JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Transformation of Benzoxazinones and Derivatives and Microbial Activity in the Test Environment of Soil Ecotoxicological Tests on *Poecilus cupreus* and *Folsomia candida*

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Benzoxazinones, such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and benzoxazolinones, such as 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA), are biologically active secondary metabolites found in cereals. Because these compounds could be exploited as part of a strategy for reducing the use of synthetic pesticides, ecotoxicological tests were performed recently. In this paper, the transformation of the compounds in the test environment of the ecotoxicological tests was studied. DIMBOA was degraded and partly transformed to MBOA during the period of ecotoxicological testing of the compounds. During testing of MBOA on *Poecilus cupreus* test media the analysis showed that at the initial concentrations of 2 and 10 mg kg^{-1} no MBOA was left after 45 days of testing, but the metabolite 2-amino-phenoxazin-3-one (AMPO) was formed. During testing of BOA on both *Folsomia candida* and *Poecilus cupreus* the more biologically active compound 2-amino-phenoxazin-3-one (APO) was formed. Thus, the ecotoxicological test results on MBOA and BOA were partly due to the microbial transformation of the compounds during the time of testing.

KEYWORDS: Ecotoxicology; microbial activity; benzoxazinoids; benzoxazolinone; benzoxazinone; transformation; degradation; cereals; allelochemicals

INTRODUCTION

Cereals, such as wheat, rye, and maize, contain benzoxazinones, such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and the benzoxazolinones 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA). The benzoxazolinones are found both in plants and in soil as degradation products of DIMBOA and DIBOA, respectively. During the past decades extensive focus has been put on the possible exploitation of the allelopathic properties of these compounds as an alternative strategy for suppressing weeds, pests, and diseases. Evaluation of the nontarget effects of these compounds was done for the first time by Coja et al. (*1*) and Idinger et al. (*2*). Ecotoxicological testing of allelochemicals is influenced by the fact that the compounds are subject to microbial transformation during the test period. Fast transformation of DIMBOA, DIBOA, MBOA, and BOA in agricultural soil has been shown recently (*3*−*6*). In all studies the transformation rate of the compounds varied when the initial concentration was changed. Understrup et al. (*6*) showed that the first-order half-life of BOA was 0.6 day in a degradation experiment with BOA in the concentration of 0.4 μg/g of soil, 3.1 days in a 0.4 mg/g experiment, and 31 days in a 4 mg/g experiment. 2-Amino-phenoxazin-3-one (APO) and 2-acetylaminophenoxazin-3-one (AAPO) were identified as transformation products of BOA in the 0.4 and 4.0 mg/g experiments. APO had half-lives of 2.7 and 45 days in the 0.4 and 4.0 mg/g experiments, respectively. The disappearance of APO was concurrent with the formation of AAPO. In MBOA degradation studies (*3*, *7*) 2-amino-7-methoxy-phenoxazin-3-one (AMPO) and subsequently 2-acetylamino-7-methoxy-phenoxazin-3-one (AAMPO) were identified as transformation products in a degradation study with an initial concentration of 0.4 mg of MBOA/g of soil, whereas no AMPO and AAMPO occurred in a MBOA degradation study at 0.4 μg/g of soil (*8*).

Because the degradation rate and the transformation pathway are influenced by the initial concentrations of the tested compounds, the determination of the disappearance of the compound and the formation of transformation products in the test media are of importance for the interpretation of the test results when ecotoxicological studies are performed.

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10.1021/jf050914a CCC: $33.50
Published on Web 01/24/2006

A number of parameters influence the degradation of a chemical compound in soil or other test media. Chemical compounds can be degraded through chemical, photolytic, and microbial processes. Of these the microbial processes are the most important. Soil from the plow layer can contain several millions of bacteria and fungi per gram, whereas artificial or air-dried media could be expected to have a lower microbial activity. Thus, the determination of microbial activity is of importance when the fate of chemical compounds in a test medium is to be evaluated. According to the literature, determination of biological activity/biomass has been made in a number of ways, and a single method cannot be considered a standard method.

Three methods, which are used very often, are the fumigation−incubation method (*9*), the fumigation−extraction method (*10*, *11*), and the substrate-induced respiration method (*12*). ATP methods (*13*−*15*), staining followed by direct counting (*16*), and determination of biomass by means of the fatty acid pattern (*17*) are other relevant methods. Martens (*18*) concluded that precise determinations of the transformation factor between the methods could not be determined.

When one adds a chemical compound to soil that is easily degraded by microorganisms, CO_2 will be formed and can be collected. The activity in different media can be compared by the rate of CO_2 formation. Anderson and Domsch (*12*) published a method, called the glucose respiration method, that builds upon the concept of collecting CO_2 formed after the addition of glucose. On the basis of a high number of replicates, an empirical correlation between CO_2 formation and biomass (milligrams of microbial C per 50 g of dry soil) was established, which has been used in many papers since the publication of the method. The method is time-consuming, and a more simplified approach can be used, in which milligrams of microbial C is not calculated, but the rate of evolution of CO_2 is used to evaluate whether differences in microbial activity are seen (*19*). If a ^{14}C-labeled compound is used, the CO_2 formation can be quantified by scintillation counting. In the method used in this comparison, sodium [^{14}C]acetate was added to the test media and the rate of evolution of $^{14}CO_2$ was measured.

The purpose of this study was to determine to which extent the chemical compounds DIMBOA, MBOA, and BOA that were tested in the ecotoxicological studies by Coja et al. (*1*) and Idinger et al. (*2*) were transformed during the test period and to determine the microbial activity in the media in comparison with natural field soils.

MATERIALS AND METHODS

Ecotoxicological Tests. The tests were performed on mature individuals of *Folsomia candida* (Willem, 1902) and 24−48-h-old first-instar larvae of *Poecilus cupreus* (L.). The study was carried out according to the international standard ISO 11267 (*20*). The test objectives were to detect potential lethal (acute toxicity; mortality) and sublethal (reproduction) effects on adults of *F. candida* and lethal (mortality) and sublethal effects (hatching weight, developmental duration, and sex ratio) on *P. cupreus* of the test substances DIMBOA, MBOA, and BOA compared with control and reference treatments. The details of the ecotoxicological test methods are described by Coja et al. (*1*), Idinger et al. (*2*), and Coja et al. (*21*).

Soil. As test medium for the *F. candida* test artificial soil was used consisting of sphagnum peat, air-dried with no visible plant remains (10%), kaolinite clay, air-dried (20%), and industrial quartz sand, air-dried (68−69%) adjusted with $CaCO_3$ (0.5−1%) to a pH of 6.0 ± 0.5. The water content during the test was adjusted to 60% of the water-holding capacity (WHC) (*1*, *2*). As test medium for the *P. cupreus* test, air-dried LUFA-Speyer soil (LUFA 2.1) was used (http://www.lufa-speyer.de/) with 1.04% of organic C, a pH value of 7.0, and a texture of 84.0% sand, 12.4% silt, and 3.6% clay. The water content of the *P. cupreus* substrate was adjusted to 35% of the WHC. The test substrate was frozen after finalization of the test (45 days for *P. cupreus* and 28 days for *F. candida*). The samples were kept frozen during transportation from the ecotoxicology laboratory to the chemistry laboratory, where they were stored at −20 °C until lyophilization. The lyophilization was performed for a period of 24 h. After lyophilization, the soil was gently crushed and frozen again at −20 °C until analysis. In *F. candida* and *P. cupreus* test media, chemical analyses were performed in the media after finalization of the ecotoxicological tests. Single analyses of the duplicated samples at each concentration were performed for *F. candida*, and duplicate analyses of single samples at each concentration were performed with *P. cupreus*. In other samples of unused artificial media determination of the microbial activity was carried out. The microbial activity was determined in natural field soil from Denmark (conventional cultivation and organic cultivation). A summary of the texture of the natural soils and ecotoxicological test media is shown in **Table 2** together with texture data for soil from various depths, for which the microbial activity was determined in an earlier study (*19*).

Chemicals. The chemicals used for the analysis were obtained from the following sources: DIMBOA was isolated from maize, purity-checked, and provided for the study by Macías et al. (*5*). MBOA (98%) and BOA (98%) were purchased from Lancaster Chemicals and ACROS Organics. APO and AAPO were synthesized and purity-checked as described in Gents et al. (*4*). AMPO and AAMPO were obtained from Dr. Francisco Macías. The synthesis methods and the purity-check were published in Macías et al. (*22*). MeOH and HPLC grade solvents were obtained from Ratburn Chemicals Ltd. (Walkerburn, Scotland), and acetic acid was from Riedel-de-Haën (Seelze, Germany). ^{14}C-Labeled sodium acetate with a specific activity of 667 μCi/mg and a radiochemical purity of 98.6% was obtained from Amersham. Sodium acetate of 99.5% purity was obtained from Merck.

Analysis. In the DIMBOA test, the following compounds were quantified: DIMBOA and the possible transformation products MBOA, BOA, APO, AAPO, AMPO, and AAMPO. In the MBOA test, chemical analysis was performed of the following compounds: MBOA and the possible transformation products BOA, APO, AAPO, AMPO, and AAMPO. In the BOA test, the following compounds were quantified: BOA and the possible transformation products MBOA, APO, AAPO, AMPO, and AAMPO. The systematic names, acronyms, and chemical formulas of both parent compounds and possible transformation products are shown in **Table 1**. The choice of transformation products to be determined was based on former results in the FATEALLCHEM (www.fateallchem.dk) project (*3*−*8*, *23*).

Extraction and Chemical Analysis of Added Compounds and Metabolites. Extraction of the compounds from the soil was performed using an accelerated solvent extraction ASE 200 system from Dionex. The principles of the extraction method are described in Understrup et al. (*6*) with the following changes: For the tests on *F. candida*, the total amount present in each sample [~24 g of test substrate (soil)] was added to the extraction cell. For the *P. cupreus* samples 10 g of test substrate (soil) was added to the extraction cell. Duplicate determinations were performed for the *P. cupreus* substrates. The *F. candida* test substrate extracts were filled to 50 mL with methanol, and the *P. cupreus* test substrate extracts were filled to 20 mL with methanol. The extracts were stored at −20 °C. A mixed standard curve containing MBOA, BOA, AMPO, AAMPO, APO, and AAPO was prepared in acidified methanol in the range between 0.25 and 400 μg/L. An individual standard curve in the same range was prepared for DIMBOA. No matrix effect was seen when standard curves dissolved in methanol and standard curves dissolved in blank soil extract were compared.

The analyses were performed in an Applied Biosystems MDS Sciex API 2000 liquid chromatography−mass spectrometry (LC-MS) system in turbo electrospray ionization (ESI) positive SIM mode for APO, AMPO, AAPO, and AAMPO, whereas DIMBOA, MBOA, and BOA were quantified in the negative mode. The chromatography of the extract was performed as described by Understrup et al. (*6*).

Validation of the Analytical Method. The detection limits in the substrates of the analytical method were the following: BOA, 0.022

Table 1. Systematic Names, Acronyms, and Formulas for the Tested Compounds and Their Possible Transformation Products

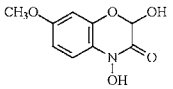
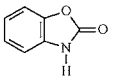
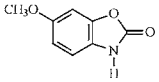
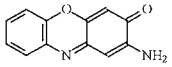
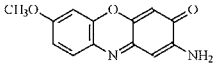
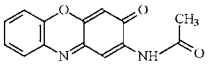
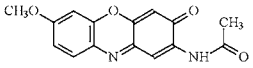
systematic name	acronym	chemical formula
2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA	
benzoxazolin-2-one	BOA	
6-methoxy-benzoxazolin-2-one	MBOA	
2-amino-phenoxazin-3-one	APO	
2-amino-7-methoxy-phenoxazin-3-one	AMPO	
2-acetylamino-phenoxazin-3-one	AAPO	
2-acetylamino-7-methoxy-phenoxazin-3-one	AAMPO	

Table 2. Texture of Soil and Rate Constants for Mineralization of Sodium [^{14}C]Acetate

	sampling depth (cm)	humus (%)	clay[a] (%)	silt[b] (%)	sand[c] (%)	pH	rate constant k_1[d] (h^{-1})	homogeneous groups for k_1
F. candida substrate	nd[e]	8.9[f]	20	0	68–69	6.0	<0.05	A
P. cupreus substrate	nd[g]	1.8[h]	3.6	12.4	84	7.0	0.28 (0.03)	B
conventional field soil	0–15	2.5	21	51	27	6.4	0.46 (0.07)	C
organic field soil	0–15	1.9	9	30	61	6.3	0.45 (0.13)	C
soil 1 (*19*)	15	2.8	3.6	2.8	91	6.9	1.10 (0.50)	D
soil 1 (*19*)	45	0.3	2.5	1.4	96	6.3	0.61 (0.14)	C
soil 1 (*19*)	75	0.1	2.1	1.4	96	6.4	0.57 (0.23)	C
soil 3 (*19*)	15	2.8	4	2.9	90	6.7	1.37 (0.55)	D
soil 3 (*19*)	45	0.9	3.5	2.4	93	5.6	0.57 (0.23)	C
soil 3 (*19*)	75	0.3	3	1.4	95	5.5	0.14 (0.02)	E

[a] Clay: <2 μm. [b] Silt: <63–2 μm. [c] Sand: <2000–63 μm. [d] Mean of four replicates with standard deviation in parentheses. [e] Not determined. The *F. candida* substrate (artificial soil) was prepared according to the standard test method ISO 11267 (1999). [f] The artificial soil was prepared with 10% sphagnum, which resulted in a content of organic C in the soil of 5.2% organic C, which is equivalent to 8.9% humus using a conversion factor of 1.72. [g] The *P. cupreus* substrate (field soil, dried) was purchased from LUFA-Speyer. [h] A conversion factor of 1.72 between organic C and humus is used; 1.04% organic C in the *P. cupreus* substrate is equivalent to 1.79% humus.

mg/kg; APO, 0.008 mg/kg; AAPO, 0.010 mg/kg; MBOA, 0.025 mg/kg; AMPO, 0.005 mg/kg; AAMPO, 0.002 mg/kg; and DIMBOA, 0.025 mg/kg. Recovery studies (seven replicates for each compound) in field soil performed at a concentration of 0.4 mg/kg showed the following recovery percentages (mean ± standard deviation): BOA, 73 ± 6.4%; APO, 60 ±2.6%; AAPO, 66 ± 2.9% (*6*); MBOA, 89 ± 3.1%; AMPO, 35 ± 6.1%; and AAMPO, 65 ± 2.8%. Additional duplicate spiked samples of the test media for *F. candida* and *P. cupreus* were analyzed, and the recovery was determined to be in the same range. The final data were not corrected for recovery.

Determination of Microbial Activity. A mixture of ^{14}C-labeled soidum acetate and nonlabeled sodium acetate was added to 50 g of freshly prepared test medium or natural field soil in a concentration of 5 mg/kg of dry soil in an Erlenmeyer flask. Additional water was added to 50% of the WHC. The Erlenmeyer flask was placed in a 2-L jar together with a beaker containing 10 mL of 1 N KOH in which the evolved $^{14}CO_2$ accumulated. The test media were incubated at 15 °C, and the KOH beaker was changed after 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h. An aliquot of the KOH solution was mixed with a scintillation liquid and counted in a scintillation counter Wallac 1409 to follow the mineralization of sodium [^{14}C]acetate. Mineralization rates of sodium [^{14}C]acetate were used as a measurement of microbial activity. Four replicate studies were performed for each test medium. Ottawa sand is considered to be chemically and microbially inert and is often used for addition of chemicals to soil in degradation experiments when the chemicals are not soluble in water. Therefore, the microbial activity in Ottawa sand was determined as well for the purpose of comparison. The determination of microbial activity did not take into consideration

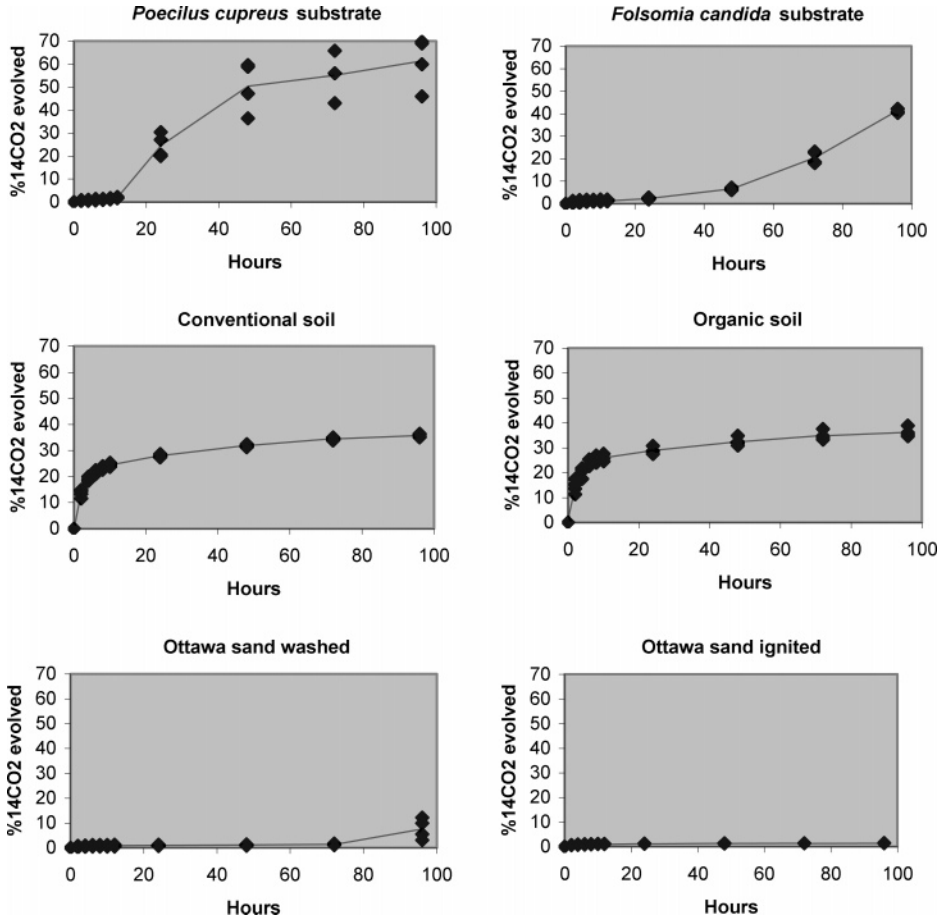


Figure 1. Mineralization, measured as accumulated evolution of $^{14}CO_2$ from sodium [^{14}C]acetate, added to test media in a concentration of 5 μg/g and incubated for 96 h: dots, individual measurements; line, mean of measurements.

that a certain proliferation of microorganisms can occur during time, when the test organisms produce feces. The influence of these microorganisms on transformation of the test compounds is considered to be of minor importance compared to the influence of microorganisms naturally present in the test media.

Modeling of Microbial Activity Data. The mineralization of sodium [^{14}C]acetate was described according to the Fomsgaard and Kristensen (*19*) modification of the Lui and Zhang (*24*) model, assuming that the formation of $^{14}CO_2$ occurred as a result of at least two processes. One process was the immediate mineralization of ^{14}C-labeled pesticide, and another process was the first-order mineralization of soil organic matter, into which ^{14}C from the pesticide had been built (or very strongly adsorbed). The model is described with the following parameters:

$$C_t = C_m + C_h \quad (1)$$

$$C_m = c_n - \frac{k_1 c_n}{(k_1 + k_2 c_n)\, e^{k_1 t} - k_2 c_n} \quad (2)$$

$$C_h = c_b(1 - e^{-k_3 t}) \quad (3)$$

In eqs 1−3 C_t = total concentration of the mineralization product ($^{14}CO_2$) formed at time t (measured as % ^{14}C evolved as %$^{14}CO_2$), C_m = % $^{14}CO_2$ formed at time t according to eq 2, C_h = % $^{14}CO_2$ formed at time t according to a first-order model (eq 3), c_n = total % of ^{14}C-labeled compound converted to $^{14}CO_2$ according to eq 2, c_b = total % of ^{14}C-labeled compound converted to $^{14}CO_2$ according to eq 3, k_1 = rate constant, k_2 = rate constant, k_3 = rate constant, and t = time in days.

Five coefficients (k_1, k_2, k_3, c_n, and c_b) of the model were estimated using a nonlinear least-squares method. As the model is nonlinear, an iterative method was used. The coefficient k_1, which describes the velocity of the initial part of the mineralization process, was used for comparison between samples.

RESULTS AND DISCUSSION

Comparison of Microbial Activity. The accumulated evolution of $^{14}CO_2$ from the test media is depicted in **Figure 1**. **Table 2** shows the texture of the test media (organic and conventional field soil and ecotoxicological test media) together with the rate constant k_1 for the sodium [^{14}C]acetate mineralization and the homogeneous groups as determined with a nonparametric Kruskal−Wallis test. The rate constant k_1 could not be determined for Ottawa sand and could only be tentatively estimated for *F. candida* substrate. The results of the determination of the $^{14}CO_2$ evolution in conventional and organic soil (**Figure 1**) showed a pattern very similar to former results in plow layer soil (*19*). In these soils a fast increment in the $^{14}CO_2$ production was seen whereupon the curve flattened out. The sodium [^{14}C]acetate can either be totally mineralized to $^{14}CO_2$ or built into humus and microorganisms. When a high number of microorganisms are present, the typical pattern results in a fast rise shortly after the addition of sodium [^{14}C]acetate. When the curve has reached the flat part, the evolution of $^{14}CO_2$ is due to a slow continued mineralization of organic matter, into which ^{14}C was built. In the *P. cupreus* and *F. candida* media the evolution of $^{14}CO_2$ during the first 12 h was minor. This indicated a very low microbial activity. After some time, the microorganisms developed even if a low activity was present in the beginning. The microorganisms developed more easily in the *P. cupreus* substrate than in the *F. candida* substrate despite the fact that the organic carbon content in the *F. candida*

Table 3. Analytical Results from the DIMBOA Test on *F. candida* after a 28-Day Test Period

replicate	added concn of DIMBOA (mg kg^{-1})	measured concn (mg kg^{-1})						
		DIMBOA	MBOA	AMPO	AAMPO	BOA	APO	AAPO
A	2	<LOD[a]	0.41	<LOD	<LOD	<LOD	<LOD	<LOD
B	2	<LOD	0.38	<LOD	<LOD	<LOD	<LOD	<LOD
A	10	<LOD	2.89	<LOD	<LOD	<LOD	<LOD	<LOD
B	10	<LOD	3.21	<LOD	<LOD	<LOD	<LOD	<LOD
A	50	0.57	7.79	<LOD	<LOD	<LOD	<LOD	<LOD
B	50	0.29	2.84	<LOD	<LOD	<LOD	<LOD	<LOD

[a] Limit of detection.

Table 4. Analytical Results from the MBOA Test on *F. candida* after a 28-Day Test Period

replicate	added concn of MBOA (mg kg^{-1})	measured concn (mg kg^{-1})						
		DIMBOA	MBOA	AMPO	AAMPO	BOA	APO	AAPO
A	2	NM[a]	1.89	<LOD[b]	<LOD	<LOD	<LOD	<LOD
B	2	NM	1.82	<LOD	<LOD	<LOD	<LOD	<LOD
A	10	NM	7.39	<LOD	<LOD	<LOD	<LOD	<LOD
B	10	NM	6.97	<LOD	<LOD	<LOD	<LOD	<LOD
A	50	NM	27.00	<LOD	<LOD	<LOD	<LOD	<LOD
B	50	NM	3.22	<LOD	<LOD	<LOD	<LOD	<LOD

[a] Not measured. [b] Limit of detection.

substrate was higher than in the *P. cupreus* substrate. However, the content of organic matter does not always correspond to microbial activity. The organic matter in the *F. candida* medium is sphagnum, which is produced by digging peat from acid water-saturated bogs, where the microbial activity is low. The air-dried field soil that formed the *P. cupreus* substrate thus had a higher microbial activity than the *F. candida* substrate. In field soil from deeper layers (45 and 75 cm) the evolution of $^{14}CO_2$ had a slower increase than in the plow layer, but after 12 h in 45 cm soil the flat part of the curve was reached (*19*). Thus, the microbial activity was lower in the *F. candida* ecotoxicological test media than in natural field soil even from a depth of 75 cm.

In Ottawa sand very reduced microbial activity was seen, which should be expected because the number of microorganisms is limited compared with live agricultural soil. The rate constant k_1 could not be determined for these substrates. In live agricultural soil, several millions of microorganisms are present in 1 gram of soil. However, when the sand is only washed and not sterilized, the few microorganisms that were present propagate slowly, and a slow evolution of $^{14}CO_2$ from sodium [^{14}C]acetate began. Sand was an ingredient in the ecotoxicological test media and is often used for the addition of chemicals to soil in degradation studies when these are to be performed with chemicals that are insoluble in water. When sand is used in this way, it is often described as chemically and microbially inert, which can be confirmed with this comparison.

DIMBOA Test. In the DIMBOA test on *F. candida* (**Table 3**), DIMBOA disappeared completely after 28 days in the concentration levels 2 and 10 mg/kg, whereas ~1% of the added DIMBOA was left after 28 days in the test at 50 mg/kg. MBOA was the only compound that was detected as transformation product. DIMBOA has been shown to degrade quickly in a soil−water suspension (*5*) with a half-life of <1 h. The amounts of MBOA as transformation product found in the studies at different DIMBOA concentrations varied. A 5 times increase in initial DIMBOA concentration from 2 to 10 mg/kg raised the amount of MBOA left after 28 days (calculated in percentage of initial DIMBOA amount) only from 2.5 to 3.9%. The presence of MBOA at levels as high as 7.79 mg/kg after 28 days might indicate that the concentration of MBOA was higher earlier in the test period. However, if MBOA never was present in higher concentrations than the ones measured after day 28, AMPO and AAMPO formation in detectable amounts should not be expected, as shown by Fomsgaard et al. (*3*, *8*). The effects of DIMBOA in the ecotoxicological tests will be of importance only for at short time interval after initiation of the test. The effects seen in the test must be considered mainly to have been effects of MBOA. However, the transformation of DIMBOA to MBOA cannot be expected to be equimolar. Thus, a lower molar concentration of MBOA than the added amount of DIMBOA will be present.

Table 5. Analytical Results from the MBOA Test on *P. cupreus* after a 45-Day Test Period

added concn of MBOA (mg kg^{-1})	measured concn (mg kg^{-1})					
	MBOA	AMPO	AAMPO	BOA	APO	AAPO
2	<LOD[a]	0.24	<LOD	<LOD	<LOD	<LOD
10	<LOD	0.44	<LOD	<LOD	<LOD	<LOD
50	6.14	1.55	<LOD	<LOD	<LOD	<LOD
100	5.62	1.65	<LOD	<LOD	<LOD	<LOD

[a] Limit of detection.

MBOA Tests. In the MBOA test on *F. candida* (**Table 4**), only MBOA was detected. A large difference was seen between the duplicate trials at 50 mg/kg concentration level. In one of the replicates 6.4% of the added MBOA was detected after 28 days, whe 54% was found in the other. This difference could be explained by the fact that in this concentration level MBOA can be distributed unevenly in the substrate, and thus the availability of the compound to the degrading microorganisms will differ between replicates. The compound can precipitate in the sample container as well. As we have only two data points (days 0 and 28), a degradation rate cannot be determined. However, in the concentration level of 2 mg/kg, 93.3% of the added MBOA was left after 28 days and in the 10 mg/kg study, 71.2% was left. Thus, the degradation was slow. In degradation

Table 6. Analytical Results from the BOA Test on *F. candida* after a 28-Day Test Period

replicate	added concn of BOA (mg kg^{-1})	measured concn (mg kg^{-1})						
		DIMBOA	BOA	APO	AAPO	MBOA	AMPO	AAMPO
A	2	NM[a]	<LOD[b]	0.07	<LOD	<LOD	<LOD	<LOD
B	2	NM	0.02	<LOD	<LOD	<LOD	<LOD	<LOD
A	10	NM	6.51	0.07	<LOD	<LOD	<LOD	<LOD
B	10	NM	9.23	0.09	<LOD	<LOD	<LOD	<LOD
A	50	NM	23.83	0.26	0.02	<LOD	<LOD	<LOD
B	50	NM	7.10	0.31	0.02	<LOD	<LOD	<LOD

[a] Not measured. [b] Limit of detection.

studies with MBOA in field soil at concentrations of 0.4 and 4 mg/kg the DT_{50} value was ≤ 2 and no MBOA could be detected after 28 days (*3*). This shows that the degradation of MBOA was much faster in active field soil than in the *F. candida* test media. In active field soil where MBOA was added at a concentration as high as 400 mg/kg, only 6% of the added MBOA was left after 28 days (*3*). Only minor concentrations of AMPO or AAMPO were measured in the present study after 28 days. In the studies by Fomsgaard et al. (*3*), AMPO and AAMPO were detected only in studies when the initial concentration of MBOA was as high as 400 mg/kg and the molar relationship between MBOA and AMPO at the time of maximum concentration of AMPO was 100:1. Fomsgaard et al. (*3*) did not detect AMPO and AAMPO in their degradation studies with initial MBOA concentrations of 0.4 and 4 mg/kg. Because the degradation rate of MBOA in the ecotoxicological tests with *F. candida* is slow, it can be concluded that no interference of metabolites needs to be considered in the evaluation of the test.

In the analysis of MBOA test media after 45 days of testing with *P. cupreus* (**Table 5**), a more advanced degradation of MBOA was seen than in the *F. candida* test after 28 days. No MBOA was left after 45 days, and AMPO was formed at the initial concentrations of 2 and 10 mg kg^{-1}. A longer period of incubation will naturally lead to a more pronounced degradation. However, the additional 17 days of incubation are not enough for explaining that the degradation is much more pronounced in *P. cupreus* media than in the *F. candida* media. The *P. cupreus* test media is a natural field soil that has been dried. Being a natural field soil the microbial activity of this soil was higher than that of the *F. candida* artificial soil (**Figure 1**), and thus the transformation of MBOA was more pronounced. It is interesting to see that AMPO was detected, even at an initial concentration of 2 mg/kg of MBOA. Fomsgaard et al. (*3*) showed that the formation of AMPO depended on the initial concentration of MBOA and that the tendency of forming AMPO increased with increasing initial concentrations of MBOA. However, this study shows that the formation of AMPO apparently was influenced by another factor as well, which probably was the microbial activity. In the *P. cupreus* air-dried substrate AMPO was formed with an initial MBOA concentration in the study of 2 mg/kg, whereas no AMPO was formed in MBOA studies at 4 mg/kg in the more microbially active fresh field soils used by Fomsgaard et al. (*3*, *8*). Although the molar relationship between added MBOA and maximum concentration of AMPO was found to be 300:1, the formation of AMPO during the *P. cupreus* test should thus be considered in the ecotoxicological evaluation together with the disappearance of MBOA. It is recommended that analysis should be performed during the whole time of testing.

BOA Tests. The transformation of BOA was more pronounced in the *P. cupreus* tests after 45 days (**Table 7**) than in the *F. candida* tests after 28 days (**Table 6**), a pattern that was similar to the MBOA pattern and again must be explained not only by the longer incubation time but also by the higher microbial activity in the *P. cupreus* media than in the *F. candida* media. In the *P. cupreus* study, only BOA amounts below or very close to the detection limit (<0.3% of the added amount) were found at all study levels. The *F. candida* test showed concentrations of BOA at the level of the detection limit for the initial concentration of 2 mg/kg. For the initial concentrations of 10 mg/kg in the *F. candida* test media, low amounts of BOA were degraded; the percentages of BOA left were 65 and 92%. In the 50 mg/kg test, a high variation between the replicates was seen. This is similar to the picture seen for MBOA as well. MBOA and BOA were added to these test media in the following way: The quantity of the test substance for the whole test run in each concentration was dissolved in acetone and mixed into the respective quantity of water that was needed to obtain the desired water content in the test medium. This solution was then mixed with the dry substrate. This was expected to lead to a uniform distribution in the test media. However, because both the MBOA and BOA tests showed this high variation between duplicates in the high concentration, it should be considered whether distribution of the compounds could be optimized in relation to the possible contact with the microorganisms. The preparation of the test media was done with the focus on distributing the compound AND obtaining a crumbly structure to enable springtails to penetrate substrate cavities. However, ISO standard 11267 (*20*), which was followed closely in the studies by Coja et al. (*1*) and Idinger et al. (*2*), does not describe in detail how the uniform distribution of the compound should be ensured in relation to a possible microbial degradation. If the microbial degradation during the test period is going to be followed, which is needed to easily degrade compounds, the homogeneous distribution should be considered.

Table 7. Analytical Results from the BOA Test on *P. cupreus* after a 45-Day Test Period

added concn of BOA (mg kg^{-1})	measured concn (mg kg^{-1})					
	BOA	APO	AAPO	MBOA	AMPO	AAMPO
2	<LOD[a]	0.16	<LOD	<LOD	<LOD	<LOD
10	0.03	0.23	<LOD	<LOD	<LOD	<LOD
50	0.03	0.22	<LOD	<LOD	<LOD	<LOD
100	0.04	0.43	<LOD	<LOD	<LOD	<LOD

[a] Limit of detection.

When BOA was partly (*F. candida*) or totally (*P. cupreus*) degraded during the test period, the formation of metabolites became important. APO was detected as the main transformation product in both tests. It was not possible to tell whether the concentration of APO was at a maximum before the endpoint of the test (45 days for *P. cupreus* and 28 days for *F. candida*)

because only the endpoint concentration was determined. However, at these endpoints the concentration of APO generally was above the NOEC and LOEC levels for APO (NOEC *F. candida* ft, 0.01 mg kg^{-1}, repro, 0.01 mg kg^{-1}) (*1*, *2*). The formation of these aminophenoxazinone compounds must thus be taken into account in the evaluation of the ecotoxicological results.

ACKNOWLEDGMENT

We thank Kirsten Jensen for doing a competent linguistic review of the manuscript and an unknown reviewer for suggestions for improvements.

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Received for review April 20, 2005. Revised manuscript received November 2, 2005. Accepted December 6, 2005. The research presented here was performed as a part of the project "FATEALLCHEM", "Fate and Toxicity of Allelochemicals in Relation to Environment and Consumer", www.fateallchem.dk. The project was carried out with financial support from the Commission of the European Communities under the Work Program Quality of Life, Contract QLK5-CT-2001-01967.

JF050914A