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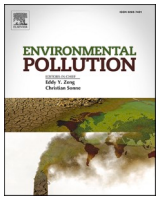

Widely used herbicide metolachlor can promote harmful bloom formation by stimulating cyanobacterial growth and driving detrimental effects on their chytrid parasites☆

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ABSTRACT

Metolachlor (MET) is a widely used herbicide that can adversely affect phytoplanktonic non-target organisms, such as cyanobacteria. Chytrids are zoosporic fungi ubiquitous in aquatic environments that parasitize cyanobacteria and can keep their proliferation in check. However, the influence of organic pollutants on the interaction between species, including parasitism, and the associated ecological processes remain poorly understood. Using the host-parasite system consisting of the toxigenic cyanobacterium *Planktothrix agardhii* and its chytrid parasite *Rhizophydium megarrhizum*, we investigated the effects of environmentally relevant concentrations of MET on host-parasite interactions under i) continuous exposure of chytrids and cyanobacteria, and ii) pre-exposure of chytrids. During a continuous exposure, the infection prevalence and intensity were not affected, but chytrid reproductive structures were smaller at the highest tested MET concentration. In the parasite's absence, MET promoted cyanobacteria growth possibly due to a hormesis effect. In the pre-exposure assay, MET caused multi- and transgenerational detrimental effects on parasite fitness. Chytrids pre-exposed to MET showed reduced infectivity, intensity, and prevalence of the infection, and their sporangia size was reduced. Thus, pre-exposure of the parasite to MET resulted in a delayed decline of the cyanobacterial cultures upon infection. After several parasite generations without MET exposure, the parasite recovered its initial fitness, indicating that detrimental effects are transient. This study demonstrates that widely used herbicides, such as MET, could favor cyanobacterial bloom formation both directly, by promoting cyanobacteria growth, and indirectly, by inhibiting their chytrid parasites, which are known to play a key role as top-down regulators of cyanobacteria. In addition, we evidence the relevance of addressing multi-organism systems, such as host-parasite interactions, in toxicity assays. This approach offers a more comprehensive understanding of the effects of pollutants on aquatic ecosystems.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), around 2.7 million tons of pesticides are globally used in agriculture, of which roughly 50 % are herbicides (FAO, 2022). Herbicides are applied to eliminate or inhibit the growth of unwanted plants both in agricultural and urban areas and can reach water bodies through agricultural or urban runoff, posing a risk to non-target organisms (Carpenter et al., 2016; reviewed in Pradhan et al., 2022). It has been estimated that only 1 % of the pesticides applied to crops reach their intended target organisms, while 99 % are dispersed to the environment (Pimentel, 1995).

Metolachlor (MET), a herbicide from the chloroacetanilide family, is one of the most widely used herbicides globally, with around 100,000 tons applied yearly (Maggi et al., 2019). The European Environmental Agency (EEA) reported MET as one of the pollutants most frequently exceeding the environmental quality standard (EQS) in surface water in the Member States, surpassing the national EQS of 0.1–1 μg L^{-1} (depending on the country) in over 100 water bodies (EEA, 2018). MET is stable to hydrolysis (Huntscha et al., 2008). The processes of

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photolysis and biodegradation may contribute to the loss of MET in surface water systems (Bech et al., 2022; Chang et al., 2020; Huntscha et al., 2008; Imfeld et al., 2018), where concentrations are often in the ng L^{-1} or low μg L^{-1} range (Correia et al., 2020; Glinski et al., 2018; Van Opstal et al., 2023). The United States Environmental Protection Agency (US EPA) and the European Food Safety Authority (EFSA) have estimated that surface water concentrations could reach between 80 and 400 μg L^{-1} after field application (EFSA et al., 2023); US EPA, 2019). In fact, concentrations between 50 and up to 400 μg L^{-1} have been detected (Battaglin et al., 2000; Székács et al., 2015; US EPA, 2019).

MET toxicity mechanism to their target organisms is associated with the inhibition of plant cell division and elongation and synthesis inhibition of other biomolecules, like fatty acids and proteins (Rose et al., 2016). MET can also negatively affect non-target aquatic organisms like fish (Rozmánková et al., 2020), crustaceans (Maazouzi et al., 2016), and phytoplankton (Fairchild et al., 1998). Toxic effects on phytoplankton include inhibition of growth and photosynthetic activity and oxidative stress (Juneau et al., 2001; Machado and Soares, 2021; Wang et al., 2017). Nevertheless, MET can also promote the growth of phytoplankton at certain concentrations (Thakkar et al., 2013; Wang et al., 2017). Effects on other less studied aquatic organisms, like fungi, remain unknown.

Phytoplanktonic organisms, like green algae and cyanobacteria, are key players in all aquatic environments. They account for 50 % of global primary productivity and thus are the basis of all trophic webs (Falkowski, 2012). Due to eutrophication (Khan et al., 2014) and other anthropogenic pressures (Ho et al., 2019), cyanobacteria increasingly dominate phytoplankton communities. Uncontrolled cyanobacterial growth in water bodies leads to the formation of cyanobacterial blooms that induce an anoxic state and affect water quality by changing its color, odor, and taste (Watson et al., 2015). Moreover, certain cyanobacterial species can produce metabolites known as cyanotoxins, which might be harmful to humans and animals (Corbel et al., 2014; Wood, 2016). Thus, cyanobacterial blooms can negatively impact the general state of the environment, the economy, and the health of living organisms.

Cyanobacterial growth in aquatic environments is regulated by a complex interplay of abiotic factors (e.g., nutrients concentration and temperature) (reviewed in Yang et al., 2008) and biotic interactions (reviewed in Gerphagnon et al., 2015). Among the latter, fungal parasitism has been increasing in importance, based on the molecular environmental surveys revealing an ubiquitous distribution of such parasites (Grossart et al., 2016). The most important phytoplankton parasites are chytrids, which are zoosporic fungi belonging to the phylum Chytridiomicota (Kagami et al., 2007). The life cycle of chytrids begins with motile flagellated zoospores, which swim to find suitable hosts. Zoospores encyst on the host and penetrate the surface with newly developed rhizoids to extract host nutrients. Encysted zoospores develop into reproductive structures called sporangia, which form new zoospores that are released upon maturation (Ibelings et al., 2004). Chytrids lethally parasitize different phytoplankton groups, including cyanobacteria. Recent studies have evidenced their pivotal role in the population dynamics, trophic interactions, and evolution of phytoplankton (Agha et al., 2016; Frenken et al., 2020a; Rasconi et al., 2014). For instance, chytrid parasites might produce massive deaths of their hosts, triggering changes in phytoplankton abundances, and the delay or suppression of cyanobacterial bloom formation (Gerphagnon et al., 2015; Gleason et al., 2015; Rasconi et al., 2012). Considering the ecological relevance of chytrid fungi and their hosts, like cyanobacteria, it is crucial to understand the impact of anthropogenic pollutants on infection dynamics.

In recent years, there has been a growing focus on investigating the effects of pollutants on host-parasite systems, particularly in crustaceans, fish, mammals, amphibians, and mollusks. These host organisms have been exposed to pollutants such as metals and pesticides, leading to varied outcomes, which include increases and decreases in parasitic infections (reviewed in Gilbert and Avenant-Oldewage, 2017; Sures et al., 2017). Furthermore, pollutants can produce multi and transgenerational effects (i.e., the detrimental effects lasting over several generations in the pollutant's absence). For instance, exposure to the fungicide tebuconazole drives multigenerational alterations in the parasite fitness in the Daphnia-Metschnikowia host-parasite system (Cuco et al., 2020). Combined effects of anthropogenic pollutants and chytrid infections are understudied, with limited research focused mainly on amphibian hosts (Buck et al., 2015; Gahl et al., 2011; Gaietto et al., 2014; Hanlon and Parris, 2014). In some of these studies, the pesticides ameliorate the effect of chytrid infection, likely due to detrimental effects on the parasite (Gaietto et al., 2014; Hanlon and Parris, 2014).

Ecotoxicological assessments of interactions between chytrid parasites and phytoplanktonic hosts are limited to only four studies. Across these investigations, consistent findings emerged regarding the impact of exposure to fungicides, diclofenac, the herbicide diuron, and nanoplastics, all of which resulted in decreased chytrid infection prevalence (Ortiz-Cañavate et al., 2019; Raman et al., 2023; Schampera et al., 2021; Van den Wyngaert et al., 2014). In addition, exposure to both nanoplastics and diclofenac hindered phytoplankton growth (Raman et al., 2023; Schampera et al., 2021). Neither of these studies evaluated the multi- or transgenerational consequences of pollutants on the associated parasites. Assessing the effect of widespread pollutants like MET on phytoplankton-chytrid interactions, particularly across multiple generations, would expand our understanding of the broader anthropogenic impact on phytoplankton and disease dynamics.

In the present study, we aim to evaluate the consequences of exposing a toxigenic phytoplankton-chytrid host-parasite system to the commonly used herbicide MET. We aimed to determine whether MET toxicity extends beyond the single organism level and alters species interactions, consequently impacting ecological and evolutionary processes. To achieve it, we quantified the effects of continuous exposure of cyanobacteria and chytrid parasites to MET on cyanobacterial growth and different parasite fitness proxies. Furthermore, we evaluated whether prior exposure of chytrids to MET produce potential multi- and transgenerational effects. Here, multi-generational effects refer to consequences observed in consecutive generations directly descended from exposed organisms, while transgenerational effects encompass impacts on non-exposed individuals descended from populations with an exposure history. Based on the existing research on the effect of other anthropogenic pollutants on phytoplankton-chytrid host-parasite systems, we hypothesized that i) continuous exposure of cyanobacteria and chytrid parasites to environmentally relevant MET concentrations hinders cyanobacterial growth, thereby indirectly exerting detrimental effects on parasites and negatively affecting the overall host-parasite dynamics, and ii) pre-exposure of chytrids to MET decreases the parasite fitness.

2. Materials and methods

2.1. Strains and culturing conditions

The host-parasite system used in the present study consists of the toxigenic filamentous cyanobacterium *Planktothrix agardhii* strain NIVA-CYA630 and its obligate chytrid parasite *Rhizophydium megarrhizum* strain Chy-Kol2008 (Sønstebø and Rohrlack, 2011), which produces lethal infections on its host. Cyanobacterial host cultures were maintained routinely in Z8 medium (Kotai, 1972) at 16 °C, under a continuous light intensity of 20 μmol photons m^{-2} s^{-1}. These conditions support the viability of the cyanobacterial cultures for over a month without collapsing. Chytrids were cultivated by transferring zoospores to uninfected cyanobacteria cultures every three weeks. The same temperature and light conditions as used for routine cultivation were used for the experiments, ensuring a comparable environment. *R. megarrhizum* generation time (i.e., the period between zoospore

inoculation to a healthy host and observation of empty sporangia) is approximately 1–1.5 days under these conditions (Agha et al., 2018).

2.2. Exposure experiments

We carried out two experiments to investigate the effect of MET exposure on the host-parasite dynamics, hereafter referred to as *continuous exposure of host-parasite* and pre-exposure of parasite experiments. In the *continuous exposure of host-parasite* experiment, we focused on the repercussions of MET-exposure on host growth and parasite fitness. In the pre-exposure of parasite experiment, we evaluated how previous exposure to MET affects parasite fitness over multiple generations.

For both experiments, chytrid zoospores were obtained by infecting an exponentially growing cyanobacterial culture 10 days before the start of the experiment. Afterward, cultures were filtered sequentially through a sterile 5 μm nylon mesh and 3 μm polycarbonate filter (Agha et al., 2018). Zoospore suspension was microscopically examined to ensure the absence of cyanobacteria filaments. Zoospores density was quantified with a Sedgewick Rafter chamber under an inverted microscope (Nikon Ti Eclypse).

Before each experiment, cyanobacterial cultures were maintained for 3 weeks as exponentially growing semi-continuous cultures. Thrice per week and at the starting day of the experiments, cultures were adjusted to an OD_{750nm} of 0.05. This optical density corresponds to approximately 10^4 filaments mL^{-1}. Based on the culture volume needed for each experiment, we incubated different numbers of "stock" cultures (final volume per culture = 160 mL): eight for the *continuous exposure of host-parasite* experiment and four for the *pre-exposure of parasite* experiment. Four out of the eight flasks of the *continuous exposure of host-parasite* experiment and all the flasks of the *pre-exposure of parasite* experiment were infected with the purified zoospore suspension to have a final concentration of 750 zoospores mL^{-1} per flask. Cultures were further incubated to let the infection establish. After six days, infected and uninfected cultures were separately pooled and distributed to the experimental units described in subsections 2.2.1 and 2.2.2. The experiments were conducted in Falcon® cell culture 50 mL bottles with a final volume of 30 mL.

2.2.1. Continuous exposure of host-parasite

We assessed the impact of continuous exposure of cyanobacteria and chytrids to MET on host-parasite dynamics. Infected and uninfected cultures were exposed to three environmentally relevant MET concentrations. The experiment had 40 experimental units including 2 conditions (infected and uninfected cyanobacteria) × 3 MET concentrations (1, 10, 100 μg L^{-1}) and 1 negative control (no herbicide) × 5 replicates (Fig. S1). Uninfected cultures were diluted to an OD_{750nm} of 0.05, corresponding to the exponential growth rate, before MET addition. All experimental units were incubated for seven days under the conditions described in section 2.1. Experimental units were sampled on days 1, 3, 5, and 7. On day 0, three samples were collected from the infected and uninfected culture pools before splitting them into the experimental units. On each sampling time, 1 mL of culture was fixed with acid Lugol and stored at 4 °C. All samples' identity were blinded and randomized before analysis.

2.2.2. Pre-exposure of parasite

Based on the results obtained in the *continuous exposure of host-parasite* experiment, we tested the effect of previous exposure of chytrids to MET on parasite fitness over multiple generations. For this assay, we tested exclusively the MET concentration that elicited the highest impact on both organisms, as concluded from the *continuous exposure of host-parasite* experiment.

The pre-exposure of parasite experiment consisted of two phases. In phase 1, infected cyanobacterial cultures were exposed to 100 μg L^{-1} MET (MET100). In phase 2, zoospores from every culture of phase 1 were collected and used to infect new cyanobacterial cultures without MET (Fig. S2).

Phase 1 consisted of 10 experimental units considering one MET concentration (100 μg L^{-1}) and one negative control without herbicide × 5 replicates per treatment. Cultures were incubated as described in section 2.1 for 14 days. We selected the exposure time based on the results of the *continuous exposure of host-parasite* experiment, where no effect of MET on parasite fitness throughout the assay was recorded, except on the last day (i.e., day 7). Therefore, we expected that long-term continuous exposure to MET could lead to detrimental effects on parasite fitness. After 14 days of incubation, 2 mL from each culture were collected and filtered through 3 μm sterile polycarbonate membranes. Zoospore suspension was microscopically examined to ensure the absence of cyanobacteria filaments. Zoospores density was quantified with a Sedgewick Rafter chamber under an inverted microscope (Nikon Ti Eclypse). Resulting zoospore solutions were used to infect *phase 2* cultures (final concentration of 750 zoospores mL^{-1}).

Phase 2 consisted of ten experimental units including two treatments: 5 cultures infected with zoospores previously exposed to 100 μg L^{-1} MET and 5 cultures infected with zoospores without previous exposure to MET (negative control). Experimental units were incubated as described in section 2.1. Samples from each bottle were collected on days 0, 1, and every two days until day 15. On each sampling time, 1 mL of culture was fixed with acid Lugol and stored at 4 °C. All samples' identity were blinded and randomized before analysis.

2.3. Recorded parameters

Cyanobacterial growth was quantified as the evolution of biovolume over time and was quantified under an inverted microscope (Nikon Ti Eclypse). Volume was estimated by measuring the filaments length in 10 fields of a Sedgewick-Rafter chamber per individual sample and applying the following formula:

$$V = \pi r^2 h$$

where r is the mean radius of cyanobacterial filaments (determined by the mean width of 100 random filaments divide it by 2) and h is the mean calculated filament length. Dead cyanobacterium filaments due to chytrid infection (i.e., empty, translucent filaments) were not included in biovolume calculations. In the *continuous exposure of host-parasite* experiment, biovolume quantification in the non-infected cyanobacterial cultures helped us to disentangle MET effects on chytrid infection from those on the cyanobacterial host. We considered cyanobacterial growth an indirect measure of the parasite performance in the pre-exposure of parasite experiment. Parasite performance involves its ability to establish in the host, exploit its resources, and reproduce (infectivity), as well as the degree of harm it produces on the host within a certain time. Given that in the pre-exposure of the parasite experiment, the host was not exposed to MET, we consider that a lower cyanobacterial growth is a sign of a better parasite performance and vice versa.

In addition, we measured three parasite fitness proxies to comprehensively evaluate the impact of exposure or pre-exposure of chytrid parasite *R. megarrhizum* to MET: 1) infection prevalence, 2) infection intensity, and 3) sporangia size. Infection prevalence represents the proportion of infected individuals within a host population and was calculated as the percentage of infected cyanobacteria (filaments with encysted zoospores or sporangia) after randomly examining 200 filaments. Infection prevalence allowed to evaluate parasite transmission in both experiments, considering transmission as the passage of the parasite from one host to another (i.e., the number of secondary infections caused by a primary infection). In addition, in the *continuous exposure of host-parasite* experiment, infection prevalence reflects the susceptibility of cyanobacteria to parasite infection under different MET concentrations (i.e., if the host was more or less prone to be infected).

Infection intensity refers to the number of parasites infecting a single host and was determined as the average infection number (i.e., encysted zoospores or sporangia) per single infected filament after examining 50 infected filaments per sample. When a single filament is infected by more than six parasites, obtaining a precise count is challenging due to the overlapping of the zoospores/sporangia. Therefore, for the analysis, infected filaments with six or more sporangia or encysted zoospores were categorized as having six infections.

The size of sporangia, which are chytrid reproductive structures, was used as a proxy of the per capita reproductive output. This approach was chosen because the sporangia size correlates with the number of zoospores (i.e., chytrid infective stage) it contains (Bruning, 1991; Gerphagnon et al., 2013; Van den Wyngaert et al., 2014). Average size was estimated from 10 fully developed sporangia per sample. Measurements were restricted to mature sporangia, identifiable by a well-developed thickened wall, and empty sporangia on hosts with single infections. The chitinaceous nature of sporangia ensured the preservation of size and structure in empty sporangia, making them eligible for quantification. Volume was calculated by measuring the two semi-axes of each sporangium, considering their rotational ellipsoid shape, and applying the following formula:

$$V = \pi/6 \times {d_1}^2 \times d_2$$

where d_1 and d_2 are the short and long semi-axes, respectively.

Cyanobacterial biovolume and infection prevalence was quantified in all samples from both experiments. Infection intensity was determined in all samples from the continuous exposure of host-parasite experiment and in samples from day five onwards from the pre-exposure of parasite experiment to ensure enough infected filaments in all samples. Sporangia size was quantified only on the last day of the continuous exposure of the host-parasite experiment and from day seven onwards in the pre-exposure of the parasite experiment to ensure the presence of enough fully mature sporangia in the samples.

2.4. MET concentration analysis

S-metolachlor (MET, CAS 87392-12-9, purity 99.1 %) was purchased from Santa Cruz Biotechnology, Inc. The stock solution was prepared in sterile Milli-Q water to a final concentration of 3 mg L^{-1} and was stored at 4 °C. We measured MET concentration at the beginning and end of the experiments to assess the MET concentration added in each experiment and verify its stability. For chemical analyses, 1 mL culture liquid was filtered using 0.22 μm regenerated cellulose syringe filters (Chromafil® Xtra RC-20/13, Macherey-Nagel) to remove bacteria and fungi and stored at 4 °C until analysis.

MET concentrations were determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 triple quadrupole mass spectrometer. Aqueous samples were analyzed using a XBridge BEH C18 column (5 cm × 2.1 mm, 3.5 μm, WATERS) equipped with a XBridge BEH C18 VanGuard cartridge (5 mm × 2.1 mm, 3.5 μm, WATERS). The eluent mixture consisted of LC-MS grade H_2O (solvent A, LiChrosolv, Sigma) and LC-MS grade methanol (solvent B, ≥ 99.95 %, Rotisolv, Roth), each acidified with 0.1 % formic acid (Fluka). The eluent gradient increased linearly from 10 % B to 95 % B in 15 min and was kept constant at 95 % B for 1 min at a flow rate of 200 μL min^{-1}. Electrospray ionization (ESI) was used in positive mode and MET was measured in multiple reaction monitoring (MRM) with a precursor mass $[M+H]^+$ of 284.1 *m/z* and two product masses $[M+H]^+$ of 252.1 *m/z* (quantifier) and 176.2 *m/z* (qualifier). MET quantification was performed using external calibration standards from 0.5 to 100 μg L^{-1} and the Agilent MassHunter Workstation software. The lowest calibration standard with a signal to noise ratio ≥10 was defined as limit of quantification.

2.5. Data analyses

We used linear mixed models to test for effects on the cyanobacteria growth and parasite fitness proxies. This approach allowed us to evaluate the effect of exposure or pre-exposure to MET on the quantified parameters at specific time points, accounting for multiple sampling per experimental unit (samples taken from the same experimental unit at different time points) and repeated measurements (multiple observations) per sample, where applicable. In the models used for the pre-exposure of parasite experiment, "MET concentrations" refer to the MET concentrations to which parasites were exposed in phase 1. All the models used in the present study are summarized in Table S1 and are described below.

We considered a two-way interaction between MET concentrations and time for the biovolume and infection intensity in both experiments and the infection prevalence and sporangia size in the *pre-exposure of parasite* experiment. The linear mixed model for infection prevalence in the *continuous exposure of host-parasite* experiment had a singular fit. Therefore, individual linear models were run to understand the effect of MET at every sampling point.

Additionally, the conditional R^2 (proportion of total variance explained by fixed and random effects) and marginal R^2 (proportion of total variance explained only by the fixed effects) was estimated for each mixed model. The distribution of the residuals was examined to determine if they met the assumption of normality with homogeneous variance. Data was log-transformed in the models where this assumption was not met to normalize the residuals.

For all models in which an interaction between MET concentration and time was included, significance of the interaction was tested using the log-likelihood ratio (LLR) of a model that contained the interaction to a model that did not. Where interactions between MET concentration and time were significant, the significance of the independent fixed factors were not tested, as this would require removing the significant two-way interaction from the model. Non-significant terms from the models were not removed as we were interested a priori in all effects.

Additionally, we used the plots of all parameters measured over time in both experiments to calculate the area under the curve (AUC) per experimental unit. The AUC approach enables us to obtain a single value that integrates the information from the entire incubation time instead of focusing on specific time points. Linear models were ran considering the AUC of each parameter as the response variable and MET concentration as the explanatory variable. The distribution of the residuals was examined to determine if they met the assumption of normality with homogeneous variance. In addition, the homoscedasticity of the residuals was evaluated with the Breusch Pagan test. In cases where the assumption of normality with homogeneous variance was not met, non-parametric linear models were performed.

2.6. Software for data processing, analysis, and visualization

We measured cyanobacterial biovolume and sporangia size with NIS-Elements BR 4.5 software. Statistical analyses and figures were performed using the R packages described below in R Statistical language (version 4.2.1; R Core Team, 2022). Linear mixed models were run using lme4 (Bates et al., 2015), *posthoc* tests were performed with emmeans (Lenth, 2022), and data visualization for normality and homogeneous variance was done with ggResidpanel (Goode and Rey, 2019). Conditional and marginal R^2 for each mixed model was estimated according to Nakagawa and Schielzeth (2013) with MuMIn (Bartoń, 2022). Breusch Pagan test was performed using lmtest (Zeileis and Hothorn, 2002). Figures were made with ggplot (Wickham, 2016), ggpubr (Kassambara, 2020) and cowplot (Wilke, 2020). Additionally, different packages from the *tidyverse* (Wickham et al., 2019) were used to import, export, tidying, and arrange data. R code used to generate the results is provided as Supplementary material. The graphical abstract was created using Inkscape free software (Inkscape Project, 2020).

3. Results

3.1. MET concentration analysis

We measured MET concentration at the beginning and end of the experiments to evaluate nominal vs real herbicide concentration in the cultures and its stability. Initial quantified MET concentrations were 0.9, 10, and 107.3 μg L^{-1}, thus very close to the targeted nominal concentrations of 1 (MET1), 10 (MET10), and 100 μg L^{-1} (MET100), respectively. MET was stable throughout the experiments with a maximum decline of 11 % at 10 μg L^{-1} MET, showing that MET did not undergo significant abiotic or biotic transformation at the tested conditions (Table S2). For simplicity, nominal concentrations are shown and discussed.

3.2. Continuous exposure of host-parasite

Cyanobacteria biovolume in uninfected cultures increased over time, reaching significantly higher levels when exposed to MET100 (from day 5 on) and MET10 (only at the end of the experiment, Fig. 1), in comparison to the non-exposed control. Fixed effects explained 81 % of the variance in our model (Table S3). Overall, cyanobacterial growth in uninfected cultures was higher when exposed to MET100 with respect to controls (AUC: $df = 27$, $t = -3.247$, $p = 0.024$; Fig. 1). In contrast, cyanobacteria growth in all infected cultures increased until day 5 and decreased afterward due to the chytrid infection. No difference was observed between the exposed cultures to MET and controls, indicating that parasitic infection overrode the growth-promoting effect of MET (Fig. 1).

Cyanobacteria were equally susceptible to chytrid infection, disregarding the presence of MET, evidenced by the similar prevalence and intensity of infection across all tested MET concentrations (Fig. 2A, B, C, D). On the other hand, sporangia on day 7 were significantly smaller at MET100 relative to control and MET1 (MET0 – MET100: $df = 16$, $t = 2.97$, $p = 0.041$; MET1 – MET100: $df = 16$, $t = 3.11$, $p = 0.031$). The reduction in sporangia size suggests a decline in the parasite's per capita reproductive output (Fig. 2). Exposure to MET explained 11.2 % of the total variance in our model (conditional R^2 - marginal R^2).

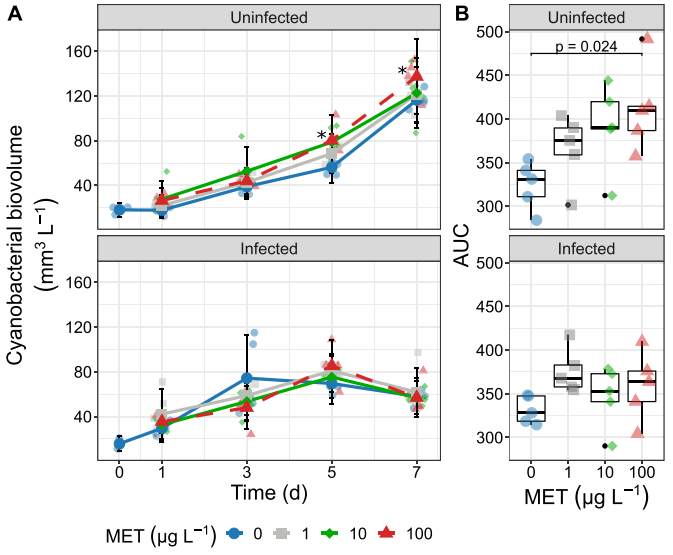

Fig. 1. Cyanobacterial growth in cultures exposed to MET for seven days. A) Cyanobacteria growth curves in infected (upper panel) and uninfected (lower panel) cultures. Cyanobacteria growth was controlled by the parasitic infection disregarding the MET presence but was promoted by MET100 in the absence of the parasite. * Indicates significant differences between setups. On day 5 MET0 – MET10: p = 0.0053 and MET0 – MET100 p = 0.0026. On day 7, MET0 – MET100: p = 0.0109. Mean values ± s.d. (n = 5). B) Area under the curve (AUC) calculated from the cyanobacterial biovolume in infected (upper panel) and uninfected cultures (lower panel). Overall growth in uninfected cultures was higher when exposed to MET100, while overall cyanobacterial growth in infected cultures was similar among all setups in infected cultures. Box indicates the upper and lower quartiles, the dark middle line indicates the median, the whiskers indicate 1.5 times the interquartile range, and black points represent any values outside this range (n = 5).

3.3. Pre-exposure of parasite

Cyanobacterial growth was significantly lower in controls from day 9 until the end of the experiment ($df = 12.6$, day 9: $t = -3.82$, $p = 0.0022$; day 11: $t = -9.05$, $p < 0.0001$; day 13: $t = -8.18$, $p < 0.0001$; day 15: $t = -2.82$, $p = 0.0147$. Fig. 3A). Fixed effects explained 76 % of the total variance in our model (Table S4). The highest cyanobacterial biovolume was quantified on day 9 in control cultures and on day 11 in cultures infected with zoospores pre-exposed to MET100. Afterward, cyanobacterial growth constantly decreased. Overall, cyanobacterial growth was higher in cultures infected with zoospores without prior exposure to MET compared to controls (AUC: $df = 8$, R^2 adjusted = 0.73, $p = 0.001$, (Fig. S3A).

Infection prevalence was higher in control cultures than in cultures infected with zoospores previously exposed to MET100 on days 5, 7, and 9 ($df = 42.2$, day 5: $t = 4.93$, $p < 0.0001$, day 7: $t = 7.1$, $p < 0.0001$, day 9: $t = 3.5$, $p = 0.0011$; Fig. 3B). Fixed effects explained 98 % of the total variance in our model (Table S4). Infection prevalence was overall significantly higher in controls than in cultures infected with zoospores exposed to MET100 (AUC: $df = 8$, R^2 adjusted = 0.54, $p = 0.009$; Fig. S3B).

We observed a higher infection intensity between days 5–11 in control cultures than in cultures infected with zoospores pre-exposed to MET100 ($df = 48$, day 5: $t = 2.52$, $p = 0.0153$; day 7: $t = 2.42$, $p = 0.0192$, day 9: $t = 3.45$, $p = 0.0012$, day 11: $t = 3.56$, $p = 0.0016$; Fig. 3C). Fixed effects explained 92.9 % of the total variance in our model (Table S4). Overall, infection intensity was significantly higher in cultures infected with zoospores without prior exposure to MET (AUC: $df = 8$, R^2 adjusted = 0.75, $p = 0.0007$; Fig. S3C).

Overall, sporangia size was comparable between controls and the cultures infected with zoospores exposed to MET100 as evidenced by the AUC (Fig. S3D). However, comparing the sporangia in the control and the MET100 from each sampling day, the sporangia were significantly smaller in the controls than in the cultures infected with zoospores isolated from MET100 on day 7 ($df = 64.9$, t = 3.6, $p = 0.0006$; Fig. 3D). Fixed effects explained 9 % of the total variance in our model (Table S4).

Given the generation time of *R. megarrhizum* (approximately 1–1.5 days), the reduction in parasite fitness was observed at sampling points where infections were produced by parasites not exposed to MET (i.e., caused by zoospores generated in MET-free cultures during phase 2, as opposed to zoospores obtained from cultures exposed to MET in phase 1). At the end of the pre-exposure of parasite *experiment,* parasite fitness was comparable between the setups infected with zoospores with and without pre-exposure to MET (Fig. 3).

4. Discussion

Our results demonstrate that exposure to MET promotes cyanobacterial growth in the absence of the parasite. Additionally, in the short-term continuous exposure (7 days) of both host and parasite, MET does not influence infection prevalence and intensity. In this context, infection-induced mortality overrides the promotion of cyanobacteria growth caused by MET. Moreover, the outcomes of this study indicate that long-term pre-exposure of chytrids to MET (14 days) exerted multi- and transgenerational negative changes, highlighting the potential for long-lasting repercussions of MET on the chytrid-parasite system. Nonetheless, parasites could recover from the detrimental effects caused by MET, indicating that the changes are temporary, reversible, and

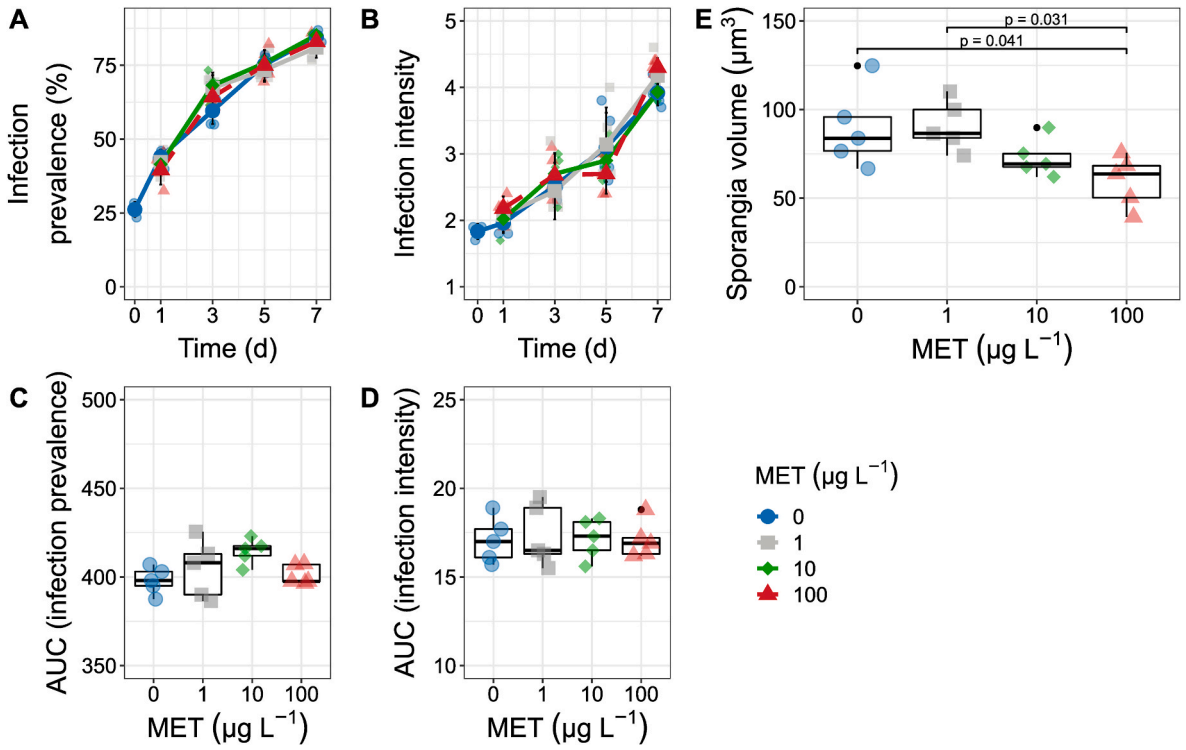

Fig. 2. Parasite fitness proxies in cultures exposed to MET for seven days. A) Infection prevalence (percentage of infected cyanobacterial filaments), B) infection intensity (mean infection number per infected filament), C) area under the curve (AUC) calculated from the infection prevalence, D) AUC calculated from the infection intensity, and E) sporangia (parasite's reproductive structures) size in cultures exposed to MET for seven days. Sporangia were measured on samples from day 7 of the *continuous exposure of host-parasite to* MET experiment, presented in Fig. 1 and in panels A and B of this figure. Infection prevalence and intensity were not significantly different among all setups. Sporangia were smaller in cultures exposed to MET100. Box indicates the upper and lower quartiles, the dark middle line indicates the median, the whiskers indicate 1.5 times the interquartile range and black points represent any values outside this range (n = 5).

likely phenotypic rather than permanent and genotypic. The outcome of our study provides evidence of how pollution by herbicides, such as MET, can negatively impact non-target organisms, like chytrid fungi, and their interactions in aquatic environments. Consequently, the present study helps to better understand the implications of water pollution by compounds with a similar mode of action as MET on various ecological and evolutionary processes, given the relevance of host-parasite interactions, such as cyanobacteria-chytrids.

At environmentally relevant concentrations, MET promotes cyanobacterial growth in uninfected cultures. Similarly, other pesticides have been also shown to boost cyanobacterial growth (Jyothi, 2016; Lin et al., 2023; Sun et al., 2013). The promotion of cyanobacterial growth by pesticides could be attributed to the direct use of organic pollutants as phosphorus or nitrogen source (Drzyzga and Lipok, 2018; Lin et al., 2023), the suppression of eukaryotic phytoplankton, indirectly favoring cyanobacteria growth (Hernández-García and Martínez-Jerónimo, 2020; Lu et al., 2019), and hormesis (Zhang et al., 2020). In the present study, MET assimilation by P. agardhii seems unlikely, given stable MET concentrations. Suppression of other phytoplankton species is ruled out due to *P. agardhii* being a monospecific culture. Therefore, hormesis is the most likely phenomenon promoting cyanobacterial growth.

Hormesis refers to the stimulatory effect at low stressor doses and the inhibitory effect at high doses (Duke et al., 2006). Cyanobacteria growth in the uninfected cultures was stimulated when exposed to MET throughout the *continuous exposure of host-parasite* experiment. Likewise, MET promoted the growth of the brown tide algae Aureococcus anophagefferens (Thakkar et al., 2013) and the cyanobacterium *Microcystis aeruginosa* (Wang et al., 2017). No inhibitory effects were observed with the tested MET concentrations, suggesting they were below the threshold to inhibit *P. agardhii* growth (Fairchild et al., 1998; Machado and Soares, 2019; Peterson et al., 1994; Thakkar et al., 2013; Wang et al., 2017). Our study does not address whether long-term exposure of the host to MET undoes the stimulatory effect on *P. agardhii* or enhances it. Therefore, further research is needed to explore the continuous long-term MET effects on the host. Based on our results, we suggest that pollutants promoting the growth of toxigenic bloom-forming cyanobacteria at environmentally relevant concentrations, such as MET, have the potential to trigger the formation of cyanobacterial blooms and increase the risk associated with them.

MET did not impact the infection prevalence and intensity during a continuous short-term exposure (7 days), enabling parasites to induce typical host mortality. Eventually, this outcome overrode the cyanobacteria growth stimulation triggered by MET. This stands in contrast to the results observed with other anthropogenic pollutants, such as the herbicide diuron (Van den Wyngaert et al., 2014), various fungicides (Ortiz-Cañavate et al., 2019), and polystyrene nanoplastics (Schampera et al., 2021), which showed diminished infection intensity and/or prevalence in phytoplankton-chytrid host-parasite systems, likely attributed to different types and mode of actions.

Continuous exposure of chytrid parasites to MET for seven days decreased sporangia size (i.e., chytrid reproductive structures). Given the correlation between sporangial size and zoospores number (Bruning, 1991; Gerphagnon et al., 2013; Van den Wyngaert et al., 2014), the sporangia size serves as a valuable proxy for determining per capita reproductive output. The presence of smaller sporangia indicates a decrease in the number of infective units available to infect new hosts, potentially hampering parasite transmission. Nevertheless, the implications of smaller sporangia on the disease outcome within the time of the *continuous exposure of host-parasite* experiment were not evidenced, and the epidemic size was comparable, disregarding MET presence. A lower zoospores number might lead to a decrease in infection metrics. However, in high-host density environments, the ease of finding new

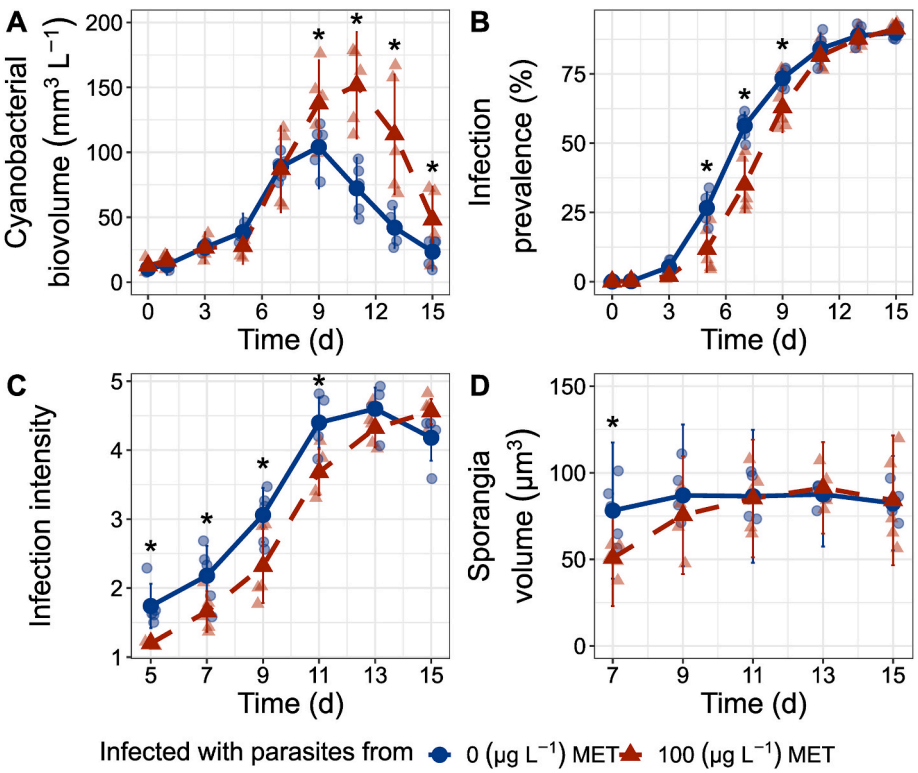

Fig. 3. Cyanobacteria growth and parasite fitness proxies in cultures infected with zoospores previously exposed to MET100. A) Cyanobacterial growth curve as an indirect measurement of parasite infectivity showing that prior exposure to MET100 for 14 days reduced parasite infectivity and thus delayed the cyanobacterial culture decline. B) Infection prevalence (percentage of infected cyanobacterial filaments), C) infection intensity (mean infection number per infected filament), and D) sporangia size. All parasite fitness proxies were lower in the cultures infected with parasites previously exposed to MET over multiple generations. Mean values ± s.d. (n = 5). * Indicates significant differences between treatments.

filaments to infect might favor the few zoospores, potentially maintaining infection levels. Consequently, MET-exposed cultures could display infection metrics comparable to unexposed cultures, sustaining a similar epidemic size.

Our findings suggest that different pollutants, even those belonging to the same category as herbicides, may not affect host-parasite interactions similarly. This highlights the importance of assessing the toxicity of pollutants with different modes of action to gain a more comprehensive idea of how pollution might affect aquatic ecosystems. In addition, considering the constant release of complex pollutant mixtures to the environment, further studies should be carried out to assess the toxicity of chemical mixtures on multi-organism systems, thereby better reflecting natural conditions. The general results of the *continuous exposure of host-parasite* experiment do not support our initial hypothesis that continuous exposure of chytrid parasites and cyanobacteria to MET hinders cyanobacterial growth, negatively impacting host-parasite dynamics.

Based on the results obtained in the *continuous exposure of host-parasite* experiment, we evaluated whether the pre-exposure of chytrids to MET would impact parasite fitness over multiple generations. We found that prior exposure to MET exerted multi- and transgenerational effects on chytrid parasites. This impact reduced the fitness of both directly exposed parasites and subsequent generations not in direct contact with MET, thereby delaying cyanobacteria culture decline. The multi- and transgenerational impact of MET on parasite fitness was evidenced by a sustained reduction of parasite performance, infection prevalence, and infection intensity, extending for more than three parasite generations (i.e., from day five onwards). In addition, the observation of smaller sporangia on day seven implies a potential decrease in the parasite per capita reproductive output during the initial phase of the experiment, indicating a likely reduction in the zoospores number released into the medium. Hence, pre-exposure to MET might affect the quantity and quality of chytrid zoospores over multiple generations. Likewise, exposure to other pollutants has been demonstrated to drive multigenerational alterations in parasite performance in the *Daphnia-Metschnikowia* host-parasite system (Civitello et al., 2012; Cuco et al., 2020). Similar trans- and multigenerational effects have been documented in various organisms exposed to pollutants (Blanc et al., 2020; DeCourten et al., 2020; Groot et al., 2016; Im et al., 2023; Rebuzzini et al., 2022).

The detrimental effects of MET on chytrids could be related to its mode of action. MET inhibits the synthesis of key biomolecules, mainly fatty acids (Rose et al., 2016) and also interferes with fatty acid oxidation (Counihan et al., 2017). Chytrids, known to uptake lipids from hosts and synthesize them de novo (Gerphagnon et al., 2019), rely on lipids for essential physiological processes, including cell membrane formation and energy storage (Pan et al., 2018). For instance, it has been suggested that lipid storage in chytrid zoospores supplies energy for swimming (Gerphagnon et al., 2019). Therefore, MET could detrimentally affect chytrid lipid metabolism, altering crucial physiological processes, including pathogenic traits related to host colonization and nutrient uptake (Ellison et al., 2017; van de Vossenberg et al., 2019). Although the exact toxicity mechanism remains unknown, MET's detrimental effects on chytrids suggest potential disruptions in natural epidemic dynamics caused by parasitic chytrid fungi, posing unfavorable ecological implications. All in all, the outcomes of the *pre-exposure of parasite* experiment support our second hypothesis that MET has a negative impact on parasite fitness.

By the end of the *pre-exposure of parasite* experiment, parasite performance was similar between the pre-exposed to MET and control treatments, suggesting that the sustained development in the absence of the stressor leads to a recovery of the parasite fitness. Hence, alterations produced by MET in parasites within the exposure time evaluated in the present study are temporary and phenotypic.

We evaluated the MET effect on the chytrid-cyanobacteria system at 16 °C, the temperature at which it has been cultivated since 2013 under our laboratory conditions. This temperature aligns with the typical temperature range in the photic zone during cyanobacteria bloom season in lakes similar to the ones where both organisms originated (Rohrlack et al., 2015). Consequently, we anticipate a comparable response from both organisms to the MET presence in natural systems. Nevertheless, sustained exposure to MET at elevated temperatures (22 °C), as estimated in the context of global warming, might intensify the reduction in parasite fitness. This is especially relevant since there is currently no evidence of adaptation of the studied chytrid to high temperatures (Schampera et al., 2022).

5. Conclusions

Altogether, the outcome of our study suggests that anthropogenic pollution of freshwaters with herbicides exerting a similar mode of action as MET can lead to the decrease of parasitic infectious diseases, thereby disrupting various ecological and evolutionary processes. First, MET pollution could contribute to the formation of harmful cyanobacteria blooms by directly stimulating toxigenic cyanobacteria growth and indirectly by affecting chytrid parasites. Chytrids are a well-known top-down control of cyanobacteria and regulator of phytoplankton community composition (Gerphagnon et al., 2015; Gleason et al., 2015; Rasconi et al., 2012). Second, the detrimental consequences exerted by MET on parasitic chytrid fungi might impact trophic interactions in aquatic ecosystems. This consideration arises from chytrids' role in establishing trophic links between primary producers and grazing organisms (Agha et al., 2016; Frenken et al., 2020b; Rasconi et al., 2014). Lastly, the harmful effects of MET on chytrids might disrupt their role as selective pressures in phytoplankton host populations (Agha et al., 2018; Gsell et al., 2013) and thus interfere with pivotal evolutionary processes. Given the crucial role that parasitic chytrid infections play in various ecological and evolutionary processes associated with phytoplankton, including cyanobacteria, it is essential to consistently evaluate the influence of aquatic pollution on host-parasite dynamics to fully understand its implications on an ecosystem level. This study offers new insights into the complex effects of anthropogenic pollution on aquatic multi-species systems, highlighting the far-reaching impacts beyond individual organisms and generations.

CRediT authorship contribution statement

Erika Berenice Martínez-Ruiz: Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Ramsy Agha:** Conceptualization, Methodology, Writing – review & editing. **Stephanie Spahr:** Methodology, Resources, Writing – review & editing. **Justyna Wolinska:** Conceptualization, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have included all my data and code to the supplementary material

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123437.

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