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Influence of the Soil Composition on the Effects of Benzoxazinoid Allelochemicals on Two Soil Nontarget Organisms

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Seven selected benzoxazinoid allelochemicals and synthetic reference compounds were tested for their lethal and sublethal effects in different field soils and standard soil on *Folsomia candida* and *Poecilus cupreus* by applying standard laboratory test procedures. The higher microbial activity in the field soils was most probably responsible for the reduced effects of test compounds on *F. candida* in the majority of all tests, whereas the higher organic carbon content in field soils was likely the reason for the reduced effects of test substances on *P. cupreus*.

KEYWORDS: Benzoxazinoids; DIMBOA; MBOA; BOA; structure-related substances; laboratory testing; side effects; *Folsomia candida*; *Poecilus cupreus*

INTRODUCTION

The cultivation of crops producing and releasing compounds with allelochemical properties to protect themselves from diseases, pests, or weeds could present an important alternative in organic farming (*1*). However, arable crops such as cereals usually cover a large production area, and the release of benzoxazinoid allelochemicals could have adverse effects on nontarget soil organisms. Up to now, no studies about side effects or potential hazards of benzoxazinoid allelochemicals on nontarget soil organisms have been reported in the literature. Thus, in the EU project FATEALLCHEM ecotoxicological trials with some benzoxazinoids and their selected degradation products on the springtail species *Folsomia candida* Willem (Collembola: Isotomidae) and the carabid beetle species *Poecilus cupreus* (L.) (Coleoptera: Carabidae) were conducted (*2*, *3*). Both selected test organisms are listed among the recommended standard test soil macroorganisms according to the Annexes to EU Council Directive 91/414/EEC (*4*) for the authorization of plant protection products.

As well as for side-effect testing with pesticides, the applied methods should be standardized and validated, both to allow the detection of potential adverse effects of the test compounds on the test organisms and to avoid false positives (*5*). Besides, the testing of compound effects on nontarget organisms in soil can be influenced by several factors, such as the composition and characteristics of the test substrate, the test duration, the developmental stage of the test organism, the characteristics and bioavailability of the test compound, and the type of application of the test compound among others (*6*−*9*). Thus, topical application of selected test compounds on the standard substrate resulted in significantly higher mortality rates of *P. cupreus* larvae than did incorporation of the test compounds into the substrate (*10*).

In the present study, the lethal and sublethal effects of selected allelochemical compounds and reference pesticides on *F. candida* and *P. cupreus* were tested in two field soils from different cultivation regimes and were compared with the effects in standard substrate from previous studies.

MATERIALS AND METHODS

Materials. *Test Substances.* As test compounds the benzoxazinoid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), its degradation product 6-methoxybenzoxazolin-2-one (MBOA), the benzoxazolinone benzoxazolin-2-one (BOA), the structure-related herbicidal compound benazolin (4-chloro-2-oxobenzothiazolin-3-ylacetic acid), and its formulated product Cresopur (4-chloro-2-oxobenzothiazolin-3-ylacetic acid) were applied. In addition, the two reference substances Betanal Plus EC (phenmedipham) (methyl-*m*-hydroxycarbanilate−methylcarbanilate) and Perfekthion S EC (dimethoate) (*O*,*O*-dimethyl-*S*-[2-(methylamino)-2-oxoethyl]phosphorodithioate) were tested compared to the water-treated control (**Table 1**).

The dose rates of the applied treatments were chosen either from results in previous studies (*2*, *3*) or because they were prescribed in the standard test methods or because they represented the maximum supposed field rates.

Test Substrate. Two field soils with different compositions, which were derived from organic and conventional wheat cultivation in Denmark, were used in the trials (**Table 2**).

Test Organisms. For the tests on Collembola, instead of juveniles (*11*), 18 ± 4-day-old mature individuals of *F. candida* (Willem, 1902) (Collembola: Isotomidae) were used to avoid a control mortality exceeding the validity criterion of 20% (*12*−*15*).

For the carabid beetle test 24−48-h-old first-instar larvae of *P. cupreus* (L.) (Coleoptera: Carabidae) from a test validation strain were used (*16*).

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Table 1. Survey of Tested Substances on *F. candida* and *P. cupreus*[a]

acronym	systematic name	chemical group	mode of action (supposed)	source	test rate in both field soils (mg/kg of dry substrate)	
					F. candida	*P. cupreus*
		Benzoxazinoids				
DIMBOA	2,4-dixydroxy-7-methoxy-2*H*-1,4-benz-oxazin-3-one	hydroxamic acid	insecticide fungicide herbicide	UCA	2.00	2.00
MBOA	6-methoxybenzoxazolin-2(3*H*)-one	benzoxazolinone	insecticide fungicide herbicide	UCA com-mercial	2.00	2.00
BOA	benzoxazolin-3(3*H*)-one	benzoxazolinone	insecticide fungicide herbicide	commercial	2.00	2.00
		Common Name Structure-Related Substances				
benazolin (ai)	4-chloro-2-oxobenzothiazolin-3-ylacetic acid	unclassified	herbicide	commercial	545.42	not tested
Cresopur (ai benazolin)	4-chloro-2-oxobenzothiazolin-3-ylacetic acid	unclassified	herbicide	commercial	592.34	500.00
		Trade Name Reference Substances				
Perfekthion S (ai dimethoate 400 g/L)	*O*,*O*-dimethyl-*S*-[2-(methyl-amino)-2-oxoethyl]-phosphorodithioate	organophosphate	insecticide acaricide nematicide	commercial	0.17	0.26
Betanal Plus (ai phenmedipham 157 g/L)	methyl-*m*-hydroxycarbanilate–methylcarbanilate	carbanilate	herbicide	commercial	104.60	1.20

[a] UCA, University of Cadiz; ai, active ingredient.

Table 2. Soil Characterization of Both Field Soils (Organic and Conventional) in Comparison with the Standard Soils for *F. candida* and *P. cupreus*

selected parameter[a,b]	unit	Danish soils		standard soils	
		org	conv	*F. candida*	*P. cupreus*
organic C	%	1.10	1.45	5.00	1.04
total nitrogen	%	0.11	0.15	0.001	0.002
phosphorus	mg/1000 g	92.00	45.00	8.09	111.92
sand (<2000–63 μm)	%	61.00	27.00	<34.00	84.00
silt (<63–2 μm)	%	30.00	51.00	>35.00	12.40
clay (<2 μm)	%	9.00	21.00	20.00	3.60
sphagnum peat	%			10.00	
water content	%	9.10	13.90	27.82	9.0

[a] Soil depth from 0 to 25 cm. [b] Analyzed according to DIN NORM.

Method and Conduct of the Test on *F. candida.* The study was carried out according to the international standard ISO 11267 (*11*). The test objective was to detect potential lethal (acute toxicity; mortality) and sublethal (reproduction) effects of the test substances on adults of *F. candida* compared to control and reference treatments applied to soil, which was derived from two fields with different cultivation. The tests were carried out in standard plastic containers (65 mm in height; 75 mm ∅ at the bottom and 95 mm ∅ at the top) sealed with a plastic lid. As standard test substrate, a mixture of spaghnum peat (10%), kaolinite clay (20%), and industrial quartz sand (68−69%), adjusted by $CaCO_3$ (0.5−1%) to a pH of 6.0 ± 0.5, and a water-holding capacity of 46−48% w/w, was used (**Table 2**). The test containers were kept under controlled climatic conditions at a temperature of 20 ± 1 °C, a relative humidity of 70−90%, and a photoperiod of 16 h ligh/8 h dark (∼400−800 lx). The validity criteria applied, which are usually prescribed for studies in standard test substrate, comprised a mean mortality of adults in the control samples of <20% at the end of the test, a minimum reproduction in each control container of ≥100 juveniles within 4 weeks, a coefficient of variation of <30% of reproduction in the control samples, and significant effects of the reference substance Betanal Plus EC (phenmedipham) at 100 and 200 mg/kg of dry substrate on the reproduction.

Range Finder Test. Because the present study was part of a research project, the applied test substances had already been tested in previous range finder tests on *F. candida* in standard soil according to ISO 11267 (*2*, *3*) prior to the present trials.

Final Test. The test concentrations representing the lowest observed effect concentration (LOEC), the next lowest LOEC, and the LC_{50} values of the test substances from the range finder tests, as well as the reference treatments and the water control, were applied to the field soils in five replicates each per treatment and soil type and filled into the test containers. Ten adults of *F. candida* per test container were exposed to the test substances. Four weeks after the start of the trial, the number of organisms was counted to evaluate the effects of the test substance on mortality and reproduction. After the test substrate of each container had been submerged in water and stirred with a spatula, the numbers of adults and juveniles (F1), which had hatched from the eggs produced by the adults, were counted separately.

Effect Values. *The LOEC* is the lowest tested concentration of the test substance at which the substance is observed to have a statistically significant effect when compared with the control.

The NOEC is the next concentration below the LOEC tested with no significant difference versus control.

LC_{50} is the estimated concentration at which 50% of the test organisms are dead at the end of the test.

EC_{50} is the estimated concentration at which the reproduction rate at the end of the test is reduced to 50% compared to the control.

The reproduction rate is the mean number of offspring per test unit after 28 days (F_1 instars emerged from eggs laid by mature adults).

Method and Conduct of the Test on *P. cupreus.* The carabid beetle larval test was carried out according to the method of ref *16*, except that soils from organic and conventional farming fields were used instead of the standard substrate (**Table 2**).

The *P. cupreus* larval test was conducted to detect lethal (mortality) and sublethal (hatching weight, developmental duration, and sex ratio) effects of all test substances compared to the water control.

As test unit a glass vial (25 mm ∅, 70 mm height, capacity of 35 mL) sealed with a plastic lid was used.

As standard test substrate, LUFA 2.1 (LUFA-Speyer) with a content of 1.04% organic C, a pH value of 7.0, and a particle proportion of 84.0% sand, 12.4% loam, and 3.6% clay was used (**Table 2**).

The test vials were filled with 25 g of the standard soil or field soil from either the organic or the conventional cultivation. For the single-dose test 30 replicates with one *P. cupreus* larva each for all tested

substances and the control were used. Each treatment was applied with a pipet to the soil surface in a volume of 100 μL per glass vial. Although the test compounds were applied to the surface, test rates were calculated in milligrams per kilogram of dry substrate due to the assumption that the larvae are very mobile and that they incorporate the substance into substrate. For a period of 30−60 min after the application, randomly selected *P. cupreus* larvae of uniform age were released into each test vial and fed with half of a frozen *Calliphora* sp. pupae. The vials were placed in a dark, ventilated chamber at 20 °C and regularly supplied with food. One week after the first pupae were observed, the vials were checked daily. Beetles hatched within 35−46 days.

The validity criteria, which are usually applied to studies in the standard test substrate, included a maximum mortality rate of 20% in the untreated control group and a mortality rate of 65 ± 35% in the toxic standard group.

Effect Values. *Mortality.* Dead larvae, pupae, and adult *P. cupreus* in or on the soil or missing test organisms at the end of the trial period were assessed as dead.

Hatched beetles were examined for abnormalities (uncoordinated movements, anatomic variations), and the sublethal parameters hatching weight, developmental duration, and sex ratio were recorded.

Hatching weight is the weight of the freshly hatched, but no longer white, beetle.

Developmental duration is the period from the start of the test until the beetle hatched (test end was defined as the 10th day after the first beetle hatched).

Sex ratio is the portion of hatched females from all hatched beetles per treatment.

Calculations. *Adjustment of Mean Mortalities.* Adjustment of results from treated samples to the control was carried out according to the method of ref *17*:

$$x\% = [1 - (t/c)] \times 100$$

In the above equation, x is the adjusted mean mortality rate, t is the mean number of living individuals in the treatment, and c is the mean number of living individuals in the control.

Statistics. Statistical analyses for significant differences between the mean mortality, the mean reproduction, the mean developmental duration, and the mean hatching weight of the different treatments were carried out. Depending on the distribution of the mean values and the number of comparisons, either parametric ANOVA and *T*-test (post hoc Bonferroni test) or nonparametric Kruskal−Wallis test (Collembola, carabid beetles) or the Bonferroni−Holm procedure (carabid beetles) was applied (SPSS 8.0 for Windows).

RESULTS

The applied validity criteria for standard substrate testing were fulfilled in all trials on Collembola with the field soils, whereas the toxic reference treatment on *P. cupreus* did not cause the required mortality rate.

F. candida. The allelochemical DIMBOA and its degradation product MBOA caused higher mortality rates in the test soil of conventional cultivation than in the soil of organic cultivation. Treatment with BOA resulted in only slightly different mortality rates in both test substrates. The structure-related compound benazolin showed higher mortality rates in the conventionally cultivated soil compared to the soil from organic cultivation, whereas the opposite was true for Cresopur (benazolin). The reference substance Perfekthion S EC (dimethoate) caused higher mortality rates in the soil from organic cultivation, whereas Betanal Plus EC (phenmedipham) resulted in mortality rates of 100% in both test substrates.

The reproduction rates in all treatments except DIMBOA and BOA were very similar in the two test soils. The reproduction rate after treatment of DIMBOA was much lower in the conventionally cultivated soil than in the soil from organic cultivation, whereas the reverse was found for BOA (**Table 3**).

Table 3. Lethal and Sublethal Effects of DIMBOA, MBOA, and BOA, Structure-Related Pesticides, as well as Reference Substances on *F. candida* in Danish Organic and Conventional Soils

test substance[a]	LC_{50} (ft)/ EC_{50} (repro)	adjusted mortality/reduction of reproduction in % of control		
		standard	org	conv
DIMBOA				
ft	>2.00	51.20[b]	6.50	26.00
repro	>2.00	79.70[b]	42.37	81.31[b]
MBOA				
ft	>2.00	70.70[b]	41.30	54.00[b]
repro	>2.00	88.40[b]	94.49[b]	93.59[b]
BOA				
ft	>2.0	73.20[b]	8.70	6.00
repro	>2.0	94.10[b]	20.48	123.47
benazolin				
ft	<545.42	100.00[b]	10.84	24.00
repro	<545.42	100.00[b]	74.00[b]	68.20[b]
Cresopur				
ft	>592.34	67.40[b]	30.43	24.00
repro	>592.34	93.00[b]	60.03	69.58
Perfekthion S				
ft	<0.17	50.00[b]	65.20[b]	44.00
repro	<0.17	58.00[b]	66.30[b]	61.53[b]
Betanal Plus				
ft	<104.60	100.00[b]	100.00[b]	100.00[b]
repro	<104.60	95.20[b]	99.86[b]	100.00[b]

[a] ft, final test; repro, reproduction. [b] Mean values significantly different from control (Kruskal–Wallis or ANOVA test; $p < 0.05$).

The pH values of the standard soil (at the start and the end of each trial) varied within a range of 6 ± 0.5 and were in general below the ones of the two field soils (**Figure 1**).

P. cupreus. Treatments with DIMBOA, MBOA, and BOA at the test rate of 2 mg/kg of dry mass did not result in a significantly different mortality rate of *P. cupreus* larvae compared to the control group (**Table 4**). No mortality of *P. cupreus* was observed after treatment with DIMBOA, MBOA, and BOA in soil from organic cultivation. The mortality rate caused by MBOA and BOA in soil from conventional cultivation was below 11%.

Cresopur (benazolin) did not cause any harmful effect on the carabid beetle larvae either, even at the application rate of 500 mg/kg of dry substrate in both field soils.

Treatment with Perfekthion S (dimethoate), the toxic reference insecticide for *P. cupreus* larvae testing, at the established LC_{50} value of 0.26 mg/kg of dry standard substrate (*2*, *3*) resulted in no or very low mortality compared to the control group in the organic soil and in the conventional soil, respectively (**Table 4**).

Betanal Plus (phenmedipham) did not show any significant effect on *P. cupreus* larvae at the test rate of 1.2 mg/kg of dry substrate (which is about the maximum predicted environmental concentration) either in conventional soil or in organic soil (**Table 4**).

No differences in the effect on the sublethal parameters (prolongation of the developmental time of larvae, reduction of the hatching weight of the beetle) could be observed between the treatments and the control group. The portion of females to all hatched beetles per test rate (sex ratio) was excluded from the analysis because of the probability for biased data in the treatments when only a small number of beetles had hatched. No other abnormalities such as uncoordinated movements or anatomic variations were observed.

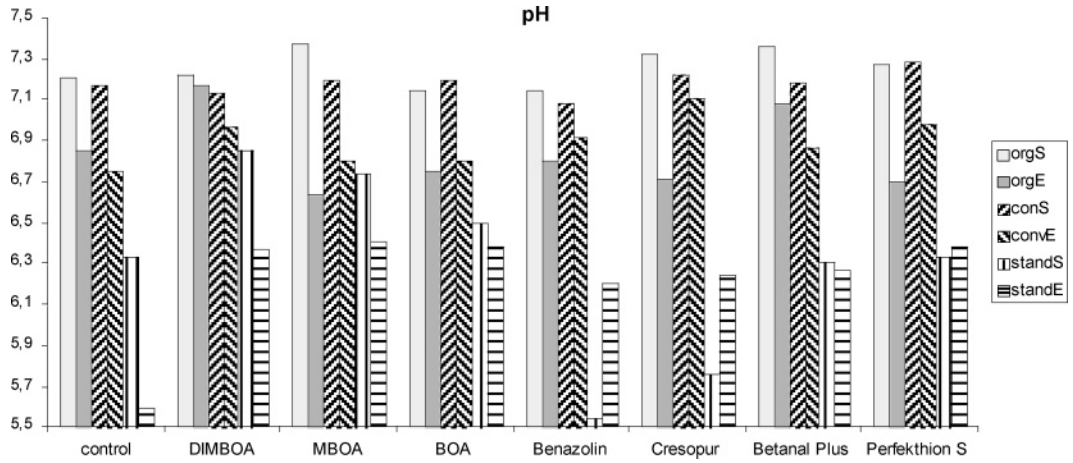

Figure 1. pH values of the two field soils in comparison with standard soil (*F. candida*): S, start of trial; E, end of trial; org, organic crop management; con, conventional crop management; stand, standard soil according to ISO 11267.

Table 4. Lethal and Sublethal Effects of DIMBOA, MBOA, and BOA, Structure-Related Pesticides, as well as Reference Substances on *P. cupreus* in Danish Organic and Conventional Soils

	test rate (mg/kg)	Abbott corrected mortality (%)			hatching wt (mg)		developmental duration (days)	
		org	conv	std	org	conv	org	conv
treatment								
control	0.0	0.0	0.0	0.0	61.1	64.3	39.5	40.0
DIMBOA	2.0	0.0	0.0	10.7	57.5	58.2	40.5	39.4
MBOA	2.0	0.0	7.1	13.8	55.2	62.6	40.3	39.7
BOA	2.0	0.0	10.7	10.7	53.6	62.3	41.4	39.5
Cresopur (benazolin)	500.0	5.3	0.0	0.0	55.2	63.4	39.6	39.8
Perfekthion S (dimethoate)	0.26	0.0	7.1	50.0[a]	59.9	62.8	40.3	38.6
Betanal Plus (phenmedipham)	1.2	10.5	0.0	5.3	61.3	59.4	39.3	39.0

[a] Mean value significantly different from control (Bonfferoni–Holm test; $p < 0.05$).

DISCUSSION

The aim of the present study was to clarify the influence of selected soil parameters on the efficacy of diverse test allelochemicals and reference compounds on two standard soil test organisms.

Because *F. candida* besides feeding on bacteria, algae, fungal hyphae, spores, and pollen also feed on dead plant materials and soil minerals (*18*−*20*), soil composition may directly influence the effects of test substances on *F. candida*. *P. cupreus* larvae have a thin cuticula and are very susceptible to substances dissolved in the soil water phase. As predators, they can be also affected by contaminated prey (for example, Collembola).

It is therefore essential to consider soil components when we compare the results of this study with the ones of former studies (*2*, *3*) conducted on standard soil according to the method of refs *11* and *16*. Special emphasis in this study was given to the potential influence of the soil pH, the microbial activity, and the content of organic C in the soils of different compositions that were used in the trials.

The soil pH is one soil factor that might influence survival and reproduction rates of *F. candida* (*21*, *22*) and was considered only in the studies with the Collembola as the standard method prescribed the adherence to a range of 6 ± 0.5.

Figure 1 shows that pH values of the standard soil both at the start and at the end of each trial for all tested substances remained in a range of 6 ± 0.5, which is below the ones of the two field soils. This range is prescribed in ISO 11267 and is also indicated by Van Straalen and Verhoef (*23*) as the optimal pH range for the subneutral species *F. candida*. As in the standard soil the mortality rates were in most cases much higher and partly the reproduction rates much lower than the ones in the two field soils (**Table 3**), the pH is probably not the most important factor for collembolan survival and reproduction under the influence of toxic substances. In this context, the microbial N/P ratio has to be mentioned, which was more positively correlated to collembolan body growth than to soil pH in the study of Kaneda and Kaneko (*24*). This parameter was not, however, a subject of this study but should be taken into account in further studies when we assume that the positive effect of a higher microbial N/P ratio on collembolan body growth implies also a general positive effect on collembolan survival. Apart from that, Kaneda and Kaneko (*24*) stated that fungi dominate at low pH, whereas bacteria dominate at neutral pH (**Figure 1**). This assumption and the statements of several authors (*24*−*26*) that Collembola are thought to favor bacteria to fungi might make us conclude that the field soils were therefore more dominated by bacteria than the standard soil because of the pH values near 7 (**Figure 1**).

As part of this EU project, Fomsgaard et al. (*27*) detected a lower microbial activity of both standard soils in comparison with the two field soils by the sodium acetate respiration method. For *F. candida* this fact seems to have influenced the population in the field soils in a more positive way than the one in the standard soil, and therefore the effects of the test substances in the field soils were less pronounced than in the standard substrate. The significantly higher mortality rates of BOA and benazolin and its formulated product Cresopur in the standard soil in comparison with the two field soils pointed out the positive effect of higher microbial activity in the field soils (*27*) on the survival of *F. candida*, especially under the influence of

toxic substances. In the tests on *P. cupreus*, the lower effect on the beetle mortality in both field soils than in the standard soil could be observed only for one substance (dimethoate) (**Table 4**), whereas the tested allelochemicals at the predicted environmental concentration of 2 mg/kg of dry substrate did not have any effect in the substrates. It could be assumed that higher microbial activity in field soils than in standard substrate, combined with higher organic C content in field soils, could be an important parameter for the reduced bioavailability and for the detoxification process in *P. cupreus* tests. Unfortunately, there are to date no literature data available dealing with microbial activity as a soil parameter in carabid beetles tests.

Despite soil pH and microbial activity in particular, the organic C content (*28*) could have influenced the mode of action of the treatments. For *P. cupreus* the importance of organic C for the efficacy of pesticide compounds could be confirmed. In the present study, the LC_{50} of the insecticide dimethoate of 0.26 mg/kg of dry standard substrate (1% organic carbon) did not have any effect on *P. cupreus* in the field soil from either organic (1.1% organic carbon) or conventional (1.45% organic carbon) farming. In a comparable study of Heimbach and Soverini (*29*), in which the same *P. cupreus* larval stage, the same application form, and the same active ingredient (dimethoate) as in the present study were applied, the increase of organic carbon by a factor 3 caused the decrease in *P. cupreus* mortality of 50%. Recent studies of Heise et al. (*30*) showed that the increase of organic carbon by a factor of 6 lowered the LC_{50} to half of the value.

Also, when the test compounds were mixed into the soil, the mortality reducing effect of increased organic matter content could be detected for dimethoate (*29*).

Even tests performed with adult beetles, which usually are not only present in the soil but on the soil surface and therefore should not be that susceptible to different soil compositions, indicated that the effects of test substances on the mortality of *P. cupreus* decreased with increased organic matter content (*6*, *31*). This could also be due to differences in the soil water content, which together with a low binding affinity of a hydrophilic compound such as dimethoate leads to a lower accumulated uptake by the carabids (*8*).

For *F. candida* the organic C content did not seem to provide greater tolerance to toxic substances, because of the higher mortality rates in the standard soil despite a much higher organic C content (5%) in comparison with both field soils (**Table 2**). For the survival of *F. candida* the microbial activity of a soil seemed to be a more relevant factor than the organic carbon content.

The differences of lethal and sublethal effects in tests with *F. candida* between the two field soils used in this study were not significant except for DIMBOA, whereas the lethal effects of all treatments in standard substrate (*3*) were more pronounced (**Table 3**). With regard to sublethal effects, no significant differences between the two field soils could be detected except after treatment with DIMBOA and BOA, causing a decrease of reproduction rate significantly higher in the soil under conventional crop management. The degrees of sublethal effects from tests in the field soils and those in the standard substrate were very similar. The ranking of the three allelochemicals regarding lethal effects on Collembola, which was DIMBOA > MBOA > BOA in ref *3*, could be confirmed only in the way that the mortality rates of the BOA treatments in both field soils were significantly lower than the ones of the DIMBOA and MBOA treatments; to the contrary, MBOA caused higher mortality rates than DIMBOA.

These results significantly revealed once again the importance and influence of the test medium on the effects of test substances on *F. candida* and confirmed the classification of the allelochemicals used to be of low risk as assumed by Idinger et al. (*3*). Furthermore, it can be assumed that under field conditions with higher microbial activity the effects of the allelochemicals would be less pronounced than under laboratory conditions and, therefore, negligible.

The present study showed that the effects of allelochemicals on the two soil nontarget organisms highly depended on the soil composition used. The higher microbial activity of both field soils is most likely the factor mainly responsible among others for the lower effects of the allelochemicals on *F. candida*, whereas the organic carbon content is probably the most important factor for *P. cupreus* survival, considering the reduced bioavailability of substances. The influence of other soil parameters, such as pH, depends on the test organism used. The results emphasized the recommendations of Martikainen (*9*) to conduct toxicity studies also in natural soil because of the differences in behavior of chemical compounds in artificial and natural soils.

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