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Antiparasitic potential of agrochemical fungicides on a non-target aquatic model (*Daphnia* × *Metschnikowia* host-parasite system)

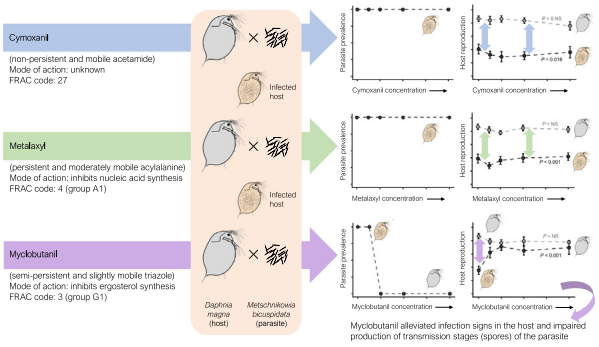
Cláudia Machado [a,b], Ana P. Cuco [a,b], Fernanda Cássio [a,b], Justyna Wolinska [c,d], Bruno B. Castro [a,b,*]

[a] CBMA (Centre of Molecular and Environmental Biology) & Department of Biology, School of Sciences, University of Minho, Braga, Portugal
[b] Institute of Science and Innovation for Bio-Sustainability (IB-S), School of Sciences, University of Minho, Braga, Portugal
[c] Department of Evolutionary and Integrative Ecology, Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany
[d] Department of Biology, Chemistry, Pharmacy, Institute of Biology, Freie Universität Berlin, Berlin, Germany

HIGHLIGHTS

- Impact of fungicides on aquatic fungi and associated functions has been overlooked.
- A *Daphnia* × parasitic yeast system was used to study effects of three fungicides.
- Myclobutanil cleared infection in the hosts and impaired parasite transmission.
- Parasite sporulation was the critical stage for antifungal action of myclobutanil.
- Azole fungicides may disrupt host-parasite interactions in natural systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticides are a major anthropogenic threat to the biodiversity of freshwater ecosystems, having the potential to affect non-target aquatic organisms and disrupt the processes in which they intervene. Important knowledge gaps have been recognised concerning the ecological effects of synthetic fungicides on non-target symbiotic aquatic fungi and the ecological processes where they intervene. The goal of this work was to assess the influence of three commonly used fungicides (myclobutanil, metalaxyl and cymoxanil), which differ in their mode of action, on a host (the crustacean *Daphnia magna*) × parasite (the yeast *Metschnikowia bicuspidata*) experimental model. Using a set of life history experiments, we evaluated the effect of each fungicide on the outcome of this relationship (disease) and on the fitness of both host and parasite. Contrasting results were observed: (i) cymoxanil and metalaxyl were overall innocuous to host and parasite at the tested concentrations, although host reproduction was occasionally reduced in the simultaneous presence of parasite and fungicide; (ii) on the contrary, myclobutanil displayed a clear antifungal effect, decreasing parasite prevalence and alleviating infection signs in the hosts. This antiparasitic effect of myclobutanil was further investigated with a follow-up experiment that manipulated the timing of application of the fungicide, to understand which stage of parasite development was most susceptible: while myclobutanil did not interfere in the early stages of infection, its antifungal activity was clearly observable at a later stage of the disease (by impairing the production of transmission stages of the parasite). More research is needed to understand the broader consequences of this parasite-clearance effect, especially in face of increasing evidence that parasites are ecologically more important than their cryptic nature might suggest.

* Corresponding author at: Department of Biology, School of Sciences of the University of Minho, Gualtar Campus, 4710-057 Braga, Portugal.
E-mail address: brunocastro@bio.uminho.pt (B.B. Castro).

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1. Introduction

Pesticides are a major anthropogenic threat to the biodiversity of freshwater ecosystems (Schäfer et al., 2019; Topping et al., 2020). A large fraction of these agrochemicals fails to reach the application target or are later transported via runoff (Bereswill et al., 2012; Schulz, 2004), thus reaching the aquatic system. Once dispersed or dissolved, they have the potential to affect non-target aquatic organisms and disrupt the processes in which they intervene (de Souza et al., 2020; Malaj et al., 2014). Recent reviews have pointed out that (i) fungicides (a class of pesticides that targets nuisance fungi and oomycetes) have been somewhat overlooked when compared to other pesticide classes (Zubrod et al., 2019), and (ii) the current ecological risk assessment for organic synthetic fungicides does not adequately consider their impacts on non-target aquatic fungi (Ittner et al., 2018; Zubrod et al., 2015). Being extensively and preventively applied in agriculture, these xenobiotics are commonly detected in surface and ground waters (Battaglin et al., 2011; Kahle et al., 2008; Wightwick et al., 2012), there representing a hazard to aquatic fungi (Dijksterhuis et al., 2011; Zubrod et al., 2015; Zubrod et al., 2019). These eukaryotic microbes are involved in important ecological processes and food web interactions in the aquatic ecosystem (Grossart et al., 2019; Ittner et al., 2018), playing roles as decomposers (Gulis et al., 2019), parasites or pathogens (Frenken et al., 2017) as well as other type of symbionts (Wilson et al., 2014).

Studies on the impacts of fungicides on non-target fungi and the processes where they intervene are heterogeneous. The majority of such studies were conducted with decomposer fungi, and reported effects include decreased sporulation (conidia production) – in the case of tebuconazole (Dimitrov et al., 2014; Pimentão et al., 2020; Zubrod et al., 2011) and pentachlorophenol (Bärlocher and Premdas, 1988) – as well as decreased growth (biomass) – in the case of tebuconazole (Artigas et al., 2012; Pimentão et al., 2020; Zubrod et al., 2011) and pyrimethanil (Abelho et al., 2016). Compositional changes were also observed on decomposer communities exposed to tebuconazole (Artigas et al., 2012; Dimitrov et al., 2014; Pimentão et al., 2020), imazalil (Flores et al., 2014) or azoxystrobin (Gustafsson et al., 2010). Only in a few cases (Artigas et al., 2012) such impacts were translated into decreased decomposition rates. Available information about the impact of fungicides on aquatic fungal symbionts is considerably less comprehensive. So far, a few studies have demonstrated that common fungicides can reduce infectivity and parasite load in a yeast that parasitizes *Daphnia* (Cuco et al., 2017a, 2017b), as well as in chytrid species that infect tadpoles (Hanlon and Parris, 2012) and cyanobacteria (Ortiz-Cañavate et al., 2019). Analogously, decreased infestation and sporulation by commensal gut fungi were correlated with fungicide body burdens in black fly larvae (Wilson et al., 2014). Further data on the impact of fungicides on parasites and other fungal symbionts are needed to consolidate these observations and to clarify the underlying mechanisms of the observed antifungal effects.

Previous results from our team have provided valuable insight on the effects of tebuconazole on the host-parasite system *Daphnia* sp. × *Metschnikowia bicuspidata*. *Daphnia* sp. are aquatic microcrustaceans that occupy a central position in aquatic systems, where they are hosts to many bacterial, microsporidian, and fungal parasites (Cáceres et al., 2014; Ebert, 2005; Wolinska et al., 2009), including the obligate endoparasitic yeast *M. bicuspidata* (Penczykowski et al., 2016; Stewart Merrill and Cáceres, 2018). This microparasite grows vegetatively in the haemolymph of *Daphnia* until it produces needle-shaped ascospores (transmission stages) that fill the entire body cavity of the host, thus killing it. Spores are then released to the environment and grazed upon by new hosts (horizontal transmission); infection occurs when spores penetrate the *Daphnia* gut wall, germinate and overcome the host's immune system (Ebert, 2005; Stewart Merrill and Cáceres, 2018). Data from our team showed that tebuconazole suppresses all signs of *Metschnikowia* infection in a concentration- (Cuco et al., 2017a) and temperature-dependent (Cuco et al., 2018) manner. Because the antifungal (or antiparasitic) action was still effective when tebuconazole was added at a later stage of infection (Cuco et al., 2017b), we concluded that the suppression of infection signs was caused by the inhibition of sporulation, i.e. the last stage of the disease. Additional support to this conclusion was provided by evidences that ergosterol biosynthesis inhibitors (like tebuconazole) cause a fungistatic response, rather than a fungicidal effect, impairing fungal growth and sporulation (Álvarez-Pérez et al., 2016). Fungicides with other modes of action may affect the host-parasite relationship differently.

The goal of this work was to assess the influence of three commonly used fungicides (myclobutanil, metalaxyl and cymoxanil), differing in their mode of action, on a host (*Daphnia magna*) × parasite (*M. bicuspidata*) experimental model. We evaluated the effect of each substance on parasite prevalence, infection intensity and host fitness (mortality and reproduction) using a set of life history experiments in the presence vs. absence of the parasite. In the case of the substances for which an antiparasitic effect was confirmed, we conducted a follow-up experiment, by manipulating the timing of application of the fungicide to understand which stage of parasite development was most susceptible to the tested substance.

2. Material and methods

2.1. Host-parasite system

Daphnia sp. are widely used as experimental models due to their advantageous characteristics, such as short life cycle and mainly parthenogenetic reproduction (Altshuler et al., 2011; Miner et al., 2012). *Metschnikowia bicuspidata* is a common and virulent parasite of *Daphnia*, reducing host fecundity and substantially shortening its lifespan (Auld et al., 2012; Cuco et al., 2016; Ebert, 2005). The *Daphnia-Metschnikowia* experimental model allows the control of parasite transmission and spore load in infection experiments (Cuco et al., 2017a; Hall et al., 2007). For this study, we used a *D. magna* genotype (E17:07; Saebelfeld et al., 2017) and an *M. bicuspidata* strain (AMME; Lohr et al., 2010) used in a previous study (Cuco et al., 2020).

Daphnia magna cultures were cyclically reared as synchronized asexual cohorts in vessels with 750 mL of a high hardness semi-synthetic medium (111 mg/L $MgSO_4{\cdot}7H_2O$, 126 mg/L $NaHCO_3$, 110 mg/L $CaCl_2{\cdot}2H_2O$, 7.45 mg/L KCl, 0.00150 mg/L SeO_2) supplemented with a standard organic additive (algal extract) and vitamins (Loureiro et al., 2011). The major ionic constituents of this reconstituted water were inspired on a high-hardness version (Baer and Goulden, 1998) of COMBO medium (Kilham et al., 1998). Medium was renewed three times per week and, at each renewal, daphniids were fed with an ad libitum suspension of the microalgae *Raphidocelis subcapitata*, previously normalized at an optical density of 0.750–0.850 at a 1:10 dilution. Cultures with synchronized females warranted regular production of asexual progeny, and offspring were culled from mothers to keep culture density constant. Neonates (individuals less than 24 h old) born between the 3rd and 5th broods were used to renew cultures and to start experiments.

The microparasitic yeast *M. bicuspidata* was cyclically maintained in infected *D. magna* (clone E17:07) cultures (under the same conditions as the healthy cultures), with the purpose of perpetuating the infection and transmission cycle, assuring the production of ascospores (Cuco et al., 2017a, 2017b). Briefly, healthy *Daphnia* neonates were exposed to *M. bicuspidata* transmission stages (spores) and reared in groups until the development of infection signs. Infected *Daphnia* (filled with yeast spores) were collected upon their death (usually after 2 to 3 weeks), and stored at 4 °C. These infected hosts represented the source of new spore suspensions to renew *M. bicuspidata* cultures and to start experiments. Spore suspensions were obtained by crushing infected *Daphnia* in culture medium and spore density was normalized after counting host homogenates with a Neubauer improved chamber. For proper control of bacteria or conspecific alarm cues in experiments in the no-parasite (NP) treatment (see Section 2.3), placebo suspensions were prepared by crushing healthy *Daphnia* in culture medium (Lohr et al., 2010).

Healthy and infected *D. magna* cultures were maintained under controlled temperature (20 ± 2 °C) and photoperiod ($16h^L$:$8h^D$) regimes.

2.2. Test chemicals

Cymoxanil (Cymoxanil PESTANAL® CAS nr. 57966-95-7 [(1E)-2-(ethylcarbamoylamino)-N-methoxy-2-oxoethanimidoyl cyanide]), metalaxyl (Metalaxyl PESTANAL®, CAS nr. 57837-19-1 [methyl 2-(N-(2-methoxyacetyl)-2,6-dimethylanilino)propanoate]) and myclobutanil (Myclobutanil PESTANAL®, CAS nr. 88671-89-0 [(*RS*)-2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)hexanenitrile]) were obtained as analytical grade compounds from Sigma Aldrich (Munich, Germany). Cymoxanil is an acetamide fungicide usually used in combination with other fungicides, and it is considered a non-persistent mobile compound (half-life of 1.1 days and K_{oc} = 43.6). Metalaxyl belongs to alanine fungicides, which act by inhibiting the activity of RNA polymerase I, affecting nucleic acids synthesis; it has a half-life of 1000 days and it is moderately mobile (K_{oc} = 163). Myclobutanil is a triazole fungicide that affects cellular membrane permeability by inhibiting ergosterol synthesis; it has a half-life of 30 days in aqueous media and it is slightly mobile (K_{oc} = 518) (Kegley et al., 2011; Kim et al., 2016).

Fungicide stock solutions were prepared weekly in absolute ethanol and stored at 4 °C. Test solutions were prepared by adding the proper amount of the respective stock solution to *Daphnia* medium, to obtain final test concentrations for each contaminant. Final ethanol concentration was equal in all experimental units (0.1 mL/L), which is innocuous to the host-parasite system (Cuco et al., 2016). In all experiments (Sections 2.3 and 2.4), ethanol was added to negative controls (0 μg/L) at an equivalent concentration.

Procedures for preparing fungicide test solutions were similar to previous studies with tebuconazole (Cuco et al., 2016, 2017a, 2017b, 2018, 2020), which warranted measured concentrations within 10–20% deviation of nominal concentrations. We conservatively assumed that a similar success would be attained for the substances here tested; all fungicide concentrations here reported therefore refer to nominal concentrations. Nonetheless, three random aliquots of freshly prepared myclobutanil concentrations were sent to an independent analytical laboratory to check the validity of nominal concentrations, because of the particular interest in this fungicide (See Results). Quantification of myclobutanil was performed by liquid chromatography mass spectrometry (LC–MS/MS), according to an internal method adapted from ISO 11369:1997. Despite some variation, analytical data confirmed a <20% deviation of nominal myclobutanil concentrations of 44.3 μg/L (37 ± 16.4 μg/L) and 59.0 μg/L (58 ± 1.3 μg/L).

2.3. Life-history experiments with cymoxanil, metalaxil and myclobutanil

Life history assays were performed with the host-parasite system to test the effect of each fungicide on parasite prevalence, infection intensity and host fitness (mortality and reproduction). For each contaminant (cymoxanil, metalaxyl or myclobutanil), a fully crossed design was used, using 5 fungicide concentrations (0, 25, 50, 100 and 200 μg/L) × 2 parasite treatments (presence or absence) × 12 replicates (15 in the controls, 0 μg/L). Selected fungicide concentrations were chosen based on our previous experience with tebuconazole, as well as preliminary experiments and literature data (to make sure concentrations were not lethal to the host). Each experiment was initiated by placing an individual *D. magna* neonate in a clear plastic tube containing 40 mL of the corresponding test solution (in a total of 126 experimental units per contaminant). Test solutions were renewed three times per week and daphniids were fed along with medium renewal (as described for the cultures). Infection was carried out on day 4, with each experimental unit receiving either an *M. bicuspidata* spore suspension (parasite presence = P treatment) or a placebo suspension (no parasite = NP treatment). On this day, test solution volume was reduced to 15 mL and spores were added to a final density of 2000 spores/mL (optimized from Cuco et al., 2020). To ensure higher filtration rates and spore encounter rates (Hall et al., 2007) no food (algal ration) was added. On the next day (day 5), feeding was resumed, and test volume was restored to 40 mL by adding freshly prepared test solutions. Assays were performed under the same light and temperature conditions as described for the cultures.

For cymoxanil and metalaxyl, life history assays lasted 21 days while for myclobutanil the experiment had to be extended until day 28, when all infected hosts died. In the presence of macroscopic signs of infection (whitish, opaque body), hosts were checked under an inverted microscope to confirm the presence of spores inside their body cavity; this procedure was also mandatorily conducted at the time of death of the hosts or at the end of the assay. The proportion of infected hosts (prevalence) as well as the proportion of dead hosts (mortality) were calculated for each contaminant, at the end of each assay. Infected hosts were collected and individually stored in ethanol at 4 °C for spore load determination. Spore load per host (a measure of infection intensity) was assessed by counting mature ascospores in homogenates obtained from each infected host. For calculations of prevalence and spore load, we excluded the few individuals that died before the infection could be visually diagnosed (i.e., before day 14). Experimental units were checked daily to account for host reproductive events, discarding offspring after counting them. Fecundity at day 14 (cumulative number of offspring per surviving female), reproductive output at day 21 (cumulative number of offspring per female, surviving or not), and the per capita intrinsic rate of population increase (*r*) were calculated at day 21. Fecundity at day 14 was estimated to exclude the influence of parasite-mediated mortality (occurring mostly from day 14 onwards), whereas reproductive output at day 21 considered the combined effects of fungicide and parasite on both survivorship and fecundity (Castro et al., 2018; Cuco et al., 2016). The intrinsic rate of increase (*r*) was determined based on survival and reproduction data using the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x$$

where *r* represents the rate of population increase per day, *x* represents the age class in days, l_x is the probability of surviving to age *x* and m_x is the fecundity at age *x*.

2.4. Follow-up experiment with myclobutanil

A follow-up experiment was conducted to further understand the influence of myclobutanil concentration and its timing of application on *M. bicuspidata* infection. Tested concentrations ranged from levels with no apparent effect on the parasite to levels that completely suppressed parasite infection in the life history experiment: 0, 21.5, 23.9, 26.6, 29.5, 32.8, 36.5, 40.5, 45.0 and 50.0 μg/L. The timing of myclobutanil application was defined according to the predicted stage of *M. bicuspidata* life cycle (Fig. 1): early - from day 4 (infection) until day 10, comprising spore germination and initial vegetative growth of the yeast; late - from day 10 until host death, comprising the final phase of vegetative growth and sporulation; continuous - from day 4 until host death. Thus, experimental design consisted in 10 myclobutanil concentrations × 3 timings (continuous, early and late) × 5 replicates.

In this assay, each experimental unit consisted of a glass vessel with 10 *Daphnia* neonates (<24 h old, born between the 3rd and 5th broods) in 150 mL of the corresponding test solution (following Cuco et al., 2017b, 2020). Infection procedure was carried out on day 4 by exposing all the experimental units to an aliquot of *M. bicuspidata* spore suspension (final concentration of 2000 spores/mL). During infection, test solution volume was reduced to 50 mL and no food was added (as previously described). On day 5, volume was increased to 100 mL and daphniids were fed accordingly. From day 6 onwards, initial conditions were restored (150 mL of test solution, feeding and medium renewal three times per week). Vessels were monitored daily to record host mortality and parasite prevalence. In the presence of infected (macroscopic signs) or dead *Daphnia*, hosts were checked under an inverted microscope to confirm the presence of spores inside their body cavity; at the end of the assay, surviving hosts were also checked for infection signs. The proportion of infected *Daphnia*

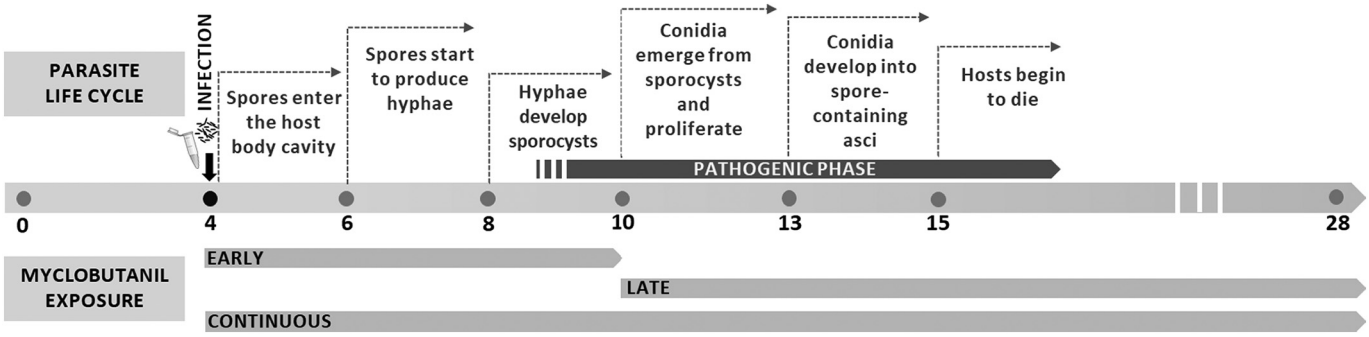

Fig. 1. Schematic representation of the predicted stage of *M. bicuspidata* life cycle and its coupling with myclobutanil application timings. The duration of the different phases of infection was estimated based on Cuco et al. (2017b), Ebert (2005), Metschnikoff (1884), Stewart Merrill and Cáceres (2018) and our observations.

(prevalence) as well as the proportion of dead *Daphnia* (mortality) were calculated for each experimental unit. Individuals which died before infection could be diagnosed (i.e., before day 14) were excluded from prevalence and spore load calculations. Infected hosts were collected and stored (pooling infected hosts from the same experimental unit) in ethanol at 4 °C, for later spore load determination. Spore load was assessed by counting mature ascospores in infected host homogenates from three (out of five) random experimental units and expressed as the number of spores per infected host (dividing the total number of spores in the host homogenate by the number of hosts, following Cuco et al., 2020). This experiment lasted 28 days and was conducted under the same temperature and photoperiod as described before.

2.5. Data analysis

All statistical analyses were performed in *R* software, version 3.5.1 (R Core Team, 2017), using the Integrated Development Environment (IDE) RStudio, version 1.1.456 (RStudio Team, 2016). Data were analysed separately for each fungicide using generalized linear models (GLM) to understand the effects of parasite presence, fungicide concentration and timing of application (as categorical predictors) on the quantified endpoints. Parasite prevalence and host mortality were modelled as binomially distributed variables (logistic model), whereas host fecundity and reproductive output were analysed as count data (quasi-Poisson model); intrinsic rate of population increase (r) and spore load were analysed with a classical linear model (Gaussian model). Spore load data were $\log_{10}$-transformed prior to analysis. To infer about the effect of the categorical predictors or their interaction, F tests were used for linear and quasi-Poisson models whereas likelihood ratio tests (χ^2) were used for binomial data, following Szöcs and Schäfer (2015) and references therein. Significance of categorical predictors and their interaction was tested via the "Anova" function from R package "car" (Fox and Weisberg, 2019).

For the life history experiment data, GLM were used to investigate the effect of parasite presence and fungicide concentration in a bifactorial design, except in the case of spore load. The following procedures were followed for fecundity, reproductive output and rate of increase: (i) in the presence of a significant interaction between parasite presence and fungicide concentration, simple main effects (Quinn and Keough, 2002) of concentration were tested in the P and NP treatments, using the function "testInteractions" from R package "phia" (Rosario-Martinez, 2015) and adjusting for multiple comparisons by controlling the false discovery rate (García, 2004; Pike, 2011); (ii) in the presence of a significant effect of fungicide concentration (but no interaction with parasite presence), significant differences relatively to control (no fungicide) were tested (Dunnett contrasts) using the function "glht" from R package "multcomp" (Hothorn et al., 2008). Because yeast spores were only produced in the P treatment, spore load data ($\log_{10}$-transformed) were analysed as a unifactorial linear model (testing the effect of fungicide concentration), followed by Dunnett test.

For the follow-up experiment data, GLM were used to investigate the effect of fungicide concentration and timing of application in a bifactorial design. As before, simple main effects of concentration within each timing were tested when a significant interaction between fungicide concentration and timing of application was found. Additionally, estimation of IC_{50} (inhibitory effect on infection) and LC_{50} (mortality of host) for each timing of application was performed by modelling prevalence and mortality data (using the R package "drc"; Ritz and Streibig, 2005) with a special case of the log-logistic dose-response model, where the asymptotes of the curve are fixed to be 1 (all organisms are infected or dead) and 0 (none are infected or dead), following the rationale of Ritz (2010).

3. Results

3.1. Life-history experiments with cymoxanil, metalaxyl and myclobutanil

Parasite (*M. bicuspidata*) caused 100% host (*Daphnia*) mortality coincident with 100% prevalence under control conditions (P treatment, no toxicant). On the contrary, in the absence of the parasite (NP treatment), no mortality was observed; this was valid for all tested fungicide concentrations in the NP treatment. The exposure to cymoxanil and metalaxyl had no effects on parasite prevalence (which remained 100%) but caused a minor decrease in spore load at intermediate concentrations (Fig. 2; Table 1). Under myclobutanil exposure, the parasite was unable to infect hosts from 50 μg/L upwards (Fig. 2; Table 1); no infection signs (i.e., absence of spores) and no host mortality were observed from this concentration upwards, in contrast to 0 and 25 μg/L.

Host fecundity prior to infection signs (measured at day 14) was negatively affected by fungicide concentration and parasite presence in an independent way (no significant contaminant × parasite interaction, Table 1). The most notorious effect was a significant decrease in host fecundity in the presence of the parasite at all fungicide concentrations (Fig. 3; Table 1). This pattern was consistent for the three fungicides.

On the contrary, distinct response patterns across fungicides were observed for reproductive output (Fig. 3): although a significant concentration × parasite interaction (Table 1) was found for the three fungicides, the meaning of such an interaction differed in each case. Offspring production in *D. magna* was slightly affected by cymoxanil and metalaxil in the presence of the parasite but not in its absence (see simple main effects of concentration in Fig. 3). In the case of myclobutanil, the response of the host-parasite system was strongly dependent from fungicide concentration: the parasite caused a reduction in reproductive output in the absence of myclobutanil (0 μg/L), but this became less pronounced from 25 μg/L upwards. Consequently, a clear reduction in offspring production of the host was overall visible in the P treatment (via parasite-induced mortality) in the case of cymoxanil and metalaxyl, whereas the effect of the parasite was alleviated in the presence of myclobutanil (compared to 0 μg/L).

The pattern of response of the rate of increase (r) was analogous to that of reproductive output (Fig. 3; Table 1): (i) cymoxanil and metalaxil had no effect on the infection but their effects on host fitness were slightly exacerbated in the presence of *M. bicuspidata*; (ii) an antiparasitic effect was observed at high concentrations of myclobutanil (≥50 μg/L).

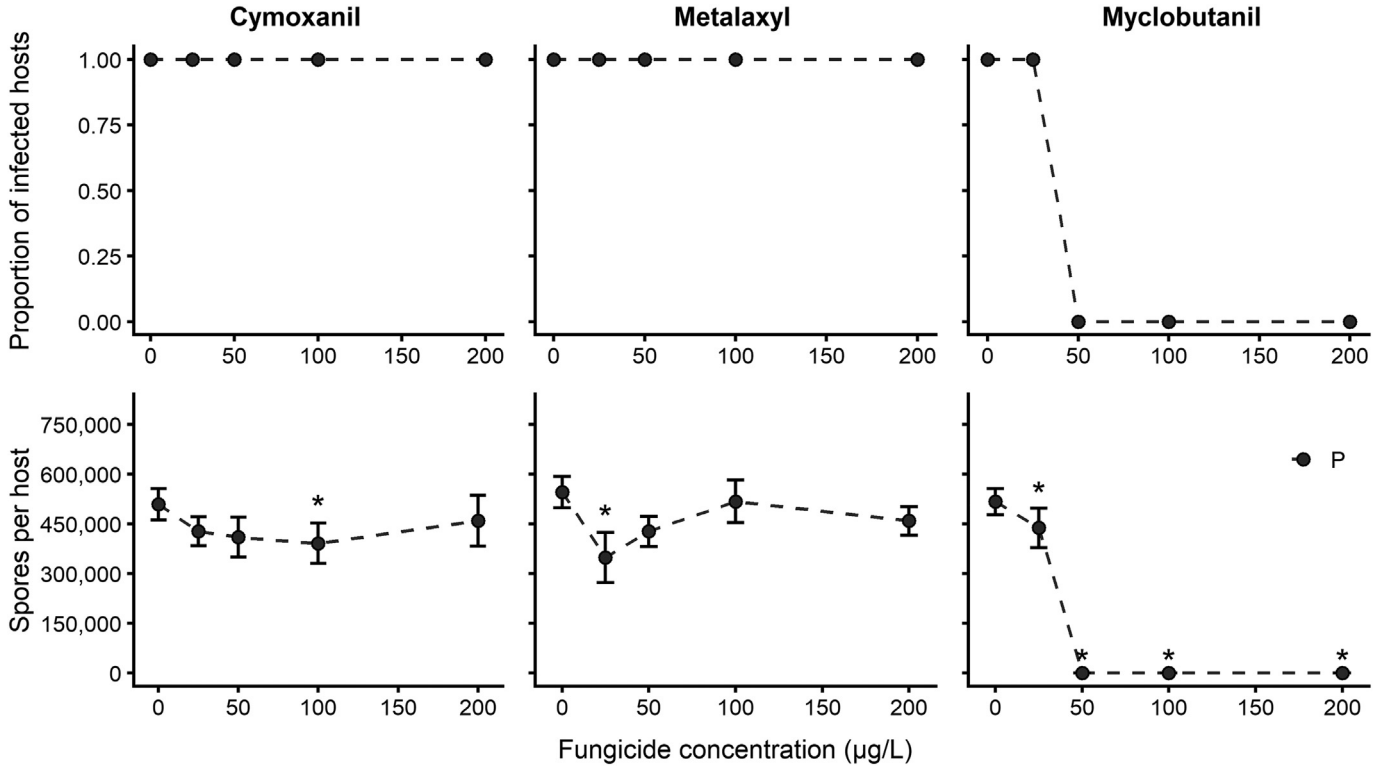

Fig. 2. Prevalence (top) and spore load (bottom) of the parasite *M. bicuspidata* in *Daphnia magna*, under increasing concentrations of cymoxanil, metalaxyl or myclobutanil (P treatment). Results are expressed as mean and respective 95% confidence interval. Asterisks denote significant differences relatively to the control in the case of spore load (Dunnett test).

3.2. Follow-up experiment with myclobutanil

A significant timing × concentration interaction was observed in the follow-up experiment with myclobutanil, for both parasite prevalence ($\chi^2_{(18,\ 120)} = 63.7$, $P < 0.001$) and host mortality ($\chi^2_{(18,\ 120)} = 32.6$, $P = 0.019$). Under a continuous exposure scenario, prevalence data confirmed the antiparasitic effect of myclobutanil, which significantly suppressed infection signs between 40.5 and 50.0 µg/L (simple main effects of concentration, $P = 0.042$). An IC_{50} of 44.3 µg/L was estimated from the data (Fig. 4). However, this effect was not observable when myclobutanil was added during shorter periods (early exposure, $P = 1.00$; late exposure, $P = 1.00$). In parallel, myclobutanil significantly reduced parasite-induced mortality when applied continuously ($P < 0.001$) or in a late ($P = 0.007$) stage of infection, but not when added at an early stage ($P = 1.00$). Furthermore, the estimated LC_{50} values for the continuous (35.6 µg/L) and late (48.9 µg/L) exposure demonstrated that a higher fungicide concentration of myclobutanil was required to achieve the same response intensity when applied at a later stage of infection (Fig. 4).

A distinct pattern of response across timings was also observed for spore load (significant timing × concentration interaction: $F_{(18,\ 60)} = 19.4$, $P < 0.001$). Whereas myclobutanil suppressed sporulation when applied continuously (simple main effects of concentration, $P < 0.001$) or at a later stage of infection ($P < 0.001$), no effects were observed in spore load when this fungicide was applied at an earlier stage ($P = 1.00$) of the parasite life cycle.

Table 1
– Summary table of the generalized linear models (GLM) applied to fecundity, reproductive output, and rate of increase to assess the effect of fungicide concentration (Conc.), parasite treatment and their interaction (Conc. × Parasite). In the case of spore load, a unifactorial linear model was applied to test the effect of fungicide concentration (Conc.) alone. Significant effects are highlighted in bold ($P \leq 0.05$).

	Cymoxanil	Metalaxyl	Myclobutanil
Spore load			
Conc.	$F_{(4,\ 58)} = 2.53$, **$P = 0.050$**	$F_{(4,\ 58)} = 7.76$, **$P < 0.001$**	$F_{(4,\ 58)} = 50{,}784$, **$P < 0.001$**
Fecundity at day 14			
Parasite	$F_{(1,\ 115)} = 141$, **$P < 0.001$**	$F_{(1,\ 115)} = 89.1$, **$P < 0.001$**	$F_{(1,\ 115)} = 67.5$, **$P < 0.001$**
Conc.	$F_{(4,\ 115)} = 5.01$, **$P < 0.001$**	$F_{(4,\ 115)} = 6.50$, **$P < 0.001$**	$F_{(4,\ 115)} = 0.68$, $P = 0.609$
Conc. × Parasite	$F_{(4,\ 115)} = 1.37$, $P = 0.249$	$F_{(4,\ 115)} = 2.15$, $P = 0.080$	$F_{(4,\ 115)} = 1.50$, $P = 0.208$
Reproductive output			
Parasite	$F_{(4,\ 115)} = 569$	$F_{(1,\ 115)} = 746$	$F_{(4,\ 115)} = 121$
Conc.	$F_{(4,\ 115)} = 1.80$	$F_{(4,\ 115)} = 3.55$	$F_{(4,\ 115)} = 7.44$
Conc. × Parasite	$F_{(4,\ 115)} = 3.01$, **$P = 0.021$**	$F_{(4,\ 115)} = 3.92$, **$P = 0.005$**	$F_{(4,\ 115)} = 29.1$, **$P < 0.001$**
Rate of increase			
Parasite	$F_{(4,\ 115)} = 253$	$F_{(1,\ 115)} = 247$	$F_{(4,\ 115)} = 49.6$
Conc.	$F_{(4,\ 115)} = 5.68$	$F_{(4,\ 115)} = 7.90$	$F_{(4,\ 115)} = 1.74$
Conc. × Parasite	$F_{(4,\ 115)} = 3.51$, **$P = 0.010$**	$F_{(4,\ 115)} = 3.16$, **$P = 0.017$**	$F_{(4,\ 115)} = 5.87$, **$P < 0.001$**

4. Discussion

Host-parasite relationships are strongly dependent on the environmental context (Wolinska and King, 2009) and can be influenced by the presence of contaminants (Lafferty and Kuris, 1999; Sures et al., 2017). In this paper, we revisit this topic by specifically focusing on the effects of fungicides on a host-parasite model (*D. magna* × *M. bicuspidata*), in which the parasite is the likely eco-receptor of the contaminants under study (following Cuco et al., 2017a, 2017b; Hanlon and Parris, 2012; Ortiz-Cañavate et al., 2019).

Contrasting results were observed across fungicides. Cymoxanil and metalaxyl were innocuous to host and parasite at the tested concentrations; however, under joint exposure to parasite and toxicant, reproductive and

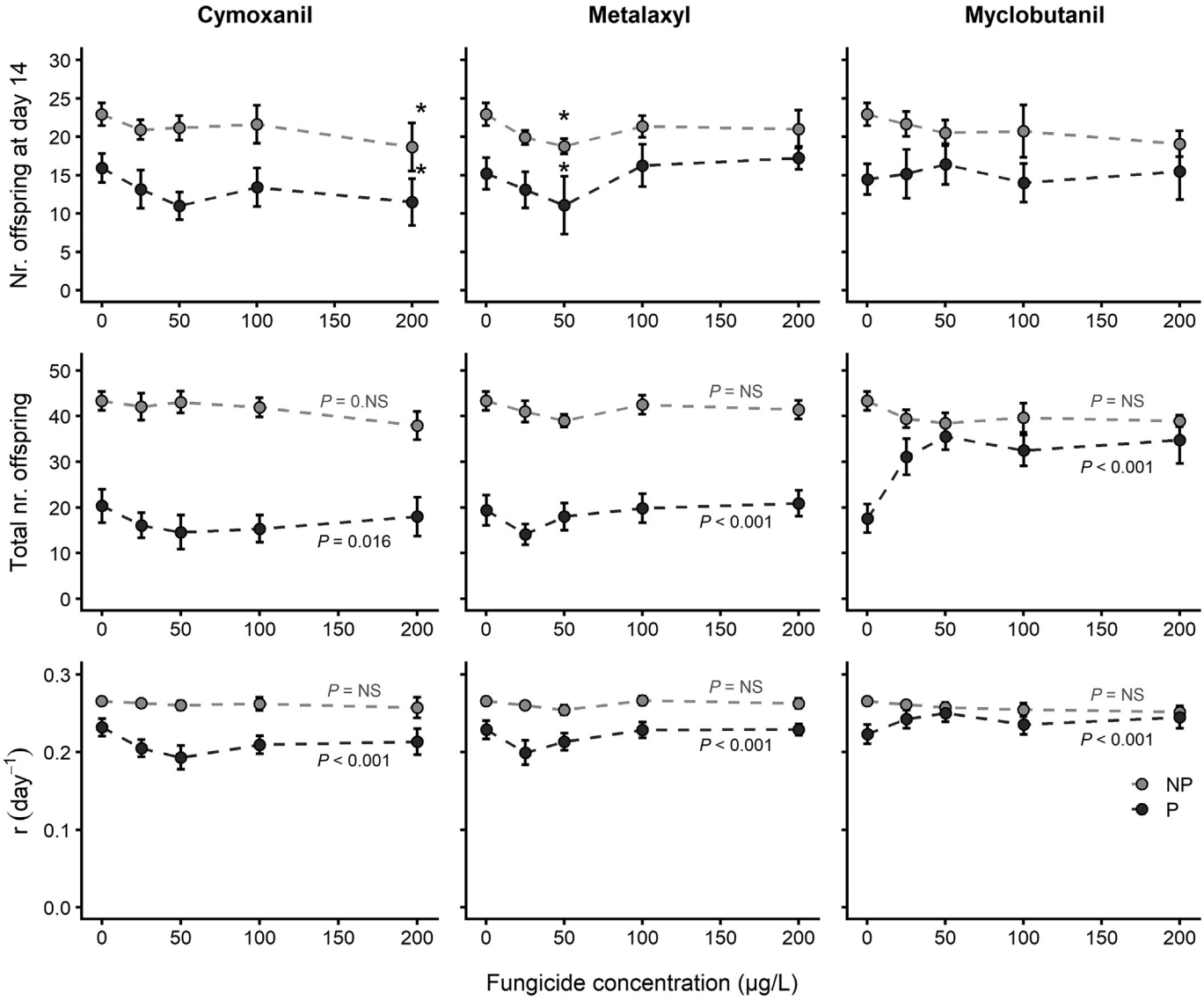

Fig. 3. Fecundity (top), reproductive output (middle) and rate of population increase (bottom) in *Daphnia magna* under increasing concentrations of cymoxanil, metalaxyl or myclobutanil in the presence (P, black circle and line) or absence (NP, gray circle and line) of the parasite. Results are expressed as mean and respective 95% confidence interval. Asterisks denote significant differences relatively to the control in the case of fecundity (Dunnett test); *P*-values are shown for simple main effects of concentration in P and NP treatments (NS = non-significant).

population parameters of the host were slightly decreased. Although this happened episodically (only in some concentrations), it could be viewed as a synergistic effect of the stressors on host fitness, caused by exacerbated impacts on the host's sensitivity to the pollutant or its susceptibility to infection (see rationale in Lafferty and Kuris, 1999). Such patterns have also been observed for insecticides (Buser et al., 2012; Coors et al., 2008) and herbicides (Kelly et al., 2010) in other host-parasite models (see also review by Blanar et al., 2009). On the contrary, myclobutanil reduced parasite fitness, decreasing infectivity (measured as prevalence) and virulence (perceived effects on host reproductive output) in a concentration-dependent manner. This beneficial effect of pollutants (from the host's perspective) has also been described for pesticides (Hanlon and Parris, 2012; Hanlon and Parris, 2014; Ortiz-Cañavate et al., 2019), metals (Blanar et al., 2010; Civitello et al., 2012), and salinity (Merrick and Searle, 2019). We have also shown analogous results in the *Daphnia* × *Metschnikowia* model for tebuconazole (Cuco et al., 2017a, 2017b), a fungicide from the same class (and mode of action) as myclobutanil. Clearly, the effects of fungicides on host-parasite dynamics are context-dependent (parasite enhances fungicide toxicity vs. fungicide alleviates parasite effects), and this must be considered when predicting the environmental impact of these substances on non-target aquatic fungi.

Albeit important, the magnitude of effects observed in the synergistic interaction between parasitism and cymoxanil or metalaxyl was subtle; in contrast, the disruptive effects of myclobutanil on the host-parasite relationship deserve further examination. Analogously to what has been described for tebuconazole (Cuco et al., 2017a, 2017b), *Metschnikowia* infection signs were suppressed and parasite virulence significantly decreased in the presence of myclobutanil (from 50 µg/L upwards); host mortality and reproductive output of the parasite (P) treatment became almost comparable to that of NP treatment (unlike in the myclobutanil-free controls). The follow-up experiment demonstrated a clear inhibition curve of parasite infectivity (prevalence) and virulence (host mortality) for myclobutanil. This suppression or clearance of infection signs occurred at concentrations higher than what is normally recorded in superficial waters (<3 µg/L; Battaglin et al., 2011; Bereswill et al., 2012; Wightwick et al., 2012), and higher than the equivalent tebuconazole concentrations causing the same effect (Cuco et al., 2017b).

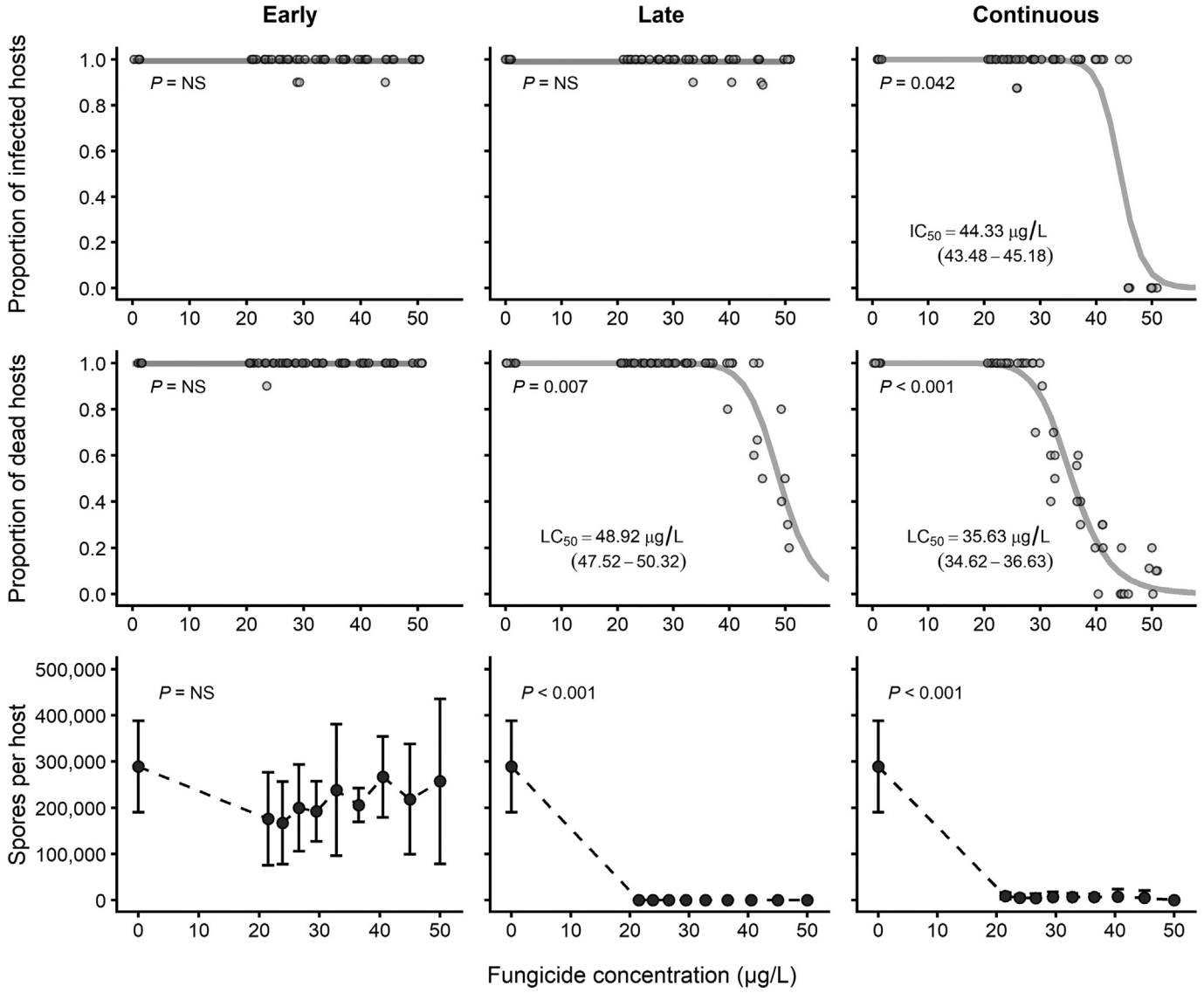

Fig. 4. Prevalence (top), mortality (middle) and spore load (bottom) of infected *Daphnia* under increasing concentrations of myclobutanil at three timings of application (early, late and continuous). Prevalence and mortality curves and respective IC_{50} and LC_{50} values were estimated with a log-logistic dose-response model. Spore load results are expressed as mean and respective 95% confidence interval. *P*-values are shown for simple main effects of concentration in each timing of application (NS = non significant).

Data from the two experiments (life history and its follow-up) demonstrated that myclobutanil does not interfere in the early stages of the disease. Indeed, before any infection signs were apparent, host fecundity at day 14 was already lower in the P treatment (significant parasite effect in Table 1), irrespective of myclobutanil concentration (a pattern that is consistent for the other fungicides that did not interfere with the outcome of the host-parasite relationship). This suggests that the pathogenic action of the parasite was not fully suppressed by myclobutanil (similarly to what was observed for tebuconazole; Cuco et al., 2017a). Additionally, when the host-parasite model was exposed to myclobutanil in an initial phase (early timing of application), the fungicide had no effect on infectivity, virulence (host mortality) or infection intensity (spore load). Altogether, these results suggest that *Metschnikowia* can germinate inside *Daphnia*, produce hyphae, sporocysts and, perhaps, conidia, irrespective of the presence of the fungicide. While doing so, the microparasitic yeast causes some damage to the host, either by forcing energy allocation to immune responses or by overtaking body regions of the host (see Stewart Merrill and Cáceres, 2018), thus affecting host reproduction (although less than other parasites; see Auld et al., 2012; Lohr et al., 2010). In a later stage of infection, *Metschnikowia* kills its host, and this is where myclobutanil had a clear protective effect.

The antifungal activity of myclobutanil was clearly observable at a later stage of the disease, by alleviating infection in the *Daphnia* hosts (decreased mortality and spore load). Previous studies from our team on aquatic fungi demonstrated that negative effects from agrochemical and pharmaceutical azoles are most evident in the sporulation phase: (i) increasing tebuconazole and clotrimazole concentrations significantly decreased spore production of aquatic hyphomycetes and led to compositional changes when assessing community composition based on spore identity and counts (Pimentão et al., 2020); (ii) tebuconazole (Cuco et al., 2017a, 2017b) and myclobutanil (this study) significantly decreased or suppressed the production of transmission stages (spores) of the microparasitic yeast *M. bicuspidata*, even when the agrochemicals were applied in a later stage of infection. By linking the timing of application of the azole fungicides with the predicted stage of parasite development in the *Daphnia* × *Metschnikowia* system, we conclude that sporulation or conidiation is the key phase in the impairment of the infection by azoles. Ergosterol depletion caused by azole fungicides may compromise conidiation of non-target fungi, reducing or suppressing the number of conidia or causing morphological abnormalities, similarly to what has been described in *Fusarium* (filamentous fungus) mutants that are defective in the synthesis of ergosterol

(Liu et al., 2013). In other fungal models, conidiogenesis and conidiation are indeed known to be dependent on various endogenous and environmental factors (Steyaert et al., 2010).

A paradoxical result in the follow-up experiment was the high prevalence observed at the late exposure scenario (no apparent effect of myclobutanil), in contrast to spore loads close or equal to 0 at all myclobutanil concentrations. This apparent contradiction can be explained by the fact that hosts had all the signs of infection (whitish body, presence of clusters of conidia) but spores were not mature, i.e., not fully developed (Green, 2010; Lohr et al., 2010; Metschnikoff, 1884). Spore (or asci) presence is the only definite criterion to diagnose infection at low magnification, but only fully mature spores (needle-shaped, housed in an ascus) are infective (Stewart Merrill and Cáceres, 2018); consequently, immature spore-forming stages (most probably conidia, according to Stewart Merrill and Cáceres, 2018) were not considered in spore load calculations. We can therefore conclude that late exposure to myclobutanil alleviated the infection and delayed host mortality, but was not able to completely suppress infection signs, suggesting it is not as effective as tebuconazole (sensu Cuco et al., 2017b).

The inhibition of complete sporulation (i.e., production of mature spores) observed under continuous and late exposure to myclobutanil – and tebuconazole (Cuco et al., 2017b) – translates into a lack of viable transmission stages. Under field conditions, this could cause the end of epidemics, interfere in disease dynamics, or compromise the coevolutionary arms race between host and parasite. Although typical field concentrations of azoles do not normally reach values in the μg/L range (see review by de Souza et al., 2020), maximum values of these substances (de Souza et al., 2020) can match or surpass the concentrations causing effects in this laboratory model (tebuconazole IC_{50} = 4.0 μg/L, Cuco et al., 2020; myclobutanil IC_{50} = 44 μg/L, this paper). In these cases, there is a real scenario of potential disturbance of epidemics and host-parasite dynamics in the field, which may be aggravated by the additive effects of azole mixtures (Kahle et al., 2008), even if low concentrations of the individual substances are recorded (ng/L range). More subtle effects can also occur, as there are evidences of negative transgenerational effects in the fitness of parasite transmission stages, even if sporulation is successful under exposure to azole fungicides (see Cuco et al., 2020). The broader consequences of such effects are still not fully understood for this host-parasite model. More research is thus needed, especially because a unifying theory is lacking about the ecosystem-level impacts of pollution and other anthropogenic changes on parasite transmission and disease (Cable et al., 2017; Wood and Johnson, 2015). This is a much-needed venture, as there is now increasing evidence that parasites are ecologically far more important than their cryptic nature might suggest (Frenken et al., 2020; Kuris et al., 2008; Wood and Johnson, 2015).

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CRediT authorship contribution statement

Cláudia Machado: Methodology, Investigation, Software, Formal analysis, Writing – original draft. **Ana P. Cuco:** Methodology, Software, Formal analysis, Writing – original draft. **Fernanda Cássio:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Justyna Wolinska:** Conceptualization, Methodology, Resources, Writing – review & editing. **Bruno B. Castro:** Conceptualization, Methodology, Software, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

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