance can be modified by disturbance type. The generally positive correlation between functional and compositional resistance was significantly shifted by the insecticide only in nutrient-enriched systems. This is because our systems were rich in species and the press disturbance exerted by the addition of nutrients selected, from the large initial pool, tolerant species which could maintain high functional level, even if community composition was severely compromised by disturbance (Allison & Martiny 2008). Theoretical and empirical studies have shown that despite large changes in community composition, ecosystem functions can be maintained (Yachi & Loreau 1999; Allan *et al.* 2011; Hautier *et al.* 2014). In our systems, the compensatory dynamics of tolerant species, which benefited by the nutrients enrichment and the decreased competition or predation promoted by the joint effect of the insecticide and the nutrients, maintained high functional levels despite a strong compositional deviation to control systems. A single mesocosm experiment is obviously insufficient to determine universal rules about the nature of the correlation between functional and compositional stability. However, our study suggests that nutrient enrichment plays a fundamental role in this relationship for species rich communities.

4.4 Recommendations for further research

To date, the majority of available studies have focused on DS at the community level and on species poor systems (Kéfi *et al.* 2019). Our study is one of the first using species-rich systems containing several trophic levels to quantify DS in its multidimensional conception. More experimental work is needed to unravel the mechanisms driving disturbance effects on DS under different ecological scenarios. In this regard, meaningful diversity manipulations across different trophic levels would greatly benefit the long-standing debate on how and to what extent biodiversity can influence ecosystem stability. Furthermore, which state variable(s) is the most appropriate to quantify multidimensional stability, especially for functional aspects, is still an open question. Indeed, functional stability's properties are mostly measured using biomass or abundance as state variable, which relate mainly with the functioning of the food-web, rather than to the ecosystem functioning processes. We argue that measuring functional stability using several ecosystem processes as state variables could be more exhaustive, and would allow to study the relationship between the multiple dimensions of compositional stability and ecosystem multifunctionality (Pennekamp *et al.* 2018). Thus, we recommend future investigations to use ecosystem (multi)functioning processes, such as chlorophyll-a concentration, organic matter decomposition, respiration, or nutrients up-take, to quantify functional stability in a multidimensional way.

5. Acknowledgments

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The supplementary information of this study may be found online at https://doi.org/10.1111/ele.13770

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Chapter 5

Food web rewiring drives long-term compositional differences and late-disturbance interactions at the community level

Francesco Polazzo, Tomás I. Marina, Melina Crettaz-Minaglia, Andreu Rico

Abstract

Ecological communities are constantly exposed to multiple natural and anthropogenic disturbances. Multivariate composition (if recovered) has been found to need significantly more time to be regained after pulsed disturbance compared to univariate diversity metrics and functional endpoints. However, the mechanisms driving the different recovery times of communities to single and multiple disturbances remain unexplored. Here, we apply for the first time quantitative ecological network analyses to try to elucidate the mechanisms driving long-term community composition dissimilarity and late-stage disturbance interactions at the community level. For this, we evaluate the effects of two pesticides, nutrients enrichment and their interactions in outdoor mesocosms containing a complex freshwater community. We found changes in interactions strength to be strongly related to compositional changes and identified post-disturbance interaction strength rewiring to be responsible for most of the observed compositional changes. Additionally, we found pesticides interactions to be significant in the long term only when both interactions strength and food web architecture are reshaped by the disturbances. We suggest that quantitative network analysis has the potential to unveil ecological processes that prevent long-term community recovery.

1. Introduction

Multiple disturbances arising from anthropogenic activities may modify the composition and functioning of ecological communities (Avolio *et al.* 2021). A recent meta-analysis on the effects of pulse disturbances on aquatic and terrestrial communities has revealed that functional and compositional responses to pulse disturbance are substantially different (Hillebrand & Kunze 2020). After a pulse disturbance, the function a community performs (typically measured as biomass) is usually recovered within a relatively short time frame, whereas community composition takes longer to recover. Moreover, in many cases, perturbed communities by pulse disturbances tend to differ further from the un-perturbed ones as time passes (Hillebrand & Kunze 2020). Such lack of compositional recovery is only evidenced using multivariate metrics (e.g., Bray–Curtis dissimilarity or ordination techniques), which account not only for changes in species number, but also for species identities and relative abundances (Komatsu *et al.* 2019). Univariate descriptors of composition, such as diversity indices, evenness and dominance, however, do not reflect such changes in community composition (Hillebrand & Kunze 2020), suggesting that species reordering after pulsed disturbance is a common phenomenon (Avolio *et al.* 2021). Despite this being consistently reported in many experiments across ecosystems (Hillebrand *et al.* 2018a) and different types and number of disturbances applied (Avolio *et al.* 2021), the mechanism(s) driving such species reorganization in multitrophic communities are anything but clear.

Recently, a new framework trying to explain the mismatch between multivariate composition and univariate functional and diversity descriptors has been proposed (Avolio *et al.* 2019). This approach links uni- and multivariate statistical techniques using rank abundance curves, which describe dynamic species-level community changes. Despite this being a stepping-stone to the understanding of disturbance impacts at the community-level, this approach provides little information on the direct and indirect mechanisms driving community changes, particularly in multitrophic communities, where species interactions may modify the overall effects of disturbance (Bruder *et al.* 2017).

The situation complicates when one tries to assess the effects of co-occurring disturbances on communities and ecosystems. At present, several syntheses have focused on summarizing multiple disturbances effects in different ecosystems (Crain *et al.* 2008; Jackson *et al.* 2016; Tekin *et al.* 2020), and in proposing methods to classify interactions between disturbances (Piggott *et al.* 2015c; Thompson *et al.* 2018a; Tekin *et al.* 2020), while the temporality of disturbances effects has received little attention (but see ref. 12 and 13).

Interactions between disturbances are temporal scale dependent (Garnier *et al.* 2017). That is, significant effects of multiple disturbances may appear only at a later stage, usually after cessation of pulsed perturbation. Time dependent interactions between disturbances have been reported across different levels of biological organization, ranging from individuals (Lange *et al.* 2018), to communities (Barmentlo *et al.* 2019), and ecosystem properties (Garnier *et al.* 2017). Recently, rapid evolution and adaptation have been proposed as mechanisms underpinning the late appearance of synergistic responses at the population level (Orr *et al.* 2021). However, we still lack a suit of methods to understand what processes may be driving the (late) disturbance interactions at the community level.

In the last years, there has been a growing interest in assessing how species interactions drive community and ecosystem responses to single and multiple disturbances (O'Gorman *et al.* 2012; Kortsch *et al.* 2015; Thompson *et al.* 2018b; Beauchesne *et al.* 2021). Disturbance-driven changes in abundance in one trophic level or in a population may alter the structure of the complex network in which species are embedded by means of its interactions (Paine 1980; Bartley *et al.* 2019). These disturbances can modify the structural properties of the food web, such as the number and configuration of interactions, as well as the biomass and energy fluxes, usually referred to as interaction's strength (O'Gorman *et al.* 2012; Kortsch *et al.* 2021). Consequently, disturbance might affect food webs' topology (that is, who eats whom) by removal or addition of links; a concept known as topological rewiring. Alternatively, or in addition, disturbance might impact the strength of the food web interactions (that is, the magnitude of the effect on the energy flow from one species to the other), which often result from changes in the consumptive demand of a consumer associated with shifts in the consumer's behavior; a concept known as interactions strength rewiring (Blanchard 2015; Kortsch *et al.* 2015; Bartley *et al.* 2019). Potentially, the interaction strength rewiring may prevent the recovery of the multivariate community composition without affecting the total number of species or community evenness (i.e., univariate descriptors and topological properties).

Here, we demonstrate how ecological network analysis may help in closing the gap between uni- and multivariate compositional descriptors of community composition by investigating the mechanisms underpinning disturbance effects at the community level. Specifically, we tested (a) whether there is a correlation between the relative change in community composition (e.g., Bray – Curtis dissimilarity) and the relative change in species interactions strength, and (b) whether long-term compositional changes in communities caused by single pulse disturbances are reflected by changes in interactions strength. Finally, we investigated (c) whether the rewiring of interactions strength may be the driving mechanism causing the appearance of late interactions between disturbances.

For this, we used data from an outdoor mesocosm experiment in which we evaluated the single and combined effects of two different pesticides, an herbicide (diuron) and an insecticide (chlorpyrifos), on freshwater communities over time under two nutrients scenarios (eutrophic and oligotrophic). The two pesticides were applied as single pulse disturbances, whereas nutrients were applied as press perturbation starting one month before the pesticides' application and being continuously applied throughout the experiment to simulate a eutrophic environment. We considered three periods representing the pre-disturbance situation (5 days before pesticides application), maximum effect phase (15 days after pesticides application) and the expected recovery phase (50 days after pesticides application). We chose those pesticides as they selectively target different compartments of the community. The pesticide concentrations used, despite being environmentally relevant (Knauert *et al.* 2008; Thompson *et al.* 2016), were expected to affect 50% of the primary producers and consumers, respectively. Thus, changes in composition and in species interactions were projected to occur significantly. Moreover, the press (continuous) addition of nutrients and the consequent eutrophication was expected to reduce richness and to modify several topological parameters of the food web (O'Gorman *et al.* 2012), allowing us to test pesticide interactions under two different environmental scenarios. Here, for the first time, we combine unweighted or topological (without considering interaction strength) and quantitative (or weighted) food web properties to study the effects of single and multiple disturbances on community composition over time.

2. Materials and Methods

2.1 Experimental design

The dataset used in this study was obtained from an outdoor mesocosm experiment performed between April and July of 2019 at the facilities of the IMDEA Water Institute (Alcalá de Henares, Spain). A full factorial design was used with eight different treatments applied in a randomized fashion and three replicates per treatment. The treatments included: the insecticide chlorpyrifos (1 µg/L and absent), the herbicide diuron (18 µg/L and absent), nutrients (750 µg/L of N and 75 µg/L of P, not added), and their binary and tertiary interactions. The nutrients treatment started one month before the application of the pesticides. Since then, N and P were applied twice a week for the whole duration of the experiment. The freshwater community tested in the experiment was composed of phytoplankton, zooplankton, and macroinvertebrates, which were sampled one time before and several times after the pesticide application. However, for the purpose of this study, we focus on days -5 (pre - disturbance condition), 15 (expected maximum effect) and 50 (expected recovery) relative to the pesticides' application. We expected recovery after 50 days based on previous mesocosm studies evaluating pulse exposure to these compounds (López-Mancisidor *et al.* 2008; Knauer & Hommen 2012). Details on the experimental systems and the sampling techniques used in the experiment may be found in (Polazzo *et al.* 2021a).

2.2 Food web construction

We used publications, personal observations, and the database built by Gray et al. (2015), which represents the largest standardized collection of trophic links for freshwater organisms, to establish the trophic interactions among the species of the experimental mesocosms. For some phytoplankton and microzooplankton species references on feeding preference were not found, limiting the possibility to add these species to the network. In such cases, we aggregated the species for which feeding information were missing together with the closest taxonomically and/or size-wise species for which feeding information were available. A detailed list of the aggregations performed as well as on links' references may be found in the Supplementary Information (SI1, Table S1 and S2). The resulting interaction list comprised 208 links and 48 trophic species (24 phytoplanktonic basal species, 12 zooplanktonic species, 9 benthonic macroinvertebrates and 3 high trophic predators; Fig. 1). Seventy-two networks were built (8 treatments including control x 3 days x 3 replicates) considering the biomass for each species. Information on biomass calculations can be found in the SI1.

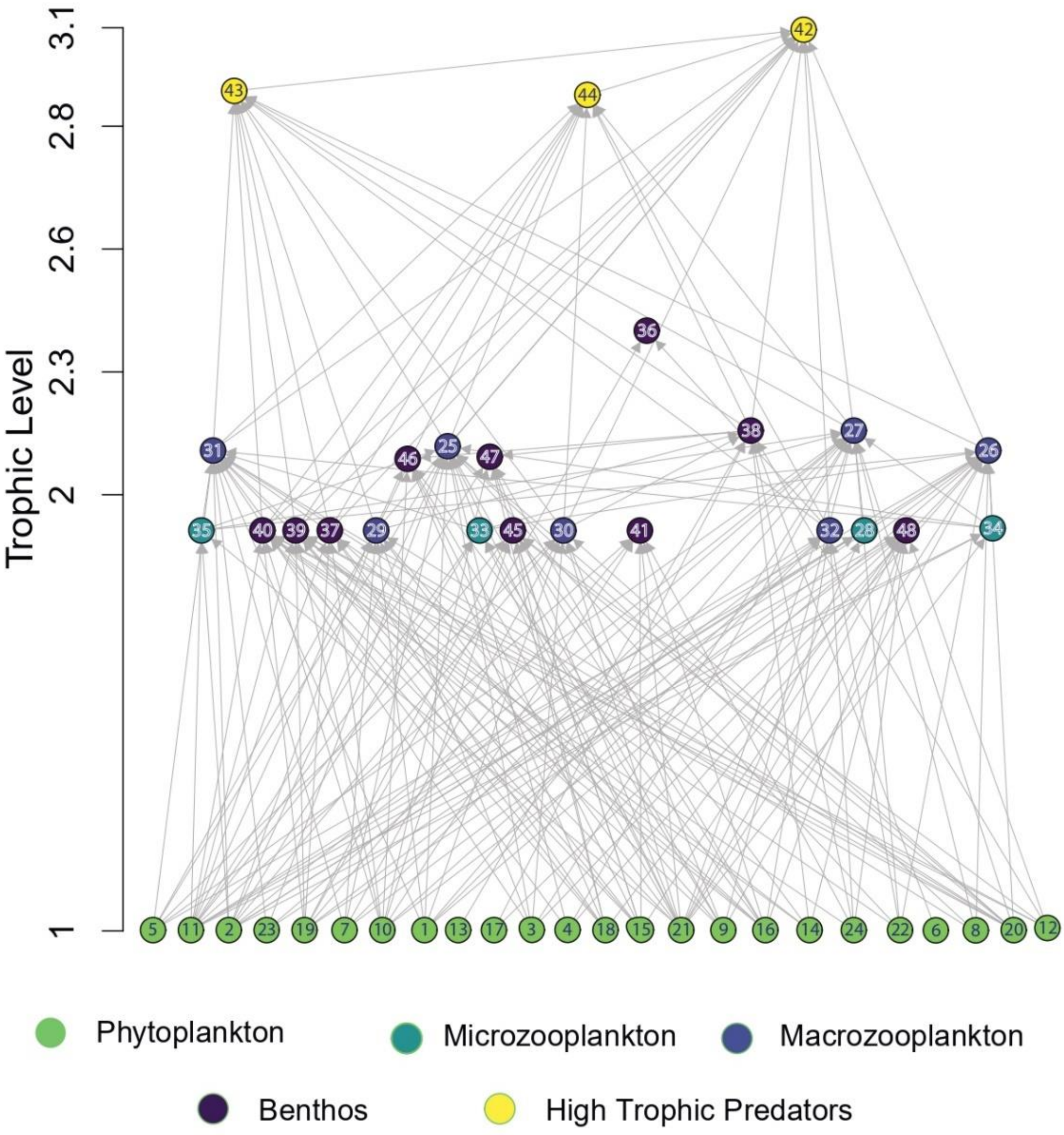

Figure 1. The mesocosms food web before the start of the experiment. (1) *Ankyra sp*.; (2) *Monoraphidium sp*.; (3) *Oocystis sp*.; (4) *Pediastrum sp*.; (5) *Cosmarium sp*.; (6) *Staurastrum sp*.; (7) *Peridinium sp*.; (8) *Rhodomonas sp*.; (9) *Achanthes sp*.; (10) *Amphora sp*.; (11) *Cocconeis sp*.; (12) *Cymbella sp*.; (13) *Epithemia sp*.; (14) *Eunotia sp*.; (15) *Gomphonema sp*.; (16) *Navicula sp*.; (17) *Rhopalodia sp*.; (18) *Rhoicosphenia sp*.; (19) *Nitzschia sp*.; (20) *Synedra sp*.; (21) *Chroococcus sp*.; (22) *Microcystis sp*.; (23) *Oscillatoria sp*.; (24) *Spirulina sp*.; (25) *Daphnia pulex*; (26) *Simocephalus sp*.; (27) Cyclopidae; (28) Nauplii; (29) *Alonella sp*.; (30) *Ion asp*.; (31) *Ceriodaphnia sp*.; (32) Ostracoda; (33) *Keratella sp*.; (34) *Polyarthra sp*.; (35) *Ascomorpha sp*.; (36) *Erpobdella sp*.; (37) Lumbriculidae; (38) Chironomidae; (39) Caenidae; (40) *Cloeon sp*.; (41) Hydrobiidae; (42) *Notonecta sp*.; (43) Anisoptera; (44) Zygoptera; (45) *Dugesia sp*.; (46) Leptoceratidae; (47) Leptoceridae; (48) Lymnaea.

2.3 Unweighted and weighted food web properties

Two types of networks were defined to characterize the food webs for each mesocosm: qualitative or unweighted, formed by the presence or absence of interactions, and quantitative or weighted, where interactions' strength was also considered. For the first, we calculated the following unweighted food web properties: number of links (L), link density (L/S, where S is the number of species), connectance (C), generality, vulnerability, number of basal and top species, mean trophic level, maximum trophic level, omnivory, path length, and clustering coefficient. We used the R package *multiweb* (Saravia *et al.* 2019) to obtain these properties. As for the weighted food webs, we used a bioenergetic approach to estimate interactions' strength, which enabled us to calculate weighted properties (Kortsch *et al.* 2021). Basically, this approach considers allometric scaling laws to quantify individual metabolic rates that are dependent on body mass and temperature, that together with losses to predation and assimilation efficiencies are used to quantify incoming fluxes (due to consumption) and outcoming fluxes (predation) for each species. Such approach assumes system's equilibrium or steady state, implying that the total amount of energy lost by a species, either by consumption or physiological processes, is exactly compensated by the metabolized energy it gains from consumption. This means that each species loss to predation and metabolism are balanced by its energetic gains, which are defined as incoming fluxes multiplied by assimilation efficiencies (Jochum *et al.* 2021). For instance, to calculate energy flux from a food chain (i.e., species A is eaten by species B, which is consumed by species C) we start from the top predator (species C in the food chain) and assess how much energy this species needs. Given the assumption of system's equilibrium, this energy needs to come out from the top predator's prey (species B). As such, it is assumed that the energy demand of species at a given trophic level must be met by the energy intake of that level. Due to ecological efficiencies (i.e., consumers cannot use all consumed energy to produce biomass, some is lost because of respiration and excretion), to fulfil its energy demands, every species needs to consume more energy from the next-lower trophic level. The energy consumed from this lower level is then treated as energy loss for the species at this level. This loss to consumption is then added to the energy demand of the prey species itself (species B) to represent the joint energy loss of this species that needs to be compensated by the next-lower level (species A), and so on. Therefore, top predators (species without predators) only have metabolic losses, while its prey, the herbivores, have both losses due to metabolism and consumption (Jochum *et al.* 2021). The interaction strength calculations in a food chain or food web begin from the top predator. In this way, the calculation of the interaction strength might be biased when there is a direct effect of the experimental treatments on the in-flux of energy. However, none of the treatments applied here (insecticide chlorpyrifos (1 µg/L and absent), herbicide diuron (18 µg/L and absent), and nutrients (750 µg/L of N and 75 µg/L of P, not added)) have been reported to directly affect the top predators of our food web (*Notonecta sp.*, Anisoptera and Zygoptera are the high-trophic predators; see Figure 1) (Brewer & Atchison 1999; Rubach *et al.* 2010). Following Kortsch *et al.*(2021) we calculated four node- and three link-weighted food web properties, where species' biomass and estimated energy fluxes were used, respectively. We calculated node-weighted connectance, generality, vulnerability and mean trophic level, whereas as link-weighted descriptors of the food web, we calculated connectance, vulnerability and generality. We used the R package *fluxweb* to estimate food web fluxes (Gauzens *et al.* 2019). Table 1 provides an overview of the definition and ecological relevance of the food web properties assessed in this study.

Table 1. List of unweighted and weighted food web properties used, definitions and relevant ecological information.

Food web	Properties	Definition / Relevance	Reference
Unweighted	Number of links	Number of trophic interactions in a food web. Represents the pathways along which matter and energy can flow.	Dunne *et al.* 2002
	Link density	Average trophic interactions per species.	Dunne et al., 2002
	Connectance	Proportion of actual interactions among possible ones. It is considered an estimator of community sensitivity to perturbations.	Dunne et al., 2002
	Generality	Number of preys of a species normalized by the average number of preys across the food web.	Bersier *et al.* 2002
	Vulnerability	Number of predators of a species normalized by the average number of predators across the food web.	Bersier *et al.* 2002
	Number of basal and top species	Species without prey (basal) and without predators (top). Basal species define the shape at the base of the food web, while top ones may have indirect effects on other species via top-down control.	Cohen & Briand 1984
	Mean and maximum trophic levels	The trophic level is an indicator of the position a species occupies in a food web resuming the distance of each species to the source of matter and energy. It represents the energy transfer efficiency from basal to top species.	Odum & Heald 1975
	Omnivory	Proportion of species that feed at different trophic levels. It provides trophic flexibility to an ecosystem.	McCann & Hastings 1997
	Path length	It is the average distance, accounted by the number of interactions, between any pair of species. Food webs with low values might face a rapid propagation of disturbances.	Albert & Barabási 2002
	Clustering coefficient	It is a measure of grouping that reflects how likely is that two connected species are part of a larger, highly connected group. High values may buffer the spread of perturbations.	Albert & Barabási 2002
Node-weighted	Connectance, Generality, Vulnerability, Mean trophic level	Species (nodes) are weighted by their biomass. For instance, mean trophic level of a food web corresponds to the average of species' trophic levels weighted by their biomass.	Olivier *et al.* 2019
Link-weighted	Connectance, Generality, Vulnerability	Interactions (links) are proportional to the magnitude of energy fluxes. Properties were calculated by estimating the average effective number of preys and predators of each taxon weighted by their relative incoming and outcoming flows.	Bersier *et al.* 2002

2.4 Statistical analyses

We analyzed the effects of single disturbances and their interactions on community composition using a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations (Anderson 2001), based on Bray-Curtis distances, obtained from log-transformed abundance data. PERMANOVA was performed using the function “adonis2” from the R package *vegan* (Oksanen *et al.* 2019). To examine patterns in the whole community changes, we performed a non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances on abundance data for 3 sampling dates, representing: the pre-disturbance condition (day -5 relative to the pesticides application), the moment of expected maximum effects (day 15 relative to the pesticides application) and the moment of expected recovery (day 50 relative to the pesticides application).

As univariate descriptors of community composition we calculated species richness, Shannon’s index and community evenness using the R packages *BiodiversityR* (Kindt & Coe 2005) and *lawstat* (Hui *et al.* 2008). Also, we studied how the function of the community was affected by the treatments using total community biomass. Differences among indexes and biomass values caused by treatments were analyzed by means of a three-way ANOVA followed by a Tukey post-hoc test in case of significant effects of the treatments. To test for significant effects of the treatments on all the different food web properties calculated, we employed again three-way ANOVAs followed by Tukey post-hoc test. To check ANOVA’s assumptions, we used the function “check_model” of the R package performance (Lüdecke *et al.* 2020). If the assumptions were not met, we log or sqrt transformed the data to better fit the assumptions. To test for a correlation between the relative change in community composition and the relative change in interactions strength, we first calculated the Log-Response Ratio (LRR) of the Bray – Curtis similarity of the community composition between each treatment and the controls: $LRR\ compostion = ln\ (\frac{BC_t}{BC_c})$. Then, we calculated the LRR of the interaction’s strength between each treatment and the controls: $LRR\ interaction\ strength = ln\ (\frac{IS_t}{IS_c})$. Finally, we calculated the non-parametric Spearman’s rank correlation between the absolute values of compositional LRR and interaction strength LRR. We used the absolute values of the LRRs as we were interested in assessing whether changes in interactions strength are well reflected by changes in community multivariate composition. Thus, both negative and positive deviations from control’s interactions strength might be translated into compositional changes. All statistical analyses and food web construction were conducted using the R software (version 4.0.3).

3. Results

3.1 Community composition

Our study shows significant differences in community composition caused by the nutrients' enrichment in all three sampling days (PERMANOVA, pre-disturbance: $F_{1,23} = 1.70$, $p = 0.022$; maximum effect: $F_{1,23} = 3.03$, $p = 0.003$; recovery: $F_{1,23} = 2.80$, $p = 0.001$; SI1, Table S2). Both the herbicide and the insecticide shifted the community composition in the maximum effect phase (PERMANOVA, herbicide: $F_{1,23} = 2.73$, $p = 0.003$; insecticide: $F_{1,23} = 6.75$, $p = 0.001$; SI1, Table S2) and in the recovery phase (PERMANOVA, herbicide: $F_{1,23} = 1.88$, $p = 0.017$; insecticide: $F_{1,23} = 2.45$, $p = 0.002$; SI1, Table S2). Interactions between pesticides were only significant in the recovery phase under non eutrophic conditions (PERMANOVA, $F_{1,23} = 2.26$, $p = 0.006$; SI1, Table S2). Generally, we did not observe compositional recovery (Fig. 2). Communities undergoing different treatments were still well distinguished from the control in the multivariate space in the recovery phase, suggesting that different treatments promoted different long-term multivariate composition (Hillebrand & Kunze 2020).

Species evenness was not significantly affected by the treatments ($p > 0.05$), whereas the Shannon's index was significantly reduced by the insecticide in the maximum effect phase (ANOVA, $F_{1,16} = 6.05$, $p = 0.021$; SI1, Table S2). Species richness was significantly reduced by the insecticide in the maximum effect phase (ANOVA, $F_{1,16} = 5.89$, $p = 0.027$; SI1, Table S2), and by the nutrients in the maximum effect (ANOVA, $F_{1,16} = 9.12$, $p = 0.008$; SI1, Table S2) and recovery phase (ANOVA, $F_{1,16} = 26.49$, $p < 0.001$; SI1, Table S2).

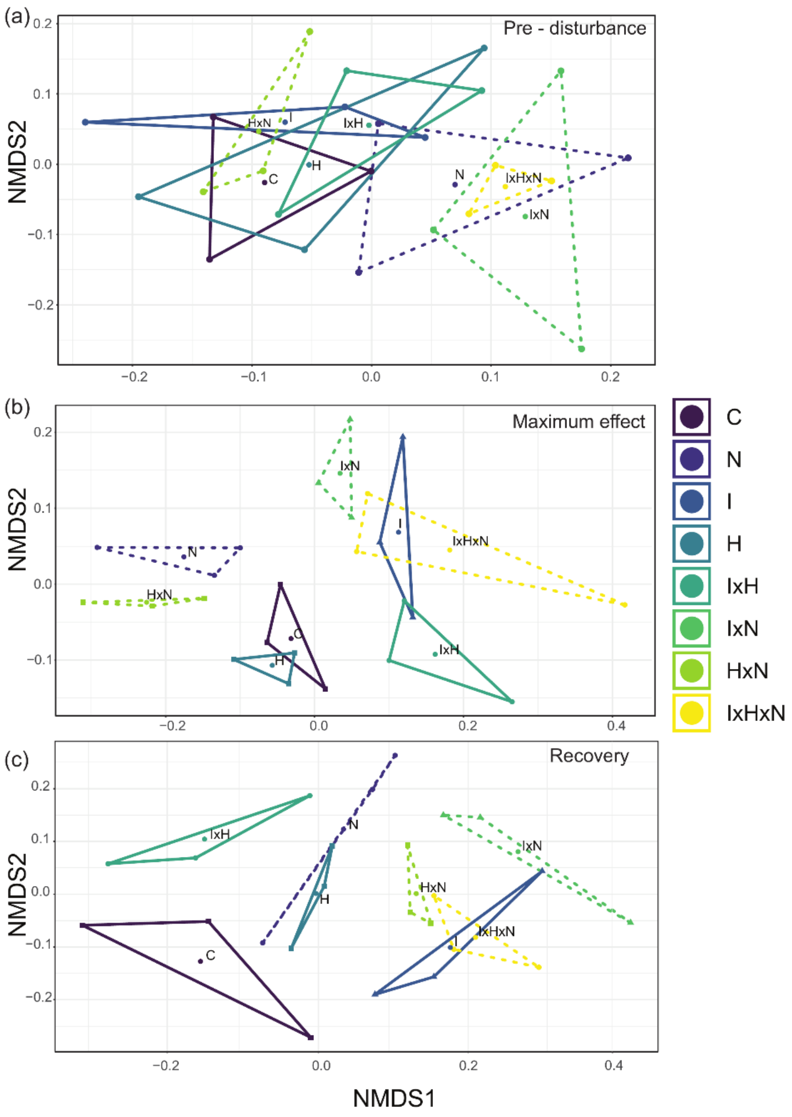

Figure 2. Non-metric Multidimensional scaling (NMDS) of the community over time. NMDS plots show differences in community composition on the pre-disturbance phase (a), maximum effect (b) and recovery phase (c). Treatments are defined by colour and eutrophic systems are characterised by a dashed line. The round dot in the middle of the three replicate of each treatment represents the centroid for the specific treatment. N: nutrients, I: insecticide, H: herbicide

Richness was also reduced by the simultaneous application of the herbicide and the insecticide under non-eutrophic conditions in the recovery phase (ANOVA, $F_{1,16}$ = 8.496, p = 0.010; SI1, Table S2).

Total biomass was significantly increased by nutrients enrichment in the pre-disturbance phase (ANOVA, $F_{1,16}$ = 2.24, p = 0.039; SI1, Table S2) and in the maximum effect phase (ANOVA, $F_{1,16}$ = 7.12, p = 0.016; SI1, Table S2), while in the maximum effect phase the herbicide decreased the total community biomass (ANOVA, $F_{1,16}$ = 5.21, p = 0.038; SI1, Table S2).

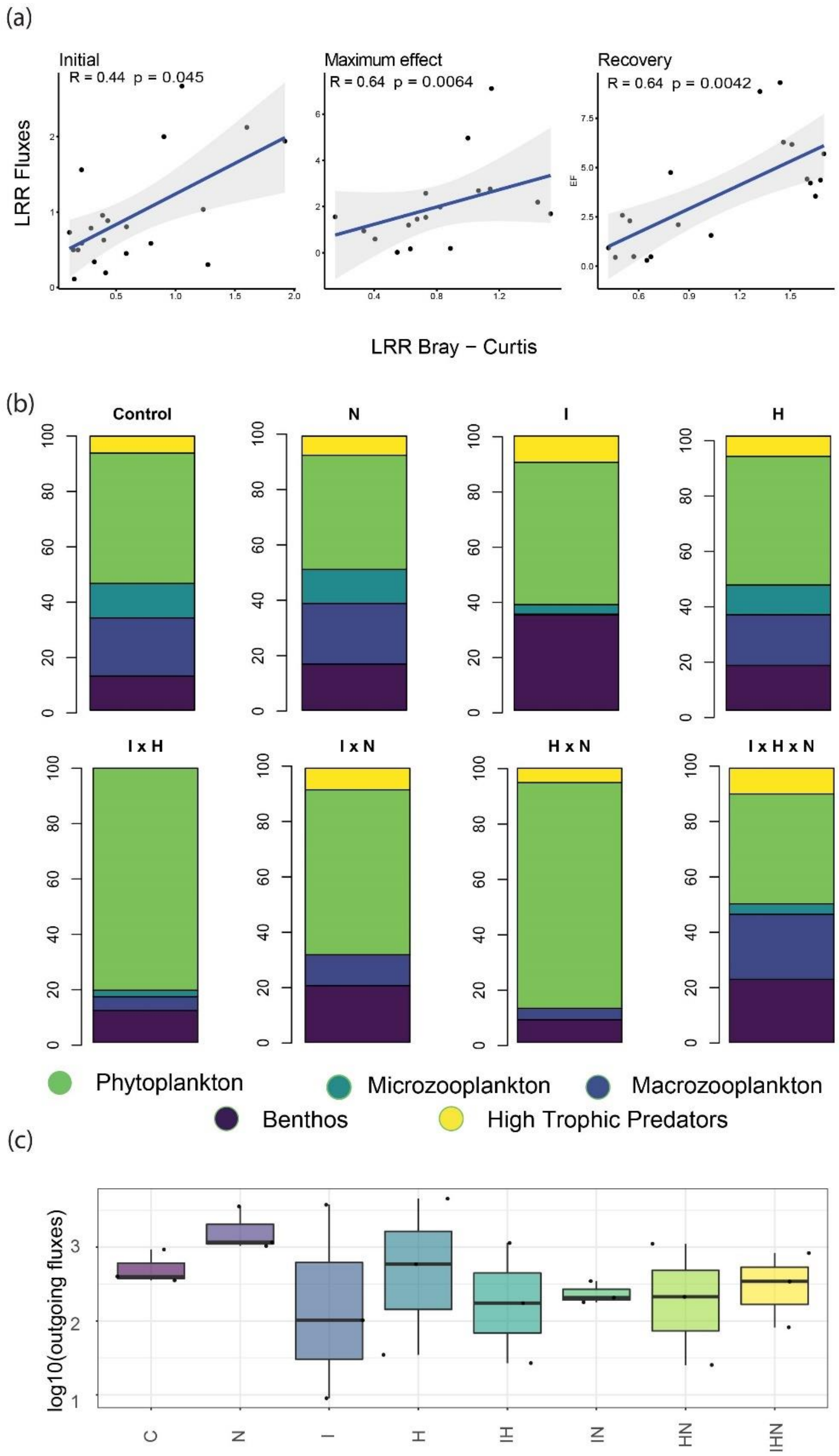

Figure 3. Effects of stressors on the energy fluxes. (a) Spearman's rank correlation between the Log response Ratio (LRR) of the energy fluxes and the LRR of the Bray – Curtis dissimilarity between the treatments and the control in each of the three experimental phases. The line represents the linear trend between the two variables and the colored area represents the confidence interval. (b) Visual representation of the proportional outgoing fluxes per trophic group in the different treatments in the recovery phase. The proportional outgoing fluxes were calculated using the mean of the three replicates of each treatment. N: nutrients, I: insecticide, H: herbicide. (c) Box and whisker plots (n = 3, for all measures) of the total outgoing fluxes in the recovery phase. The center line indicates the median, while the bottom and top hinges of the box correspond to the 25th and 75th percentiles, respectively. Every replicate of each treatment is shown as an individual dot.

3.2 Food web properties

Unweighted topology-based food web properties (number of links, link density, number of basal species, path length, generality, and vulnerability) were generally negatively influenced by nutrients enrichment in the maximum effect and recovery phase (SI1, Table S3). In the maximum effect phase, also the insecticide significantly affected several food web properties (SI1, Table S3), while the herbicide did not. In the recovery phase, neither the insecticide nor the herbicide modified these metrics. Disturbances interactions on food web properties appeared only in the recovery phase between the herbicide and the insecticide under non-eutrophic conditions. The pesticide mixture significantly decreased the number of links (ANOVA , $F_{1,16} = 5.01$, $p = 0.039$), link density (ANOVA , $F_{1,16} = 3.12$, $p = 0.036$), generality (ANOVA, $F_{1,16} = 6.32$, $p = 0.023$), and increased the number of basal species (ANOVA, $F_{1,16} = 6.63$, $p = 0.020$).

Node-weighted properties were never modified by the insecticide, whereas the herbicide significantly decreased connectance in the maximum effect phase (ANOVA, $F_{1,16} = 7.63$, $p = 0.037$; SI1, Table S4). Nutrients' enrichment decreased connectance in the pre-disturbance phase (ANOVA, $F_{1,16} = 1.41$, $p = 0.036$), and generality and vulnerability in the recovery phase (ANOVA, $F_{1,16} = 9.56$, $p = 0.006$ and $F_{1,16} = 15.75$, $p = 0.001$, respectively). Significant interactions between disturbances were reported only in the recovery phase, when the mixture of pesticides (herbicide x insecticide) significantly decreased connectance (ANOVA, $F_{1,16} = 5.14$, $p = 0.037$).

Link-weighted connectance was increased by the herbicide and nutrients as single treatments in the maximum effect phase (ANOVA, $F_{1,16} = 11.18$, $p = 0.006$ and $F_{1,16} = 5.74$, $p = 0.029$, respectively; SI1, Table S5) and by the herbicide as single treatments in the recovery phase (ANOVA, $F_{1,16} = 4.46$, $p = 0.036$, respectively). Conversely, the insecticide and the mixture of the pesticides in the recovery phase decreased link-weighted connectance (ANOVA, insecticide: $F_{1,16} = 3.32$, $p = 0.048$ and insecticide x herbicide: $F_{1,16} = 8.12$ $p = 0.011$). Link-weighted generality was increased by the nutrients in the pre-disturbance and recovery phase (ANOVA, $F_{1,16} = 6.89$, $p = 0.018$ and $F_{1,16} = 6.80$, $p = 0.019$). Linked-weighted generality was also significantly decreased by the insecticide in the maximum effect phase (ANOVA, $F_{1,16} = 11.18$, $p = 0.006$), and increased by the herbicide, but decreased by the combination of the pesticides in the recovery phase (ANOVA, $F_{1,16} = 4.32$, $p = 0.046$ and $F_{1,16} = 4.94$, $p = 0.040$, respectively).

We also found a significant correlation between the compositional LRR and the interaction strength LRR of all sampling days Fig. 3).

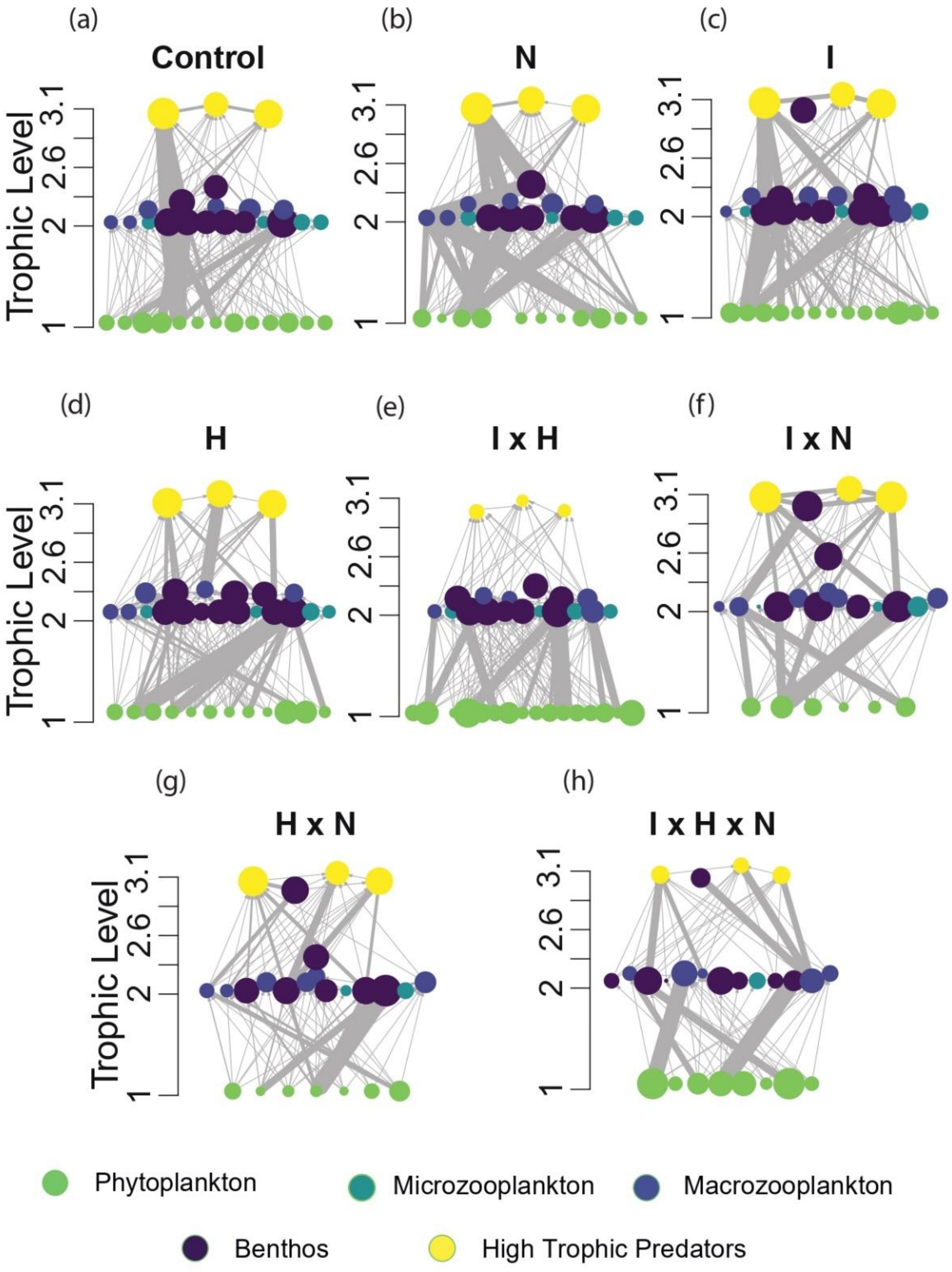

Figure 4. Link-weighted representation of the trophic network in the recovery phase. The networks, weighted links and nodes were built using the average values of the fluxes of the three replicates per treatment. N: nutrients, I: insecticide, H: herbicide.

4. Discussion

4.1 Effects of single pulse disturbances

In this study we assessed the value of ecological network analyses to better understand the differences between univariate and multivariate compositional descriptors of community composition and to identify ecological processes that may be responsible for long-term dissimilarity and late interactions between disturbances at the community level. Our study shows that changes in multivariate community composition were linked to changes in community biomass or diversity indexes after the disturbances' application (day 15, maximum effect phase). After 50 days (recovery phase), all univariate descriptors of composition and function did not differ anymore from the control, indicating potential recovery. Yet, the analysis of multivariate composition showed that communities treated with a pulse application of insecticide or herbicide as single disturbance were still significantly different from the control. Long-term compositional dissimilarities at the community level after pulse disturbance were reported in another study using aquatic communities (Barmentlo *et al.* 2019), and have been observed in a wide range of ecosystems (Hillebrand & Kunze 2020). Analysing the food web properties and interactions strength dynamics, we found a coherent explanation for this apparent mismatch between uni- and multivariate descriptors of community composition, which has been reported extensively in the literature (Chase & Knight 2013; Chase *et al.* 2018; Hillebrand *et al.* 2018a).

In the maximum effect phase, topological properties were strongly influenced by the insecticide. Such properties are based on presence-absence interactions, thus heavily affected by the number of species (i.e., richness), which was indeed significantly reduced by the insecticide in the maximum effect phase. Node-weighted properties (based on biomass) in the maximum effect phase were significantly influenced by the herbicide. Consistently, nutrients enrichment and the herbicide were the only treatments modifying total community biomass on the same phase. In the recovery phase, when the effects of the pesticides were expected to be recovered, not a single property of the unweighted nor of the node-weighted food webs was affected by the pesticides in isolation and no univariate descriptor of community composition was significantly impacted.

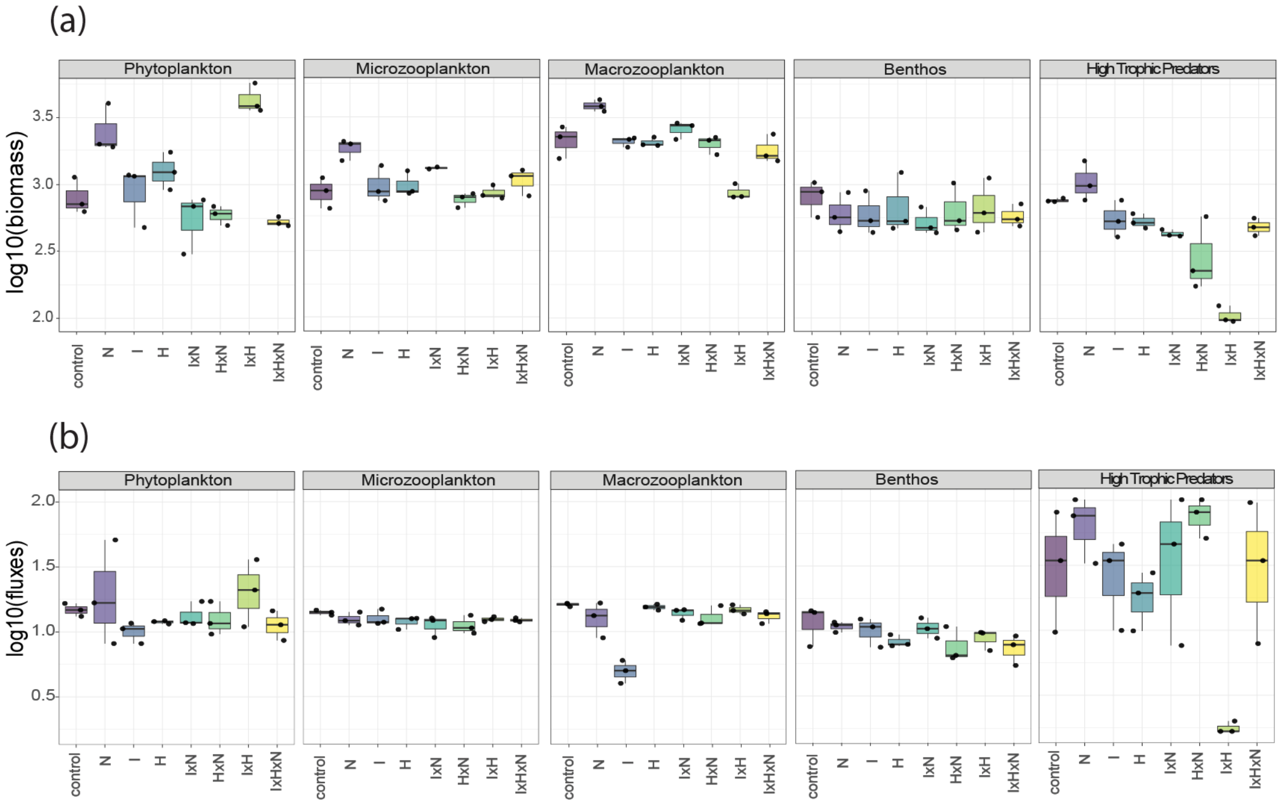

Figure 5. Effects of the treatments on community biomass and interactions strength in the recovery phase. Box and whisker plots (n = 3, for all measures), where the centre line indicates the median, the bottom and top hinges of the box correspond to the 25th and 75th percentiles, respectively. Every replicate of each treatment is shown as an individual dot. (a) Log10 transformed biomass values for the different trophic groups across the treatments in the recovery phase. (b) Log10 transformed values of the sum of incoming and outcoming energy fluxes per trophic group across the treatments divided by the number of nodes/species contained in each trophic group. Such values represent the average interaction strength per each trophic group and how it varies in the different treatments. N: nutrients, I: insecticide, H: herbicide.

Yet, looking at the link-weighted food web properties, we found that connectance and generality were significantly modified by both pesticides applied as single disturbance in the recovery phase. An increase in link-weighted connectance suggests that several nodes/species have relatively high and similar energy flows (i.e., species interactions strength), which increases link-weighted food web complexity (Kortsch *et al.* 2021). Oppositely, a decrease in link weighted connectance indicates an uneven distribution of energy flows, which may be clustered in a specific portion of the food web and disconnected from the whole network. In our model ecosystems, the herbicide increased link-weighted connectance, showing an even distribution of energy fluxes across the feeding guilds (Figure 3b). This even distribution of interactions strength was also mirrored by the increase in link-weighted generality, which indicates an increased number of preys for each predator. Conversely, the insecticide caused a rewiring of the interactions strength that promoted the compartmentalization of energy fluxes (significant reduction in link-weighted connectance), reducing the outgoing energy fluxes from the macro-zooplankton compartment, while expanding the outgoing fluxes from basal species and benthic organisms (Figure 3b). Subsequently, this increased the incoming flow to high trophic predators feeding on benthic organisms (Figure 4).

Overall, the analysis of single pulse disturbance on our experimental systems shows that, after pulse disturbance, communities may change in their uni- and multi-variate composition as well as in their functional parameters. However, biomass and diversity may be recovered, but the rewiring of interactions strength prevents communities to re-gain their concise composition. This was further sustained by the significant correlation between the relative changes in multivariate composition and the relative changes in the interactions' strength. Indeed, large compositional dissimilarities from the control were positively correlated with large differences in the interactions' strength, suggesting that changes in the intensity of species interactions may shape community composition. We propose that such rewiring of the interactions' strength is linked to the different sensitivities of the taxa to a specific disturbance. Our systems were populated by several species with different sensitivities to the disturbances applied. The initial decrease in univariate descriptors of composition was mainly caused by the decline of sensitive species (Van Den Brink *et al.* 1996; Van Wijngaarden *et al.* 2005; Knauert *et al.* 2008; Hasenbein *et al.* 2017). Later, through re-colonization (particularly of sensitive Ephemeroptera taxa, such as *Cloeon sp*. and Caenidae) or the development of resistance forms (e.g., ephippia and eggs in case of Cladocera) the total number of species could be regained. Similarly, total biomass was recovered by compensation dynamics, where tolerant species increased their abundance at the expenses of declined sensitive species (Gonzalez & Loreau 2009). Nonetheless, the pre-disturbance relative abundances of all taxa were not recovered, as indicated by the multivariate analysis of composition. We suggest that such community dissimilarity was driven by the tolerant species that persisted in the environment for the whole experimental time, causing the rewiring of the interactions strength in the food web highlighted by our analysis. Thus, compositional recovery was stonewalled by what could be called the "post-disturbance priority effect". The "priority effect" (Fukami 2015) suggests that the order and timing of species immigration during community assembly may affect species abundances and cause historical contingency in the structure and function of the community. We build on this concept and suggest that, after a disturbance large enough to cause the decline of some populations within a community, a similar process drives the rewiring of interactions strength. The niche pre-emption and niche modification (Fukami 2015) carried out by the tolerant species that persisted in the community hampered the recovery of declined population through competitive exclusion and/or strong predation pressure (Relyea & Hoverman 2006). This was particularly evident in our systems, where the insecticide almost completely eradicated all Cladocera (e.g., Daphnia sp., Ceriodaphnia, Simocephalus, Alona, and Alonella) and Ephemeroptera (Cleon sp. and Caenidae) taxa in the maximum effect phase (Fig. S1). Yet, the Ephemeroptera taxa could recolonise the mesocosms successfully, whereas the Cladocera taxa re-gained abundance level comparable to the control's one only under eutrophic condition. Non-eutrophic systems treated with the insecticide showed a dominance turnover in favour of the Cyclopoid copepods from the maximum effect phase onwards. Probably, in eutrophic systems the zooplankton suffered less from competition, as the nutrients treatment assured higher energy provision after the insecticide pulse that led to an increased over all zooplankton biomass in the recovery phase (Fig. 5a). In turn, this decreased the phytoplankton biomass owing to the increased grazing pressure. Similarly, the herbicide treatment strongly reduced the abundance of the Chlorophyta after its application. This promoted a compensatory dynamic that increased Dinophytes abundance. Subsequently, Dinophytes competitively excluded some the Chlorophyta, which never re-gained the pre-disturbance dominance (Fig. S1).

We can conclude that interactions strength was rewired, which shifted network connectance and modified further single population dynamics without modifying topological properties of the food web or univariate descriptors of community composition and function. Although these mechanisms have been long considered in theoretical ecology, this is the first time such rewiring of species interactions is quantitatively assessed with empirical data and used to explain the reported delayed (or completely missing) compositional recovery of freshwater communities after pulse disturbance.

4.2 Effects of nutrients enrichment

Nutrients enrichment caused a significant shift in several unweighted and node-weighted properties throughout the experiment. Eutrophic systems, despite showing a simplification of the food web, which was reflected by the changes in the architecture of the network and by univariate descriptors, were characterised by a general increase in biomass (Figure 5a) and interaction strength in the long term (Figure 4). Eutrophication is known to increase the energy fluxes due to higher resources availability (Binzer *et al.* 2016). Consequently, eutrophication increases the bottom-up energy supply, which may lead to the "paradox of enrichment" (Rosenzweig 1971) and to a following decline in richness (Nessel *et al.* 2021).

However, in the context of this study, it should be noted that nutrients were applied before and throughout the experiment as a press perturbation, thus significant effects for the whole experimental time were expected. The nutrients treatment was designed to look for possible interactions between pulse (pesticides) and press (eutrophication) disturbances, which were not identified in this study.

4.3 Multiple disturbance interactions

Pulse disturbance interactions on multivariate community composition only appeared in the recovery phase in the herbicide-insecticide treatment under non-eutrophic conditions. It is unlikely that this compositional change was driven by the sensitivity of the different taxa towards the disturbances, as in the recovery phase the concentration of the pesticides was too low to be harmful for aquatic organisms (Knauert *et al.* 2008; Huang *et al.* 2020). The analysis of the link-weighted properties highlighted that in the recovery phase the connectance and the generality of the food web were decreased by the mixture of the pesticides. Figure 3b shows that the outgoing energy fluxes in this treatment was dominated (> 80%) by the basal species, whereas the outgoing fluxes from higher trophic levels were drastically reduced, with no outgoing fluxes from the high trophic predators. At the same time, Figure 5 (b) shows that the per-capita average interaction strength (sum of in- and outgoing flows divided by number of node/species) for top-predators was extremely low compared to the other treatments, where high trophic predators exerted strong interactions. Although both strong and weak interactions are essential in promoting the stability of ecological networks (Mccann 2000; Allesina & Tang 2012), the disruption of strong trophic interactions has been previously shown to produce drastic cascading effects on food webs which lead to changes in community structure (O’Gorman & Emmerson 2009). The loss of strong interactions from high trophic predators, despite being well reflected by the changes in connectance and generality, also resulted in significant changes of the node-weighted properties. Node-weighted connectance was decreased by the pesticides mixture, driven by a reduction of high trophic predators and macro-zooplankton biomass (Figure 5a). Probably, the reduced grazing pressure derived from the pesticide-driven decrease in macro-zooplankton biomass triggered an expansion at the base at the food web, which was reflected as an increased phytoplankton biomass (Fig. 5a) resulting from decreased top-down control by the zooplankton, and as an increase in diversity of basal species (Table S3). Despite the increased number of basal species, the mixture of pesticides reduced the total richness of the network in the recovery phase (SI1, Table S2). Such reduction was then translated into significant changes in the topological properties of the food web (reduction in the number of links, link density, and generality; Table S3). Particularly, the large changes in the relative abundances of macroinvertebrate composition (Fig. S1), coupled with the reduced biomass of the zooplankton might have reduced the availability of preys for the high trophic predators. High order consumers are known to suffer disproportionately from instability at lower trophic levels (Greig *et al.* 2021). The changes at lower trophic level may thus have led to a strong biomass reduction of the high trophic predators.

That the loss of high trophic predators may trigger rapid changes in community structure (O'Gorman & Emmerson 2009) and may lead to secondary extinctions (Donohue *et al.* 2017) was already known. However, here we show for the first time that the rewiring of the trophic interactions' strength, which led to a loss of strong interactions with high trophic predators without causing their extinction, is sufficient to trigger a complete change in the food web (that is, topological as well as of interactions strength).

4.4 Outlook and future directions

In this study we investigated the differences between functional and compositional dynamics under different disturbance regimes. We show that functional and multivariate compositional responses to disturbance are substantially different. Importantly, we demonstrate that community biomass can be recovered despite significant changes in community composition, supporting the idea that functional redundancy can ensure similar level of functioning even if species composition changes (Hillebrand & Kunze 2020; Avolio *et al.* 2021). In line with other empirical studies (Hillebrand *et al.* 2018a; Komatsu *et al.* 2019), our work suggests that, under changing environment, are actually shifts in composition that allow maintaining biomass stable. Indeed, in field or semi-field experiment (i.e., high richness), sensitivity-driven species turnover and changes in dominance after disturbance allow maintaining (or recovering) ecosystem functioning through species asynchrony (Bruder *et al.* 2017; Avolio *et al.* 2019) (Figure S1). In this context, the eutrophication treatment deserves a particular mention. The press nutrients enrichment determined a reduction of species richness, but a general increase in biomass production. Nutrients' addition favours the growth of primary producers with high nutritional demands and fast uptake strategy. In turn, the fast-growing species cause an increase in competition for other resources (e.g., light, space) which determines the decline of the other slower-growing species. Thus, under eutrophic conditions, functioning levels can increase despite changes in composition, and a diversity decline (O'Gorman *et al.* 2012).

Recently, energy fluxes going from one trophic compartment to another have been proven to be a sensitive and meaningful way to measure ecosystem functioning in multitrophic systems (Barnes *et al.* 2018, 2020; Eisenhauer *et al.* 2019), and have been used to quantify the effect of biodiversity loss on ecosystem functions (Barnes *et al.* 2014). However, how different measurements of functional and compositional stability (Donohue *et al.* 2013) relate to the temporal variation in fluxes has not yet been investigated. Further investigation in this direction may arise new hypotheses and shed light on the relationship between functional and compositional aspects of stability (Hillebrand *et al.* 2018b; Polazzo & Rico 2021). Regarding the frequent disturbance-driven compositional shift we found, our study suggests that the rewiring of the interactions' strength may be driving long-term compositional dissimilarity at the community level under both single and multiple disturbances. Interestingly, the only significant interaction between pulse disturbances on the community composition appeared at a late-stage and was associated with a complete change in the ecological network, which produced an expansion of the bottom of the food web and a decrease in high-order consumers. We identify interactions strength (or energy fluxes) rewiring as the driving mechanism of the restructuring in the food web architecture. Consequently, it appears that changes in trophic structure and energy fluxes are key elements to understand, and potentially explain, the mechanisms driving the late appearance of interactions between disturbances at high levels of biological organization (Christensen *et al.* 2006; Garnier *et al.* 2017; Nagelkerken *et al.* 2020).

We do recognize, however, that our approach has some limitations. First, this study includes only negative trophic interactions. Non-trophic and positive interactions have been shown to play a role in determining the overall effects of interacting disturbances (Thompson *et al.* 2018b) and in shaping food webs (Kéfi *et al.* 2015). Additionally, although our work involves a relatively large number of species and links for experimental systems, it is still incomparable with real-world ecosystems, where food webs are larger and more complex. Future research that may succeed to include the analysis of non-trophic (competition, facilitation) and positive trophic interactions (mutualism) in food webs containing a larger number of high trophic predators (i.e., vertebrates) has the potential to eviscerate the mechanisms driving communities' responses to disturbance at higher spatial resolution, as well as those causing the large compositional turnover reported at the global scale (Dornelas *et al.* 2014).

We are aware that the analysis of ecological networks poses great challenges, as the construction of food webs may be time and resource consuming. Moreover, resolving complex food webs at a high spatial scale is extremely laborious, if not impossible. Nonetheless, the application of food web analyses to model ecosystems exposed to different types and gradients of disturbance may unveil mechanisms that explain ecological responses in natural ecosystems (Gray *et al.* 2014; Bruder *et al.* 2019). Communities and ecosystems are the primary target of management and policy makers (Donohue *et al.* 2016; Orr *et al.* 2020), thus understanding the processes causing (unexpected) long-term interactions between disturbances is the first step towards increasing our predictive capacity of ecosystems response to multiple anthropogenic perturbations.

5. Acknowledgement.

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Chapter 6

Extreme climatic event impacts on trophic network complexity and community multidimensional stability

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Abstract

Untangling the relationship between network complexity and ecological stability under climate change is an arduous challenge for theoretical and empirical ecology. Even more so, when considering extreme climatic events. Here, we studied the effects of extreme events (heatwaves) on the complexity of realistic freshwater ecosystems using topological and quantitative trophic network metrics. Next, we linked changes in network complexity with the investigation of four stability components (temporal stability, resistance, resilience, and recovery) of community's functional, compositional, and energy fluxes stability. We found reduction in topological network complexity to be correlated with reduction of functional and compositional resistance. However, temperature-driven increase in link-weighted network complexity increased functional and energy fluxes recovery and resilience, but at the cost of increased compositional instability. Overall, we propose an overarching approach to eviscerate the effects of climate change on multidimensional stability through the lens of network complexity, providing helpful insights for preserving ecosystems stability under climate change.

1. Introduction

Species composing ecological communities are interconnected in intricated networks of interactions, which may comprise competition, predation, mutualism and parasitism (Thomas *et al.* 2009; Pilosof *et al.* 2017). Species embedded in an ecological network exchange signals, materials and energy (Montoya *et al.* 2006). The way ecological networks are organised (that is, their structure and complexity) depends on the type of interactions linking the nodes of the network, on the strength and allocation of those interactions, and on a wide array of biotic and abiotic factors. The overall structure and complexity of the network determine how an ecosystem functions (Thébault & Fontaine 2010).

How network complexity influences ecosystem's stability has been hotly debated in ecology over the past half-century (MacArthur 1955; May 1972; Pimm 1984; McCann *et al.* 1998; Mccann 2000; Montoya *et al.* 2006). Both theoretical and empirical evidence have provided insights on the relationship between stability and network complexity. Yet, how network complexity correlates with stability remains divisive (MacArthur 1955; May 1973; Toju *et al.* 2017; Landi *et al.* 2018).

Ecological stability is the core notion to understand ecosystems' potential to withstand and/or to recover from perturbations. It underlies our ability to understand today's ecosystems' response to disturbance and to predict future ecosystems dynamics. Ecological stability may be the key to an effective ecosystem management and to the development of meaningful policies against rapid environmental change (Donohue *et al.* 2016; Kéfi *et al.* 2019). With the increasing pressure exerted by human activities upon ecosystems, understanding whether and how ecological network properties are related to stability in face of disturbance has become a central matter in ecology (Nagelkerken *et al.* 2020; Yuan *et al.* 2021). At the same time, ecological stability has recently emerged as a multidimensional construct (Donohue *et al.* 2013, 2016). That is, several metrics (including temporal variability, resistance, resilience and rate of recovery) are needed to fully describe the stability of a system in response to a given disturbance, as no single metric can adequately capture the different response aspects composing stability (Hillebrand *et al.* 2018b; Polazzo & Rico 2021).

Global climate change is one of the most pervasive anthropogenic disturbances to our planet (IPCC 2013). Global surface temperature has risen by roughly 1°C since the pre-industrial era, and it is projected to increase an additional 1.5 – 4.5°C (depending on RCP-scenario) by the end of the century (IPCC 2021). Owing to its profound implication for species metabolism and ecological dynamics, temperature affects all levels of biological organisation (Brown *et al.* 2004). Increasing temperature is, thus, expected to deeply alter ecological network structure and the nature and intensity of species interactions, influencing the overall stability of ecosystems. Experimental (Nagelkerken *et al.* 2020), theoretical (Petchey *et al.* 2010; Fussmann *et al.* 2014), and mixed approaches (O'Gorman *et al.* 2019) have investigated the effects of warming on trophic networks structure, interactions strength, and stability. Results showed that consumers, due to a temperature-driven increase in metabolism, exert stronger top-down control on the biomass stocks of lower trophic levels, increasing the overall consumer-resource biomass ratio (O'Connor *et al.* 2009; Fussmann *et al.* 2014; O'Gorman *et al.* 2019). However, consumers might struggle to meet their rising energy demands under warming (O'Connor *et al.* 2009; Fussmann *et al.* 2014). This may lead to shorter and simpler food webs (Fussmann *et al.* 2014; O'Gorman *et al.* 2019), reduced energy fluxes efficiency (Barneche *et al.* 2021), altered distribution of biomass and interactions strength throughout the food web (Nagelkerken *et al.* 2020), and potentially to a food web collapse (Ullah *et al.* 2018).

Most of the experimental and theoretical work done in this area has dealt with the effects of increasing mean temperature on ecological stability and network structure. Yet, climate change is composed of different processes and phenomena (IPCC, 2021), including extreme climatic events, such as heatwaves (HWs; Jentsch *et al.* 2007). HWs are of particular concern because their magnitude, duration, and frequency are predicted to increase in the future (Meehl & Tebaldi 2004; Woolway *et al.* 2021, 2022). Still, how HWs affect trophic networks and ecological stability has received little attention (Polazzo *et al.* 2021b). The few empirical evidence of the effects of HWs on multitrophic systems reported that HWs may affect both compositional and functional stability of freshwater communities, particularly in the absence of top predators (Ross *et al.* 2021).

To understand whether and how HWs affect trophic network complexity and community stability of freshwater ecosystems, we monitored the dynamics of a realistic community composed of periphyton, phytoplankton, zooplankton, and macroinvertebrates in an outdoor pond mesocosm experiment, simulating two temperature scenarios: a long but moderate HW and three strong reoccurring HWs (Fig.1). The long HW lasted for 40 days and was characterized by a temperature increase of +4°C from the control. The reoccurring HWs treatment consisted of three HWs lasting 7 days each, followed by 7 days of ambient temperature control conditions. In the reoccurring HWs, temperature was risen by +8°C versus the estimated mean temperature of controls. After the temperature manipulation phase, we kept monitoring network and community dynamics for 40 days. We employed two different HW scenarios as recent projections suggest that future HW may be stronger and more frequent (reoccurring HWs), but also longer-lasting (long HW) (Woolway *et al.* 2021, 2022). Thus, focusing on only one scenario might exclude important insights on whether and how extreme climatic events affect network complexity and community stability.

Specifically, our main research questions were: (a) whether and how extreme climatic events impact trophic network complexity; (b) whether and how extreme climatic events influence multidimensional stability; and (c) whether and how changes in trophic network complexity are related to altered community multidimensional stability. To answer these questions, we recorded over time the abundance and biomass of all major components of the food web (that is, periphyton, phytoplankton, zooplankton, and macroinvertebrates). We then studied the dynamics of trophic networks complexity using unweighted/topological metrics (based only on presence/absence of species) and link-weighted metrics (considering interaction strength). Finally, the relationship between those dynamics with four stability components (temporal variability, resistance, resilience, and recovery) of community's functional, compositional, and energy fluxes stability were studied. The term "network" is here used for simplicity since our research only focused on trophic interactions, and was hence limited to trophic networks (e.g., food webs).

2. Materials and methods

2.1 Mesocosm experiment

We performed an outdoor mesocosm experiment at the facility of the IMDEA Water Institute (Alcalà de Henares, Spain) between April and August 2021. Each mesocosm was filled with approximately 40 cm of sediments and 850 L of water from an artificial lagoon. The community, composed of basal species (periphyton and phytoplankton), zooplankton, and macroinvertebrates was allowed to establish and homogenize among experimental units for two months prior to the start of the experiment.

We experimentally simulated two heatwave (HW) scenarios following a full factorial design (n = 4): a long moderate HW and three strong repeated HWs (Fig.1). Four control mesocosm were kept at ambient temperature for the whole experimental duration. The long HW lasted for 40 days and was characterized by a temperature increase of +4°C above the control temperature, whereas the reoccurring HWs treatment consisted of three HWs lasting 7 days each and separated one another by 7 days of ambient temperature. In the reoccurring HWs treatment, the temperature was +8°C above the control temperature. After the end of the temperature manipulation phase, we monitored the recovery of the systems for 40 days. All temperature manipulations and recordings were carried out using a transportable temperature and heatwave control device (TENTACLE) applicable for aquatic micro- and mesocosm experiments (Hermann *et al.* 2022).

2.2 Sampling of the freshwater community

The whole community was sampled on days -4, +10, +24, +38, +52, +66, and +80 relative to the start of the HWs. Samplings of phytoplankton, zooplankton, and macroinvertebrates composing the freshwater community were done following Polazzo *et al.* (2021). Periphyton colonization in substrates (25 cm^2) was measured using transparent polypropylene strips placed in an upright position 20 cm from the water surface. Strips were taken on days -4, +10, +24, +38, +52, +66, and +80 relative to the start of the temperature manipulations using a tube of 50 mL filled with water. Samples were placed protected from light and temperature until they were analysed.

2.2 Quantification of the biomass

Every basal, zooplankton, and macroinvertebrate node was digitally photographed with scale reference using a camera Samsung 12 mp (4032 x 3024, JPG format), and measured using Image J software (Schneider *et al.* 2012). The biovolume (µm^3/org) of the basal, zooplankton, and macroinvertebrate was calculated using geometric models according to Sun & Liu (2003), Hillebrand *et al.* (1999), Ruttner-Kolisko (1977) and Alcaraz *et al.* (2003), and Bernardini *et al.* (2000). Biovolume was transformed to fresh weight (FW) using the following ratio 1 µg = 10^6 µm^3 (Salamanca Villate 2017), assuming specific weight of water = 1. Colonized periphyton was washed and concentrated by centrifugation (Ependorf 5810R at 3500 rpm during 20 min, 4 ºC). Fresh pellets were weighed using a balance: Denver instrument (precision = 0.005 g). Results were expressed as g/cm^2. Finally, fresh biomass of every community (i.e., periphyton, phytoplankton, zooplankton, and macroinvertebrates) was calculated per mesocosm (g/mesocosm).

2.3 Trophic network construction

We used publications, personal observations, and the database built by Gray et al. (2015), which represents the largest standardized collection of trophic links for freshwater organisms, to establish the trophic interactions among the species of the experimental mesocosms. The resulting interaction list comprised 1202 possible links and 142 trophic species (91 basal species, 23 zooplanktonic species, 23 benthonic macroinvertebrates and 3 high trophic predators). Eighty-four networks were built (3 treatments including control x 7 sampling days x 4 replicates) considering the biomass for each node.

2.4 Unweighted and weighted trophic network properties

Two types of networks were used to characterize the food webs for each mesocosm: topological or unweighted, formed by the presence or absence of nodes and relative interactions, and quantitative or link-weighted, where interactions' strength was also considered. For the first, we calculated the following unweighted network properties: number of links (L), link density (L/S, where S is the number of species), connectance (C), generality, vulnerability, number of basal and top species, mean trophic level, maximum trophic level, omnivory, path length, and clustering coefficient. We used the R package *multiweb* (Saravia *et al.* 2019) to obtain these properties.

As for the weighted food webs, we used a bioenergetic modelling approach to estimate interactions' strength (as energy fluxes), which enabled us to calculate three link-weighted properties: connectance, generality, and vulnerability (Kortsch *et al.* 2021). We here used the bioenergetic food web model developed by Gauzens *et al.* (2019) as implemented in the R package *fluxweb.* Shortly, this approach considers allometric scaling laws to quantify individual metabolic rates that are dependent on body mass and temperature, which together with losses to predation and assimilation efficiencies are used to quantify incoming fluxes (due to consumption) and outcoming fluxes (due to predation) for each species. The main assumption of this approach is system's equilibrium or steady state (Barnes *et al.* 2018), implying that the total amount of energy lost by a species, either by predation or physiological processes, is exactly compensated by the metabolized energy it gains from consumption. This is, species loss to predation and metabolism are completely balanced by energetic gains, which are defined as incoming fluxes multiplied by assimilation efficiencies (Jochum *et al.* 2021).

To calculate the metabolism of each node, species-specific metabolic rates were derived from body mass metabolic relationships using the allometric equation developed by Brown *et al.* (2004).

$$X_i = (X_0 * M_i^a * e^{\left(-\frac{E}{BT}\right)}) * Bi$$

Where X_i is the metabolic loss of species *i*, X_0 is the organism-specific normalization constant, M_i the body mass of species *i* (individual/g), a is the allometric scaling constant (a=−0.29), *E* is the activation energy (0.69 eV, Brown *et al.* 2004), *B* is the Boltzmann's Constant (8.61x10^{-5} eV/K), *T* is the temperature (K), and Bi is the biomass of species i (g/m^3).

The X_0 normalization constants are the intercepts of the body mass-metabolism scaling relationship for invertebrates and vertebrates presented in Brown *et al*. (2004), which correspond to 15.85 for unicellular organism, 17.17 for invertebrates and 18.47 for vertebrates. Since our study only involved unicellular basal species and invertebrates, we used the normalization constants for these specific classes of organisms.

The metabolic rate increases exponentially with temperature (Brown *et al.* 2004). Thus, temperature difference between different temperature treatments were accounted for in the bioenergetic model. We did that by using the average temperature recorded in each treatment in the time interval separating one sampling point from another. For the sampling point (e.g., before the beginning of the temperature manipulation), we took the average temperature measured in the three weeks before the start of the temperature treatments.

Finally, we estimated the different assimilation efficiencies depending on prey type: 0.906 for animal preys (Gauzens *et al.* 2019) and 0.77 for basal or primary producer preys (Landry *et al.* 1984).

The biomass of each taxon was calculated in grams per m^3. As such, the units of the calculated energy fluxes are joules per m^3 per second.

2.5 Stability properties

We quantified four stability properties: resistance, recovery, resilience, and temporal stability. We calculated the four stability properties for composition, function, and energy fluxes. Compositional stability was calculated using the Bray – Curtis similarity as a state variable (Donohue *et al.* 2013; Radchuk *et al.* 2019), whereas functional stability was based on total biomass (Hillebrand *et al.* 2018b; White *et al.* 2020). The stability of energy fluxes was calculated using the total energy fluxes derived from the bioenergetic model. To account for potential differences in sensitivity to the temperature manipulations of the different organism groups, we calculated the four stability properties separately for three different organism groups: basal (phytoplankton and periphyton), zooplankton and macroinvertebrates.

Resistance. Resistance is defined as the ability of a system to withstand disturbance (Hillebrand *et al* 2018). Hence, resistance was measured for each replicate as the difference between the value of the state variable of a replicate and that of the mean of the controls divided by the value of the state variable of the mean of the controls, all measured at the first sampling after the end of the temperature manipulation phase:

$$Resistance = \left(\frac{T_{HW3} - C_{HW3}}{C_{HW3}}\right)$$

T_{HW3} and C_{HW3} are the treatment and control values of the variable measured right after the end of the temperature manipulation phase (Fig. 1), which corresponded to day 38 relative to the start of the temperature manipulations.

Resistance values of 0 reflect maximum resistance (i.e., biomass, energy fluxes, or Bray – Curtis distances in treatment and control are the same). A negative resistance value means low resistance as compared to the control. Resistance higher the 0 indicates overperformance (increase in biomass or energy fluxes).

Recovery. Recovery is here defined as the capacity of a system to return to undisturbed state following a disturbance (Ingrisch & Bahn 2018). We quantified recovery for each replicate as the difference between the value of the state variable of a replicate and that of the mean of the control divided by the value of the state variable of the control, all measured at the last day of the experiment:

$$Recovery = \left(\frac{T_{Rec3} - C_{Rec3}}{C_{Rec3}}\right)$$

T_{Rec3} and C_{Rec3} are the treatment and control state variables measured at the end of the recovery period, which corresponded to day 80 relative to the start of the temperature manipulations. The interpretation of the recovery values follows that of resistance.

Resilience. We defined resilience following the definition of Pimm (1984), also known as engineering resilience. This resilience conception measures the speed of recovery after perturbation, and was quantified for each replicate as the slope of the ln-response ratio of the value in a treatment (T) compared to control (C) over time (Hillebrand *et al.* 2018b):

$$Resilience = \frac{\ln\left({}^{\mathrm{T}}/_{\mathrm{C}}\right) + i}{\mathrm{t}}$$

where i = intercept, t =time
The interpretation of the resilience values follows that of resistance.

Temporal stability. The temporal stability of each replicate was calculated as the inverse of the coefficient of temporal variation in biomass (functional), Bray – Curtis distances (compositional), or energy fluxes.

$$Temporal\ Stability = \frac{1}{Xcv}$$

Where Xcv is the variable coefficient of variation (ratio of the standard deviation to the mean of the variable). The larger the value of the temporal stability is, the higher is its temporal stability (i.e., less fluctuating over time). The value was biomass in case of functional recovery, Bray – Curtis distances in case of compositional recovery, and energy fluxes in case of energy fluxes recovery.

Overall stability. Following Pennekamp *et al.* (2018), we quantified the overall functional, compositional, and energy fluxes stability of each organism group. This is a more integrative approach which, similarly to ecosystem multifunctionality, integrates variation in multiple stability components into a single measurement. Overall, functional, compositional, and energy fluxes stability were calculated using the same methodology, which follow the same procedure used to calculate multifunctionality with the averaging method (Maestre *et al.* 2012):
OS = scale(resistance) + scale(resilience) + scale(recovery) + scale(temporal stability)

The function “scale” subtracts the mean and divides by the standard deviation. In this way, all stability components were standardized. Standardization before summing implies that all stability components have the same weight in the overall stability calculation.

2.6 Statistical analyses

Analysis of unweighted and link-weighted network properties was performed using linear regression models, with the reoccurring HWs and long HW as predictors. To check that linear models’ assumptions were met, we visually examined for the homogeneity of variances and normality using the R function *check_model* of the R package *performance* (Lüdecke *et al.* 2020), and found models to follow model assumptions. The linear models were constructed using R with the *lm* function of the package *stats* (R Core Team 2021).

Analysis of single stability components and of overall functional, compositional, and energy fluxes stability was performed using linear mixed-effects models, with reoccurring HWs (factor) and long HW (factor) as fixed effects. Mesocosm identity and organism group were included as random effects to account for potential idiosyncratic effects of experimental units (mesocosms) and for potential different responses between organism groups, respectively. We used the package *nlme* for all the linear mixed-effects model (Pinheiro *et al.* 2021). Again, the R function *check_model* of the R package *performance* was used to visually examined for the homogeneity of variances and normality, finding that models’ assumptions were satisfied.

To assess whether communities undergoing different HW treatments were different in the different phases of the experiment, we performed two non-parametric multivariate analysis of dissimilarity, permutational multivariate analysis of variance (PERMANOVA, function *adonis2* of the R package *vegan* (Oksanen *et al.* 2019)) and analysis of similarity (ANOSIM, function *anosim* of the R package *vegan* (Oksanen *et al.* 2019)), both with 999 permutations and based on Bray – Curtis distances.

Finally, to analyze the relationship between stability components and between stability components and network properties we calculated Pearson correlation coefficients.

All statistical analyses and trophic networks construction were conducted using the R software (version 4.1.2, R Core Team 2021).

3. Results and discussion

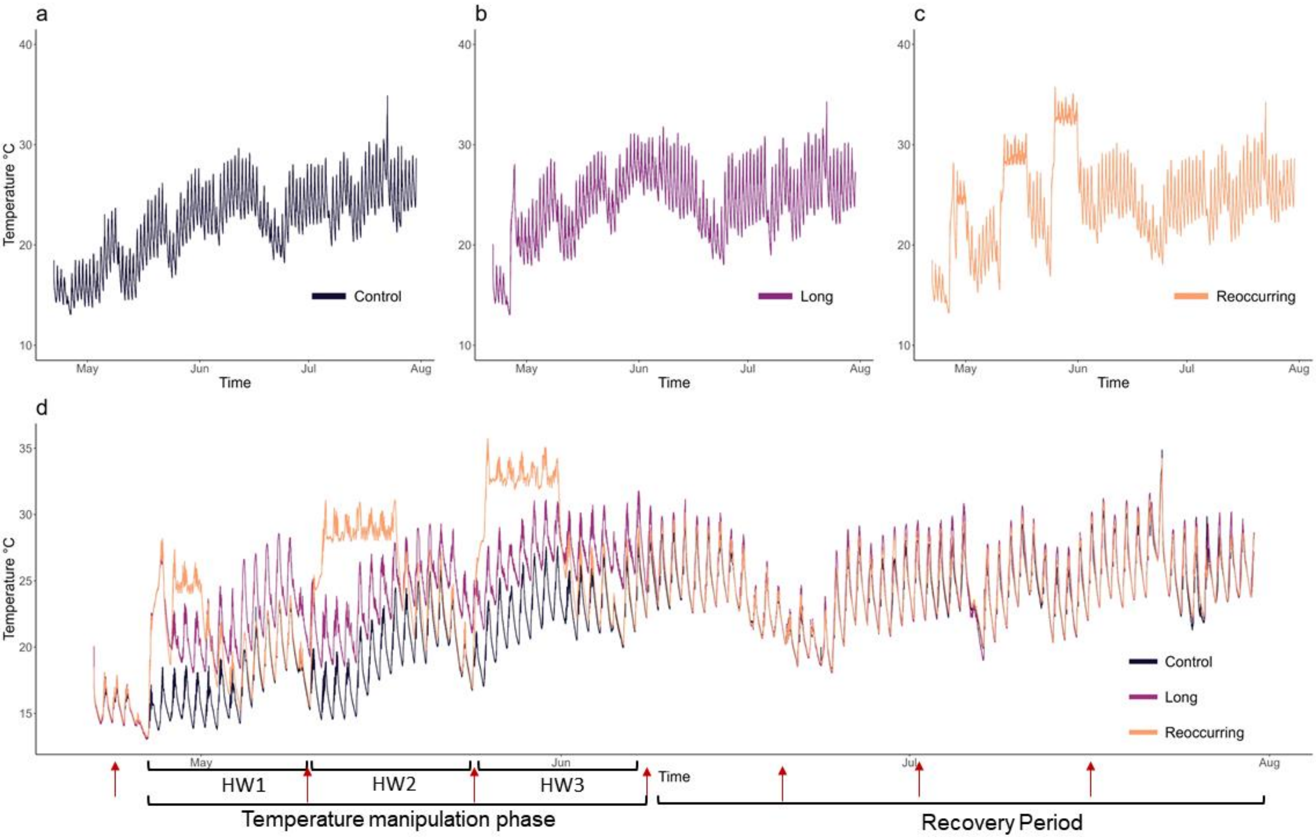


Fig. 1| Experimental temperature manipulations and timeline. a, Temperature dynamics recorded in the ambient temperature mesocosms not undergoing temperature manipulation (control). **b,** Temperature recorded in mesocosms undergoing the long HW treatment over the experimental time. **c,** Temperature recorded in mesocosms undergoing the reoccurring HWs treatment over the experimental time. **d**, Combination of the different temperature treatments over the experiment, and different experimental phases. Red arrows indicate biological samplings. HW: heat wave.

Before the start of the temperature manipulations, community composition was indistinguishable between control and treatments (PERMANOVA, p-value >0.05), and so was biomass (Linear mixed-effects model, LMM, p-value >0.05) and total energy fluxes (LMM, p-value >0.05). Consistently, all unweighted and link-weighted network proprieties were not different between the control and the treatments.

At the end of the temperature manipulation phase (Fig. 1), size (Linear Model, LM, t = -2.282, p = 0.048), number of links (LM, t = -2.49, p = 0.018), number of basal species (LM, t = -2.93, p = 0.016), unweighted generality (LM, t = - 2.83, p = 0.019) and link-weighted vulnerability (LM, t = -3.366, p = 0.008) were significantly decreased by the reoccurring HWs treatments, whereas unweighted mean trophic level was increased (LM, t = - 2.801, p = 0.020) (Fig. 2). By contrast, the long HW treatment did not affect any unweighted or link-weighted network property right after the end of the temperature manipulation phase.

At the end of the recovery period, no significant difference between control and treatments was found for any unweighted network property. Yet, the reoccurring HWs treatment significantly increased link-weighted generality (LMM, t = 2.518, p = 0.032), while the long HW increased both link-weighted generality (LMM, t = 2.296, p = 0.047) as well as link-weighted connectance (LMM, t = 2.625, p = 0.027).

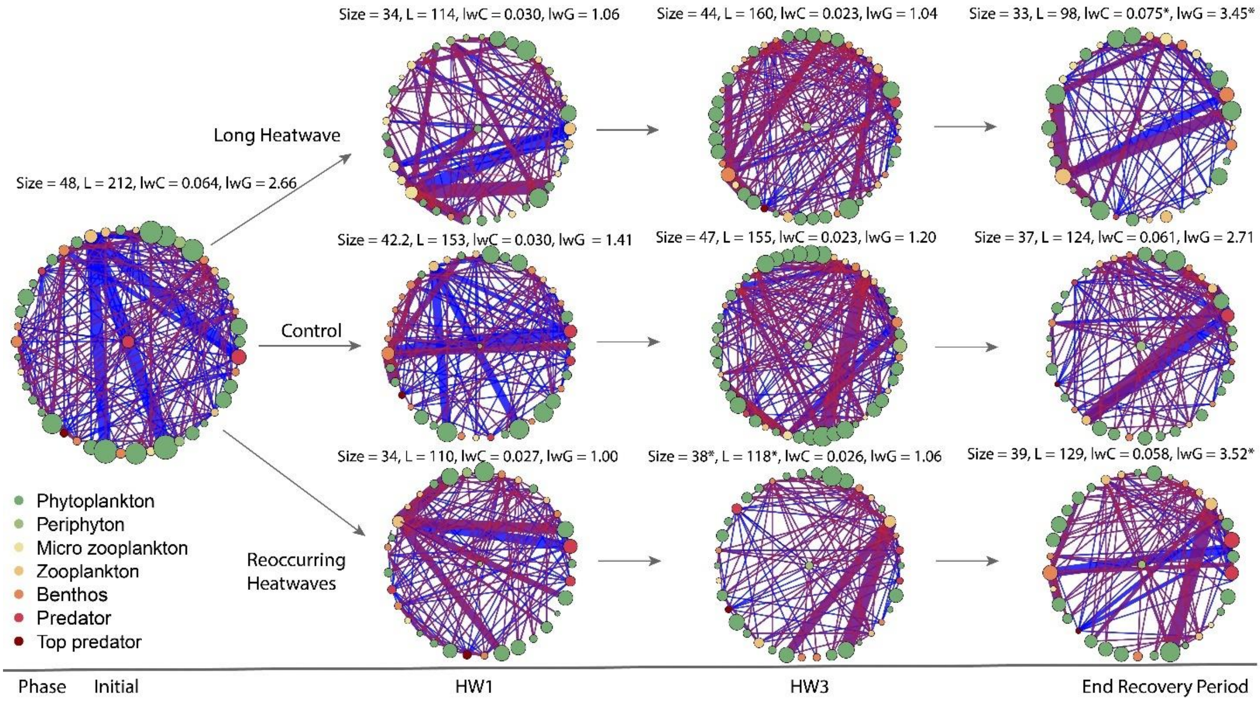


Fig. 2| Trophic networks dynamics over time. Visualisation of constructed trophic networks in different experimental phases (Initial = before the start of the temperature manipulations, HW1 = 10 days from the beginning of temperature manipulation, HW3 = end of the temperature manipulation period; End Recovery Period = last day of the experiment). Size is the average number of nodes composing the networks for a specific temperature treatment. L is the average number of links. lwG is the average link-weighted generality, whereas lwC is the average link-weighted connectance. Red links show fluxes going from primary producers to primary consumers, whereas blue links represent fluxes going from primary consumers to predators. Asterisks indicate significant differences in the metric according to linear regression models.

The reoccurring HWs significantly affected multiple aspects of compositional, functional and fluxes stability (Fig. 3). Resistance of function (LMM, t = -2.58, p = 0.015) and of composition (LMM, t = -5.43, p-value<0.001) were reduced by the reoccurring HWs. Compositional recovery (LMM, t = -5.00, p-value < 0.001) and functional temporal stability (LMM, t = -3.22, p = 0.003) were also reduced by the reoccurring HWs, whereas the resilience of function (LMM, t = 3.71, p = 0.001) and of energy fluxes (LMM, t = 2.80, p = 0.009) were significantly increased.

Although the long HW generally decreased biomass in the temperature manipulation phase (Fig. 1 of the Appendix of this Thesis), it only caused a significant increase in functional resilience (LMM, t = 2.75, p = 0.01). The long HW also reduced compositional recovery (LMM, t = 3.35, p = 0.002), whereas it increased energy fluxes resistance (LMM, t = 4.75, p < 0.001), recovery (LMM, t = 3.77, p = 0.001), and resilience (LMM, t = 2.96, p = 0.006).

Finally, following Pennekamp *et al.* (2018), we calculated the overall functional, compositional, and fluxes community stability. This is a more integrative approach which, similarly to ecosystem multifunctionality, considers variation in multiple stability components in a single measurement (see methods for details). Neither of the HW treatments affected the overall ecosystem functional stability, whereas both the long HW (LMM, t = -2.91, p = 0.007) and the reoccurring HWs (LMM, t = -5.21, p < 0.001) decreased overall community compositional stability. Either treatment also significantly increased overall community energy fluxes stability (Long HW, LMM: t = 8.38, p < 0.001; Reoccurring HWs, LMM: t: 3.71, p = 0.001).

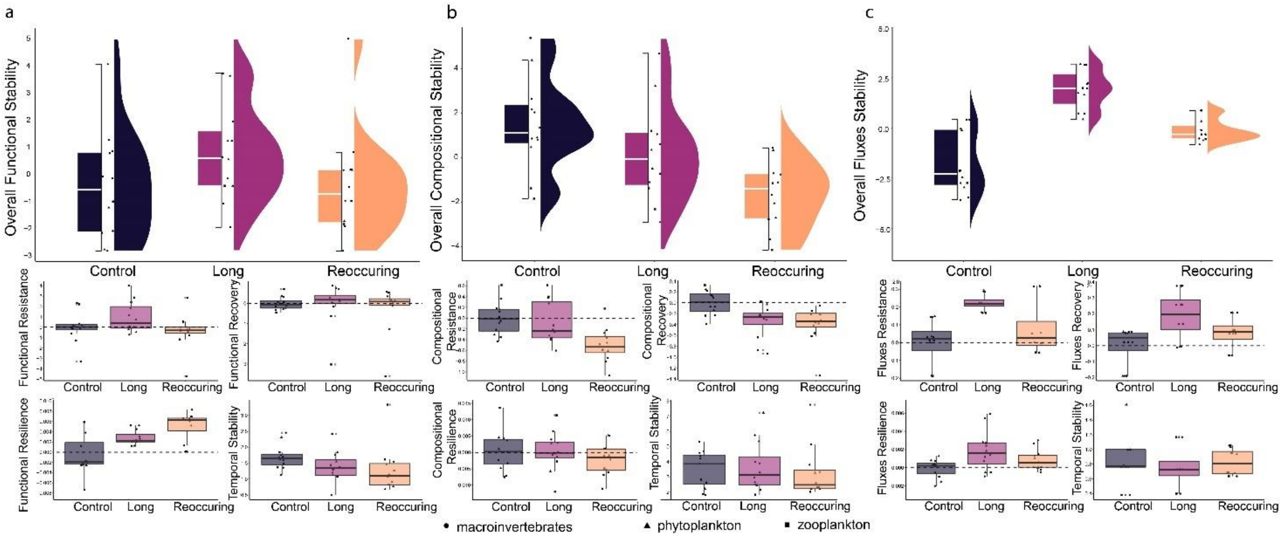


Fig. 3| Multidimensional stability response. Effects of experimental temperature manipulation on different stability components of **a** functional stability, **b** compositional stability, and **c** energy fluxes stability. In the box and whisker plots (*n* = 12, for all measures), the centre line indicates the median, while the bottom and top hinges of the box correspond to the 25th and 75th percentiles, respectively. Individual dots show the different stability properties calculated for basal species (*n* = 4), zooplankton (*n* = 4), and macroinvertebrates (*n* = 4).

Our study shows that repeated extreme climate events may reduce the complexity of the unweighted network structure in the short term. Yet, at least in our simplified freshwater lentic ecosystem, those effects were short-lived. At the end of the experiment, no single unweighted network property was still affected by the reoccurring HWs, suggesting that the food web's unweighted structure may be regained after extreme climatic events. The initial reduction in unweighted network complexity, though, was positively correlated with decreased functional resistance (Fig. 4). Topological network complexity after experimental perturbation was also positively correlated with compositional resistance. That is, compositional and functional resistance were high where topological network complexity remained high. Consistently, in the reoccurring HWs treatment, a decline in unweighted network complexity was reflected by significant reduction in compositional resistance. Two additional non-parametric multivariate dissimilarity analyses (permutational multivariate analysis of variance (PERMANOVA and analysis of similarity (ANOSIM), Table 1 of the Appendix of this Thesis) confirmed that, after the temperature manipulation phase, the composition of the community that underwent reoccurring HWs was significantly modified.

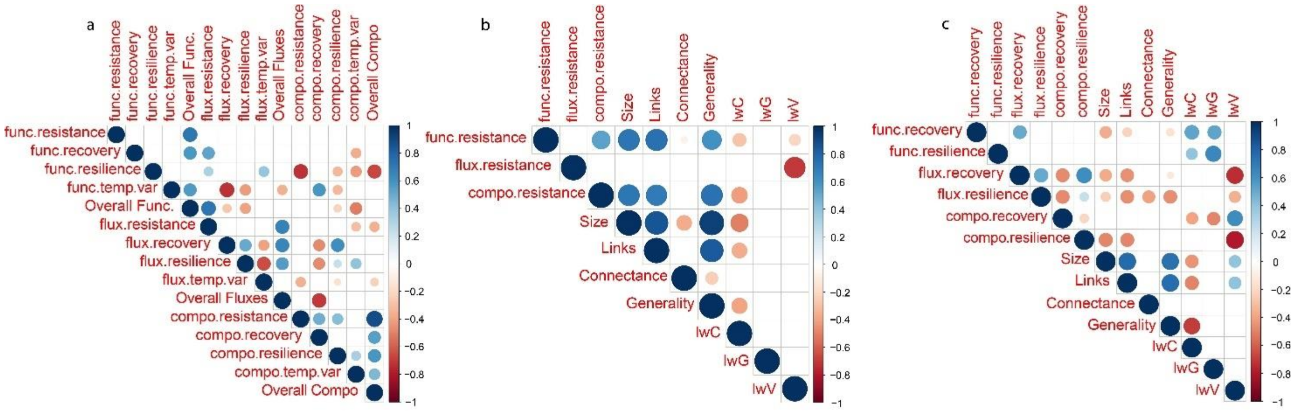


Fig. 4| Correlation between stability components and trophic networks properties. a, correlation between the different stability metrics of functional, compositional, and energy fluxes stability. **b**, correlation between trophic network structure metrics measured on phase HW3 and the resistance of functional, compositional and energy fluxes stability. **c**, correlation between trophic network structure metrics measured at the end of the recovery phase and the recovery and resilience of functional, compositional and energy fluxes stability. The circle size and colour indicate the size and sign of the correlation. Only significant correlations (p-value of the Pearson's correlation coefficient < 0.05) are shown.

Those results are in line with the body of theoretical and empirical work that has linked reduced network complexity to a decline in ecosystem functioning (Morris 2010; Eisenhauer *et al.* 2019; Sebastián-González *et al.* 2020; Yuan *et al.* 2021). Additionally, a recent study has shown that an experimental reduction in unweighted trophic network complexity (removal of high-level consumers) can significantly reduce both compositional and functional resistance (White *et al.* 2020). As such, compositional and functional resistance appear to be positively correlated across ecosystem types and despite the nature of disturbance applied (Hillebrand *et al.* 2018b; White *et al.* 2020; Polazzo & Rico 2021; Urrutia-Cordero *et al.* 2022). On the other hand, we did not find any correlation between trophic network complexity and the resistance of energy fluxes immediately after the temperature manipulations.

HWs may act essentially as pulse disturbances. A recent synthesis of the effects of pulse disturbance on community multidimensional stability has shown that communities' functional responses might be initially severely impacted by pulse perturbation, but function is generally recovered within the experimental time (Hillebrand & Kunze 2020). Conversely, community multivariate composition appears to recover substantially slower, or to not recover at all, after a pulse perturbation (Hillebrand & Kunze 2020), suggesting that compositional and functional stability dynamics may follow different pathways (Hillebrand & Kunze 2020; White *et al.* 2020; Ross *et al.* 2021).

Recently, new experimental work has shown that changes in trophic network link-weighted properties (that is, accounting for species interactions strength and their organisation within the network) might promote long-term compositional dissimilarity after pulse disturbance, thus, impeding compositional recovery (Polazzo *et al.* 2022).

In our study, we observed an overall increase in link-weighted network complexity in both temperature treatments by the end of the experiment (Fig. 2). This increase in link-weighted complexity was found to be negative correlated with compositional recovery (Fig. 4). Consistently, either HW treatments significantly reduced compositional recovery, and community composition was significantly modified by both treatments according to ANOSIM and PERMANOVA at the end of the experiment (Table 1, Appendix of this Thesis). Previous studies have shown that changes in composition are actually needed to achieve a complete functional recovery after pulse disturbance (Hillebrand *et al.* 2018; Polazzo *et al.* 2022). This is because pulse disturbances may select for different compositions which can maintain functional level under disturbance if there is functional redundancy (Yachi & Loreau 1999). Yet, we also show that the compositional change promoted by both types of heatwaves is associated with an increase in link-weighted network complexity. Furthermore, the recovery of energy fluxes (a proxy for interactions strength) was negatively correlated with compositional recovery (Fig. 4). In other words, if total energy fluxes increase over time, community composition will further depart from control conditions, supporting the hypothesis that changes in interactions strength drive compositional divergence (Polazzo *et al.* 2022).

All the different components of the energy fluxes stability were negatively correlated with link-weighted vulnerability, which declined in both treatments following an increase in energy fluxes, although not significantly (Fig. 6, Appendix of this Thesis). Yet, excluding vulnerability, we found no significant correlations between any other energy fluxes stability property and link-weighted connectance and generality. The absence of a significant relationship between these link-weighted network properties and the stability components of the energy fluxes suggests that both link-weighted network complexity and total energy fluxes might influence the community composition independently. Therefore, the way interactions strength is organised within the network (i.e., link-weighted network complexity), and the total interactions strength (how much), both, shape community composition after pulse disturbance and influence functional levels. Such mechanism was particularly evident for the reoccurring HWs treatment in our experiment. Reoccurring HW disturbance caused an initial significant decline in functional resistance. Still, heat stress fostered a shift in community composition associated to an increase in link-weighted generality. Such increase in link-weighted network complexity consequently increased functional resilience through which functional recovery could be achieved within the experimental time.

Similarly, the long HW treatment reduced (even though not significantly) total biomass during the temperature manipulation phase (Fig. 1 Appendix of this Thesis). However, a biomass level similar to the control could be regained at the end of the experiment thanks to an increase in functional resilience, which could only be achieved through an increased trophic network complexity (e.g., link-weighted connectance and generality), increased energy fluxes, and a significant compositional shift.

Finally, our analysis of the overall functional, compositional and of energy fluxes stability summarises our findings obtained when studying the different stability components. Overall functional stability was not affected by either treatment, however this was achieved by significantly reducing the overall compositional stability in both temperature treatments. This is, composition had to be modified to maintain functional stability. The changes in overall compositional stability were paired to an increase in overall fluxes stability. Hence, communities exposed to extreme climatic events modify their composition and increase the overall energy fluxes within the food web, fostered by the temperature-induced increase in metabolism. This produces an increase trophic network complexity and preserves functional stability.

Our findings have important implications for ecosystem management and for projecting the effects of (future) extreme climatic events and climate change on trophic networks and community stability. First, preserving topological network complexity appears crucial to protect both compositional and functional stability, as reductions in topological complexity were associated with a reduction in functional as well as compositional resistance. Second, functional recovery and resilience were positively associated to an increase in link-weighted network complexity, which however, came at the cost of increased compositional dissimilarity (e.g., reduced compositional recovery). Given the different management goals of different stakeholders (i.e., agriculture or aquaculture managers will likely privilege functional stability over compositional stability, whereas managers of natural protected areas might want to maintain compositional stability), tailoring specific protection measures to different objectives does seem a promising way, although needing careful planning. Finally, even though the effects of the reoccurring HWs on compositional and functional stability were somehow stronger compared to the long, moderate heatwave, the latter had a larger effect on the stability of energy fluxes and network complexity. These observations suggest that constant warming, via its effects on metabolism, causes a larger increase in the individual energetic demand compared to intermittent heat pulses. This is translated into stronger energy fluxes over time and increased network link-weighted complexity.

This study shows that all the nuances of climate change are likely to have profound implications for community stability and trophic network complexity. Yet, here we present an overarching approach to eviscerate the effects of environmental change on multidimensional stability through the lens of trophic network complexity. The results of this study can be used to evaluate policy-targeted endpoints and provides helpful insights for the preservation of ecosystems stability under global change.

Supplementary information:
The supplementary information of this study may in the appendix of this Thesis.

Chapter 7

Conclusions

The ultimate goal of multiple stressor's research is to develop accurate predictions on how several global change drivers interact with each other and affect biological systems. To reach this overarching goal, a mechanistic understanding of how different stressors act in isolation and when combined is needed. As of today, most multiple stressor studies have investigated stressors' interactions on low levels of biological organisation, particularly on individuals and populations. This approach has provided invaluable insights on how multiple stressors affect physiological, growth and reproductive endpoints, but has still rendered a poor understanding of how multiple stressors affect communities and ecosystems. Direct effects on the physiology or development of sensitive individuals and populations may result in indirect effects for other organisms, which complicates our understating as well as our predictive capacity of effects on complex communities.

From this thesis, the following conclusions were derived:

- The knowledge on the combined effects of heatwaves and chemicals is extremely scarce. Thus, it resulted difficult to summarise consistent trends at any level of biological organisation. The imbalance found towards single species and population experiments results in a knowledge gap for relevant community and ecosystem level endpoints. This appears particularly problematic, as it prevents the exploration of important indirect effects that can compromise food web stability. (Chapter 2).

- The individual application of nutrients and pesticides can affect community structure and species richness. Ecosystem functioning is generally less sensitive to chemical stress than community structure, while eutrophication may foster the dominance of species that are more resilient to pesticides. (Chapter 3).

- Stressor interactions may be significant at different time points, with late stressor interactions affecting the recovery of community composition. The correlation between biodiversity and ecosystem functioning can be shifted from positive to negative under particular stress conditions. (Chapter 3).

- Pesticides did not affect the dimensionality of compositional stability, whereas the dimensionality of functional stability was significantly increased by the insecticide only in non-enriched systems. Stressor interactions may act non-additively on single stability dimensions as well as on the dimensionality of functional stability. Single and multiple stressors have been proven to be able to shift the correlation between functional and compositional aspects of stability. (Chapter 4).

- The quantitative network approach revealed that rewiring of species-interactions strength may be the main mechanism driving differences between structural and functional recovery. Additionally, interactions between multiple disturbances appeared in the long term only when both species interaction strength and food-web architecture changed significantly. (Chapter 5).

- Reduction in topological network complexity was found to be correlated with a reduction of functional and compositional resistance after exposure to heatwaves. Heatwaves caused an increase in link-weighted network complexity which promoted functional and energy fluxes recovery and resilience, but at the cost of increased compositional instability. (Chapter 6).

- Overall, the overarching approach presented in Chapter 6, allows to eviscerate the effects of climate change on multidimensional stability through the lens of network complexity, providing helpful insights for preserving ecosystems stability under climate change.

2. Future directions

2.1 Filling the gaps

This thesis has highlighted that the knowledge on the combined effects of heatwaves and chemical contaminants is extremely scarce (Chapter 2). An obvious avenue for future research will be to explore more the potential interactions between extreme climatic events and chemicals. Both classes of disturbances directly influence individuals’ survival and metabolism, with high potential for interactions. Effects at the individual level are then translated into density dependent effects at the population and community level. However, the implication of the combined effects of chemicals and extreme events at the community level are largely unknown. Future research needs to address and understand the consequence of the interactions between these two classes of stressors at high levels of biological organisation.

Interactions between multiple stressors can appear at different time points at all levels of biological organisation. Chapter 3 shows that stressors' interactions emerged at different times at the population, community, and ecosystem level. However, the temporal scale dependency of stressors' interactions remains somewhat overlooked. Understanding the mechanisms driving the appearance of non-additivity between stressors will enable research to increase the predictive power of the overall effects of stressors' interaction, allowing better protection measures. Additionally, understanding the drivers of time-dependent non-additivity, would allow to understand and predict stressors effects on different stability dimensions.

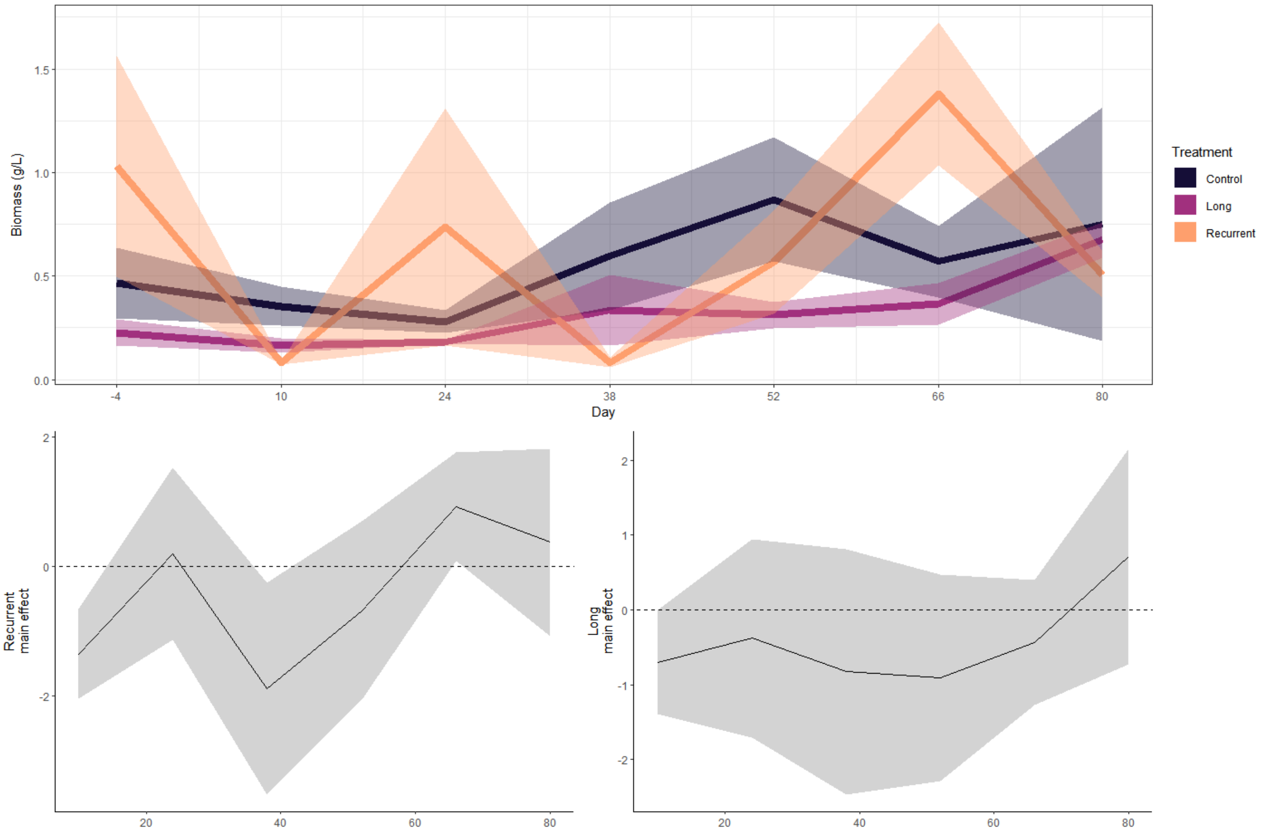
This thesis has shown that multiple stressors can interactively modify the dimensionality of ecological stability (DS) (Chapter 4). In this regard, more experimental work is needed to unravel the mechanisms driving disturbance effects on DS under different ecological scenarios. Meaningful diversity manipulations across different trophic levels would greatly benefit the long-standing debate on how and to what extent biodiversity can influence ecosystem stability. Moreover, future research measuring functional stability using several ecosystem processes as state variables would allow to study the relationship between the multiple dimensions of compositional stability and ecosystem multifunctionality, an aspect that has not been investigated so far.

Generally, multiple stressors research could greatly benefit from adopting a network approach (Chapter 5, 6) and by investigating stressors' interactions through a multidimensional stability approach (Chapter 6). Future research could use the tools and the framework implemented within this thesis to advance our mechanistic understanding of how stability is shaped in a stressed world.

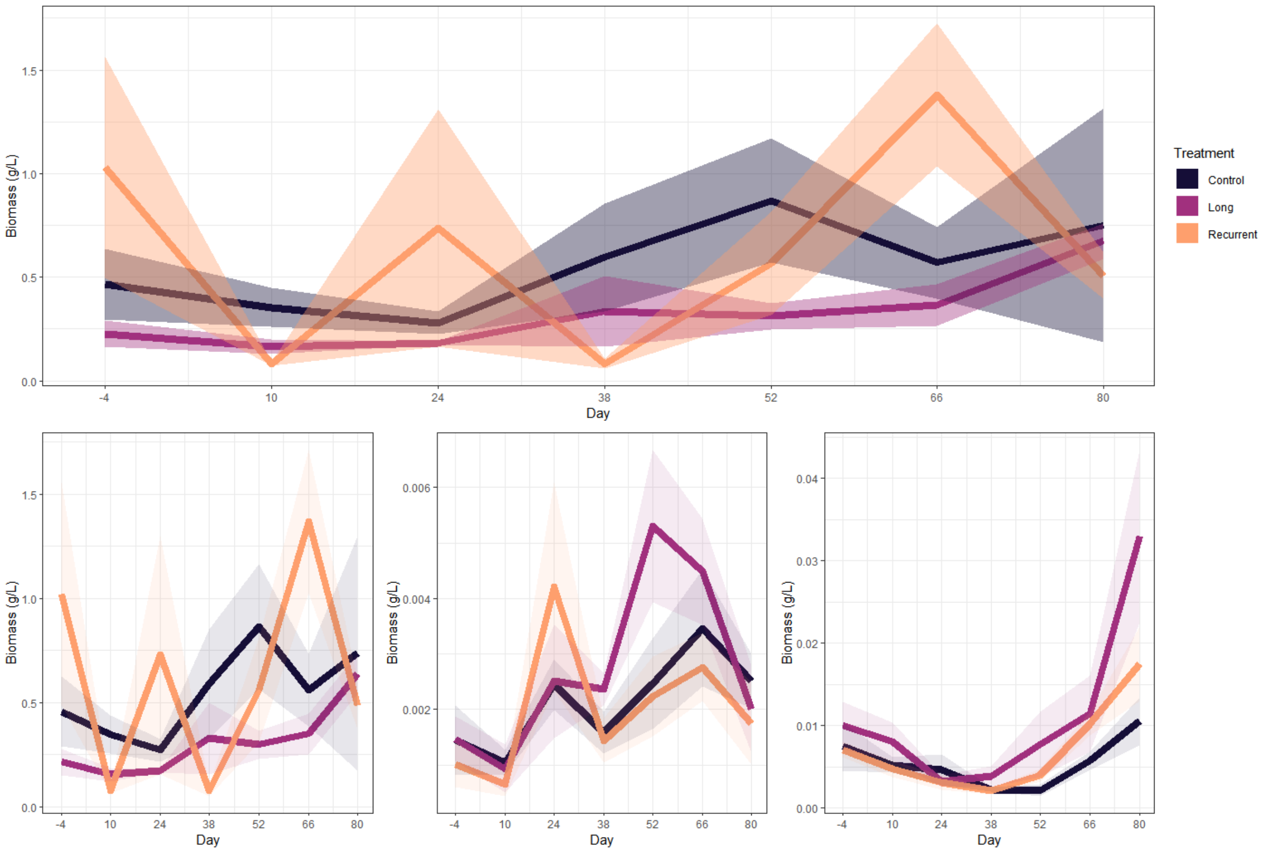
2.2 Moving forward

Multiple stressors research, as well as research dealing with ecological stability, has largely ignored how evolutionary processes might influence communities and ecosystems responses to environmental change. Rapid evolution should be considered as a key process driving adaptation to multiple stress sources. Therefore, a promising avenue for future research will be considering how eco-evolutionary processes shape organisms' and communities' responses to multiple drivers of environmental change. Studying eco-evolutionary processes appears particularly important when a community is exposed to constant, press stressors such as climate change or eutrophication. Long-lasting disturbances might shape community's traits through evolutionary pathways, which, in turn, may result in higher or lower sensitivity to additional pulse stressors. Moreover, eco-evolutionary processes determined by environmental change can select for specific phenotypes within a network. Such evolutionary pressure, determined by environmental change, could produce changes in network characteristics. As this thesis has showed, changes in network characteristics will inevitably influence multiple dimensions of ecological stability across levels of biological organisation. Yet, all the studies analysing the effects of environmental change on network complexity and community stability have only studied the ecological dynamics driving the observed responses. At present, an empirical investigation of the evolutionary mechanisms driving network and community level dynamics in face of multiple disturbances is lacking. Nevertheless, in a stressed world, disturbance-driven selective pressures are likely to shape phenotypes, which might in turn generate new biotic conditions altering network structure, leading to eco-evolutionary feedback loops. Phenotypes link ecological and evolutionary dynamics within ecological networks (particularly in food webs). From an ecological perspective, phenotypes regulate population-level processes, such as per-capita assimilation, consumption, birth, and death rates. Phenotypes also determine the strength and organization of species interactions within ecological networks, and determine how individuals respond to the environment they live in. From an evolutionary perspective, transmissible phenotypes that influence the overall fitness are exposed to evolutionary changes and natural selection. The evolution of phenotypes may therefore influence multiple ecological processes. On the other hand, species interactions can determine ecological dynamics which, in turn, mediate the pace and direction of phenotypic change. Yet, the role of evolution in determining the structure and dynamics of ecological networks, and how the structure of these networks set the pace and the direction of phenotypic change under a changing environment are largely unknown. Experiments involving organisms with fast life cycle have been used successfully to study eco-evolutionary processes in controlled systems. Yet, this has been done mainly for single species or populations. Evaluating adaptation and evolutionary processed under multiple stress factors seems a promising way to shed light on how eco-evolutionary dynamics modify network properties, ecosystems responses, and ecological stability.

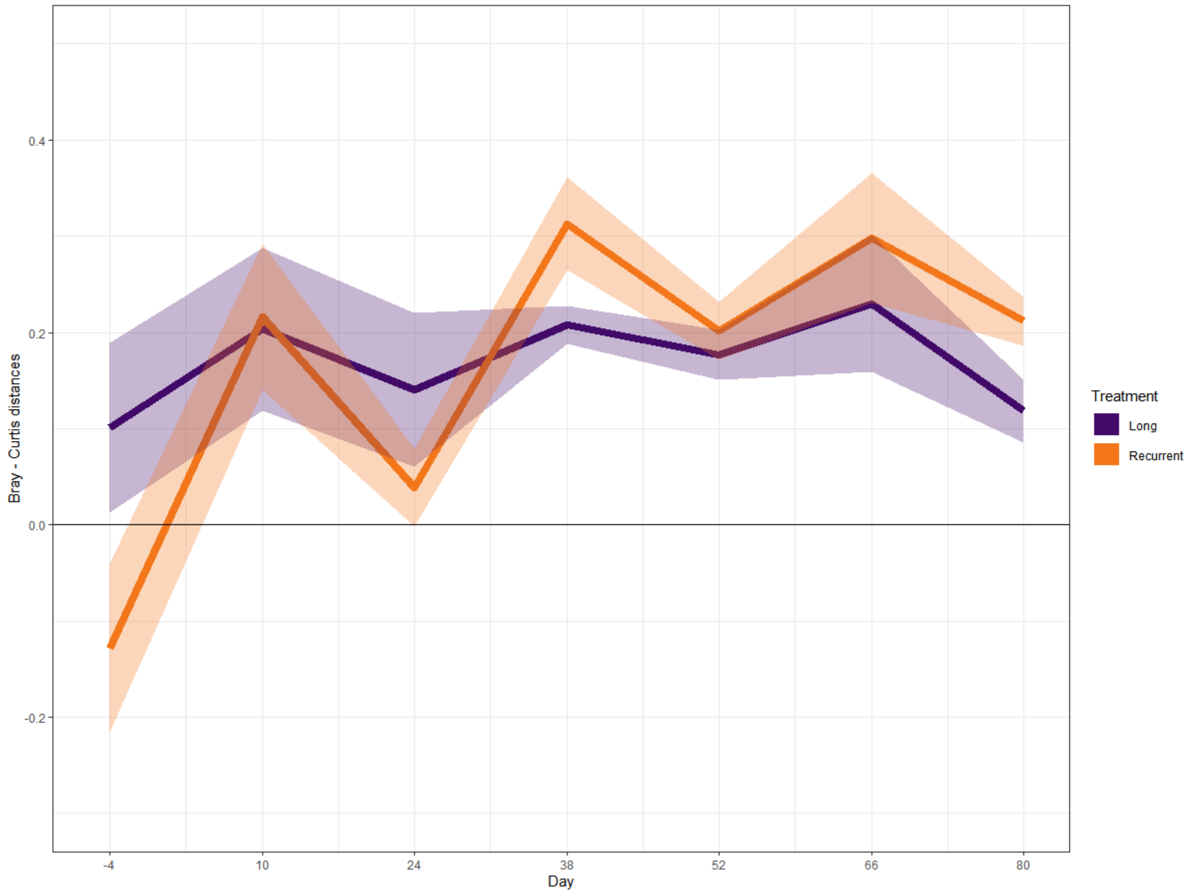
Appendix A: SI Chapter 6



Extended data Fig. 1 | Biomass dynamics over the experimental time.
Upper panel: Total biomass dynamics over time. Lower panels: Temporal dynamics of effect sizes of the treatment (95% confidence intervals) of a linear mixed effects models (LMM) of total biomass. LMM had long and reoccurring HW as fixed effects whereas mesocosm ID and day were used as random effects.

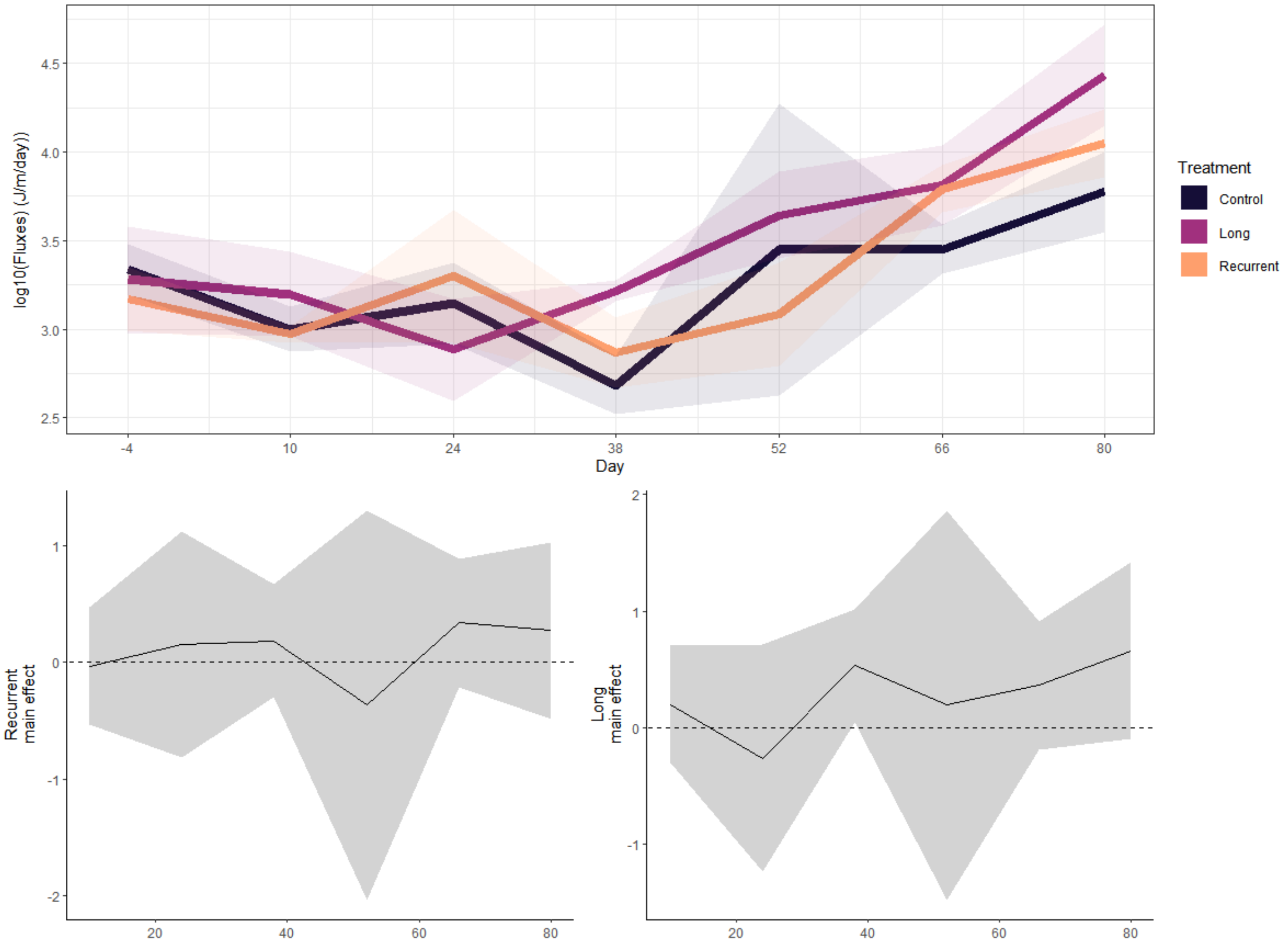


Extended data Fig. 2 | Biomass dynamics of the different organism groups. Upper panel: Total biomass dynamics over time. Lower panels: Temporal dynamics biomass of basal species, zooplankton and macroinvertebrates

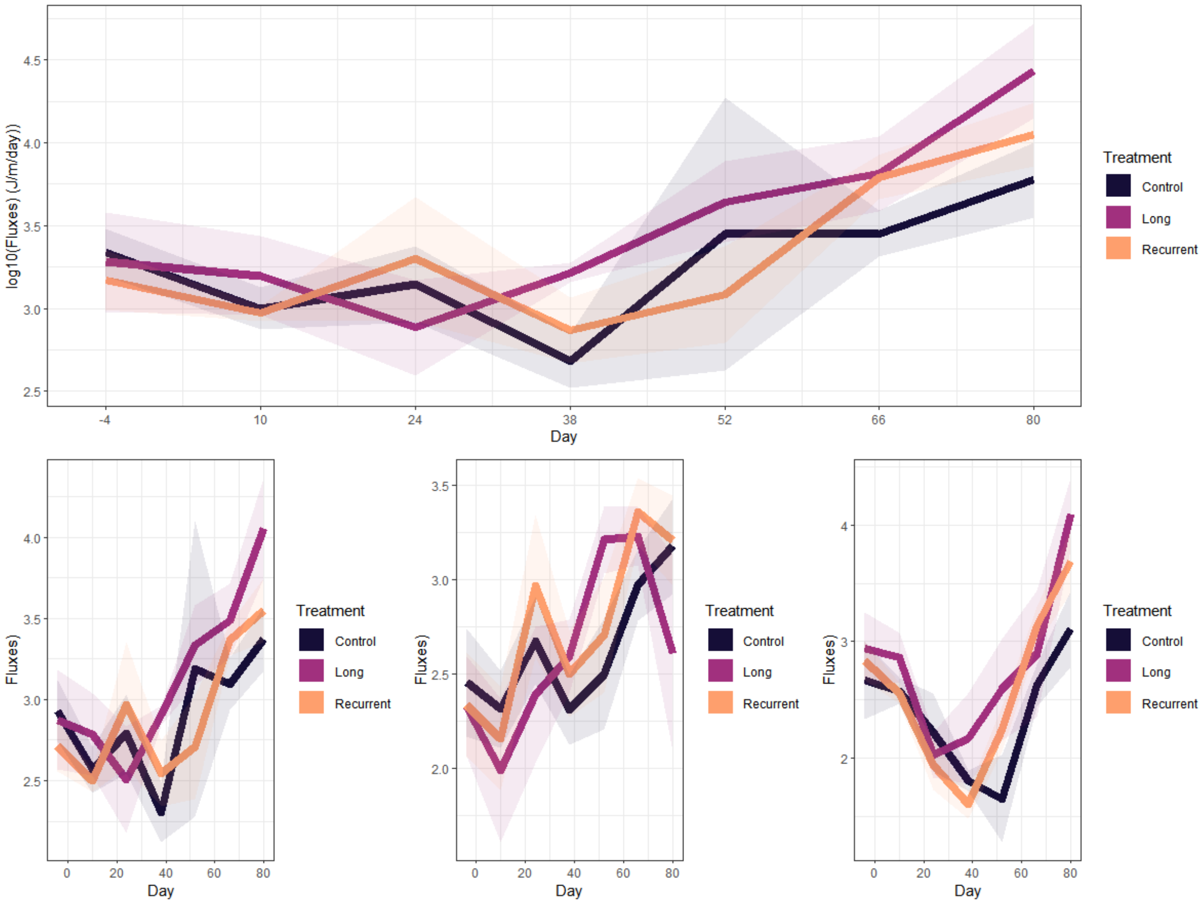


Extended data Fig. 3| Bray - Curtis dynamics over time.

Bray – Curtis distances from the control over the experimental time for the whole community.



Extended Data Fig. 4| Energy fluxes dynamics over the experimental time.
Upper panel: Total energy fluxes dynamics over time. Lower panels: Temporal dynamics of effect sizes of the treatment (95% confidence intervals) of a linear mixed effects models (LMM) of total energy fluxes. LMM had long and reoccurring HW as fixed effects whereas mesocosm ID and day were used as random effects.



Extended data Fig. 5 | Energy fluxes dynamics of the different organism groups. Upper panel: Total energy fluxes dynamics over time. Lower panels: Temporal dynamics energy fluxes of basal species, zooplankton and macroinvertebrates

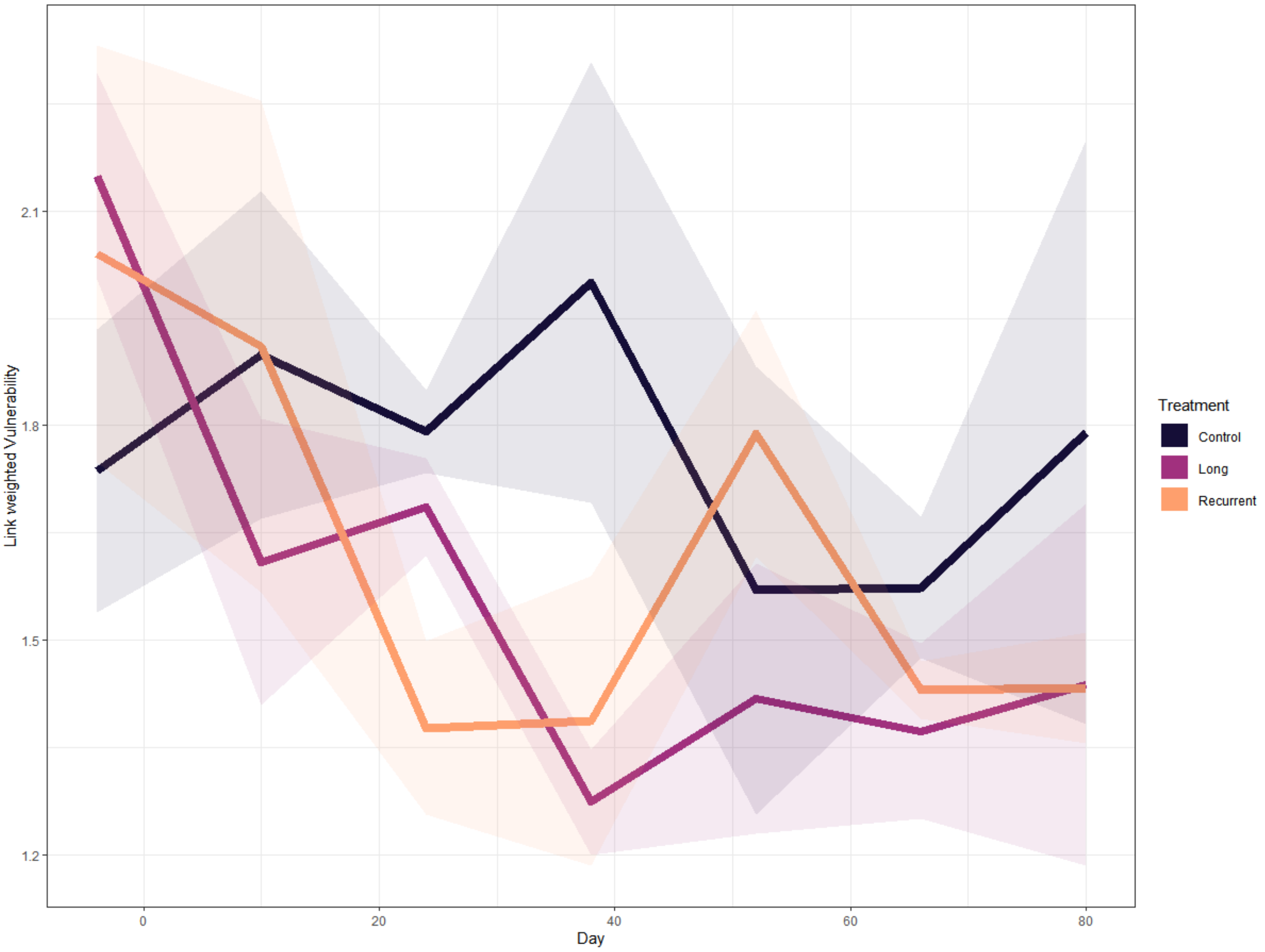


Fig. 6 Extended data | Link-weighted vulnerability dynamics over time.

Table 1| Extended data. Effects of the temperature treatments on community composition

		PERMANOVA		ANOSIM	
Phase		F	P	r	P
Pre	Reoccurring	0.65	0.78	0.21	0.11
	Long	0.87	0.83	0.13	0.17
HW3	Reoccurring	**3.18**	**0.002**	**0.53**	**0.008**
	Long	1.54	0.12	0.07	0.62
Recovery	Reoccurring	**1.76**	**0.035**	**0.48**	**0.02**
	Long	**1.42**	**0.043**	**0.35**	**0.04**

Two different permutation tests (PERMANOVA and ANOSIM) were conducted on Bray - Curtis distances

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