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Effects of Seven Fungicides on Non-Target Aquatic Fungi

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Abstract Aquatic risk assessments for fungicides are carried out without information on their toxicity to non-target aquatic fungi. This might cause an underestimation of the toxic effects to the aquatic fungal community. This study focuses on the question whether recently derived concentrations limits for fungicides considered to protect populations of primary producers and (in)vertebrates also do protect the aquatic fungi. A panel of fungal species and Oomycetes was isolated and identified from unpolluted surface waters in the Netherlands. Toxicity tests were used to determine effects of seven fungicides with different modes of actions. For the triazoles epoxiconazole and tebuconazole, the chronic lowest observable effect concentration was lower than the regulatory acceptable concentration based on acute HC5 values.

Keywords Fungicide · Risk assessment · Azoxystrobin · Aquatic fungi · Epoxiconazole

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1 Introduction

Fungicides are meant to control fungal pathogens, but effects on non-target aquatic fungi are not taken into account in risk assessments underlying the authorization of these chemicals. Only few studies give some information on this topic (Bärlocher and Premdas 1988; Chandrashekar and Kaveriappa 1994). Risk assessments often just assume that effects on non-target fungi can be extrapolated from data obtained from the routinely tested aquatic organisms. This in contrast with the scrutiny common for assessments of ecotoxicological effects for other groups of organisms. Some fungicides are non-specific and also affect other organisms (Van den Brink et al. 2007; Stenersen 2004) while others as imidazoles and triazoles specifically affect the biosynthesis of ergosterol. As ergosterol only occurs in fungal membranes, the toxicity of triazoles to non-target fungi should be taken seriously.

Recent work (Maltby et al. 2009) studies which of the three taxonomic groups used in pesticide risk assessments (vertebrates, invertebrates, primary producers) should be the prime target for fungicide studies. They concluded that the derived lower-limit HC5 or the median HC5 divided by an assessment factor of 3 were always protective of adverse ecological effects on aquatic primary producers, invertebrates and litter breakdown in semi-field studies. However, the risk for the fungal community is still a point of concern, and the authors conclude

that further research on the effect of fungicides to non-target fungi is necessary.

In this study, we performed toxicity tests with six non-target aquatic fungi and two oomycetes (*Pythium*) and determined the effect of seven fungicides with different modes of actions. Our results demonstrated that fungicide HC5 values calculated on basis of species sensitivity distributions (SSDs) constructed with acute toxicity data of other aquatic organisms than fungi do not always protect the aquatic non-target fungi.

2 Materials and Methods

Samples were obtained from four surface waters in The Netherlands at large distances from known sources of fungicides such as glasshouses and agricultural crops. Samples were collected in 1-L sterile flasks and subsequently filtered through a 0.45-μm membrane filter (Whatman, Dassel, Germany) and plated out on growth media including malt extract agar (MEA), dichloran 18% glycerol agar and oatmeal agar (OA) (Samson et al. 2004), some of them including antibiotics. All cultures were incubated for 5–14 days at 24°C in the dark. Pure cultures of dominant species were used for DNA isolation and the internal transcribed spacers were obtained using the V9G en LS266 primer set (Gerrits van den Ende and De Hoog 1999). The obtained sequences were used in similarity searches on the NCBI nucleotide database and internal databases of the CBS Biodiversity Centre. Oomycetes were isolated by means of baits (snakeskin or hemp seeds) in water samples (from Crous et al. 2009).

For toxicity testing, four abundant fungal species were selected and deposited in the CBS culture collection. In addition, two aero-aquatic fungi (from the CBS culture collection and originating from The Netherlands) and two isolates of *Pythium* spp. were added to the test panel.

Seven fungicides were used: carbendazim, chlorothalonil, fluazinam, imazalil, epoxiconazole, tebuconazole, and azoxystrobin. Stock solutions were made in demineralised water (carbendazim, fluazinam, chlorothalonil) or DMSO (imazalil, epoxiconazole, tebuconazole, azoxystrobin). Solvent controls were included. Maximum test concentrations were based on available ecotoxicity information. An overview of all species and fungicides (and their mode of action) is presented in Table 1.

Table 1 Overview of species and fungicides used, including the maximum concentration tested (mg/l)

Fungal species		Fungicides							
		Medium	Carbendazim	Chlorothalonil	Fluazinam	Imazalil	Epoxiconazole	Tebuconazole	Azoxystrobin
Mode of action	Phylum		1	2	3	4	4	4	3
Cryptococcus flavescens	Basidiomycetes	MM MEB	8.2	0.26	0.26	210	315	262	235
Trichoderma hamatum	Ascomycetes	MM MEB	8.2	0.26	0.26	210	315	262	235
Fusarium sporotrichioides	Ascomycetes	MM MEB	8.2	0.26	0.26	210	315	262	235
Mucor hiemalis	Zygomycetes	MM MEB	8.2	0.26	0.26	210	315	262	235
Pythium spp isolate 1	Oomycetes	PDA	5.0	0.2	0.2	10	10	10	5
Pythium spp isolate 2	Oomycetes	PDA	5.0	0.2	0.2	10	10	10	5
Helicoon richonis	Ascomycetes	MEA	–	–	–	10	10	10	5
Helicodendron tubulosum	Ascomycetes	MEA	–	–	–	10	10	10	5

MM minimal medium revised from de Vries et al. 2004, with $(NH_4)_2SO_4$ instead of $NaNO_3$ and supplemented with a Vischniac trace elements solution; *MEB* malt extract broth (Samson et al. 2004, p. 380); *MEA* malt extract agar; *PDA* potato dextrose agar; – not tested; *1* cell division; *2* multisite; *3* energy production; *4* ergosterol synthesis

Test series consisted of 12–24 different concentrations using a dilution factor of 2 in either minimal medium or a complex-rich medium (malt extract broth). As light causes degradation of fluazinam and azoxystrobin, all tests were carried out in the dark. Precultures of *Cryptococcus flavescens*, *Trichoderma hamatum*, and *Mucor hiemalis* were grown on MEA and *Fusarium sporotrichioides* on OA for 12–14 days at 24°C in the dark. Conidia, sporangiospores, or yeast cells were harvested after addition of ice-cold ACES buffer (pH 6.9, with 0.05% Tween-80), filtered through sterile glass wool and washed twice in buffer and 10^4 spores/cells were added to each well. The fungal cells were incubated for 24 h at room temperature before addition of the fungicides to evaluate the effect on growing fungal cells. All tests were, at least, carried out in duplo. Growth was qualitatively assessed using four categories (no growth at all; some growth; medium growth; no inhibition of growth). Data were used to determine NOEC and EC_{100} values. It is possible to derive an extrapolated EC_{50} as the average of the NOEC and the EC_{100}, but this is not the same quantitative EC_{50} with confidence intervals. EC_{10} values are even more difficult to extrapolate and are regarded as outside the scope of this note.

The *Pythium* isolates and the aero-aquatic fungi had to be tested on agar plates, since no dense spore solution could be produced. Small pieces (diameter 3 mm) of an active growing culture on potato dextrose agar (*Pythium*; 12–14 days old) or 0.1% malt extract agar (*H. richonis* and *H. tubulosum*; 16–21 days) were used as test inoculum on agar plates amended with fungicides. Agar plates were incubated at room temperature, and growth was visually evaluated after 3–6 days (*Pythium*) or after 7–14 days of growth (aero-aquatic species).

3 Results and Discussion

Table 2 summarizes the effect of seven fungicides on a panel of six species of water-associated fungi and two isolates of *Pythium* sp. Negative effects on the growth of different fungi were observed in 48 out of the 74 toxicity tests performed. In 35, the concentration range was wide enough to estimate both NOEC and EC_{100} values. For chlorothalonil, no NOEC could be estimated. This study aimed to be a first overview to see which fungicides and fungal species might need most attention in further research. Despite this limitation, data can be used to derive NOEC and EC_{100} values and compare them with the threshold levels derived by Maltby et al. (2009).

The tested organisms were least sensitive to carbendazim and imazalil, with NOEC values generally in the range of 0.1–25 mg/L. Lowest effect ranges for fluazinam, epoxiconazole, tebuconazole, and azoxystrobin were often one to two orders of magnitude lower and generally varied between 1 μg/L up to 1 mg/L. Fungi were especially sensitive for epoxiconazole, since three species even showed effect in the lowest concentration tested (1 μg/L).

Our data concerning NOEC and EC_{100} values for aquatic non-target fungi are compared to the range of median EC_{50} values for fish, invertebrates and primary producers. The broad-spectrum fungicides carbendazim, chlorothalonil and fluazinam are characterized by NOEC and EC_{100} values which were generally in line with the data provided by Maltby et al. (2009). Van Wijngaarden et al. (2010) addressed the effect of fluazinam indirectly on the ecological activity of fungi in microcosmos studies and measured an effect on the decomposition of leaf material with a NOEC at the 50 μg/L level after 28 days. The HC5 value of these anti-fungals is indeed protective enough since all NOEC values for the non-target aquatic fungi are well above the HC5 value.

For epoxiconazole, tebuconazole and azoxystrobin, some of the tested species showed a higher sensitivity with NOEC and even some EC_{100} values below the median EC_{50} values as reported by Maltby et al. (2009), which unfortunately did not present data on imazalil, for which this comparison was therefore not possible. Four out of the six fungal species studied (*Pythium* is an oomycete and does not contain ergosterol, whose synthesis is the primary target of those triazoles) showed NOEC values below the HC5 value. This difference was more than a factor of 10 for either three (epoxiconazole) or two (tebuconazole) fungal species.

The two *Pythium* isolates showed a relatively low sensitivity for fungicides with a marked exception for azoxystrobin where *Pythium* was clearly the most sensitive organism. The two aero-aquatic fungi (*H. richonis* and *H. tubulosum*) exhibited relatively high NOEC values for the triazoles and azoxystrobin. Differences between media were also observed;

Table 2 Results of the toxicity tests, specifying NOEC and EC_{100} (mg/l)

Fungal species	Fungicides								
	Medium	Effect level	Carbendazim	Chlorothalonil	Fluazinam	Imazalil	Epoxiconazole	Tebuconazole	Azoxystrobin
Median HC5[a] (mg/L)			0.008	0.006	0.008	–	0.014	0.238	0.042
Cryptococcus flavescens	MM	NOEC	>8.2	>0.26	0.06	26	**<0.001**	**0.008**	0.46
		EC_{100}			>0.26	210	19	33	235
	MEB	NOEC	>8.2	>0.26	>0.26	–	–	–	–
		EC_{100}							
Trichoderma hamatum	MM	NOEC	0.26	>0.26	0.06	0.41	**<0.001**	**0.008**	0.46
		EC_{100}	2.0		>0.26	52	0.039	8.2	59
	MEB	NOEC	0.26	>0.26	0.13	0.41	0.039	**0.008**	0.9
		EC_{100}	2.0		>0.26	52	79	8.2	117
Fusarium sporotrichioides	MM	NOEC	1.0	>0.26	0.06	3.3	**<0.001**	**0.13**	**0.029**
		EC_{100}	4.1		>0.26	105	1.2	4.1	117
	MEB	NOEC	2.0	>0.26	0.13	3.3	**0.010**	**0.032**	0.12
		EC_{100}	4.1		>0.26	26	79	131	>235
Mucor hiemalis	MM	NOEC	>8.2	>0.26	0.06	26	**0.005**	0.5	0.23
		EC_{100}			0.26	>210	>79	131	15
	MEB	NOEC	>8.2	>0.26	>0.26	26	0.31	**0.07**	**0.014**
		EC_{100}				210	79	131	235
Pythium spp isolate 1	PDA	NOEC	>5.0	>0.2	0.1	>10	>10	>10	**0.002**
		EC_{100}			>0.2				5.0
Pythium spp isolate 2	PDA	NOEC	>5.0	>0.2	0.1	>10	>10	>10	**0.002**
		EC_{100}			>0.2				0.10
Helicoon richonis	MEA	NOEC	–	–	–	0.5	1.2	0.5	>5.0
		EC_{100}				10	10	10	
Helicodendron tubulosum	MEA	NOEC	–	–	–	0.1	0.2	0.5	>5.0
		EC_{100}				>10	>10	>10	

NOEC values in bold are lower than derived HC5 values

MM minimal medium; *MEB* malt extract broth; *MEA* malt extract agar; *PDA* potato dextrose agar; – not tested

[a] See Maltby et al. 2009

NOEC values for the toxicity tests in malt extract broth were higher in 10 out of 18 valid comparisons (five cases were equal, three lower).

For epoxiconazole and tebuconazole, it can therefore be concluded that the HC5 values as derived using acute toxicity data for other taxa (Maltby et al. 2009) are not protective enough for the fungal community. Based on the general principles for decision-making concerning the risk of plant protection products (EC 1107/2009), it is therefore advised to continue and expand the research on toxicity testing with non-target aquatic fungi and especially focus on the question whether the risk assessment for triazoles should not be based on a fungi-specific SSDs. For example, more aquatic fungi can be isolated and added to our panel of test organisms. There is as yet no member of the Phylum *Chytridiomycota* in the panel, while it is known that these fungi, which are avid cellulose degraders, must be present in the aquatic environment (Wong et al. 1998).

Fungi and oomycetes play a key role in decomposition and nutrient recycling processes in aquatic ecosystems (Wong et al. 1998; Bärlocher 2005; Shearer et al. 2007). Further, these organisms have specific effects on aquatic organisms as parasites and predators of fish, nematodes or other fungi (Dijksterhuis et al. 1994). Given their importance, there is a clear caveat in the knowledge of the effect of fungicides on non-target aquatic fungi.

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