The predictability of mixture toxicity of demethylase inhibiting fungicides to *Daphnia magna* depends on life-cycle parameters

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Abstract

A variety of different fungicides is found simultaneously in surface waters, among which demethylase inhibitors (DMIs) are a major group. The joint toxicity of four DMIs from different chemical classes (Fenarimol, Prochloraz, Triadimefon and Pyrifenox) was investigated in the reproduction test with *Daphnia magna*, following an extended protocol according to ISO 10706. We assessed the toxicity of the DMI mixtures across different endpoints and effect levels and evaluated the predictability of their joint action using Concentration Addition (CA) and Independent Action (IA). The mixture reduced fecundity, delayed molting and caused characteristic malformations in offspring in a concentration-dependend manner which is possibly due to an anti-ecdysteroid action, as previously described for individual DMIs. However, also mixture-specific effects were observed: exposed daphnids reached sexual maturity already after the third juvenile molt, and thus significantly earlier than unexposed daphnids, which needed four juvenile molts to reach maturity. This effect is not caused by any of the DMIs alone. Additionally, the percentage of aborted broods was synergistically higher than expected by either CA or IA. IA underestimates the mixture toxicity for all parameters. The predictive quality of CA differed between life history responses, but was always within a factor of two to the observed toxicity. The parameter “fecundity reduction, counting only normally developed offspring” was the most sensitive endpoint, while the parameter “fecundity reduction, counting all living offspring” was slightly less sensitive. The mixture caused a 90% reduction in fecundity at

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individual concentrations that only provoke 7% effect or less, which calls for a mixture-specific toxicity assessment of DMI fungicides.

Keywords: Mixture toxicity, DMI fungicides, *Daphnia magna*, Concentration Addition, Independent Action, anti-ecdysteroids

1. Introduction

Fungicides are applied in huge amounts to prevent crop losses in agriculture. However, not even one percent actually reaches the target organisms and the remainder enters aquatic ecosystems, for example through run-off events from the field (Racke 2003). Fungicides are also ubiquitously used to treat fungal infections in human and veterinary medicine and may reach surface waters trough municipal effluents (Bodey 1992). As a result, aquatic organisms are exposed towards various fungicide cocktails.

Nowadays, antifungal compounds with diverse modes of action are applied. Fungicides that interfere with sterol biosynthesis, especially the demethylase inhibiting fungicides (DMIs), occupy the most important position on the world fungicide market (Tsuda *et al.* 2004; Krämer 1986). DMIs are a chemically heterogeneous group comprising imidazoles, triazoles, pyrimidines, piperazines and pyridines (Kuck *et al.* 1995). All of them prevent fungal growth by blocking a specific demethylation step in ergosterol biosynthesis, which is driven by 14-α-demethylases. DMIs from several classes are often found together in environmental samples (Wogram 2001; Ewald & Aebischer 2000; Kahle *et al.* 2008; Battaglin *et al.* 2010), also because DMIs are increasingly applied in sequence or in tank mixtures to optimize efficacy (Matthiessen *et al.* 1988; Hollomon & Kendall 1997). Still, the joint ecotoxicology of such DMI cocktails on non-target organism are largely unknown. So far investigations on the combined effects of DMIs focused largely on the design of effective pesticide formulations against target pests, the reduction of resistance development or the discovery of specific mixture ratios producing a higher effectiveness (Karaoglanidis & Karadimos 2006; Hollomon & Kendall 1997).

Ecological risk assessment of pesticides routinely focuses on the evaluation of single substances, providing for example also the basis for water quality criteria (European Commission 2002). However, the relevance of chemical mixtures is increasingly acknowledged (Scientific Committee on Health and Environmental Risks (SCHER) 2010; European Commission 2012b; European Commission 2012a). While the direct testing of mixtures is feasible for selected cases, especially the setting of environmental quality criteria

has to rely for the most part on component-based approaches that use knowledge on the toxicities of the mixture components to predict their joint toxicity. Only these approaches allow a broad prospective toxicity assessment of the multitude of detected or conceivable environmental mixtures. Two approaches based on different conceptual ideas are established for this purpose: Concentration Addition (CA) and Independent Action (IA) (Faust *et al.* 2000; Grimme *et al.* 1996; Backhaus *et al.* 2003; Boedeker *et al.* 1993). CA is based on the idea that all components of a mixture act similarly, having a common mode of action. The concept has its origin in the works of Loewe and Muischnek (1926) and was described by Berenbaum (1985) for a mixture with *n* compounds as

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad (1),$$

where c_i denotes the individual concentrations of substances *1 – n* in the mixture and ECx_i is the effect concentration that alone would cause the same quantitative effect *x* as the mixture. The quotient ci/EC_{xi} is also known as a toxic unit (TU) (Sprague 1970).

The alternative concept of Independent Action assumes that components of a mixture act dissimilarly, having different target sites but are still triggering a common toxicological endpoint. This concept was first formulated by Bliss (Bliss 1939) for binary mixtures and later extended for multiple substance combinations to

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad (2),$$

(e.g. (Backhaus *et al.* 2000a). $E(c_{Mix})$ is the predicted effect of a *n*-component mixture with a total concentration of c_{Mix}, c_i is the individual concentration of compound *1-n* in the mixture and $E(c_i)$ the effect of this concentration if the compound is applied alone.

It has been shown that CA accurately predicts the toxicity of mixtures of strictly similarly acting substances (Faust *et al.* 2001; Altenburger *et al.* 2000; Backhaus *et al.* 2000b), as well as of compounds with a narcotic mode of action (e.g. (Hermens *et al.* 1984). IA has gained substantially less attention in ecotoxicological studies. However, a few studies have shown that it is superior to CA for multi-component mixtures of strictly dissimilarly acting substances (Faust *et al.* 2003; Backhaus *et al.* 2000a). For binary mixtures, IA and CA often predict virtually identical toxicities (Belden *et al.* 2007; Backhaus *et al.* 2004). In particular CA has gained large acceptance and has been proposed as reasonable default approach for regulatory purposes, providing precautious estimates even for chemically heterogeneous mixtures (Faust *et al.* 2000; Junghans *et al.* 2006; Kortenkamp *et al.* 2009; Scientific Committee on Health and Environmental Risks (SCHER) 2010; Backhaus & Faust 2012).

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Despite their known common mechanism of action in fungi, the mechanism of action of DMIs is largely unknown for non-target organisms. There is growing evidence that DMIs interfere with aromatase activity, the enzyme that is responsible for the balance between androgens and estrogens in vertebrates (Zarn *et al.* 2003). Therefore, demethylase inhibiting fungicides have been discussed as endocrine disrupters, interfering with steroid synthesis pathways in non-target organisms (Sanderson 2006). In invertebrates, such as crustaceans, a possible mechanism of action of DMIs is the interference with ecdysteroids commonly known as molting hormones. In a previous study we demonstrated that DMI fungicides belonging to different chemical classes delayed molting and development, reduced fecundity and produced developmental abnormalities among offspring of the freshwater crustacean *Daphnia magna*, presumably related to an anti-ecdysteroid action (Hassold & Backhaus 2009). However, four of the investigated DMIs, namely the pyridine Pyrifenox, the imidazole Prochloraz, the triazole Triadimefon and the pyrimidine Fenarimol, differed clearly in their toxicity profiles: the piperazine Triforine did not exert any toxic effect on *Daphnia magna*, while Fenarimol and Triadimefon (but none of the other DMIs) caused eye malformations in offspring. Because of such clear differences in the toxicity profiles we suggested at least partially dissimilar mechanisms of action for the investigated DMIs (Hassold & Backhaus 2009). This might be important for the predictive accuracy of the presented concepts and raises the question whether CA would be adequate to predict the mixture toxicity of DMIs or whether IA would be superior. It is known that the choice of endpoint may determine the outcome and quality of predictions due to differences in the susceptibility of physiological processes affecting the endpoint (Cedergreen & Streibig 2005; Barata *et al.* 2006; Jonker 2003). Moreover, the predictive ability of both concepts might be hampered as DMIs are known to interact, causing synergistic or antagonistic combination effects as shown by several authors (Hollomon & Kendall 1997; Noergaard & Cedergreen 2010; Cedergreen *et al.* 2006).

The aim of the present study was therefore to investigate the joint toxicity of representatives from the four main DMI classes (the pyridine Fenarimol, the imidazole Triadimefon, the triazole Prochloraz and the pyrimidine Pyrifenox) with presumably diverse mechanismus of action to *Daphnia magna.* The class of Piperazines were not included, as the only representative of this class, Triforine was non-toxic to *Daphnia magna* in concentrations up to its water solubility (Hassold & Backhaus 2009). We comparatively assessed the predictive accuracy of the concepts CA and IA across different life history parameters, analyzing fecundity reduction, percentage of malformed offspring, percentage of aborted broods as well as the developmental delay (molting and maturity).

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2. Materials and Methods

2.1 Culture conditions and test procedure

Experiments were conducted with *Daphnia magna* Straus from a synchronous laboratory culture (clone B, Bayer, Monheim, Germany, obtained from the Helmholtz - Centre for Environmental Research - UFZ in Leipzig, Germany). Single substance and mixture experiments were conducted on the basis of an extended three week reproduction test according to ISO guideline 10706 (ISO 2000). A detailed description of culturing and test procedures is provided in Hassold & Backhaus (2009).

2.2 Test substances

Fenarimol, Triadimefon, Prochloraz and Pyrifenox were obtained from Riedel de Haën as Pestanal® analytical standards (stated purity 90 - 99.8%). For both the single substances and the mixture, appropriate geometric dilution series were prepared in HPLC-grade Methanol and stored at -20°C. Aliquots of these methanolic solutions were evaporated under a gentle stream of nitrogen and subsequently re-dissolved in M7 medium. Hence, no additional solvent was used for the preparation of test medium to prevent unwanted combination effects. Concentrations were checked regularly using rp-HPLC. Nominal concentrations were in overall agreement with measured concentrations and proved to be stable over time in single substance and mixture experiments. For the mixture, measured concentrations of the highest concentration tested were in overall agreement with the nominal concentrations (Fenarimol:126.5%, Triadimefon 96.6%, Pyrifenox 90,9% and Prochloraz 110.6% of the nominal concentrations). Details on the preparation of test solutions, the analytical validation of the test concentrations and results from the chemical analyses for the single substances are provided in (Hassold & Backhaus 2009). Throughout the paper we refer to nominal concentrations.

2.3 Experimental Design

Single substances were previously tested in 3 to 4 independent experiments, providing complete concentration-reponse relationships (Hassold & Backhaus 2009).

The mixture was tested following a fixed ratio design, by keeping the molar ratio (p) of the substances constant throughout the experiments and varying the total concentration of the mixture systematically. Components were mixed in relation to the previously determined EC_{50} estimates of the single substances (fecundity reduction considering normally developed

offspring after 21 days of exposure). EC_{50} were 0.76 µM for Prochloraz, 1.14 µM for Fenarimol, 3.15 µM for Pyrifenox and 5.13 µM for Triadimefon (Hassold & Backhaus 2009). Hence, the mixture was composed of 7.5 % Prochloraz, 11.2 % Fenarimol, 30.9% Pyrifenox and 50.4 % Triadimefon.

Mixture toxicity testing was identical to the single substance experiments. For the mixture 12 different concentrations between 0.19 and 5.68 µM were tested, covering the concentration range between EC_1 and EC_{99} as predicted by both concepts (see below). 5 replicates were used for the treated samples, 15 replicates of the untreated controls were set up and the following life history traits were recorded: number of normally developed offspring, number of malformed offspring and number of fully aborted broods during the exposure time of 21d, as well as the time needed to complete the juvenile molts, to reach maturity (deposition of eggs in brood pouch for the first time), and to release the first brood. All offspring were inspected under a binocular microscope and classified either as normally or abnormally developed. They were judged abnormally developed, when the shell spine was not fully extended, antennae were not fully developed and/or the eye was missing or malformed (see figure 5 B-E). In some cases broods were completely aborted in an early developmental stage without living individuals (Figure 5F), which was recorded as well.

The observed life-cycle characteristics were condensed into the following test parameters: the cumulative number of living offspring produced after 21 days (fecundity reduction relative to controls), the fraction of malformed offspring in the total number of neonates, the fraction of fully aborted broods (with dead, not developed offspring) in the total number of broods per individual as well as days to reach maturity, time to the first reproductive event, or to complete the first four molts, respectively (all parameters expressed as delay, relative to controls).

2.4 Data analysis

Data was checked for normal distribution and homogeneity of variances using SPSS® 15.0 (SPSS, Chicago, IL, USA). Statistical significances for the % individuals that reached maturity after 3 days were checked using the Kruskal Wallis test and the Mann and Witney *U* test for pairwise comparisons.

Data was normalized to the arithmetic mean of the controls. For the parameters fecundity reduction, the percentage of malformed offspring and of aborted broods, data was fitted with a two-parametric Weibull model ($E(conc)=1-\exp(-\exp(\theta_1+\theta_2 \log_{10}(conc)))$). Data for the time needed to reach maturity, complete four molts or release the first brood were expressed as

time delay in days, relative to the controls and fitted with a two-parametric Power model (E(*concn*)= $\theta_1 \bullet concn^{\theta 2}$). All fits were implemented in SAS proc nlin (Cary, US, vers. 9.2). Predictions of mixture toxicity according to CA and IA were conducted according to Backhaus et al. (2000a).

The fits of the experimentally determined mixture toxicity were compared to both predictions. For the delay data only CA predictions were calculated, as IA conceptually assumes input data on a 0-1 scale (0 to 100% probability). In order to calculate confidence intervals for the predictions of the test parameter fecundity reduction, a bootstrap method was used to estimate the distribution for both predictions on the basis of the empirical data (Scholze *et al.* 2001), using SAS (Cary, US, vers. 9.2). The index of prediction quality (IPQ) was used as measure for deviations of the experimental data from the predictions for a better comparison at different effect levels according to Grimme et al. (1998). IPQs were calculated as EC_{pred}/EC_{obs}-1 if the predicted effect was bigger or equal to observed values and $-EC_{obs}/EC_{pred}$ + 1 otherwise.

3. Results

3.1 Fecundity reduction

According to the chronic reproduction test with daphnids (ISO 107069), the inhibition of reproduction is usually expressed on the basis of the total number of living offspring produced during 21 days of exposure. Due to the occurrence of malformed neonates after exposure to the DMI fungicides, we decided to differentiate between the total number of all living offspring (i.e. including living malformed individuals) and the number of normally developed living offspring. The latter might be more relevant for assessing the impact on the ecological fitness of a population of daphnids.

Concentration response curves for the 4 individual DMIs as well as the experimentally determined mixture toxicity for the parameter fecundity reduction (considering only the normally developed offspring) are presented in Fig. 1 and Table 1. For the mixture an experimental EC_{50} of 2.86 µM was estimated, which falls between the EC_{50}s of the most toxic DMI, Prochloraz (EC_{50}: 0.76 µM) and the least toxic DMI, Triadimefon (EC_{50}: 5.13 µM). This effect concentration for the mixture (experimental EC_{50} of 2.86 µM), is quite well predicted by CA (predicted EC_{50} value of 2.55 µM). In contrast, IA clearly underestimates toxicity with a predicted EC_{50} value of 6.76 µM. At lower effect levels CA overestimates the mixture toxicity slightly with a predicted EC_{10} of 1.31 in comparison with the experimental EC_{10} of 2.09].

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The parameter fecundity reduction was the most sensitive of the investigated endpoints, when only normally developed offspring were considered. The alternative endpoint fecundity reduction based on all living offspring (according to ISO 10706, i.e. including malformed living individuals) was slightly less sensitive and deviations of experimental data from predictions of CA were somewhat larger (Table 1). Although the differences are significant on the level of the EC50 and EC90 (table 1), the absolute differences are (with a factor of less than 1.5) rather small.

3.2 Percentage of malformed living offspring

The DMIs caused concentration-dependent malformations in the F1 generation. The resulting concentration response curves for both single substances and the mixture were extremely steep (Figure 2). An EC_{50} of 3.31 µM was determined for the mixture, which is slightly higher than the EC_{50} for the parameter fecundity reduction (2.86 µM). Again, the mixture EC50 falls into the span between the lowest (1.01 µM for Prochloraz) and the highest EC_{50} (6.8 µM for Triadimefon). CA provided very accurate predictions of the mixture toxicity over the entire concentration range with a predicted EC_{50} of 3.37 µM. In contrast, IA clearly underestimated toxicity at all effect levels with a predicted EC_{50} of 9.90 µM (Figure 2 and Table 1).

3.3 Completely aborted broods

Exposure to any of the individual DMIs, except Triadimefon (which was applied in concentration up to 10 µM), lead to an arrest of offspring development in very early stages and a complete abortion of a certain percentage of broods in a concentration-dependent manner (Figure 3A) (Hassold & Backhaus 2009). This effect was also observed after exposure to the mixture (Figure 3B). Again, concentration response curves were very steep. An EC_{50} of 3.6 µM was calculated on the basis of the experimental data. This is clearly lower than the predicted EC_{50} of 5.3 for CA respectively 14.5 µM for IA, indicating synergistic effects, i.e. higher toxicities than expected by both concepts (Figure 3 and Table 1).

3.4 Delay to complete juvenile molts and reach maturity

Unexposed daphnids reached sexual maturity, i.e. depositing eggs in the brood pouch for the first time, with the completion of the fourth molt. Exposure to any of the four DMIs delays the time to complete the fourth molt (Figure 4A). Also the DMI-mixture delays the fourth molt in a concentration-dependent manner, an effect which is well predictable by CA (Figure

4B and Table 1). At the highest tested mixture concentration (5.7 µM), the 4th molt was delayed by 4 days.

But the mixture delayed the onset of sexual maturity (deposition of eggs in the brood pouch) at this concentration by only 0.5 days (Figure 4C and Table 1). The time at which the fourth molt is completed and the time at which sexual maturity is reached diverge, because mixture-exposed daphnids skip one molt and reach sexual maturity already with the third juvenile molt. The percentage of animals showing this behavior follows a clear concentration dependence (Figure 4D). It should be emphasized that in none of the single substance tests daphnids reached maturity already with the third juvenile molt, and as a result CA fails to predict the effects of the DMI mixture for the parameter "delay to reach maturity" (Figure 4C).

3.5 Developmental malformations

The DMI mixture provoked all the different types of embryo abnormalities that were also observed in the single substance experiments (Figure 5) and are in accordance with the abnormalities observed by Kast-Hutcheson and his coworkers for the DMI fungicide Propiconazole (Kast-Hutcheson *et al.* 2001). Minor embryo abnormalities such as not fully extended shell spines (Figure 5E) were already observed at mixture concentrations ≥1.04 µM. The concentrations of the single substances present in the mixture at this concentration (0.12 µM Fenarimol, 0.08 µM Prochloraz, 0.32 µM Pyrifenox and 0.52 µM Triadimefon) did not provoke any embryo abnormalities if applied singly. The lowest individual concentrations that cause unextended shell spines are 0.15 µM for Fenarimol, 0.25 µM for Prochloraz, 1.02 µM for Pyrifenox and 3.49 µM for Triadimefon (Hassold & Backhaus 2009).

Eye malformations, which are characteristic for an exposure to Fenarimol or Triadimefon, were observed at mixture concentrations ≥2.74 µM, corresponding to ≥0.31 µM Fenarimol and ≥1.38 µM Triadimefon and were hence already caused at lower single substance concentrations when present in the mixture than when tested singly: In single substance experiments eye malformations occurred first at concentrations ≥1 µM Fenarimol or ≥4.4 µM Triadimefon, respectively. The observed eye malformations ranged from an eye that was either not developed at all, a tiny black spot or protruding eyes (Figure 5 B, D, and E), indicating different or disrupted stages of development as also observed by (Champlin & Truman 1998). At the two highest mixture concentrations (4.5 and 5.7 µM) offspring were completely aborted in early developmental stages (Figure 5F).

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3.6 Contributions of single substance concentrations to the mixture toxicity

Figure 6 compares the overall mixture effect with the individual effects that the single substances would provoke if applied singly at the concentration at which they are present in the mixture. At a mixture concentration of 3.1 µM 50% of the neonates showed malformations, while the underlying single substance concentrations (0.23 µM Prochloraz, 0.30 µM Fenarimol, 0.96 µM Pyrifenox and 1.6 µM Triadimefon) did either not provoke any malformation at all (Fenarimol, Pyrifenox) or provoke malformations in less than 0.5% of the population (Prochloraz, Triadimefon) (Figure 6A). Even at the EC90 of the mixture a similar picture emerges: each individual compound provokes less than 1% effect at the concentration at which it is present in the mixture (Figure 6A). Also for the parameter "fecundity reduction" concentrations that individually caused a maximum of 7% effect resulted in 90% effect of the mixture (Figure 6B).

4. Discussion

4.1 Qualitatively new mixture effects

Although the DMI mixture triggered mostly the same fundamental life history responses as each of the single fungicides, it caused a qualitatively new mixture effect not observed in any of the experiments with individual DMIs: mixture exposed daphnids skipped one juvenile molt and deposit their first brood already with the completion of the third molt. This results in an earlier onset of reproduction and enhanced offspring production during the exposure time of 21d at lower (non-embryotoxic) mixture concentrations, which followed a clear concentration-dependent pattern. This novel effect type indicates an interference with egg maturation and development and points towards a specific interaction of the components in the mixture. Furthermore, the synergistic (more than additive or expected) joint effect observed for the parameter "percentage of aborted broods" after exposure to the DMI-mixture indicating an increased embryotoxicity at higher concentrations supports the assumption of interactions between the components, which could take place at the toxicokinetic or toxikodynamic level.

Several azole fungicides (i.e. imidazoles and triazoles) have already been reported to interact in mixtures, enhancing their combined toxicity beyond additivity at certain mixture ratios in target-organisms (Hollomon & Kendall 1997). Other DMIs, (Prochloraz and Propiconazole) have also been reported to act synergistically in combination with other pesticides on non-target organisms (Thompson 1996; Cedergreen *et al.* 2006; Schmuck *et al.* 2003; Pilling & Jepson 1993; Levine & Oris 1999; Bjergager *et al.* 2011). Andersen et al. (2009) showed that

Prochloraz enhances the toxicity of the pyrethroid Esfenvalerate 7 fold in daphnids in relation to predictions by CA (Bjergager *et al.* 2012). A common hypothesis for these synergisms is the inhibition of cytochrome P450-driven insecticide biotransformation by the DMI, leading to an increased concentration of the insecticide at the target site under conditions of mixed exposure (Rider & LeBlanc 2005; Thompson 1996).

However, should similar interactions take place in the present fungicide mixture (i.e. a mutual inhibition of the biotransformation of the DMIs in the mixture), deviations from the conceptual predictions should consistently occur across all endpoints, which was not the case. Hence, an interaction with biotransformation processes does not seem to be a likely explanation for the observed deviations from the predictions provided by CA and IA for the endpoints “aborted broods” and “time to sexual maturity”. Instead, a counteraction of the components on the receptor, hormone or enzyme level as proposed by (Mu & LeBlanc 2004) seems a more likely explanation for the unexpected mixture effects. As it was shown in our previous study (Hassold & Backhaus 2009) and also indicated by others, (Ankley *et al.* 2005; Kinnberg *et al.* 2007), DMIs are known to elicit complex patterns of actions and differ with respect to their effects produced possibly due to slightly differing mechanisms of action. Therefore, they might affect a larger variety of targets sites and different ecdysteroids that are responsible for different developmental processes.

A visual inspection of the neonate individuals indicated severe developmental malformations ranging from unextended shell spines to missig or protruding eyes. At high mixture concentrations a high number of broods was fully aborted and the aborted broods comprised only dead and poorly developed individuals. It is hence reasonable to assume that the broods are aborted not because of effects on the mother animal, but because of effects on the embryo itself. Taking this high embryotoxicity together with the observed synergisms with respect to the parameter “percentage of aborted broods”, the higher occurence of eye malformations after exposure to the mixture and the enhancing effect on egg development and onset of reproduction, strongly indicates that specific interactions between the DMIs take place during the early developmental stages. This concurs with the known specific interferences of DMIs with ecdysteroid-mediated processes, which are largely responsible for regulating the major developmental processes in daphnids, including egg maturation, embryonic development, molting, growth and reproduction (Subramoniam 2000). This is also in concordance with the observation that DMIs may induce the maturation of oocytes in vertebrates (Monod et al. (2004) and might interfere with egg development of arthropods, which is under regulation by ecdysteroids (Subramoniam 2000; Barata & Baird 2000).

4.2 Environmental hazard assessment of DMI mixtures

Pronounced mixture effects of DMIs on reproduction were caused by concentrations, at which the individual DMIs would not have exerted any or only minute effects. This demonstrates once more that it is insufficient to set water quality criteria on the basis of single substance assessments as e.g. discussed by (Vighi *et al.* 2003). Recently, this was also acknowledged by the (Scientific Committee on Health and Environmental Risks (SCHER) 2010) and the communication of the EU commission (European Commission 2012a), as well as the concepts for mixture assessment were included in the draft guidance to derive environmental quality standards in the context of the water framework directive (European Commission 2011).

Despite the clear differences in toxicity profiles of the four differing DMI fungicides (Hassold & Backhaus 2009), Concentration Addition provided a sound and accurate prediction of mixture toxicity for the majority of test parameters. In particular the endpoints “percentage of malformed offspring” as well as the “time delay to complete the 4^{th} molt” were perfectly described by CA at all effect levels. Independent Action did never provide a good prediction of the experimental mixture toxicities. On the contrary, it consistently underestimated the actual toxicity of the mixture.

Applying CA to the standard endpoint that is suggested by ISO 10706 (fecundity reduction counting all living offspring) results in an estimated mixture EC50 of 3.2 µM, while the observed EC50 is 3.0 µM. However, the endpoint “fecundity reduction counting only normally developed offspring” is slightly more sensitive, the observed and predicted EC50 values are 2.9 and 2.6 µM (observed and predicted, see table 1). Although all those values are not significantly different from each other, this higher sensitivity indicates that care should be taken in future studies to appropriately include sublethal developmental effects in the toxicity assessment. This is in particular true as the endpoint “fecundity reduction counting only normally developed offspring” is more ecologically relevant, assuming that alive but malformed offspring might not contribute to the stability of a daphnia population in the wild. Although a clearly synergistic toxicity was observed for the endpoint “percentage of aborted broods”, the absolute value (3.6 µM) is higher than the CA-based EC50 for fecundity reduction. It can hence be tentatively concluded that the cumulative and quantitative hazards of DMI fungicides for daphnids can be estimated by CA, even though the DMIs belong to different chemical classes.

Mixtures containing DMI fungicides warrant assessment as the compounds are ubiquitously used in fungicide mixtures and are known to reach environmental compartments together with a number of other chemicals. Concentration Addition provided sound and very accurate predictions of mixture toxicity for the standard endpoint "fecundity reduction" describing chronic toxicity towards daphnids – even despite the differences in toxicity profiles and interactions among DMIs. The analysis of different endpoints was nevertheless crucial in this study as it revealed qualitatively novel adverse effects that would not have been discerned during the standard test protocol and furthermore revealed interactions between the mixture components. It seems crucial to consider possible embryo malformations as well as alterations of developmental time and molting for substances suspected to interfere with ecdysteroid-mediated processes although it would in a standard test normally not justify the extensive effort needed for visually inspecting all neonates. However, interactions either between DMI fungicides as shown in this study or between fungicides and insecticides (see above) certainly warrant further investigation. There is a clear need to further refine the limits of the application of CA for mixtures involving DMIs, as all studies consistently show that the presence of these compounds violates one of the fundamental assumptions of CA, i.e. that no interactions between the mixture components occur.

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Figures and Tables

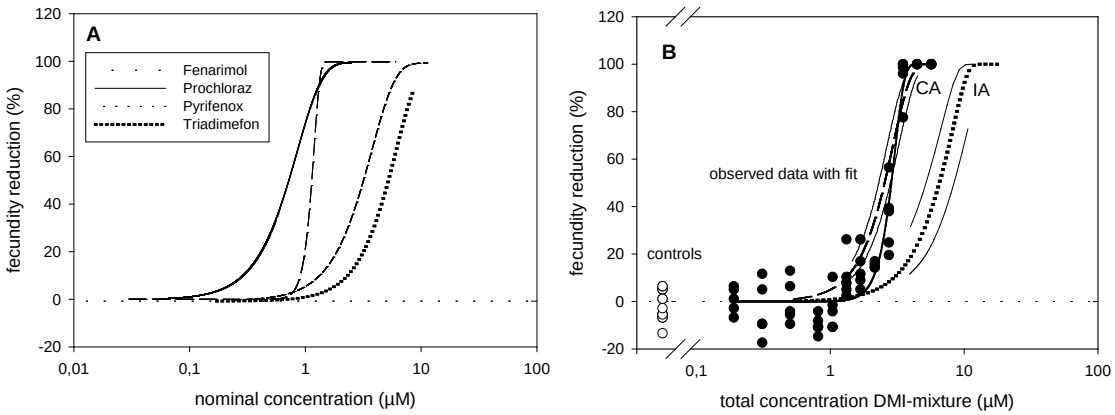
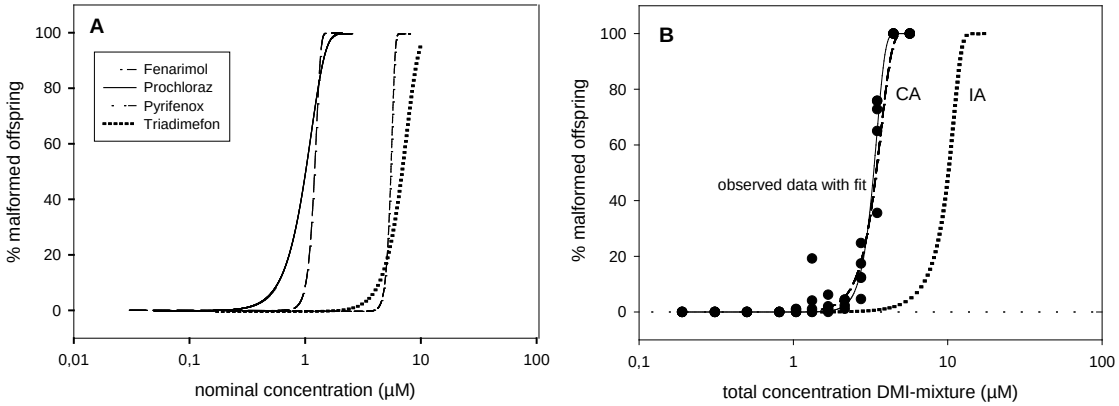


Fig. 1. Single substance and mixture toxicity for the parameter fecundity reduction, considering the cumulative number of normally developed offspring after 21 days of exposure. **A** Concentration response curves of the single test substances, for details see Hassold & Backhaus, 2009. **B** Experimental data for the 4-compound mixture. Solid line: fit to the data, dashed line: CA-prediction, dotted line: IA-prediction (both with estimated confidence bands at the 95% percentile).



673
674
675 **Fig. 2.** Single substance and mixture toxicity for the percentage of malformed living offspring during 21 days of
676 exposure. **A** Concentration response curves for the single substances. **B** Data points and fitted curve for the
677 mixture experiment with predicted concentration effect curves, provided by CA (dashed line) and IA (dotted
678 line).
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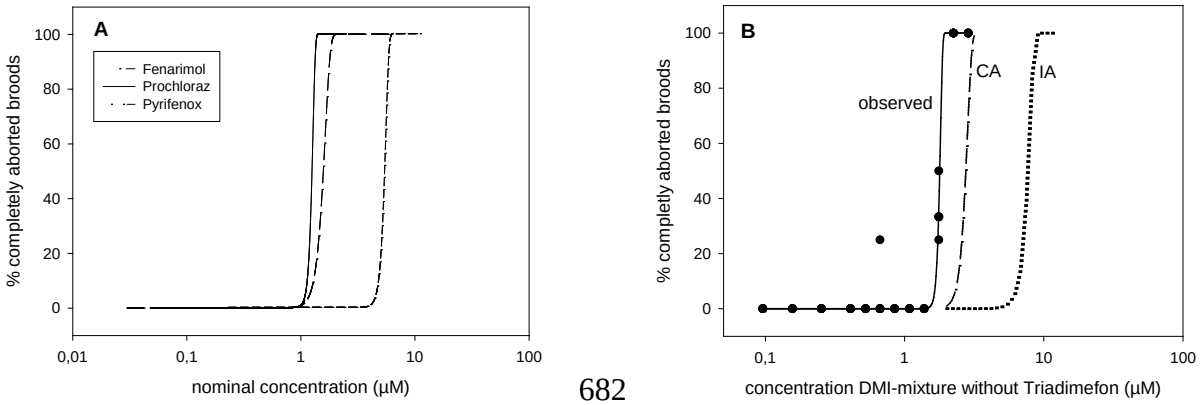

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684 **Fig. 3. Single substance and mixture toxicity for the percentage of completely aborted broods A**
685 Concentration-response relationships for the single substances. **B** Experimental data fit and predictions provided
686 by CA and IA. As Triadimefon did not cause completely aborted broods at the tested concentrations it was not
687 considered in the predictions.

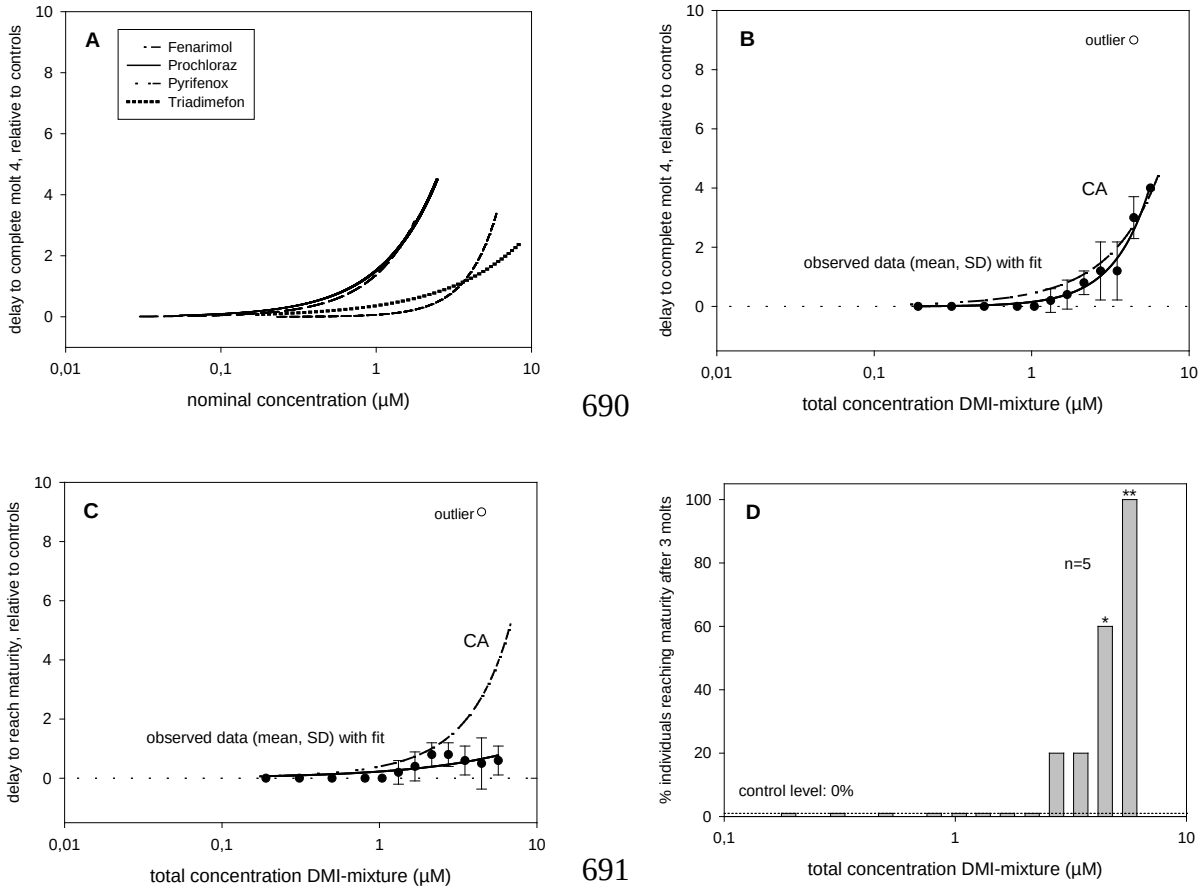

Fig. 4. A Concentration-response relationships for the single substances for the parameter "delay to complete 4 molts, relative to controls. Curves are identical for the parameter "delay to reach maturity" (indicated by the first deposition of eggs in the brood pouch). **B** Experimental data from mixture experiment and predictions based on CA for the "delay to complete 4 molts", relative to controls (controls needed 6 ± 0 days). **C** Experimental data from mixture experiment and predictions based on CA for the "delay to reach maturity". **D** Percentage of individuals that left out one molt and reached maturity after 3 molts during the mixture experiment. Significant differences/trends in comparison with controls are indicated with asterics (U-test, **p<0.001, respectively *p=0.056).

710 **Table 1.** Observed and predicted mixture toxicity at different effect levels, regarding different endpoints. Data was either fitted with a two parametric Weibull model respective a
711 two-parametric Power model. Effect concentrations (EC_{10}, EC_{50}, EC_{90} as well as concentrations needed to produce a delay of x day(s)) are given in µM with confidence intervals
712 at the 95% percentile in brackets. Predicted effect concentrations were based on CA and IA. Confidence intervals for the predictions were only obtained for the most sensitive
713 parameter fecundity reduction (normal developed offspring). The deviations from observed mixture toxicity are indicated with the "Index of Predictive Quality" (IPQs) for better
714 comparison ("-": under-estimation, "+": over-estimation).

Effect level	**Effect concentrations (µM)**			**IPQ**		**Model parameter**	
	Observed [95% CI]	**CA**	**IA**	**CA**	**IA**	$\hat{\theta}_1$	$\hat{\theta}_2$
Fecundity reduction (normal developed offspring)							
EC_{10}	2.09 [1.91–2.56]	1.31 [-1.31-1.45]	2.97 [1.91-2.56]	-0.60	+0.42	-6.6584	13.7806
EC_{50}	2.86 [2.77– 2.96]	2.55 [2.31-2.81]	6.76 [5.2-8.4]	-0.12	+1.36		
EC_{90}	3.50 [3.30– 3.67]	3.75	9.90	+0.07	+1.83		
Fecundity reduction (all living offspring)							
EC_{10}	2.28 [2.01–2.49]	1.27	2.14	-0.80	-0.07	-6.8616	12.8820
EC_{50}	3.19 [3.08–3.30]	2.97	6.54	-0.07	+1.05		
EC_{90}	3.96 [3.76–3.97]	4.68	10.54	+0.18	+1.66		
% malformed offspring							
EC_{10}	2.60 [2.46 – 2.74]	2.38	6.76	-0.09	+1.6	-9.6816	17.9217
EC_{50}	3.31 [3.25–3.38]	3.37	9.90	+0.02	+1.99		
EC_{90}	3.86 [3.76 – 3.97]	4.13	11.97	+0.07	+2.10		
% aborted broods							
EC_{10}	3.30	4.63	12.00	+0.40	+2.64	-32.8675	58.9974
EC_{50}	3.56	5.34	14.52	+0.50	+3.08		
EC_{90}	3.73	5.85	15.97	+0.57	+3.28		

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Table 1 continued

Delay time to complete 4 molts, relative to controls							
0.5 day	1.68 [1.22-2.27]	1.12	-	-0.50	-	0.2030	1.7840
1 day	2.46 [1.94-3.00]	2.01	-	-0.22	-		
2 days	3.62 [3.17-4.02]	3.49	-	-0.04	-		
Delay time to reach maturity, relative to controls							
0.5 day	3.08 [1.91-x 5.68]	1.23	-	-1.50	-	0.2275	0.7073
1 day	-	2.14	-	-	-		
2 days	-	3.58	-	-	-		
Delay time to release first brood, relative to controls							
0.5 day	0.93 [0.47-1.49]	1.12	-	+0.20	-	0.5200	0.5569
1 day	3.25 [2.49-4.72]	2.01	-	-0.62	-		
2 day	-	3.43	-	-	-		

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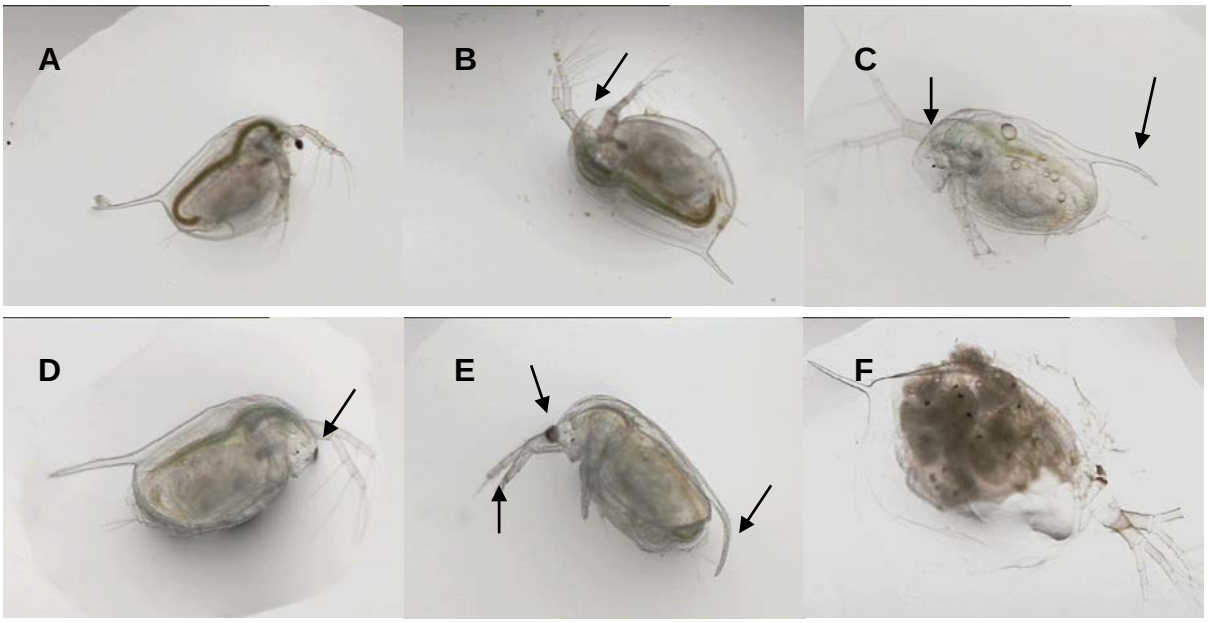

Fig. 5. Neonates, exposed *in mater* to the DMI-mixture, with developmental malformations (photographed at age of approx. 3 days). **A** normally developed control daphnid. **B** eye apparently not developed. **C** eye apparently not developed, tiny spot, slightly curved shell spine. **D** Eye not fully developed, small spot. **E** eye protruding, sticking out, poorly extended shell spine, underdeveloped antennae. **F** exuvium with aborted embryos in earlier stage (eye is developed).

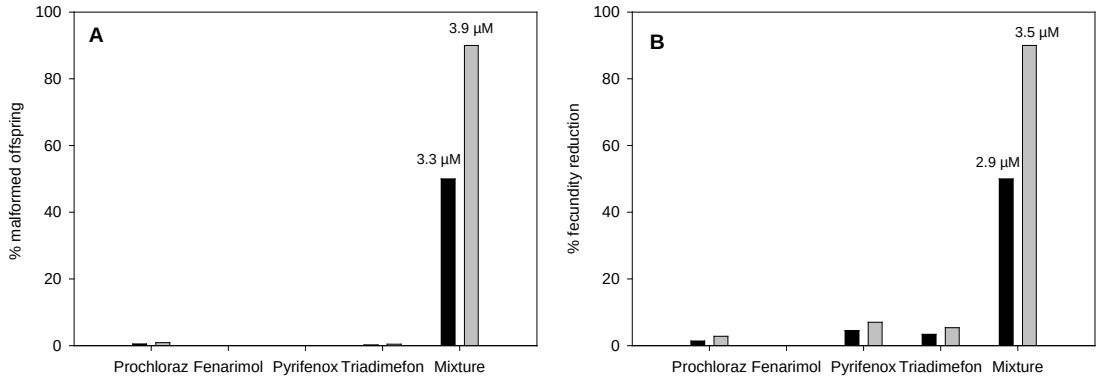

Fig. 6. Comparison of the individual effects, caused by each of the DMIs at those concentrations at which they are present in the mixture (7.5 % Prochloraz, 11.2 % Fenarimol, 30.9% Pyrifenox and 50.4% Triadimefon) and their joint effect at the indicated effect levels 50% (black) and 90 % (grey) for the parameters malformed offspring (**A**) and fecundity reduction (**B**).

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