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# Concentrations and Allelopathic Effects of Benzoxazinoid Compounds in Soil Treated with Rye (*Secale cereale*) Cover Crop

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**ABSTRACT:** The concentration of benzoxazinoids (BX) was measured in field soils at selected intervals after rye residue was either incorporated or left on the soil surface. The spectrum of compounds arising in the soil persisted approximately two weeks and was dominated by methoxy containing BX compounds, which were only minor components of the rye foliage. Growth assays with lettuce and smooth pigweed species showed inhibition when treated soils were tested during the first two weeks after rye applications; however, there were no sufficient concentrations of any one BX compound in the soil to explain these affects. Solution applications of two pure BX compounds, benzoxazolin-2(3*H*)-one (BOA) and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA), to the surface of soils revealed that movement into the soil column was minimal (greater than 70% BOA and 97% MBOA remained in the top 1-cm of soil profiles) and that the time course for their complete dissipation was less than 24 h.

**KEYWORDS:** *benzoxazinoids, allelopathy, rye cover crop, field exposure, soil*

## INTRODUCTION

Many cereals produce secondary metabolites that are important in their natural defense against pests, diseases, and weeds. A major chemical group responsible for this activity in rye, wheat, and corn is the benzoxazinoid group (BX compounds)[1] with the base skeleton structure of 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one that is demonstrated in most of the compounds included in this study (Figure 1). The potential for these BX compounds to contribute to the allelopathic potential of rye cover crops has long been suggested.[2] Chemical analyses in soil for the causative agents responsible for rye allelopathy have been recently reported by two research groups.[3,4] In these studies, measured soil concentrations of many of the BX-family of compounds were reported after treating soil with plant isolates of DIBOA-Gluc (**7**), DIBOA (**3**), and BOA (**1**)[4] or 10-day old rye sprouts;[3] however, these soil studies were observed under controlled laboratory conditions and in one case with unnaturally high initial concentrations of the compounds.[4] Interesting, however, was evidence from these studies that the more persistent APO (**10**) was formed as a degradate, and this compound is one of the more toxic of the BX compounds[5]

Glucosylated benzoxazinoid forms are known to be the precursors of the benzoxazinoids expected to be present after release from the plant tissues; therefore, the glucosylated forms (DIBOA-Gluc (**7**), HBOA-Gluc (**8**), and DIMBOA-Gluc (**9**)) need to be considered when investigating the chemical basis for rye mulch allelopathy.[6] It is widely accepted that these glucosylated forms lose the glucose moiety and release the base structures, HBO (**2**), DIBOA (**3**), HMBOA (**5**), and DIMBOA (**6**), when the plant cells are disrupted.[1] These products readily degrade to other structurally related products, especially the benzoxazolinones, **1** and MBOA (**4**), and even further to the more toxic aminophenoxazinones where two phenoxy rings of the benzoxazinoid structures are joined together across bridging oxygen and nitrogen atoms APO (**10**), AAPO (**11**), and AMPO (**12**).[5,7]

The main goal of this study was to determine the quantity and composition of benzoxazinoid compounds found in soil after treatment with rye cover crop vegetation either as incorporated or as surface-applied residues. We applied new and sensitive analytical methods for benzoxazinoid determinations in soil and quantitated the concentrations of the known set of BX compounds discussed above and shown in Figure 1. A further goal was to observe the activity of benzoxazinoid products in two different soil types over two seasons. The quantitated amounts were compared with the relative phytotoxity of these soils to determine the potential contribution of BX compounds to rye allelopathy.

## MATERIALS AND METHODS

**Field Experiments.** Field experiments were conducted on two field sites at the USDA-ARS Beltsville Agricultural Research Center, USA. The first field site was characterized by a Christiana silt loam (fine, kaolinitic, mesic Aquic Hapludults; soil moisture field capacity 26%) and was located adjacent to our Farming Systems Project (designated FSP). The second field site was characterized by a Keyport fine sandy loam (fine, mixed, semiactive, mesic Aquic Hapludults; soil moisture field capacity of 16%) located on our North Farm (designated NF). In September of 2005 and 2006, each field was planted with a rye cover crop at a sowing rate of 101 kg/ha using a locally grown Abruzzi rye cultivar that is commonly grown in the mid-Atlantic area. Four 6.1 by 6.1 m plots in each field served as no-rye control plots after killing off rye plants by paraquat application in late winter for the 2006 experiments or in late fall for the 2007 experiments. These control areas were rotovated in early April and then treated as described below.

Rye above-ground biomass was sampled from four 0.25 $m^2$ quadrants located in the periphery of each experimental field just before they were treated with 0.56 kg/ha of paraquat on April 26, 2006

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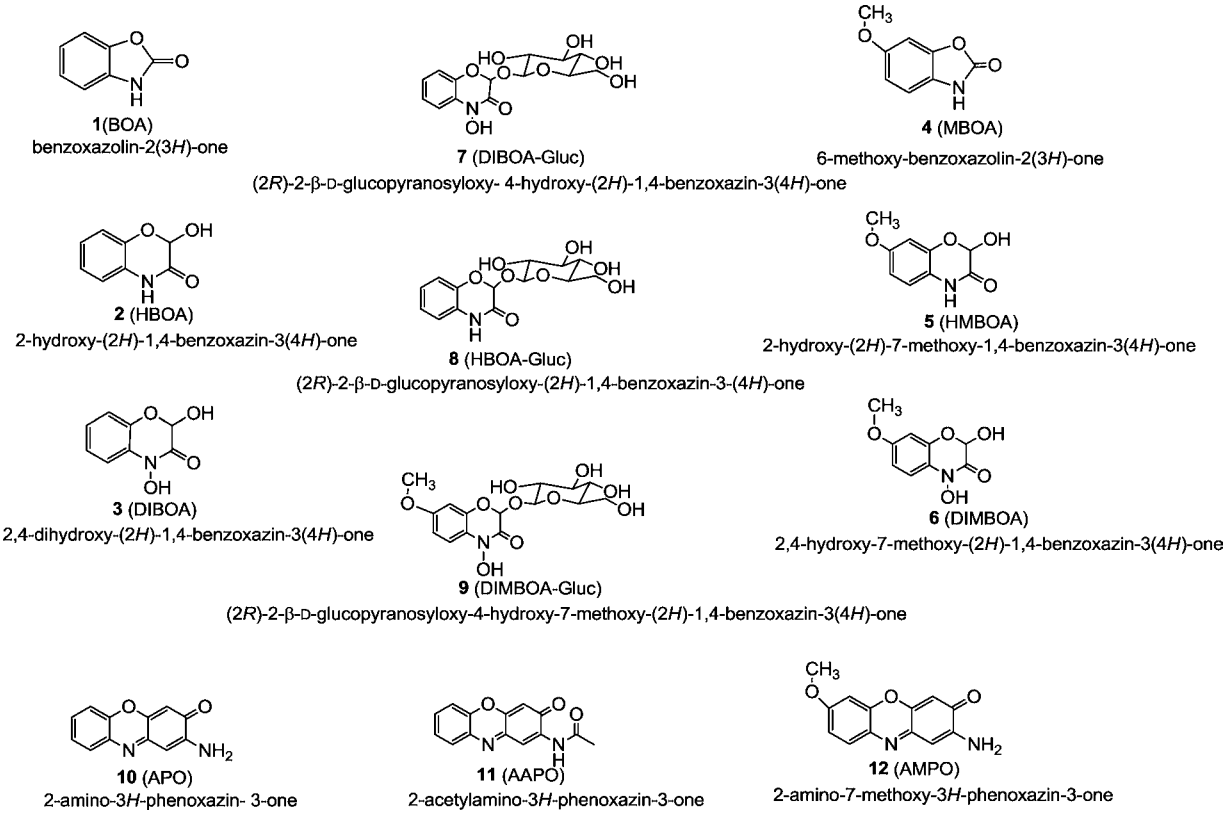


**Figure 1.** Chemical structures for the family of 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-ones (benzoxazinoids) contained in cereals and other plants.

and May 2, 2007. Rye was in the boot stage in 2006 and the early heading stage in 2007 when terminated. These plant samples consisted of above-ground materials cut just above the soil line that were either quickly frozen for later extraction to assess their benzoxazinoid content or oven-dried for biomass determination. The next day, three treatments were differentiated: (1) the existing no-rye control; (2) rye incorporated (INC) approximately 15 cm into the soil; and (3) rye no-till (NT) with residue left on the soil surface. The no-rye control and incorporated rye plots were rotovated twice and then tilled with a spader that firmed up the soil surface. The untilled rye was mowed with a flail mower that shredded the rye and dropped the residue back in place. After treatment establishment, there were four 6.1 by 6.1 m plots of each of the three treatments in each field arranged in a randomized complete block design. Tractor traffic lanes for all operations were arranged so as not to impact areas where samples and assays would later be conducted. The days these treatments were performed, April 27, 2006, and May 3, 2007, were considered day 0 of the respective experiments. Samples for analysis of soil BX were taken on the following schedule for 2006, day 0, 2, 4, 6, 7, and 19, and for 2007, day 0, 4, 11, 18, and 26. On each sampling date, 10 soil samples were taken to a 10 cm depth with a 1.9 cm diameter corer within each plot, composited into one sample, and approximately 100 g was removed and placed into whirl pack bags where they were stored frozen for later extraction of their benzoxazinoid content.

*Phytotoxicity Assay.* Bioassays were conducted as weather permitted at 0, 7, and 19 days after treatment in 2006, and at 0, 11, 18, and 26 days after treatment in 2007. This field assay consisted of planting 200 seeds each of Great Lakes lettuce and locally collected smooth pigweed in 76 cm long rows in each treatment area of each field. Residue was moved aside in the untilled rye treatment so that planting could be performed between and parallel to stumps of former rye rows, followed by replacement of residue. Plant emergence was assessed weekly. After enumeration, plants were pulled except for eight healthy plants in the row, which were allowed to grow to assess plant growth. Emergence was assessed for five weeks, after which the plants that had been allowed to grow were clipped at soil level, and dry weight was determined. Total number of emerged plants and average weight per plant within each plot were divided by the average value of the control treatment across all reps of each date/location/year to express data as a percent of control. Data by year were subjected to an analysis of variance with rye treatment and planting date as fixed factors and field site and rep nested within field site as random factors. A preliminary analysis of data revealed that results were similar at the two sites in each year, so the site was treated as a random factor. Variance was partitioned to deal with the heterogeneity of variance found in this data.

**Laboratory Experiments.** Two sets of experiments were performed where pure aqueous solutions of **1** (BOA) and **4** (MBOA) were applied to soil. In the first experimental trial, concentrated solutions of **1** and **4** were added to the surfaces of FSP and NF soil in 3 cm deep plastic food storage containers (80 × 80 × 60 cm) to achieve concentrations of 1.0 and 10 mg/kg soil. Since the primary question was to assess migration into the soil column, small core samples were taken with a cork borer (1 cm diameter), and these were sectioned into upper, middle, and lower layers and analyzed for BX content. In the other experimental trial, a concentrated aqueous solution of **1** was added to FSP and NF soil at field capacity moisture content to achieve 10 mg/kg (ppm) and rapidly mixed to ensure uniform distribution. Then sampling and analysis of the BX soil content was carried out at timed intervals, 0, 1, 2, 4, 6, 8, and 24 h, over a 24-h period to follow availability and loss.

**Extraction and Quantitation for BX Determinations.** The plant samples were extracted by accelerated solvent extraction as modified from Krogh et al.[3] as follows: the frozen leaves (approximately 5 g) were first ground in liquid nitrogen using a mortar and pestle. The powder was transferred to a labeled plastic bag and stored frozen for later pressurized solvent extraction with a Dionex Solvent Extractor (Dionex Corp., Sunnyvale, CA, USA). Each extraction cylinder was loaded with two filter membranes, a mixture of 5 g of sand that had been baked at 400 °C for 5 h and 0.5 g of ground plant material. The rest of the cylinder was then filled with additional sand. The solvent used in the extraction was a methanol/

$H_2O$/glacial acetic acid mixture (80:19:1). The ASE was set to the following extraction sequence: preheat for 5 min to 80 °C and then hold for 5 min, fill with solvent and leave static for 3 min; flush 80% of the cell volume, then purge for 60 s four times at 1500 psi at 80 °C. The collected extracts were dried in an evaporator (TurboVap, Uppsula, Sweden) at 40 °C and 5 psi for 4 h until the volume was approximately 10 mL. These extracts were analyzed using a procedure similar to that employed for soil extracts.

For extraction of the soils, the method was modified from our previous method.[8] Two grams of wet soil (about 25% of field capacity) was mixed with 5 mL of acidified water (1% glacial acetic acid). This slurry was then extracted by mixing with ethyl acetate (5 mL), which was separated by centrifugation and decanted off as the upper solvent layer. This ethyl acetate mixing and removal was repeated two more times and the three volumes combined as one BX-containing extract. The extracts were then reduced to dryness with a stream of nitrogen gas and made up to 2 mL with a water and methanol (1:1) mix and injected into an LC column which was interfaced to an LC/MS-MS system. For extracting soils dosed with pure **1** and **4**, the above soil extraction procedure was modified by the extraction of 1 g of moist soil with two sequential 3 mL extractions with ethyl acetate.

It was important to ensure that our extraction methods were adequate to account for the existence of these BX compounds in the soil and plant matrices that were being tested. Initial methods for soils utilizing ASE extraction methods were found to be inadequate to ensure acceptable recoveries; furthermore, methods had to be developed for soil especially in order to detect the low concentrations that were expected. After considerable trial and error, it was determined that an adaptation of the slurry method reported by Macias et al.[4] produced consistent recoveries of all the expected analytes. To ensure continued performance of this method, frequent spikes, blanks, and duplicates were carried out throughout the soil extraction process in order to monitor performance.

**Compounds Analyzed.** The structures for the respective compounds analyzed in this study are shown in Figure 1: benzoxazolin-2(3*H*)-one (**1**); 1,4-benzoxazin-3-(4*H*)-one (**2**); 2-hydroxy-(2*H*)-2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (**3**); 6-methoxy-benzoxazolin-2(3*H*)-one (**4**); 2-hydroxy-(2*H*)-7-methoxy-1,4-benzoxazin-3(4H)-one (**5**); 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (**6**); (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (**7**); (2*R*)-2-*β*-D-glucopyranosyloxy-(2*H*)-1,4-benzoxazin-3-(4*H*)-one (**8**); (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (**9**); 2-amino-3*H*-phenoxazin-3-one (**10**); 2-acetylamino-3*H*-phenoxazin-3-one (**11**); and 2-amino-7-methoxy-3*H*-phenoxazin-3-one (**12**).

The chemical purity of **1** was 98% and that of **4** was 97%; both were purchased commercially from Sigma-Aldrich (St. Louis, MO, USA); compounds **6** and **9** (estimated purity >90%) were isolated from corn according to the methods described by Klun et al.;[9] and **7** and **3** (both of estimated >95% purity) were synthesized following the methods of Sicker et al.[10] for **3** and Kluge et al.[11] for **7**. Compounds **10**, **11**, and **12** (estimated purities of 100%). were obtained from F. Macías (University of Cádiz, Spain). Synthetic **2** was kindly provided by D. Sicker (Institute of Organic Chemistry, University of Leipzig, Germany). Both **5** and **8** were kindly supplied by I. Fomsgaard, Danish Institute of Agricultural Sciences, Slagelse, Denmark.

**Instrumental Analyses.** The extracts were analyzed as described previously[12] utilizing initially a Quattro- and later an Ultima-LC benchtop triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK). Additional analytes were added to those analyzed earlier[12] in order to include the following BX compounds: **2**, **6**, **10**, **11**, and **12**. The conditions utilized to detect these additional compounds were similar to those used by Krogh et al.,[3] with the exception that an MRM method based on MS/MS methods rather than a single MS system was employed. Therefore, the parent to daughter transitions for the new compounds were as follows: $m/z$ 164 >108.4 and $m/z$ 326 >164 (negative electrospray ionization) and $m/z$ 213 > 185.5, 243 > 228, and 255 > 213, (positive electrospray ionization), respectively, for **2**, **6**, **10**, **12**, and **11**; and these compounds had the following LC-retention times, 8.6, 8.0, 22.2, 22.8, and 23.8 min. To improve separation of all analytes, methanol was replaced with acetonitrile in the running solvent, and the following gradient programs were utilized. Program 1 for all analytes: initial 100% A (30% acetonitrile mixed with 70% of a 1% formic acid solution) which was gradient programmed to 20:80 A/B (B, distilled deionized water) at 6 min then gradient programmed to 40:60 (A/B) at 15 min; followed by 60:40 A/B in 20 min when it is returned to initial conditions and held for 9 min before the next run. Program 2 was used just for the aminophenoxyphenones (**10**, **11**, and **12**) which were started at 80:20 A/C (C, 100% acetonitrile) and gradient programmed to 57:43 A/C in 12.5 min, then programmed to 80:20 A/C at 13.5 min when conditions were returned to initial settings for 6.5 min in preparation for the next injection.

**Quality Assurance/Quality Control Results.** For each set of samples, blanks, spikes, and duplicate analyses were included. For the plant results, the average recoveries for the following matrix spikes were as follows: **1**, 125%; **3**, 85%; **7**, 100%; **6**, 103%; **4**, 105%. The average relative percent differences for duplicate analyses were as follows: **1**, 14% ; **3**: 15%; **7**, 17%; **6**, 40%; **2**, 15%; **8**, 14%; **4**, 22%; **10**, 16%, and **12**, 56%. Blank values were low for all analytes, except **1** where the average was 60 ng/g dry weight; however, these were not high enough to affect the total concentration detected in the samples, and corrections were not made. For the soil results, the detection limits (in ng/g dry wt) for the analytes were as follows: **5**, 1.78; **3**, 0.83; **2**, 1.74; **6**, 0.67; **1**, 0.74; **4**, 1.05; **10**, 0.003; **11**, 0.017. The average recoveries for spiked analytes were as follows: 72 to 90% for **1**, **2**, and **3** and 60 to 66% for **4**, **5**, and **6**. Recovery of **10** was lower, averaging 49%. Duplicates averaged 21% relative percent difference (RPD) for the duplicate pairs for the combined 2006 and 2007 soil data. This variation is not unusual for soil samples.

## RESULTS AND DISCUSSION

**Plant Result.** The total BX concentration and biomass results for rye vegetative tissue grown on each soil for 2006 and 2007 are show in Table 1. The total concentrations of benzoxazinoids were significantly lower in 2007 rye samples versus those used in 2006. Even with these large differences, these concentrations are typical for rye measured by other researchers.[12−14] The lower BX contents in 2007 appear to reflect a later harvest which has been noted by other researchers, namely, the BX content declines as rye plants mature.[14,12] The relative composition of BX constituents in the separate plant collections for the NF and FSP soil sites for each of the sampled years is shown in Figure 2. The composition in 2006 shows that compound **3** (DIBOA) predominates (average of 58%), with **1** (BOA) next at 14%, and all the remaining compounds at less than 10% (especially **4** (MBOA), which was near 6% of the total). For 2007, the breakdown was similar except for rye collected at the FSP soil site, which had only 38% of compound **3** (DIBOA). The numerous replicate analyses carried out in 2006 ($n$ = 5) showed that variability in

**Table 1. Rye Biomass and Total BX Content of the Rye Plant Material Applied in 2006 and 2007 at Each of Two Soil Sites**[a]

| rye biomass and total BXs content | | | |
|---|---|---|---|
| | | BX content | |
| year and soil | biomass (kg/ha) | mg/kg dry wt | SD |
| 2006 FSP | 3950 | 145.0 | 59.1 |
| 2006 NF | 6350 | 161.1 | 33.8 |
| 2007 FSP | 6790 | 55.4 | 39.4 |
| 2007 NF | 5680 | 12.2 | - |

[a]In 2006, the rye was at boot stage and in 2007 in the early heading stage.

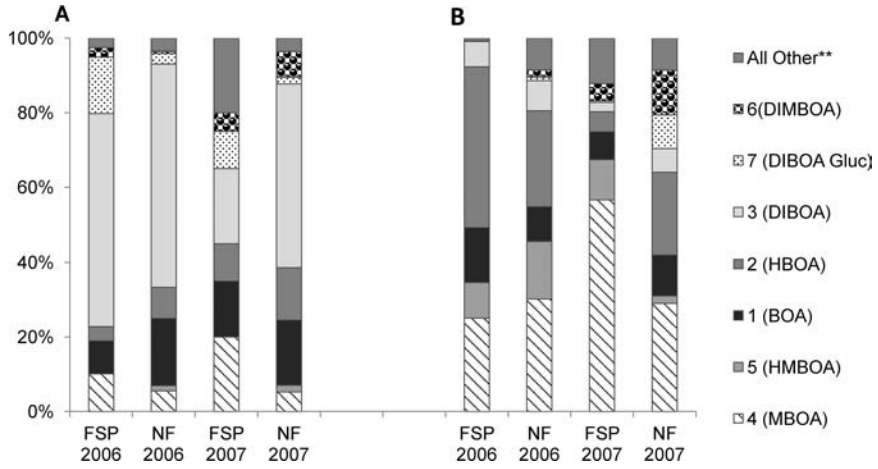


**Figure 2.** Percent benzoxazinoid (BX) composition in (A) rye plant tissues at boot stage in 2006 and early heading stage in 2007; and percent BX composition in (B) soil treated with rye cover crop in 2006 or 2007 in silt loam (FSP) or sandy loam (NF) soil types. All other = the total concentrations of **9**, **10**, **11**, and **12**.

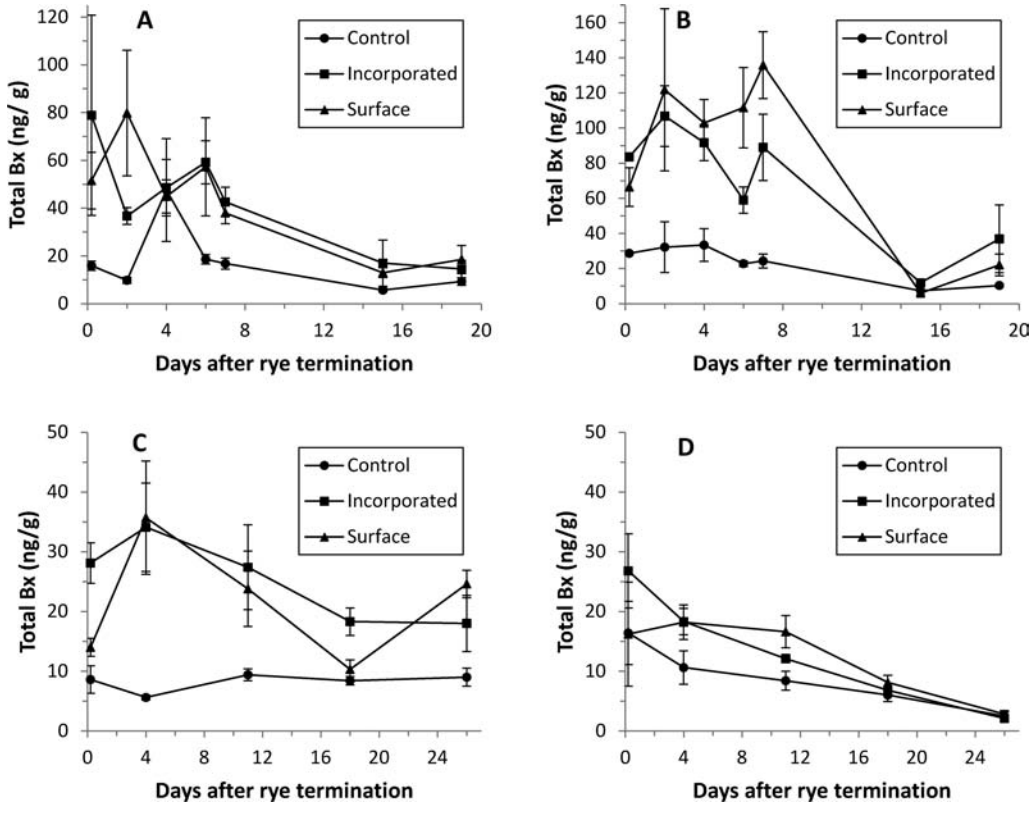


**Figure 3.** Concentration of total benzoxazinoid (BX) species in the top 10 cm of two soil types (FSP and NF) as a function of days after rye cover crop application in 2006 (A,B) and 2007 (C,D). Treatments were control (rye-free), incorporated (rye mixed into the soil), or surface (rye residue left on the soil surface or no-till). Standard error bars are presented with each mean.

concentration of the plants grown over the field plots did exist (standard deviations 33.8−59.1, (Table 1)) but that relative BXs compositions remained similar and agree with those found by other researchers. Krogh et al.[3] reported the following composition for rye sprouts (83% for **3**; 7%, **9** (DIMBOA-Gluc); 4.1%, **7** (DIBOA-Gluc); and 4% for **6** (DIMBOA)); and Carlsen et al.,[6] who analyzed several cultivars at growth stages typical for use as cover crops (Feekes stages 7−8), noted that **3** was the major component, averaging about 80% of their total BX vegetative samples. Timper et al.[13] measured levels of BX compounds in different rye cultivars and found 68% for **3**, 17% **1**, and 9% **2** (HBOA) as well as totals that ranged from 8.6 to 105 mg/kg.

**Background BX Concentrations in Control Plot Soils.** There was detectable background concentrations of total BX compounds in the soils collected from the no rye control plots (Figure 3). Concentrations in these control soils ranged from 10 to 30 ng/g total BX compositions in 2006 (with the exception of one aberrant spike 4 days after rye termination at the FSP site) and from 5 to 15 ng/g in 2007. These background BX levels in control soils may have been released during mineralization of organic matter following the tillage of these


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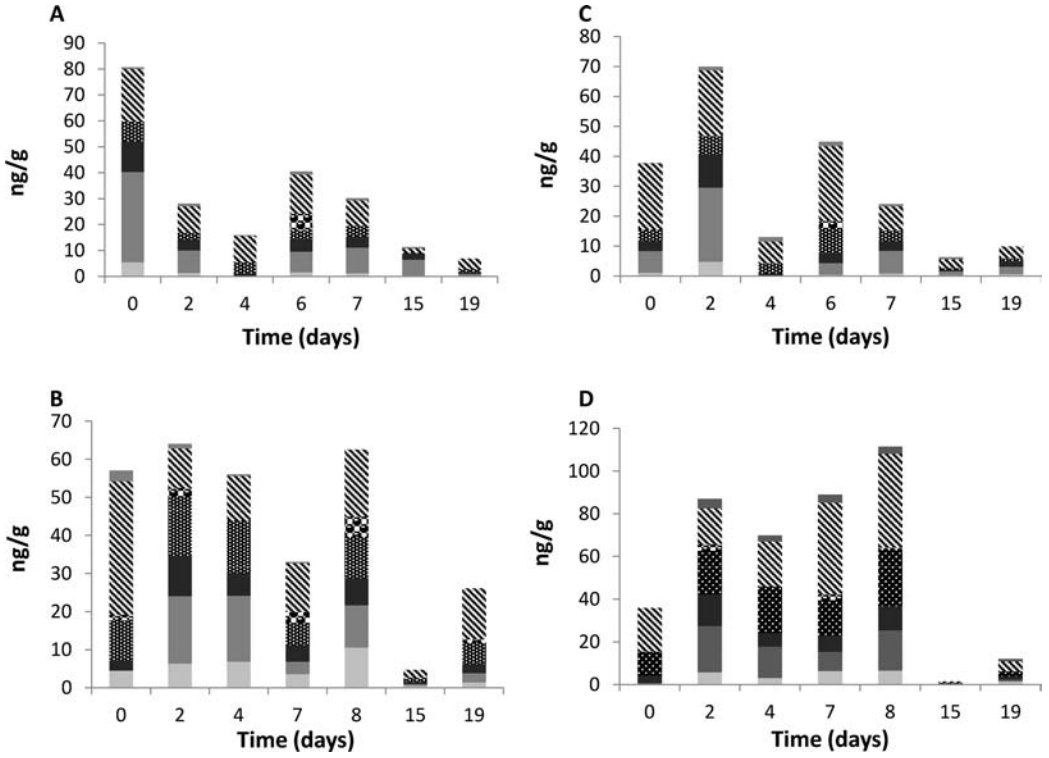


**Figure 4.** Average benzoxazinoid concentration in soil in 2006. (A) FSP soil and incorporated treatment, (B) NF soil, incorporated treatment, (C) FSP soil, no-till treatment, and (D) NF soil, no-till treatment. Dark gray bar, all other; diaganonally lined bar, **4** (MBOA); checkered bar, **6** (DIMBOA); dotted bar, **5** (HMBOA); black bar, **1** (BOA); medium gray bar, **2** (HBOA); light gray bar, **3** (DIBOA). All other" = the total concentrations of **7**, **8**, **9**, **10**, **11**, and **12**.

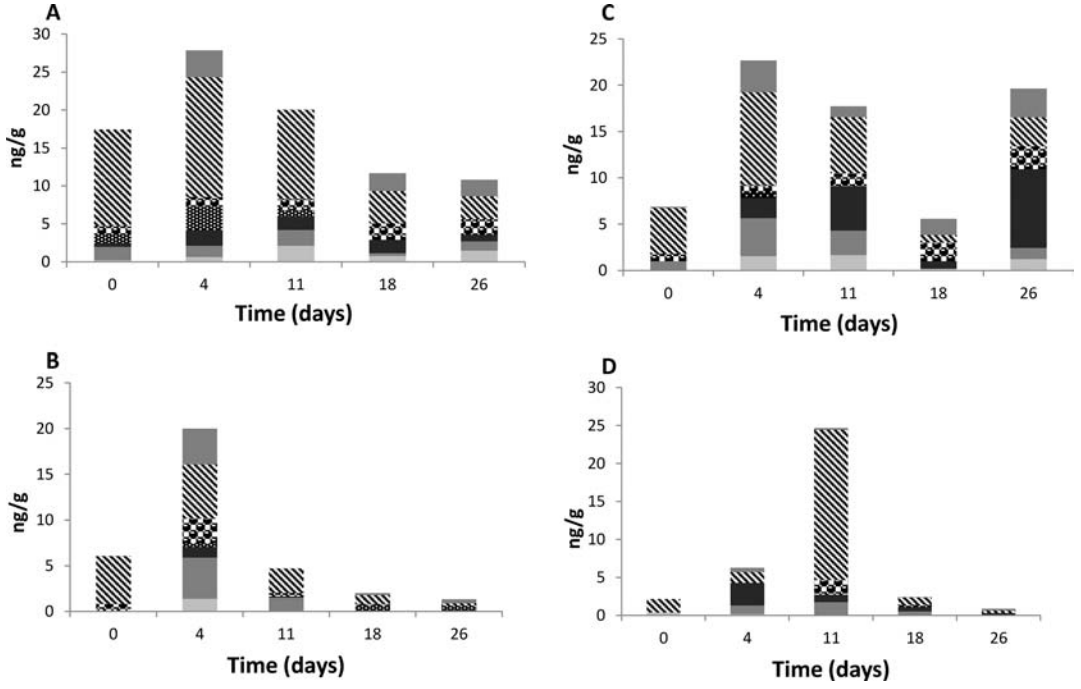


**Figure 5.** Average benzoxazinoid concentration in soil in 2007. (A) FSP soil and incorporated treatment, (B) NF soil, incorporated treatment, (C) FSP soil, no-till treatment, and (D) NF soil, no-till treatment. Dark gray bar, all other; diaganonally lined bar, **4** (MBOA); checkered bar, **6** (DIMBOA); dotted bar, **5** (HMBOA); black bar, **1** (BOA); medium gray bar, **2** (HBOA); light gray bar, **3** (DIBOA). All other = the total concentrations of **7**, **8**, **9**, **10**, **11**, and **12**.

soils at the initiation of the experiment. This organic matter was derived from a history of grain and rye cover crops grown on these fields. Krogh et al.[3] also alluded to finding BX "signals naturally present in the soil." Such passive and active organic matter pools are reported to exist in soils.[15] More research is required to understand their contribution to the spring-tillage


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activated releases believed to be causing these background signals. Because of these background concentrations, it was decided that subtracting them from the corresponding time and soil-matched rye treatments was necessary in order to properly assess the contribution from the rye cover crop.

**Benzoxazinoid Concentration in Cover-Crop Treated Soils.** The soil concentrations of all BX species (sums of all measured BX compounds) in all of the soil treatments (FSP and NF for 2006 and 2007) are shown in Figure 3. Generally, there were higher levels of BX in the soils after rye treatments than in control soils, and these elevated amounts lasted for approximately 2 weeks after rye termination. Maximum total soil concentrations were 80 and 136 ng/g in FSP and NF soils, respectively, in 2006, and 36 and 27 ng/g in FSP and NF soils, respectively, in 2007. Lower soil concentrations in 2007 are probably related to the corresponding lower BX concentrations in rye tissue in that year as described earlier. All of the BX compounds and their degradation products were observed in these soils. The average concentrations (background subtracted) for the major BX compounds occurring in the soil samples in each soil type and each soil treatment (incorporated (INC) and no-till (NT)) are shown as bar graphs of their stacked sums at each sample interval for 2006 (Figure 4) and 2007 (Figure 5). During this period, the dominant BX species were **2** (HBOA), **5** (HMBOA), **1** (BOA), and **4** (MBOA) which combined to comprise 67 to 92% of the total BX over both years (Figure 2). For 2006, **4** averaged 32 to 46% of the total BXs, and **5** was 14 to 22% of total BXs; notably, these are both methoxy-substituted BX compounds which were minor constituents in the plant material. For the nonmethoxy (demethoxy) compounds in the soil, **2** and **1** were the dominant members, each contributing between 14 to 27% of total BXs, and they sometimes ranked second in abundance to **4**. With the 2007 soil data, generally a similar trend for relative abundance of the BX composition was observed as in the 2006 soil samples, e.g., **4** was even more dominant (36 to 53% of total BXs), while second in abundance were often **1** and **2** (5 to 20%). The levels of **10** (APO) rarely exceeded 3 ng/g in 2006 or 1 ng/g in 2007. These overall results are similar to those of Krogh et al.[3] who found that incorporation of rye yielded primarily **2** and **4** during the first 4 days after incorporation; trace levels of **10** (APO) were noted, and minimal BX compounds were present 10 days after incorporation.

The pattern of BX species distribution remained relatively similar across all sample dates. There was no evidence of sequential transformations from one BX species to another across the sampling period contrary to what has been noted by other researchers.[3,4] However, these observations came from more controlled studies performed in soil slurry incubations[4] or using young rye sprout in potted systems placed in a greenhouse.[3] Our study has more direct relevance to the field situation, which we believe increases the impact of these observations.

In general, the NT treatment attained maximum BX values later and maintained higher concentrations longer than the INC treatment, especially in the north farm NF/NT plots for 2006 and 2007, (Figures 4 and 5). The extended duration of higher levels for the no-till situation versus the incorporated treatment seems logical since the incorporated rye would be expected to degrade and become released faster than the no-till rye, where surface residue decomposition would be driven more by intermittent wetting and drying patterns. These patterns of release were also demonstrated by An et al.[16] where they developed a mathematical model to describe the effects of allelopathy when comparing incorporated versus surface applied materials in cover cropping systems.

**Plant Composition versus Soil Composition Considerations.** The data presented in Figures 2 clearly show that the compositional mix of the BX source material is different from the BX compositional patterns measured in the soil. Compound **4** (MBOA) showed the most striking overall increase in the soil versus what was measured in the plant (10% mean in plant versus 35% mean in soil), and compound **2** (HBOA) also showed a substantial increase in soil (9% in plant versus 24% in soil). While compound **2** (HBOA) has been suggested to arise from the breakdown of **3** (DIBOA),[3] which was readily abundant in the plant material, it is more difficult to determine likely sources for compound **4**. Furthermore, it is generally assumed that the demethoxy forms do not interchange with the methoxy-substituted forms during plant decomposition, suggesting that some additional sources for the methoxy-substituted forms must exists. There was no sufficient methoxy-substituted quantities (**4**, **5**, **6**, or **9**) measured in our foliar plant analyses to produce the quantities of **4** that were observed in the soil. This mysterious observance of **4** after the addition of rye tissue to soil was also observed by Krogh et al.[3] It is our hypothesis that a likely source for this **4** could have been the rye roots that remained buried in the plots and were not accounted for in our rye tissue analyses. Root portions of rye are reported to contain higher proportions of methoxy-substituted BX compounds than above ground tissues. Carlsen et al.[6] reported that greater abundance of compounds **5** (HMBOA) and **4** were present in roots of several Danish cultivars of rye versus foliage. Also Rice et al.[12] found evidence for higher concentrations of methoxy-substituted BX compounds in root tissues versus the foliage of mature rye plants. Other explanations also could account for the high soil amounts of **4** in our study. For example, this form may persists longer in soil since **4**, as previously noted by Macias et al.,[17] has a half-life of 5 days versus the shorter half-life values measured for both **1** of 2.5 days[4] and **3** of 18 to 22 h[8]. It also could be postulated that we incurred selectively higher losses for **5** (HMBOA) and **6** (DIMBOA) (known precursors for **4**) during the frozen storage of our soil samples. Support for this storage loss is suggested by reports by Elljarat et al.[18] where analytical reference standards of **5** and **6** were found to be unstable even when stored at −20 C°, where they showed 20% loss in 7 days, while **2** and **3** showed losses of only 10%. Another possibility would be selective immobilization on the soils of certain species. Krogh et al.[3] also offered the possibility that their observations of extra methoxy-substituted forms in their potted soil studies may "originate from a compound in the plant that was not quantified." Additional research is required to determine these potential sources for elevated **4** in soil.

**Aminophenoxazinone Concentrations.** Only trace amounts of **10** (APO) were observed, rarely exceeding 3 ng/g in 2006 and 1 ng/g in 2007, and even more negligible amounts of **12** (AMPO) or methoxy-substituted aminophenoxazinones were found. Krogh et al.[3] also found minimal **10** at less than 3 ng/g of soil after the addition of rye plants to soil. In experiments where pure BX compounds were incorporated in soil, BX compounds degraded within days to **10**, which was found to be highly persistent in soils.[4,19,20] However, in all of these experiments, persistent **10** was only found in soils in which BX compounds were originally added at high concentrations (approximately mg/g amounts). Under-

strup et al.[20] showed that as the **1** (BOA) concentration added to soil decreased from 30,000 to 3 nmol/g, the **1** half-life decreased by approximately 50-fold, and the persistence of **10** declined from greater than 90 days to undetectable at any time. Macias et al.[4] suggest that the persistence of **10** resulting from high concentrations of exogenous BX may be the result of toxicity to soil microorganisms by the relatively high levels of **10** that were produced, which had essentially prevented further microbial transformations from occurring. Since BX transformations do not occur in sterile soil,[19,4] it is reasonable that the high persistence of **10** is probably an artifact of the high BX concentrations used for these degradation experiments and that relatively negligible **10** levels are actually present in soils when BX inputs are at natural levels produced by field grown cereals as shown in our research and that of Krogh et al.[3]

The **10** patterns for occurrence were more pronounced in NF soil than in FSP soils, but in all cases, the measured levels decreased to below the detection limit values after 10 days. One additional possibility to explain our somewhat low values for these **10** could be the fact that our average recovery for **10** was only 49%; however, even doubling our results would still produce very low concentrations. Additionally, **10** is less water soluble than the other BX compounds[5] making it more likely to be immobilized by soil absorption, although this was not tested by us. Thus, despite the fact that **10** is the most phyotoxic BX degradation product,[5] our soil data suggests that **10** and related aminophenoxazinones are present in such low concentrations that they probably do not contribute to BX-mediated allelopathy.

**Phytotoxicity Assay.** Plant dry weight of lettuce and pigweed proved to respond more consistently than emergence to rye treatments, so only plant dry weight testing results are shown (Table 2). Incorporated rye treatments inhibited dry weight increases of both species when planted the same day as or 7 days after the incorporation in 2006. Dry weight was only significantly inhibited when planted on the same day as the incorporation in 2007, although there was evidence of inhibition when planted at 11 days after treatment. Generally, there was little or no inhibition by incorporated residue if assay species were planted greater than two weeks after incorporation. Thus, the period of growth inhibition by incorporated rye residue coincided with the period of BX availability in soil after incorporation (Figure 3), which circumstantially suggests that BX compounds may have been responsible. However, the most prevalent BX species, **1** (BOA), **2** (HBOA), **4** (MBOA), and **5** (HMBOA), are among the least toxic benzoxazinoids,[5] whereas the most toxic potential metabolite, **10** (APO), was present in negligible amounts. Further research is needed to address whether BX compounds persist long enough and at high enough concentrations to account for observed inhibition or whether other compounds released at the same time as BX can better account for the inhibition observed.

**Table 2. Dry Weight of Lettuce and Smooth Pigweed Planted at Specified Days after Treatment of Soil Where the Rye Residue Was Either Incorporated or Remained on the Soil Surface**

| | | dry weight (% of control)[a] | | | |
|---|---|---|---|---|---|
| | | lettuce | | smooth pigweed | |
| year | days after treatment | incorporated rye | surface rye | incorporated rye | surface rye |
| 2006 | 0 | 34* | 4*† | 23* | 6*† |
| | 7 | 55* | 5*† | 33* | 3*† |
| | 19 | 142 | 5*† | 73 | 2*† |
| 2007 | 0 | 7* | 2* | 2* | 1* |
| | 11 | 58 | 13* | 44 | 3*† |
| | 18 | 89 | 10*† | 89 | 19*† |
| | 26 | 118 | 36 | 127 | 45*† |

[a]Values for terminated rye are expressed as a percentage of the value of the bare-soil control without rye. * indicates the value is significantly different from that of the control ($P < 0.05$). † indicates that the surface rye treatment value is significantly different from the corresponding incorporated treatment value ($P < 0.05$).

Rye residue on the soil surface was highly suppressive of dry weight increases of both species regardless of assay planting dates from 0 to 26 days after treatment (Table 2). This result is consistent with physical suppression by the surface residue, which remained intact for this time period and is inconsistent with the pattern of BX soil concentration, which was most pronounced during only the first two weeks (Figure 3). If there was any allelopathic contribution by BX compounds, it would have been masked by the dominating physical suppression of growth throughout this assay. Previous research by Teasdale and Mohler[21] showed that weed suppression by several mulching materials including rye was explained more by the physical properties of these materials than by allelopathy.

**Laboratory Experiments.** If one considers the physical properties of the BX compounds, especially their relatively high water solubilities (**1** (BOA) is 8.9 g/L,[20,22] and **4** (MBOA) is 0.54 g/L (measured by us using official OECD methods[23])) and their low soil partitioning tendencies (e.g., **1**, log $K_{oc}$ 1.16[22]), then they should exist largely in the solution phase of soils and should move freely in this aqueous phase. To support this generalization, these physical properties can be compared to those of pesticides which have relatively low solubilites, < 0.1 g/L, and generally higher log $K_{oc}$ values, >2.0.[24,25] Pesticides are generally observed to adsorb to soil and move sparingly through the vadose zone in the soluble phase.[26] On the basis of the above considerations, it was surprising that very little of the BX chemicals released by the rye applications were measured in the soil samples. This same general observation of low availability in the soil was supported by our laboratory dosing studies with pure materials. We conducted two experiments where this was observed. In the first set of experiments, **1** and **4** solutions were added to the surface of soils, and it was observed that mobility even in these moist (about 75% of field capacity) soils was limited (Table 3). The data show a general lack of diffusive mobility for both compounds with the majority of dosed surface material remaining in the top 0−1 cm (71−97% of the total measured) over a 3 cm deep soil column. With

**Table 3. Distribution of the Total Recovered 1 (BOA) and 4 (MBOA) in the Soil Profile after Surface Additions of Concentrated Solutions to Achieve 1.0 and 10 ppm Soil Concentrations[a]**

| | **1** (BOA) | | **4** (MBOA) | |
|---|---|---|---|---|
| soil layer | FSP | NF | FSP | NF |
| top (0 to 1 cm) | 84% | 71% | 97% | 97% |
| middle (1 to 2 cm) | 11% | 19% | 3% | 3% |
| bottom (2 to 3 cm) | 5% | 10% | 0% | 0% |

[a]Each of these values is an average of 6 measurements, 3 reps at each concentration.

these surface applications, there were small differences between **1** and **4**, where **1** appeared to migrate deeper, which likely relates to its greater water solubility. However, the general lack of diffusive mobility for both compounds is an important property for explaining the environmental impacts of these compounds. Lack of mobility could result in BX compounds concentrating near the soil surface in surface applied treatments, which could lead to potentially higher effective concentrations in this zone. This suggests a role of BX during the early days of inhibition of dry weight increases observed in our seedling assay with surface residue (Table 2).

In the second set of experiments on the availability of BX compounds after dosing, **1** was added to soil as concentrated aqueous solutions and mixed thoroughly into soils wetted to near their maximum water holding capacity. Analyses of **1** in these samples showed that availability was much less than 50% of that predicted even shortly after mixing (Figure 6). We

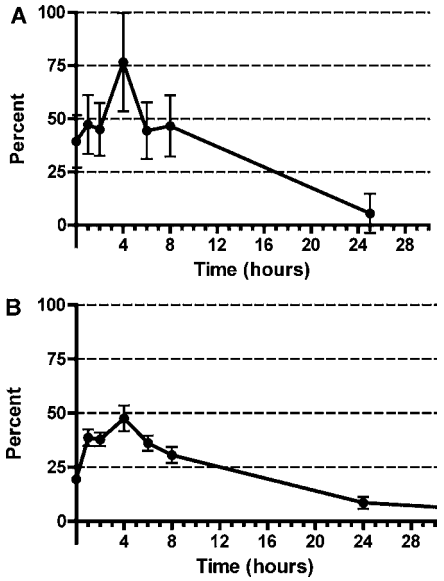


**Figure 6.** Timed measurement of the percent availability of **1** (BOA) uniformly mixed at 10 mg/kg in two soil types (A) silt loam soil, FSP, and (B) fine sandy loam soil, NF.

believe this apparent enhanced binding is connected to the water content of the soil since our experiments to improve analytical recovery revealed that we were able to overcome the problems of poor recovery of **3** (DIBOA) and **6** (DIMBOA) that were reported by Krogh et al.,[3] if the water content in the soil is increased well above it field capacity. In these tests, we found that it took about a 2:5 soil to water volume ratio to improve recoveries to near 70%. These unexpected observations show how important soil interactions appear to be for these BX compounds and highlight the importance of conducting more careful studies to determine true exposure concentrations in soils by these BX compounds.

The time course for the availability of **1** was also followed in the uniformly-mixed dosing experiments with **1**. The concentrations measured in these samples started at an initial amount of 19 and 40% of added **1** that increased to maxima of 48 and 78% after 4 h, respectively, for FSP and NF soils (Figure 6). Apparently, the FSP soil tied up less of the **1** than the NF soil. Similar binding behavior was observed by us[8] when **3** (DIBOA) was added to soil in a study of nematode affects. Here, the initial levels varied between 20 and 36% of added material after which the levels rose to slightly higher amounts, 6 h later. Another important observation from the time course data with the uniformly-mixed **1** experiments was that once the maximum values were achieved, then concentrations fell off rapidly to near zero after 24 h in both soil types. Analyzing the losses shown in the curves in Figure 6 using a first-order decay curve-fitting procedure showed that after the maximum was achieved half-life values of 3.5 to 6 h were observed, which were shorter than the half-life values of 14.4 h for low initial concentrations and 3 to 3.1 days for higher concentrations of **1** by Understrup et al.[20] We conducted numerous additional laboratory assays using pure treatments with **1** and **4** (MBOA) (0.1 to 10 mg/kg) (unpublished) to soil which further supported the almost complete loss of these compounds in 24 h. All of these observations, therefore, would support our field measurements where we found concentrations relatively lower than those predicted from the plant material, and these low amounts persisted for several days during rye decomposition followed by rapid disappearance from soil after about two weeks. These results could be accounted for by continuous release of BX during rye decomposition over a two week period followed by relatively rapid disappearance and perhaps also coupled with possible immobilization of the BX materials once released into the soil environment.

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### Notes

The authors declare no competing financial interest.

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