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Fungicides at environmentally relevant concentrations can promote the proliferation of toxic bloom-forming cyanobacteria by inhibiting natural fungal parasite epidemics

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Abstract

Fungal parasites of the phylum Chytridiomycota (chytrids) are increasingly recognized as potent control agents of phytoplankton, including toxic bloom-forming cyanobacteria. We experimentally tested whether agricultural fungicides can interfere with natural epidemics caused by parasitic chytrid fungi and thereby favor cyanobacterial bloom formation. Specifically, we exposed the toxic bloom-forming cyanobacterium *Planktothrix* and its chytrid parasite *Rhizophydium megarrhizum* to different concentrations of the widely used agricultural fungicides tebuconazole and azoxystrobin, as well as the medical fungicide itraconazole (the latter was included to test its potential to suppress infection *in vitro*). Environmentally relevant concentrations of tebuconazole (20 - 200 µg/L) and azoxystrobin (1 - 30 µg/L) significantly decreased infection prevalence over a timespan of seven days, while not affecting the growth of uninfected cyanobacteria. Itraconazole suppressed infection completely. Our findings demonstrate that agricultural fungicide run-off has the potential to inhibit natural chytrid epidemics, and thereby to promote the proliferation of toxic cyanobacteria.

Keywords: azoxystrobin, chytrid, cyanobacterial blooms, fungicides, host-parasite, itraconazole, *Planktothrix*, *Rhizophidium*, tebuconazole

1. Introduction

Cyanobacterial blooms raise serious public health concerns, as many cyanobacterial taxa produce diverse toxic metabolites with hepatotoxic, cytotoxic, neurotoxic or tumor promoting effects (Falconer, 2005, Araoz *et al.*, 2010) that can accumulate along the food chain (Ibelings & Chorus, 2007). The management of cyanobacterial blooms has hence become an important priority for environmental agencies, water authorities and health organizations (Huisman *et al.*, 2018)). Extensive research has demonstrated that the intensity, frequency and toxicity of cyanobacterial blooms has increased over recent decades, chiefly due to over–supply of phosphorous and nitrogen as a result of anthropogenic eutrophication, together with global warming (Wagner & Adrian, 2009, Paerl & Otten, 2013). Yet, relatively sharper increases in cyanobacteria biomass have also been reported in low-nutrient alpine water bodies as opposed to nutrient-rich low-land systems (Taranu *et al.*, 2015), suggesting the existence of other under-recognized factors contributing to the proliferation of harmful cyanobacteria.

Cyanobacteria are naturally targeted by a number of biological antagonists. In addition to the extensively studied effects of zooplankton grazing (Ger *et al.*, 2016) or viral infections (Suttle, 1994), cyanobacteria are lethally parasitized by chytrids, a group of primitive aquatic fungi (phylum *Chytridiomycota*; Frenken *et al*. 2017). An increasing number of environmental molecular surveys have repeatedly reported a so-far disregarded diversity and widespread distribution of chytrids in aquatic ecosystems worldwide (e.g. Lefèvre *et al.*, 2008, Hassett & Gradinger, 2016, Ortiz-Álvarez *et al.*, 2018). Chytrid infection of phytoplankton is now considered an omnipresent phenomenon, which often reaches epidemic proportions (Frenken *et al.*, 2017). As lethal parasites, chytrids control the abundance of their phytoplankton hosts and delay or even suppress bloom formation (Rasconi *et al.*, 2012, Gerphagnon *et al.*, 2015). In addition to direct effects on the timing and intensity of blooms, chytrid parasites seem to play more profound roles in the functioning of aquatic ecosystems, for instance by establishing alternative trophic pathways between primary and secondary production in aquatic food webs (Kagami *et al.*, 2014, Agha *et al.*, 2016) and promoting genetic diversity in phytoplankton populations (Gsell *et al.*, 2013, Agha *et al.*, 2018).

The use of fungicides has more than doubled since the 1950s. An estimated 300,000 tons of fungicides are used annually in agriculture worldwide to fight fungal pests and maximize food production (De *et al.*, 2014). Maximum residual levels are usually set for these compounds to ensure consumer safety and plant protection. However, events such as overspray and drift lead to leaking of fungicides into nearby surface waters, potentially affecting non-target organisms, sometimes with unexpected outcomes (e.g. Rohr et al., 2017). Under controlled conditions, we tested the hypothesis that agricultural fungicides at environmentally relevant concentrations can indirectly promote harmful cyanobacterial blooms by inhibiting infection by their natural fungal antagonists.

2. Materials and Methods

2.1 Experimental setup

A laboratory experiment was conducted to analyze the effects of environmentally relevant concentrations of the agricultural fungicides tebuconazole (CAS nr. 107534-96-3), and azoxystrobin (CAS nr. 131860-33-8) on the spread of the chytrid parasite *Rhizophydium megarrhizum* (strain Chy-Kol2008; Sønstebø & Rohrlack, 2011) in populations of the toxic bloom-forming cyanobacterium *Planktothrix rubescens* (strain NIVA-CYA 98), as well as on the growth of uninfected populations. Additionally, the effectiveness of the medical fungicide itraconazole (CAS nr. 84625-61-6) against chytrid infection *in vitro* was evaluated.

The experimental setup included 78 experimental units: 2 treatments (infected & non-infected cyanobacteria) × 10 types of fungicide concentrations (4 tebuconazole, 4 azoxystrobin and 2 itraconazole), 2 positive controls (ethanol without fungicide: a) ethanol concentration of tebuconazole and azoxystrobin treatments and b) ethanol concentration of itraconazole treatment), 1 negative control (no fungicide and no ethanol) × 3 replicates. Non-infected (only cyanobacteria) treatments were included in order to disentangle the effects of fungicides on chytrid infection from those on cyanobacterial hosts. Four concentrations of tebuconazole and azoxystrobin were tested (20, 200, 2000, 20000 µg/L and 1, 30, 300, 3000 µg/L, respectively). The two lowest concentrations represent an environmentally relevant range of concentrations, whereas higher concentrations were set to test for the ability of fungicides to completely suppress infection. For itraconazole, two concentrations were tested (100 and 1000 µg/L) that were proven successful in eradicating an amphibian chytrid parasite infection *in vitro* (Garner *et al.*, 2009). Ethanol was used as solvent for the fungicide solutions, reaching a final concentration of 0.4 mL/L in tebuconazole and azoxystrobin treatments. Ethanol final concentration in the itraconazole treatment was 4.8 mL/L, due to its lower solubility. Positive controls (ethanol without fungicide) were included in order to disentangle detrimental effects of ethanol. Before starting the experiment, eight replicate cyanobacterial cultures were acclimated under 20 ^{o}C and 20 µmol photons m^{-2} s^{-1} for two weeks as exponentially-growing semi-continuous cultures. A chytrid zoospore suspension was obtained after Agha *et al*. (2018) and used to infect four out of the eight cyanobacterial cultures (final zoospore concentration 930 mL^{-1}). After a 4-day incubation, the pooled infected and uninfected cultures were each redistributed as 30 mL aliquots into tissue culture flasks (72 flasks, see above) and the respective fungicide (or control) treatment was applied. Experimental units were kept for 7 days under the above temperature and light conditions.

2.2 Recorded parameters and data analysis

Samples of uninfected and infected cultures (1 mL) were collected at day 1, 3, 5, and 7. Optical density at 750nm (OD_{750}) in the non-infected treatments was measured and used as a proxy of cyanobacterial biomass. Samples of the infected treatments were fixed with 2% formaldehyde and prevalence of infection was determined microscopically as the percentage of infected cyanobacterial filaments, after inspecting 200 filaments for the presence of attached chytrid sporangia. For each fungicide concentration, plots showing the change in optical density or prevalence of infection over time were used to compute their respective area under the curve (AUC) (Purves, 1992). This approach allowed collapsing the response variables over time (i.e. optical density and prevalence of infection) down to a single value that integrates information from the entire incubation period. To evaluate the effects of fungicide concentrations on the growth of uninfected hosts and prevalence of chytrid infection, a one-way ANOVA was performed for each fungicide, testing for fixed effects of fungicide concentration on the respective AUC. Tukey HSD post hoc tests were used to identify significant differences between individual fungicide concentrations. To test for effects of ethanol addition, AUCs were compared between negative and positive controls (t-test).

3. Results and discussion

The experiment confirmed the hypothesis that agricultural fungicides have the potential to promote the growth of cyanobacteria by inhibiting infection by their fungal parasites. Environmentally relevant concentrations of tebuconazole (20 - 200 µg/L) and azoxystrobin (1 - 30 µg/L) significantly reduced infection by chytrids (compared to controls without fungicides) without hampering cyanobacterial growth (Fig. 1a, b). These results strongly suggest that, under natural conditions, the sustained presence of fungicides in waters could suppress initial chytrid infections, ultimately preventing the development of epidemics. This conclusion is particularly supported by the well-established level of infection at the onset of the experiment (35% of the population was infected when fungicides were added), and by the observed reduction of infection spread even at low fungicide concentrations. The highest concentrations of tebuconazole, azoxystrobin, and itraconazole completely suppressed infection but still did not have any significant effect on cyanobacterial growth (except for 20000 µg/L tebuconazole; Fig. 1). The use of positive and negative controls with and without ethanol allowed us to unequivocally disentangle the inhibitory effect of the different fungicides from those of ethanol (used as solvent for fungicides), which slightly inhibited chytrid inhibition at low concentrations (0.4 mL/L; Fig. 1a,b), and completely at higher concentrations (and 4.8 mL/L; Fig. 1c). Toxic effects of tebuconazole and itraconazole on chytrids are attributable to the disruption of sterol biosynthesis, specifically the inhibition of sterol 14α-demethylase, which is responsible for the conversion of lanosterol into other sterols, including cholesterol (Risley, 2002). In turn, azoxystrobin acts as an inhibitor of mitochondrial respiration (Tomlin, 2009). Since cyanobacteria are prokaryotic organisms and do not produce sterols, the absence of effects on cyanobacteria is not surprising.

These findings indicate that widely used agricultural fungicides might promote harmful algal blooms by inhibiting cyanobacterial natural antagonists. This exemplifies how environmental stressors other than nutrient enrichment or increased temperatures, e.g. pollution by persistent organic pollutants, can also impact the frequency and/or intensity of harmful algal blooms in unexpected ways.

These results call for further field studies addressing the impact of widely used fungicides on the proliferation of toxic cyanobacteria under natural conditions. We argue that the consequences of fungicide run-off may go beyond alleviating natural top-down control of cyanobacteria, and extend to disrupting chytrid-mediated transfer of energy and matter in aquatic ecosystems. Due to their inedibility, toxicity and low nutritional value, cyanobacterial blooms cause trophic bottlenecks, where carbon flow between phytoplankton primary producers and zooplankton consumers is disrupted (Frenken *et al.*, 2017). When infecting phytoplankton, chytrids repack and upgrade autochthonous carbon fixed by primary producers and convey it to zooplankton consumers in the form of easily edible and highly nutritious zoospores, thereby establishing an alternative trophic link between primary and secondary production and alleviating such bottlenecks (Agha *et al.*, 2016, Gerphagnon *et al.*, 2018). In addition, chytrid fungi with saprophytic lifestyles are abundant in aquatic ecosystems, where they act as important degraders of otherwise persistent allochthonous carbon sources (e.g. pollen; (Wurzbacher *et al.*, 2014). This inaccessible carbon is conveyed to consumers as edible chytrid zoospores and thereby made available to the aquatic food web (Kagami *et al.*, 2017). Overall, by inhibiting growth of parasitic (and possibly, saprophytic) chytrids, fungicide run-off may have far-reaching cascading effects on the functioning of aquatic ecosystems that demands further research.

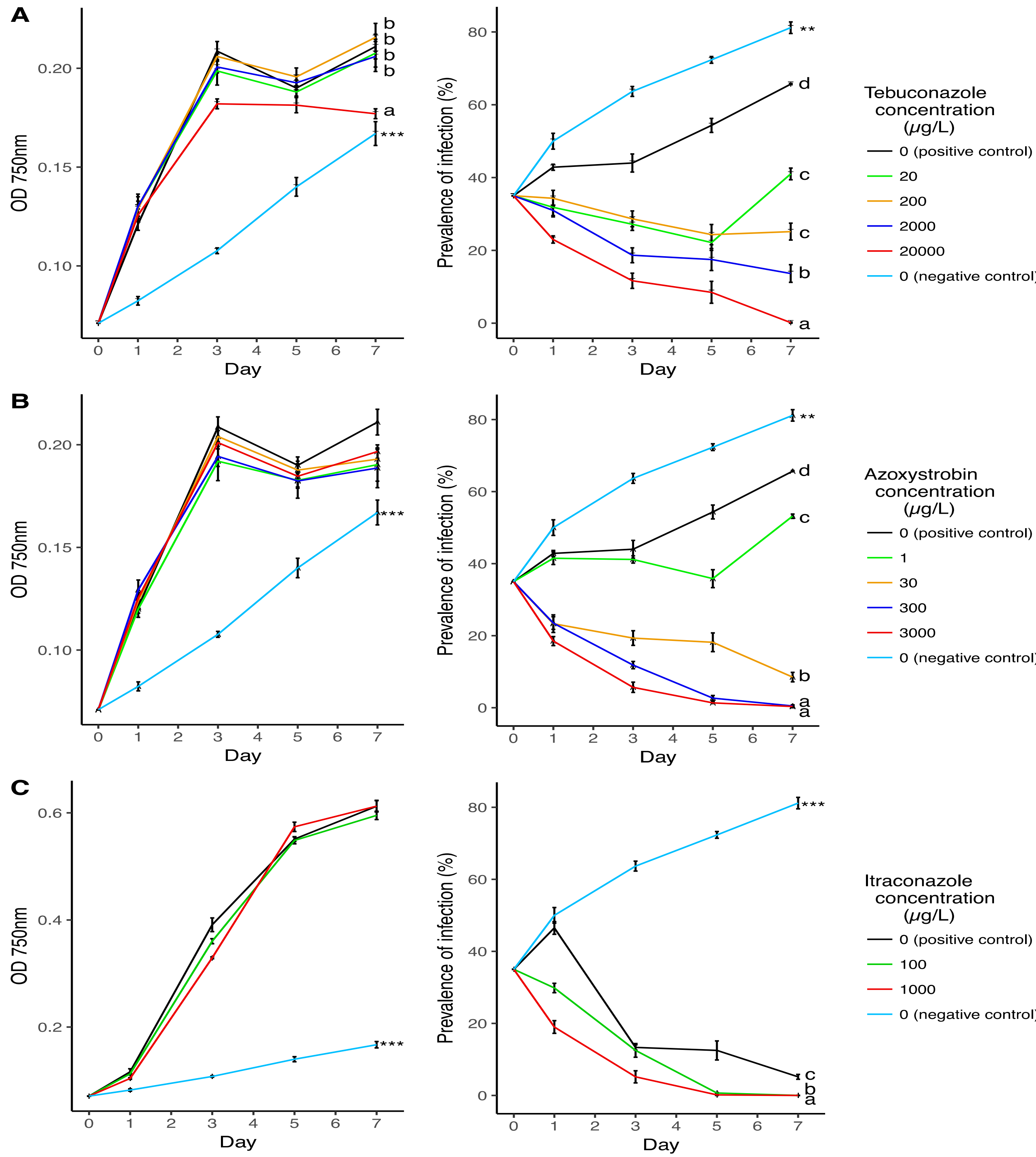

Figure 1. Changes in OD_{750} of uninfected cyanobacterial cultures (left panel) and prevalence of chytrid infection in infected cyanobacterial cultures (right panel) over time and under different concentrations of tebuconazole (A), azoxystrobin (B), and itraconazole (C) (mean ± S.E.) Negative control refers to no fungicide and no ethanol, whereas positive controls contain equal amounts of ethanol as in the fungicide treatments, i.e. 0.4 mL/L for tebuconazole and azoxystrobin, and 4.8 mL/L for itraconazole. Lines sharing the same letter are not significantly

different (Tukey HSD posthoc test, $p < 0.05$). Asterisks indicate a significant difference (t-test) between negative and positive controls: *** $p < 0.0001$, ** $p < 0.001$.

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Conflict of interest

The authors declare no conflict of interest.

Contributions

RA conceived the experiment. All authors designed the experiment. BOC conducted the experiment and analyzed the data, with the help of RA. BOC wrote the initial version of the manuscript which was then reviewed and edited by JW and RA.

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