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Ecotoxicological Effects of Benzoxazinone Allelochemicals and Their Metabolites on Aquatic Nontarget Organisms

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Before natural plant allelochemicals can be exploited as biological pesticides against weeds and for disease control, more than the effect on target organisms needs to be known. This study presents results of aquatic biotests using four organisms, namely, a water flea, a freshwater alga, a soil alga, and a luminescent bacterium. The tested substances were 10 benzoxazinone derivatives, 3 of them known to be wheat allelochemicals, benzoxazolin-2(3*H*)-one (BOA), 6-methoxybenzoxazolin-2(3*H*)-one (MBOA), and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA), and 7 identified degradation intermediates and metabolites. For comparison, two commercial pesticide formulations (BAS, Betanal) were tested by applying the same set of biotests. The data set produced could be seen as an ecotoxicological evaluation for effects of allelochemicals against nontarget organisms and as a base for further risk assessment.

KEYWORDS: Benzoxazinones; secondary metabolites; aquatic ecotoxicity; daphnia; freshwater algae; luminescent bacteria

INTRODUCTION

Many scientific facts are known about allelochemicals, especially about their biosynthesis, their appearance in plants, and their effect toward identified target organisms (*1*, *2*). In contrast, little is known about effects on nontarget organisms (no literature found). No broad risk assessment, covering nontarget organisms, has been done for wheat allelochemicals before this study. As long as such substances are not used in a technical scale for plant protection and pest control, it does not appear to be necessary. In the course of an EC-funded scientific project ("FATEALLCHEM"), wheat benzoxazinones were investigated for their suitability as pest control. Therefore, the need to analyze ecotoxicological effects of those chemicals on aquatic organisms arose.

The three already known wheat allelochemicals [benzoxazoline-2(3*H*)-one (BOA), 6-methoxybenzoxazolin-2(3*H*)-one (MBOA), and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA)] and seven identified degradation intermediates and metabolites were tested for their ecotoxicity. For comparison, two commercial pesticides (trade names BAS and Betanal) were analyzed as well.

Four aquatic organisms were chosen, including the marine bacterium *Vibrio fischeri*, the freshwater alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), the soil alga *Chlorella pyrenoidosa*, and the water flea *Daphnia magna*. The stability of the allelochemicals was evaluated by chemical analysis on one of the algae test setups.

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NOEC and EC_{50} values were calculated as typical endpoints, whenever an organism showed a regular dose−response relationship against a test substance. Those ecotoxicity data could and should be used as a base for a risk assessment before wheat benzoxazinoids are exploited in the agricultural practice. However, the final step, a comprehensive risk assessment, was not part of this work.

MATERIALS AND METHODS

The test substances (allelochemicals) are listed in **Table 1** with acronyms used in the following text and in the figures as well as with the full chemical name. The substances were selected after the degradation schemes for BOA and MBOA, as described by Fomsgaard et al. (*3*) and Zikmundová et al. (*4*). The two commercial pesticides were obtained from the Austrian Health Agency (AGES), Vienna. The allelochemicals were obtained from different sources: BOA was ordered from Fluka (article 12825); DIMBOA, MBOA, and all metabolites were obtained from the University of Cadiz (UCA), Institute for Organic Chemistry. Metabolites deriving from the degradation pathways of two of the three parent substances had been selected depending on their availability [synthesis possibilities by UCA, described by Macías et al. (*5*)]. Therefore, not all of the known metabolites could be tested.

Sample Preparation for Use in the Biotests. From preliminary trials it was known that the allelochemicals were not sufficiently water soluble. Ecotoxicity tests could not be performed by dissolving or suspending the substances directly in water. Therefore, the substances were dissolved in ethanol (96 vol %, p.A. grade) or DMSO (p.A. grade). Stock solutions of 40 mg of substance in 5 mL of solvent (equal to 8 g/L) were prepared. From those solutions, dilution series were made in solvent. From each of the prediluted solvent solutions, an amount of 25 μL was added to 100 mL of biotest medium and shaken well. If precipitations were observed after homogenization, such suspensions

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Table 1. Overview of Substances (Allelochemicals, Their Metabolites, and Commercial Pesticides) That Were Tested for Their Toxicity against Aquatic Organisms[a]

acronym/common name	chemical name	MW	type
BAS	dimethoate	229	commercial pesticide
Betanal	phenmedipham	300	commercial pesticide
BOA	benzoxazolin-2(3*H*)-one	135	wheat allelochemical
MBOA	6-methoxy-benzoxazolin-2(3*H*)-one	165	wheat allelochemical
DIMBOA	2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one	211	wheat allelochemical
AP	2-aminophenol	109	BOA metabolite
HPAA	2-acetamidophenol	151	BOA metabolite
HPMA	*N*-(2-hydroxyphenyl)malonamic acid	195	BOA metabolite
APO	2-aminophenoxazin-3-one	212	BOA metabolite
AAPO	2-acetamidophenoxazin-3-one	254	BOA metabolite
AMPO	2-amino-7-methoxyphenoxazin-3-one	242	MBOA metabolite
AAMPO	2-acetamido-7-methoxyphenoxazin-3-one	284	MBOA metabolite

[a] The commercial pesticides were obtained as solutions: Betanal, 157 g/L; BAS 400 g/L. The purity of allelochemicals was ≥98%; their synthesis is described in Macías et al. (*5*).

were not used for testing. The highest concentration without visible precipitation was the individual limit for each substance. With the transfer from solvent into biotest medium, the final concentration of each allelochemical in the test was reached and the same amount of organic solvent (0.025 vol %) was present in each of the test vessels. Due to the weak solubility of the allelochemicals, the upper concentration limit for the biotests was reached at ∼100 μmol/L. Some of the metabolites, especially HPAA and AAMPO, could be tested only in concentrations of <10 μmol/L because the solubility in the organic solvent was already limited. References and controls, which received pure mineral medium with 0.025% pure organic solvent, were analyzed together with the samples in each batch of tests.

Test Organisms. The marine bacterium *V. fischeri* was obtained together with the ready-to-use test kit LumisTox from Dr. Lange (article LCK 486). The freshwater green alga *P. subcapitata* was obtained from ATCC (article 22662, ordered as *Selenastrum capricornutum*). The soil green alga *C. pyrenoidosa* was isolated from a standard soil of the University of Applied Sciences, Vienna, and characterized for morphology and physiological properties according to the method of Bellinger (*6*; procedure described in ref *7*). The freshwater microcrustacean *Daphnia magna* STRAUSS was obtained from the Higher School for Chemistry, Vienna, and kept in continuous culture at our own laboratory. Those four test species were chosen because they are typical representatives of three different trophic levels and were selected to indicate unwanted effects of the allelochemicals on nontarget aquatic organisms. Those organisms are widely used for ecotoxicity and risk assessment in routine analysis, and the test procedures are known to be reproducible (*8*). As an additional benefit, the new results could be compared with a very large set of available data from the literature (*9*).

Standardized Biotests. The ecotoxicity of the allelochemicals and their degradation intermediates was tested using standardized methods. Only minor changes of the standardized working procedures were made (see **Table 2**). Reference substances of known toxicity were potassium chromate ($K_2Cr_2O_7$) for algae and daphnia tests and a 7% sodium chloride solution for the LumisTox test. Together with controls they were used as validity criteria for the physiological status of the test organisms, strictly in accordance with the standard methods. Results obtained from invalid test batches (between 5 and 30% of all trials) were discarded and never used for any further calculation.

The marine bacterium, *V. fischeri*, was exposed to the test substances for the very short time of 30 min. That test has no practical relevance for a soil ecosystem with properties as expected for agriculture. However, the LumisTox biotest should be seen as a model for interactions of substances with the biological energy pathway and can be a representative example for short-term interactions and modes of membrane passages (*8*, *10*).

Because the exposure time in the algae biotest was 72 h, when the algae should have grown through at least six generations, this test could be seen as a chronic test. The green algae are plant homologue microorganisms and could be understood as models for the detection of plant-specific interactions, including the photosynthesis process. The freshwater alga *P. subcapitata* was used for comparability with many data in the literature (*9*), whereas the soil alga *C. pyrenoidosa* could be seen as relevant for the soil ecosystem. The test setup was miniaturized according to the method of Blaise (*11*) and adapted to be applied in 24-well titer plates.

Table 2. Biotests Used for Determination of the Ecotoxicity of Pesticides, Allelochemicals, and Their Degradation Products[a]

test type	method	remark
bacteria, light emission 30 min of contact time	LumisTox (equal to EN ISO 11348-3)	no changes to standard
Ps. sub., growth inhibition 72 h of contact time	OECD 201 (equal to DIN 38412 L33)	2 mL test vol (*11*)
Chl. pyr., growth inhibition 72 h of contact time	OECD 201 (equal to DIN 38412 L33)	2 mL test vol (*11*)
Daphnia, immobilization 48 h of contact time	OECD 202 (equal to DIN 38412 L11)	no changes to standard

[a] The standardized methods (*22*, *23*) were followed for organism conditioning, test size, number or amount of organisms, contact time, and overall physical and chemical properties during the test runtime. All general recommendations were followed (*24*).

The daphnia biotest exposed the organisms to the test substance for 48 h. Compared to the generation time, this is to be classified as an acute test. Again, the model character and the comparability with other literature data were the most important reasons for the selection.

Calculation of Endpoints. EC_{50} values were calculated as typical endpoints using the Weibull eq 1 for curve fit (according to ref *12*). By using the curve parameters received from the fitting function, EC_{50} values could be calculated according to eq 2. NOEC values were read out of the data points and were chosen as the highest test concentration at which a substance did not show any inhibition effect higher than the blind in the same batch.

$$y = 100 \times [a + (1 - a)^*(1 - \exp(-x/b)^c] \quad (1)$$

$$EC_{50} = b \times \ln 2^{1/c} \quad (2)$$

In eqs 1 and 2, y is the inhibition in %; x is the concentration of substance in mol/L; and a, b, and c are equation constants, obtained by curve fitting.

The curve fits were done by including all single-test data, which were between 35 and 112 per biotest type and substance. The reliability of the results could be expected to be very high, although remarkable deviations were observed.

Table 3. NOEC Values (All Data in Micromoles per Liter) As Directly Read from the Results of the Aquatic Biotests[a]

substance	*Da* (48 h)	*Chl* (72 h)	*Pse* (72 h)	*Vib* (0.5 h)
dimethoate (BAS)	2.2	4.4	4.4	8.7
phenmedipham (Betanal)	1.7	0.0067	0.0067	0.033
DIMBOA	9.5	0.0047	0.0047	0.047
MBOA	12.1	1.21	12.1	0.030
AMPO	41	0.21	0.041	0.021
AAMPO	7.0	7.0	0.35	0.018
BOA	15	7.4	7.4	15
AP	0.92	92	0.46	9.2
HPAA	13.3	3.3	13.3	6.6
HPMA	10.3	10.3	10.3	5.1
APO	0.24	0.47	0.094	0.24
AAPO	0.39	0.039	0.39	0.079

[a] *Da*, *Daphnia magna*; *Chl*, *Chlorella pyrenoidosa*; *Pse*, *Pseudokirchneriella subcapitata*; *Vib*, *Vibrio fischeri* (LumisTox).

Table 4. EC_{50} Values (All Data in Micromoles per Liter) As Calculated from the Results of the Aquatic Biotests[a]

substance	*Da* (48 h)	*Chl* (72 h)	*Pse* (72 h)	*Vib* (0.5 h)
dimethoate (BAS)	4.0	>4.4	>4.4	>8.7
phenmedipham (Betanal)	5.3	35	0.34	0.94
DIMBOA	>9.5	>47	>47	44
MBOA	>12	>61	>61	1.3
AMPO	>41	8.8	0.22	68
AAMPO	>7	>7	>7	>7
BOA	>15	>74	>74	>15
AP	3.5	>92	3.0	>92
HPAA	>13	>13	>13	>13
HPMA	>10	>51	>51	>51
APO	1.5	1.3	0.73	9.4
AAPO	10.1	27	1.7	11

[a] *Da*, *Daphnia magna*; *Chl*, *Chlorella pyrenoidosa*; *Pse*, *Pseudokirchneriella subcapitata*; *Vib*, *Vibrio fischeri* (LumisTox).

Chemical Analysis of the Test Substances. Two algae biotest setups were done for the purpose of analyzing the allelochemicals and for estimating the stability and the resulting concentration gradient over the test run time. The two setups were done in parallel, both inoculated with the freshwater algae. The first was interrupted and frozen at −20 °C immediately after preparation. The second was operated as usual (including measurement) and was frozen at −20 °C immediately after the final measurement, 72 h after the setup. The samples were stored frozen and transported over dry ice to be analyzed via HPLC-MS in the laboratory of the Centre d'Investigació i Desenvolupament (CSIC), Barcelona, Spain (*13*).

RESULTS

The results obtained from the aquatic biotests are summarized as NOEC (**Table 3**) and EC_{50} values (**Table 4**) whenever possible. To demonstrate some irregular dose−response relations, for which no or no reliable endpoints could be calculated, some graphs were added.

The dose−response relations of the three wheat allelochemicals are presented in **Figure 1**. BOA caused significant inhibitions to both algae species but without a regular relationship. It did not inhibit the luminescent bacteria and daphnia even at the highest tested concentration, so that no EC_{50} values could be calculated or estimated (data excluded from the figure). Also, DIMBOA showed no regular relationship for the soil algae and did not inhibit daphnia (data points also not shown). A reliable curve fit was obtained for the luminescent bacteria, but because of highly deviating data points only a very weak curve fit for the freshwater algae could be calculated. MBOA caused no inhibition to the daphnia (again, data points were excluded)

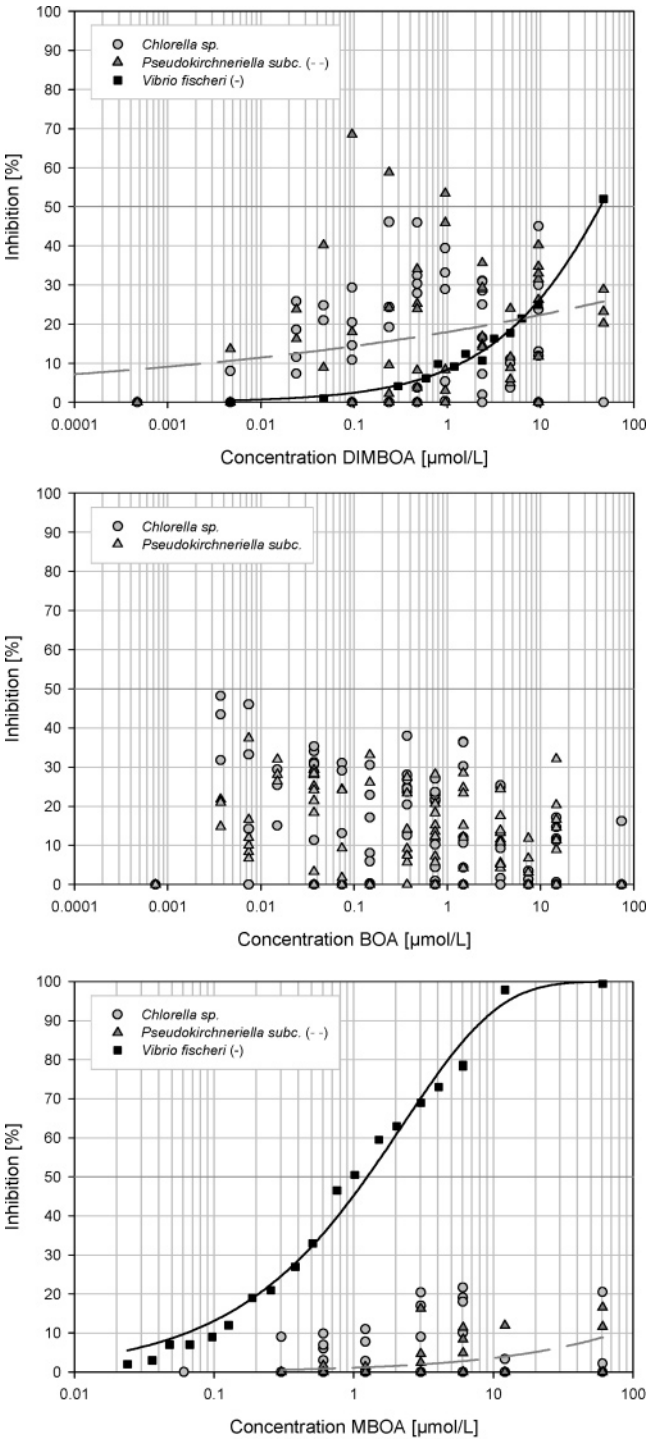

Figure 1. Graphical presentation of the dose–response relationships of the wheat benzoxazinoids DIMBOA, BOA, and MBOA.

and resulted in an irregular pattern for the soil algae. It affected only the luminescent bacteria in a very highly reproducible dose−response relation. The freshwater algae were slightly inhibited by MBOA but, even at the highest tested concentration, not enough to allow the calculation of a reliable EC_{50} value.

In **Figure 2** the inhibition patterns of AMPO and AAMPO, both degradation metabolites of MBOA, are shown. Neither substance affected daphnia. Dose−response relationships of AMPO could be drawn for the two algae, whereas this was not possible for the acetylated metabolite AAMPO. The luminescent bacteria were inhibited slightly in the presence of 0.04 μmol/L of AMPO or 0.035 μmol/L of AAMPO but with no further increase (all points ∼25% inhibition) at higher concentrations.

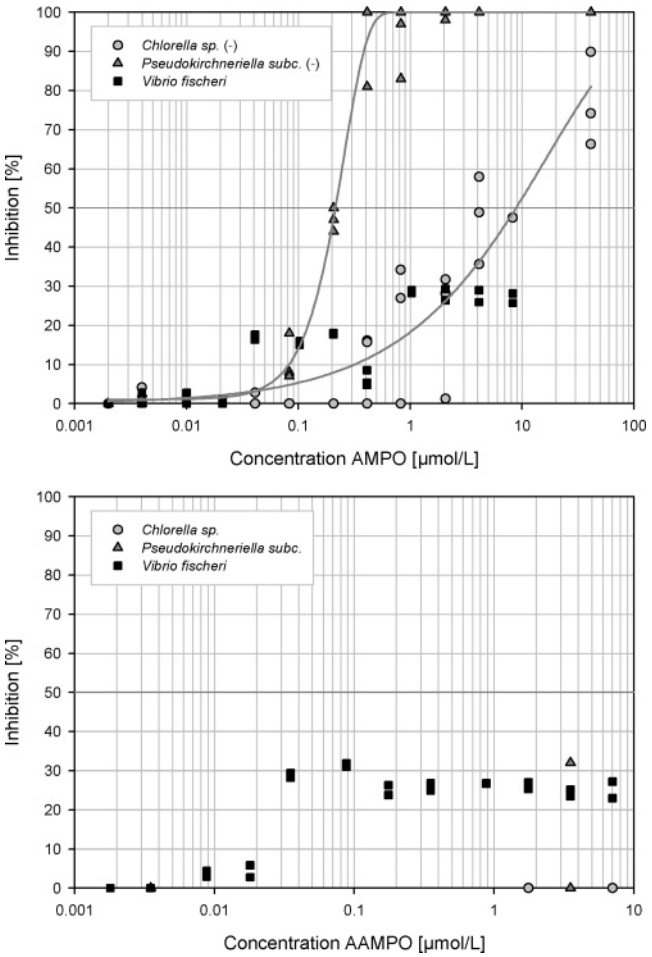

Figure 2. Graphical presentation of the dose–response relationships of the MBO degradation metabolite AMPO and its acetylated follow-up product, AAMPO.

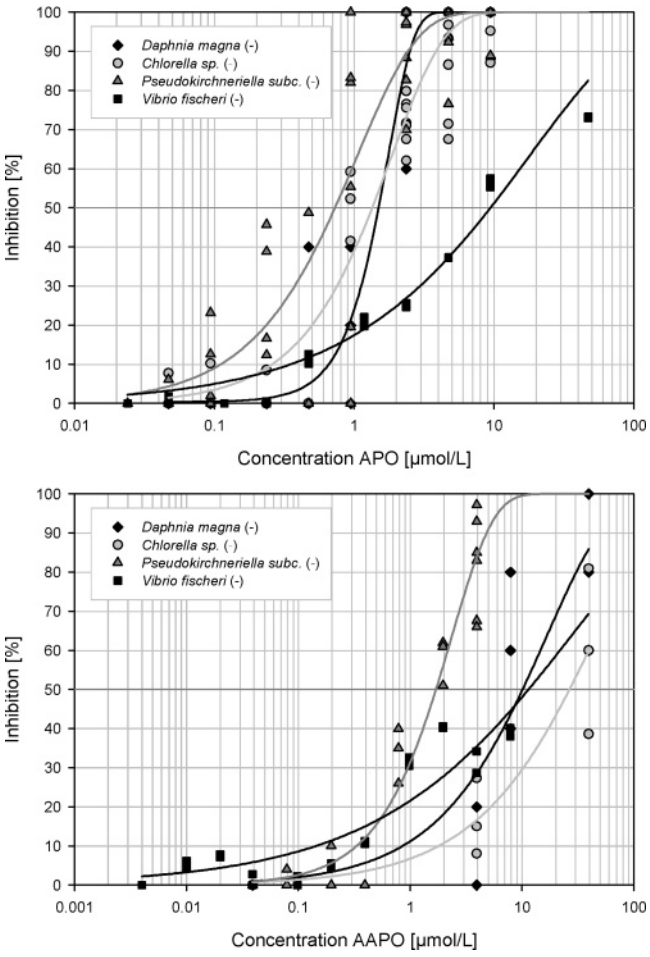

Figure 3. Graphical presentation of the dose–response relationships of the BOA degradation metabolite APO and its acetylated follow-up product, AAPO.

Neither HPAA nor HPMA affected any of the four organisms significantly, although some very weak inhibition effects to algae and luminescent bacteria appeared at the highest possible test concentration. The degradation intermediate AP caused a very steep dose−response relation for daphnia but almost no inhibition for the soil algae and luminescent bacteria. An irregular peak-shaped relationship was obtained for the freshwater algae: a maximum inhibition of 100% was obtained at 10 μmol/L but lower inhibitions appeared at higher and lower concentrations.

Finally, in **Figure 3** the dose−response relationships for APO and AAPO are shown. Clear relationships were obtained, and EC_{50} values could be calculated for all four organisms against both substances. The acetyl-APO (AAPO) generally caused lower inhibitions than APO.

The results of the HPLC analysis of allelochemicals and metabolites from the algae test are shown in **Tables 5** (initial) and **6** (after 72 h of biotest run time). The initial concentration was 2 mg/L for all substances. All substances that could be detected and quantified in the initial setup could also be detected and quantified in the setup after 72 h of run time. Only HPMA and MBOA could be analyzed in concentrations >50% of the initially added amount in both measurements. AMPO, APO, BOA, and DIMBOA were found at low concentrations but were stable over the run time. AP could not be detected, but APO was formed even during the very short time of ∼15 min needed to prepare the test setup.

Table 5. Analysis Values of an Algae Test Setup Immediately after Preparation (Initial Values)[a]

substance	AAMPO	AAPO	AMPO	AP	APO	BOA	DIMBOA	HPMA	MBOA
AAMPO	nd	nd	nd	nd	nd	nd	nd	nd	nd
AAPO	nd	nd	nd	nd	nd	nd	nd	nd	nd
AMPO	nd	nd	0.3	nd	nd	nd	nd	nd	nd
AP	nd	nd	nd	nd	0.35	nd	nd	nd	nd
APO	nd	nd	nd	nd	0.9	nd	nd	nd	nd
BOA	nd	nd	nd	nd	nd	0.73	nd	nd	nd
BW	nd	nd	nd	nd	nd	nd	nd	nd	nd
DIMBOA	nd	nd	nd	nd	nd	nd	nq	nd	0.1
HPMA	nd	nd	nd	nd	nd	nd	nd	1.38	nd
MBOA	nd	nd	nd	nd	nd	nd	nd	nd	1.28

[a] Every well contained one of the allelochemicals or metabolites at a concentration of exactly 2 mg/L. The first column describes the substance initially put into the test; the other columns show the HPLC analysis results. nd, below detection limit (0.02–0.1 mg/L); nq, detected but not quantified; BW, blind test, no substance added.

DISCUSSION

The commercial pesticides showed clear dose−response relationships, as expected. The insecticide dimethoate affected specifically the daphnia with a steep dose−response relationship. The herbicide phenmedipham affected all organisms, especially the freshwater algae; surprisingly the least affected were the soil algae.

The three parent benzoxazinone derivatives, BOA, MBOA, and DIMBOA, showed almost no inhibition effects to all four

Table 6. Analysis Results of the Test As Described in **Table 5** but after 72 h of Test Run Time

substance	AAMPO	AAPO	AMPO	AP	APO	BOA	DIMBOA	HPMA	MBOA
AAMPO	nd	nd	nd	nd	nd	nd	nd	nd	nd
AAPO	nd	nd	nd	nd	nd	nd	nd	nd	nd
AMPO	nd	nd	0.13	nd	nd	nd	nd	nd	nd
AP	nd	nd	nd	nd	0.39	nd	nd	nd	nd
APO	nd	nd	nd	nd	0.91	nd	nd	nd	nd
BOA	nd	nd	nd	nd	nd	0.76	nd	nd	nd
BW	nd	nd	nd	nd	nd	nd	nd	nd	nd
DIMBOA	nd	nd	nd	nd	nd	nd	nd	nd	0.48
HPMA	nd	nd	nd	nd	nd	nd	nd	1.63	nd
MBOA	nd	nd	nd	nd	nd	nd	nd	nd	1.29

test organisms except DIMBOA and MBOA, which caused concentration-dependent inhibitions to the marine bacterium *V. fischeri*. Because of the very short contact time of only 30 min, it can be assumed that those compounds were stable enough to show such an effect. For the other tests, which lasted either 48 or 72 h, the allelochemicals may not have been stable enough to affect the daphnia and algae significantly. In those tests, only irregular dose−response relationships (at a low effect level) were observed. It is assumed that metabolites were formed in a not reproducible way, which caused the deviating results (see also below).

After further testing of seven degradation metabolites deriving from two of the three wheat allelochemicals, significantly higher toxicity was observed for some of them. It was observed that especially the phenoxazinones (APO, AAPO, and AMPO) showed the highest inhibition effects, affecting all four test organisms.

Acetyl-APO (AAPO) as a degradation product of APO and acetyl-AMPO (AAMPO) as a degradation product of AMPO in soil have been detected by several authors (*4*, *14*−*19*). In this study the acetylated compounds showed a lower inhibition effect than their respective nonacetylated parents. For plants a similar result was obtained by Macías (*20*, *21*), suggesting a more general rule in the effect mechanisms of degradation products deriving from benzoxazinones.

The 2-aminophenol affected daphnia and, even with a strange dose−response relationship, also the freshwater algae. During only a few minutes a remarkable amount of APO was formed in the algae biotest environment. Because APO is stable during the test run time, it was most probably the cause for the observed inhibition.

Only some of the used test substances were stable during the 72 h biotest run time; those were MBOA, AMPO, HPMA, and APO. The six other benzoxazinone derivatives were not detectable in the biotest solutions, either out of the initial setup or after 72 h. The analysis results did not allow differentiation between chemical or biological degradation and sorption effects (at the well walls). Some of the substances showed an inhibition effect even though they were not detectable by HPLC. There is no satisfying scientific explanation for that, but it is highly probable that the substances affect the algae cells either immediately after contact or after other metabolites were formed, which caused the observed inhibition but had not been detectable by the chosen chromatographic method.

The original wheat benzoxazinone derivatives, namely, BOA, MBOA, and DIMBOA, showed very low or even no ecotoxicity against the four test organisms (see **Figure 4**). They may be used like natural pesticides with not quantified, but most probably low, risk to the aquatic environment. Especially APO, AMPO, and, to a lower extent, AAPO caused significant ecotoxic effects to the four aquatic organisms. Those three

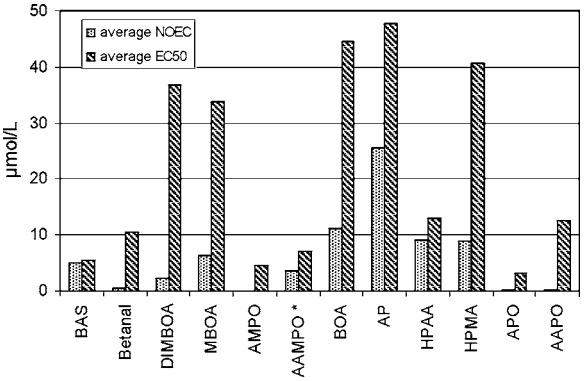

Figure 4. Summary of the endpoints of all four biotests (equally weighted average) for each of the 12 test substances. The lower the bar, the higher the ecotoxic or inhibitory effect. * The bar for AAMPO appears to be very low because the highest possible test concentration (for reasons of solubility) did not cause any significant effect.

substances are documented degradation products of the parent benzoxazinones, but did not appear as such in the biotests. Their formation in soil should be considered, even if only the parent substances are applied. Nevertheless, this conclusion is based on only one analytical follow-up in the course of this study; no final conclusion could be drawn now, and more experiments will be necessary.

In conclusion, first and foremost, it should be mentioned that procedures able to evaluate the ecotoxic potential of allelochemicals and their natural degradation products have been described. Irregular dose−response relationships were obtained in those cases when allelochemicals with limited stability had been tested. Uncertainties about degradation pathways demonstrated the need for a much closer analytical follow-up as it had been possible to do in the course of this study. The 10000-fold concentration between NOEC and EC_{50} values for DIMBOA may underscore this argument. To obtain even more applied results, other test setups could be necessary, maybe adding an inoculum of soil bacteria to the biotest, simulating the possible bioactivation of wheat allelochemicals by the soil microflora. Nevertheless, the data generated in this study could be used for a preliminary risk assessment of wheat allelochemicals if exploited as natural pesticides.

No final risk assessment could be done by the authors because of two facts: first, the degradation pathway described for soil was not reproducible in the aquatic biotests and, second, the applied amounts of the substances in the field and the form of application are not specified now.

It is currently unclear which organisms are the natural targets of the investigated wheat benzoxazinone derivatives. It is further unclear what the ecological meaning of the bioactivation of the allelochemicals could be for the soil environment, because some of the more stable metabolites showed significantly higher toxicities than the parents. It is suggested that further research should be done to fully clarify the degradation pathway of those allelochemicals in the natural soil as well as in the aquatic environment. Specific conditions and the degrading microorganism population in the compartment rhizosphere should be investigated in detail. Finally, mobilization or immobilization due to changes in the polarity of some metabolites may occur in soil, influencing the transport mechanism of allelochemicals into groundwater or other aquatic environments.

ABBREVIATIONS USED

AAMPO, 2-acetamido-7-methoxyphenoxazin-3-one; AAPO, 2-acetamidophenoxazin-3-one; AMPO, 2-amino-7-methoxyphenoxazin-3-one; AP, 2-aminophenol; APO, 2-aminophenoxazin-3-one; BO, benzoxazolin-2(3*H*)-one; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one; EC_{50}, concentration of test substance with 50% effect to test organisms; HPAA, 2-acetamidophenol; HPLC, high-pressure liquid chromatography; HPMA, *N*-(2-hydroxyphenyl)malonamic acid; MBOA, 6-methoxybenzoxazolin-2(3*H*)-one; NOEC, no observed effect concentration.

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