

# ACQUISITION, TRANSFER AND METABOLISM OF [ $^{14}\text{C}$ ] IMIDACLOPRID AMONG WORKERS OF THE SUBTERRANEAN TERMITE, *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE)

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**Abstract** Imidacloprid, the active ingredient in Premise<sup>®</sup>, is a slow-acting, non-repellent termiticide used to control termites in residential and commercial applications. Workers acquire imidacloprid from contact with treated soil, and then transfer the AI among individuals throughout the colony. However, the mechanisms of acquisition, distribution, and metabolism of this AI in individuals, as well as the transfer of this AI among termites within the colony, are not clear. We found that workers of the subterranean termite *Reticulitermes flavipes* continuously exposed to 50 ppm [ $^{14}\text{C}$ ] imidacloprid-treated sand (w/w) for 48 hr, had 5 to 10-fold greater amounts of imidacloprid (IMI) internally (about 1,750 pg) than on the cuticle (about 250 pg). Untreated workers exposed to treated workers in various ratios groomed the intoxicated workers and ingested toxic amounts of AI. Soldiers were not observed to groom nestmates and did not acquire significant or toxic amounts of imidacloprid from workers topically applied with AI. Topically applied imidacloprid appears stable on the cuticle surface under the conditions of the experiment, but is metabolized internally to possibly 11 compounds that are more polar than the parent molecule. Six of these have been identified (from least to more polar): olefin-IMI (major), 5-OH IMI, 4,5-di-OH IMI, desnitro olefin IMI, desnitro IMI, and 5-chloronicotinic acid (tentative). In termites exposed to metabolite-treated sand, we found that both the olefin-IMI and 5-OH IMI exhibited significant activity in workers, but were about 10-fold less toxic than imidacloprid. The other metabolites were non-active at the highest dose tested (100 ppm) by this method of exposure. Termites excreted these metabolites onto the substrate. Those termites that were removed from the AI-treated sand and allowed to recover eventually had undetectable levels of the olefin-IMI, 5-OH IMI and 4, 5-di-OH IMI in their bodies, whereas only the desnitro IMI compounds and 5-CNA remained.

**Key Words** Grooming, non-repellent termiticide, radiolabel

## INTRODUCTION

Effective termiticides are non-repellent, slow acting, and transferred from intoxicated workers to naive nestmates. Non-repellency is important so that the termite is not inhibited from either tunneling into a treated area or from remaining in contact with the active ingredient (AI) long enough to acquire an incapacitating dose. A slow or delayed acting AI allows the contaminated termite to maintain its normal behaviors for an extended period so as to be able to transport AI away from the treated area and transfer it to nestmates via contact or trophallaxis thus spreading the toxin throughout the population.

A few studies show that imidacloprid is both non-repellent and slow acting, and is transferred among workers *in vitro*. Thorne and Breisch (2001) found that naive termites neither avoid nor are repelled by imidacloprid-treated soil, and those termites, which have recovered from imidacloprid intoxication, do not avoid re-entering a treated area. In addition, they found that termites remain alive for days on imidacloprid-treated sand, and if removed from the treatment, are able to recover. Theirs and other studies have shown a transfer effect where untreated nestmates exhibit symptoms of intoxication when exposed to contaminated workers, e.g., *Coptotermes formosanus* (Shelton and Grace, 2003) and *Reticulitermes virginicus* (Thorne and Breisch, 2001). The curious observation that imidacloprid-intoxicated workers of *Coptotermes formosanus* (Osbrink and Lax, 2003) were found 46 m from a treatment site suggests either the delayed toxicity of imidacloprid, which allowed workers time to travel that distance from the treatment, or an efficient transfer of AI from workers that were at the treatment site to workers away from the site.

The above studies raise many questions concerning what actually happens to the termites and to the termiticide during the time after they make contact in the treatment zone. Here we report on some of the dynamics of the termite-imidacloprid interaction that result when termites contact this AI in a treated area with regards to the acquisition of imidacloprid from the substrate, transfer of the AI to nestmates, and metabolism.

## MATERIALS AND METHODS

*Reticulitermes flavipes* workers were collected from traps placed on the North Carolina State University campus and used for all of the following experiments. Each trap consisted of 4 in. diameter x 12 in. sections of PVC pipe placed upright 6 in. into the ground, and contained two 6 in x 3.5 in. diameter moistened, rolled corrugated cardboard cylinders. Termites were removed from the cardboard, placed into 150 x 15 mm Petri dishes with two sheets of moistened 125 mm diameter Whatman #1 filter paper, and kept in the dark at ambient temperature until used.

To determine how much imidacloprid termites acquired from treated sand, workers were placed on sand containing 50 ppm [methylene-<sup>14</sup>C] imidacloprid (w/w) (25.32 mCi per mmol; 99.0  $\mu$ Ci per mg; chemical and radiochemical purity >99%) in the wells of a 12 well microtiter plate. To obtain the 50 ppm (w/w) concentration, 400  $\mu$ L of 250 ppm [<sup>14</sup>C] imidacloprid in water was added to 1 mL (1.6 gm) of dry 60-mesh play sand. After 48 hr of continuous exposure at room temperature, 18 live workers were removed from the sand and placed individually in 1.5 mL microcentrifuge tubes. To determine the amount of imidacloprid on the cuticle of each termite, individuals were washed 2 x 150  $\mu$ L 95% ethanol. The washes for each were combined into separate 7 mL scintillation vials so that, when finished, there were 18 vials each containing 300  $\mu$ L of EtOH wash. To determine the internal amount of imidacloprid in each termite, carcasses were placed individually into 7 mL scintillation vials to which 250  $\mu$ L of tissue solubilizer (Solvable) was added. Each termite was macerated with the end of a pipette tip, and incubated at 55° C for at least 12 hr to extract the internalized radiolabel. Four mL of scintillation fluid (Ultima Gold) were added to each of the wash and extraction vials, and the contents mixed by vortexing for 20 sec. Samples were placed in the scintillation counter for about 20 min to allow the bubbles to dissipate, and then counted for 5 min.

To determine how much AI was transferred from contaminated to uncontaminated individuals, donor termites were exposed continuously to 50 ppm [<sup>14</sup>C] imidacloprid sand for 48 hr as described above to acquire radiolabel. These were placed with untreated nestmates (recipients) in 2:1, 4:1, and 8:1 donor to recipient ratios. Eight recipients were used per ratio. After 24 hr, the amount of cuticular and internal radiolabel was determined for each recipient as described above.

To uncover any transfer behaviors, 100 ng of [<sup>14</sup>C] imidacloprid was applied topically in 200  $\mu$ L of 500 ppm [<sup>14</sup>C] imidacloprid in 50% EtOH with 0.01% Nile Blue A to the dorsal abdomen of donor termites. After the application fluid had dried and the termite had developed symptoms, two donors were placed in the well of a six well microtiter plate containing eight soldier nestmates. After 1 hr, four worker nestmates were added. Soldier and worker behavior was observed and videotaped intermittently over the course of several hours. After 24 hr, soldiers and workers were analyzed for external and internal radiolabel as described above.

The metabolic fate of imidacloprid was determined in termites that had been topically applied with 100 ng of [<sup>14</sup>C] imidacloprid and incubated for 10 d. Thirty-six live termites were placed into a single 1.5 mL microcentrifuge tube and washed with 4 x 1 mL acetone to remove the cuticular imidacloprid. The radiolabel was extracted by first adding 1.2 mL solvent to the termites, then grinding the termites with a pestle, sonicating the homogenate for 15 sec, and centrifuging at 12,000 rpm for 2 min. The supernatant was removed, placed into a 7 mL scintillation vial, and the pellet resuspended in another 1.2 mL solvent. This extraction sequence was performed 2 x 1.2 mL acetone and then 2 x 1.2 mL methanol. The supernatants were combined and then the solvents evaporated using a gentle stream of nitrogen in a warm glass bead bath. The residue was dissolved in 200  $\mu$ L 95% EtOH, and the constituents chromatographed on thin layer silica gel plates (Whatman HPTLC 20 x 20 cm silica gel plate (Fisher #05-713-323)) using 8:1 or 9:1 chloroform/methanol. Radiolabeled components were detected with a TLC imaging scanner (Bioscan). Metabolite identification was performed using HPLC-MS (Bayer CropScience, MO).

The toxicity of the metabolites relative to imidacloprid and water (control) was tested by exposing termites to sand containing 100, 50, 5, and 0.5 ppm (w/w) of either IMI, olefin-IMI, 5-OH IMI, 4,5-di-OH IMI, desnitro olefin IMI, or desnitro IMI (metabolites provided by Bayer CropScience). Twenty termites were used per compound per dose, and their mobility was assessed after 7 d of continuous exposure.

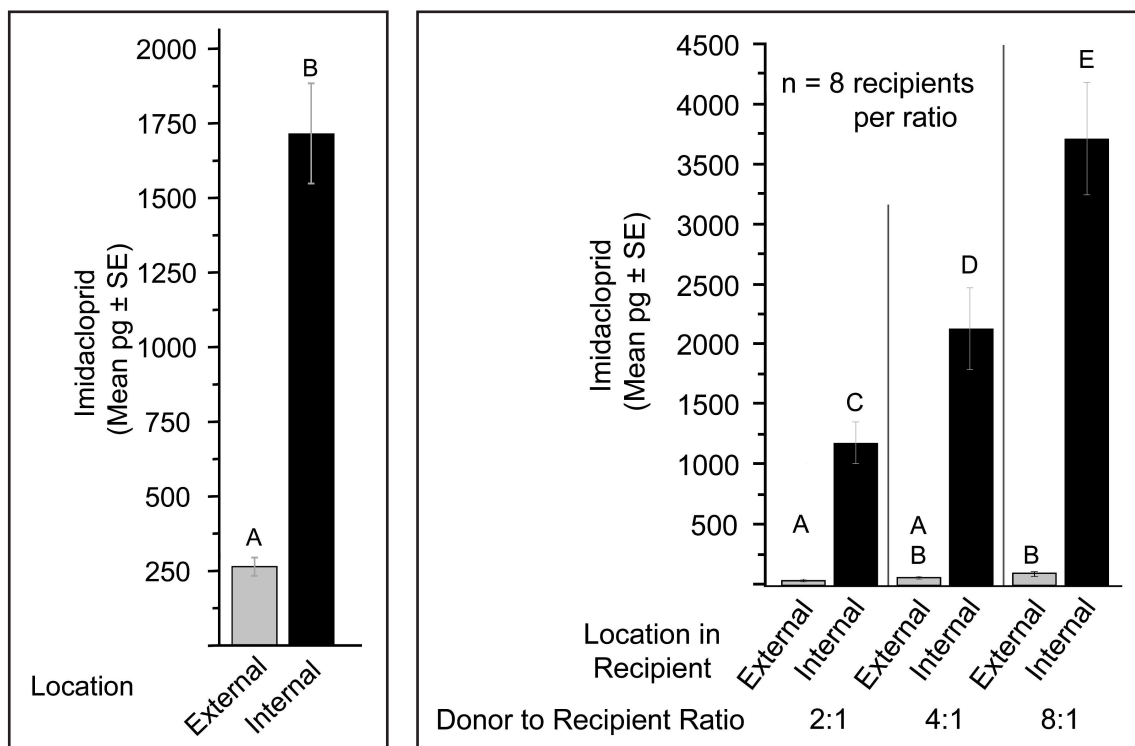
To see if termites excrete metabolites during recovery, 16 termites were transferred to each of three clean wells containing a piece of moistened filter paper and allowed to recover for 4 d after a 48 hr period of continuous exposure to sand containing 50 ppm [<sup>14</sup>C] imidacloprid (w/w). The radiolabel from 48 termites was extracted as described above. Wells and filter papers were rinsed 3 x 500  $\mu$ L 95% EtOH. Samples were processed for TLC as described above.

**Data Analysis.** The numbers of termites used for the treatments of each experiment are given in the corresponding figures or figure legends. All statistical analyses were performed using JMP Release 5.0.1.2 (SAS Institute, Inc.).

## RESULTS

Termite workers acquired significant amounts of radiolabel after a continuous 48 hr exposure to 50 ppm [<sup>14</sup>C] imidacloprid-treated sand (w/w). These termites were alive, but exhibited tremors and were immobilized due to imidacloprid intoxication. At this full label rate in the substrate, workers had a total of about 2,000 pg of which greater than 80% was internal, about 1,750 pg, compared with about 250 pg on the cuticle (Figure 1).

Untreated workers, exposed to treated workers in various ratios, groomed the intoxicated workers, ingested toxic amounts of AI, and exhibited symptoms of imidacloprid intoxication. The recipients acquired increasing amounts of radiolabel with increasing ratio. Though the amount on the cuticle did increase with increasing ratio, most of the radiolabel was internal in all ratios. Recipients in ratio 2 acquired a total about 1,200 pg of radiolabel. Recipients in ratio 4 acquired a total of about 2,200 pg of radiolabel, and is nearly double that of ratio 2. Recipients in ratio 8 acquired a total of about 3,700 pg of radiolabel (Figure 2).

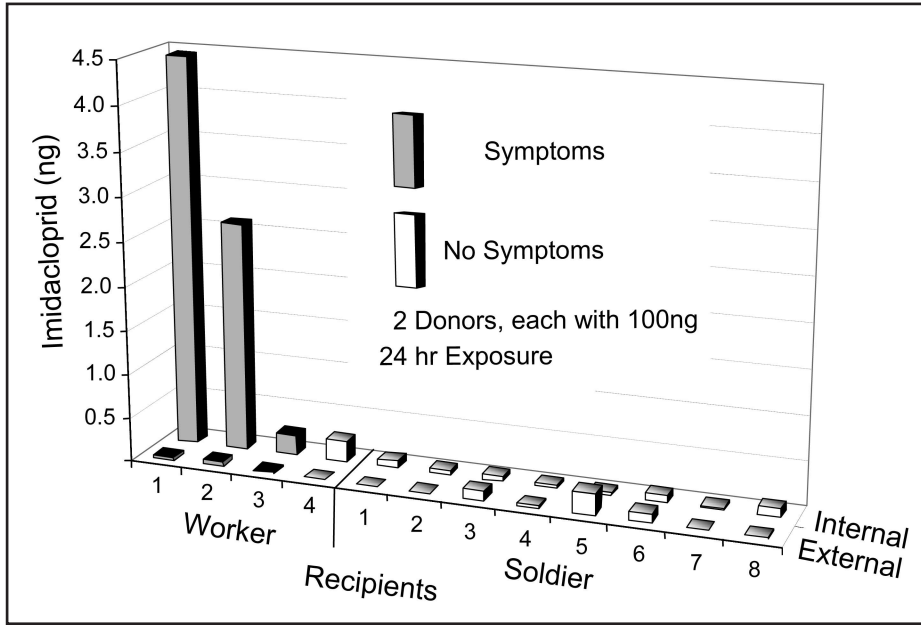


**Figure 1.** Termite acquisition of imidacloprid after a 48 hr exposure to sand containing 50 ppm [<sup>14</sup>C] imidacloprid (w/w). Means with the same letter are not significantly different,  $\alpha = 0.05$ .

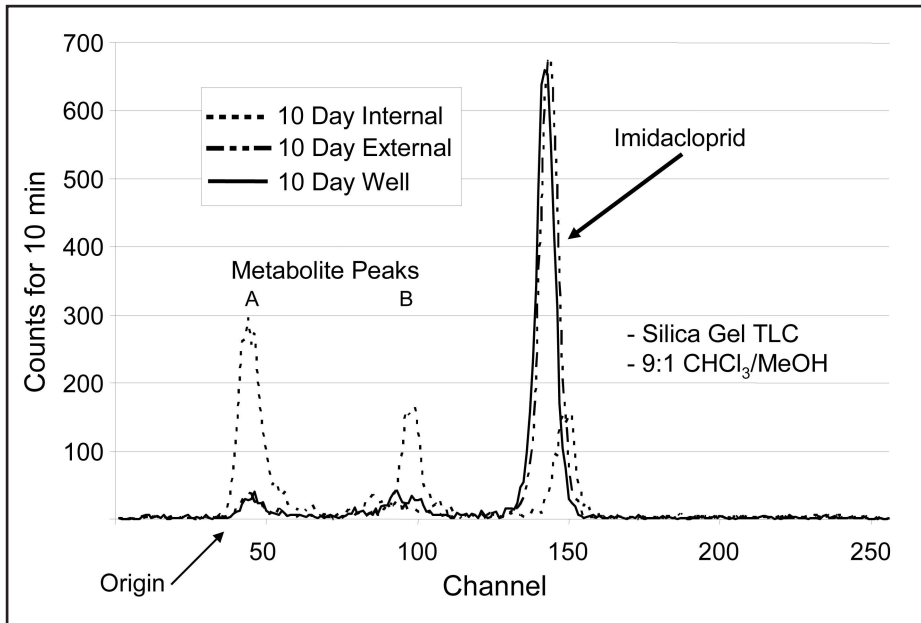
**Figure 2.** Termite acquisition of imidacloprid after 24 hr continuous exposure to donors that had been continuously exposed for 48 hr to sand containing 50 ppm [<sup>14</sup>C] imidacloprid (w/w). Means with the same letter are not significantly different,  $\alpha = 0.05$ .

Soldiers were not observed to groom nestmates and therefore did not acquire significant or toxic amounts of imidacloprid from workers that had been topically applied with AI (Figure 3).

Topically applied imidacloprid appears stable on the cuticle surface under the conditions of the experiment, but is metabolized internally to possibly eleven compounds that are more polar than the parent molecule. Six of these have been identified (from least to more polar): olefin-IMI (major), 5-OH IMI, 4,5-di-OH IMI, desnitro olefin IMI, desnitro IMI, and 5-chloronicotinic acid (tentative) (Figure 4).

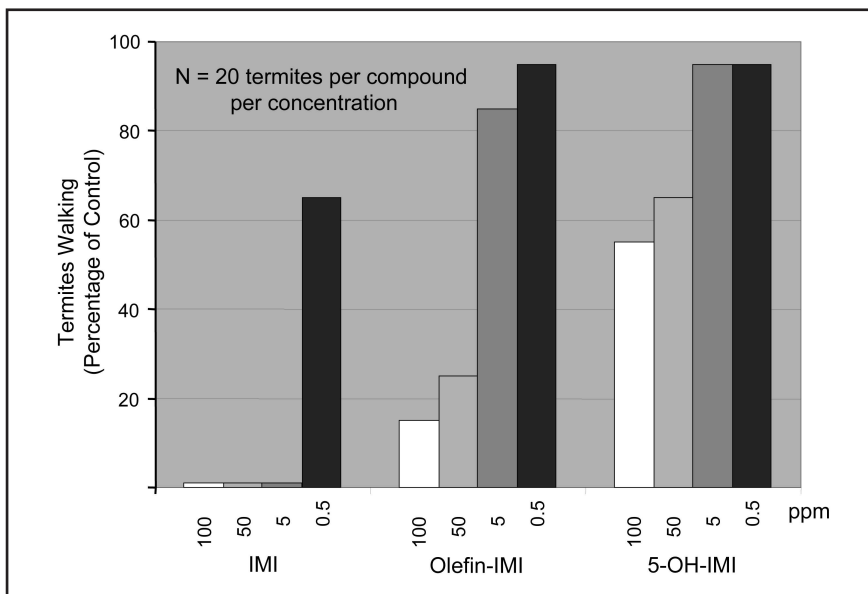


**Figure 3.** Worker and soldier acquisition of imidacloprid after a 24 hr exposure to two workers topically applied with 100 ng of [<sup>14</sup>C] imidacloprid.



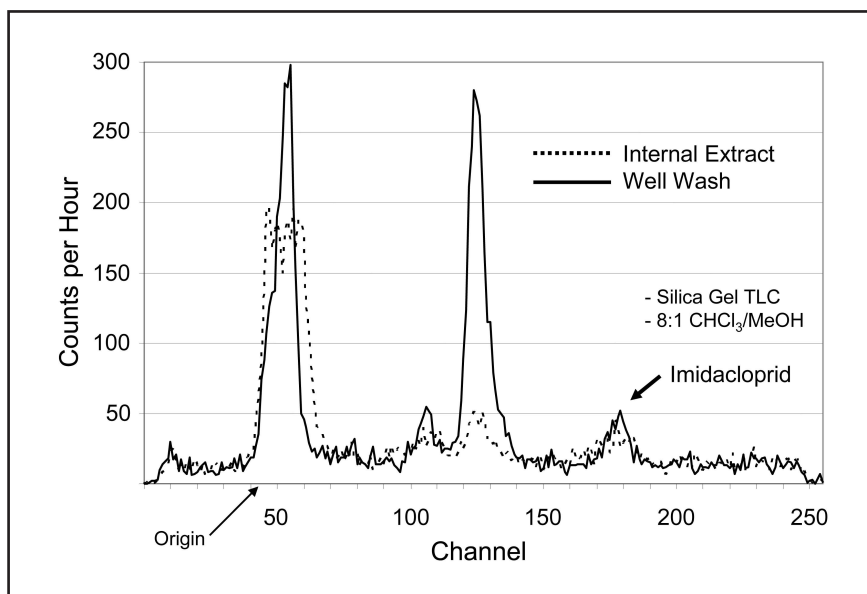
**Figure 4.** Metabolism of imidacloprid in *Reticulitermes flavipes* workers 10 days after topical application with 100ng [<sup>14</sup>C] imidacloprid.

In termites exposed to metabolite-treated sand, we found that both the olefin-IMI and 5-OH IMI exhibited significant activity in workers, and were about 10-fold less toxic than imidacloprid. The other metabolites were not toxic at the highest dose tested (100 ppm) by this method of exposure (Figure 5).



**Figure 5.** Toxicity of imidacloprid metabolites to *Reticulitermes flavipes* workers after 7 days continuous exposure to treated sand. The data for 4,5-di-OH IMI, desnitro olefin IMI, and desnitro IMI were indistinguishable from control, and therefore not included.

Termites excreted metabolites onto the substrate. Well and filter paper washes contained peaks that corresponded to metabolite compounds. Those termites, which were removed from the AI-treated sand and allowed to recover, had mostly the desnitro IMI compounds and 5-CAN, and very low levels of the olefin-IMI and 5-OH IMI (Figure 6).



**Figure 6.** Metabolism of imidacloprid and excretion of metabolites by *Reticulitermes flavipes* workers after a 2 day exposure to 50 ppm [<sup>14</sup>C] imidacloprid-treated sand and 4 day recovery.

## DISCUSSION

The process of termite acquisition of an AI during normal behavioral repertoires is the first component of a series of events leading to successful structural protection. The non-repellent and slow-acting properties of some of the current termiticides are important to enable the termite to contact and immerse itself in the AI long enough to obtain initially a debilitating dose, and then for the affected termite to be able to engage in behaviors that allow it to transfer the AI to its nestmates. In placing termites on sand at the full label of 50 ppm imidacloprid, we can see what is probably the maximum amount that they would acquire over a certain period of time. Then, by determining the compartmentalization of the AI, i.e., whether it is external or internal, we can surmise the availability of the AI to nestmates, and by what behaviors it might be transferred. For example, if high amounts are found on the cuticle, then transfer may be more via grooming of the AI from the cuticle. If high amounts are found internally, then transfer may be more via trophallaxis or grooming of regurgitated gut contents. Also, internal imidacloprid may be metabolized and excreted as constituents, which may be picked-up from the soil by other nestmates. Therefore, we undertook some initial studies to determine the mechanisms of termite acquisition of the non-repellent and slow-acting termiticide, imidacloprid.

Termites acquired significant amounts of imidacloprid both internally and on the cuticle during forced contact with treated sand containing the full label rate of imidacloprid. That greater than 80% of this total amount is found internally is probably due predominantly to oral ingestion when the termites handle soil particles with their mouthparts during tunneling and, to a lesser extent, permeation through the cuticle. Termites, containing about 2 ng of imidacloprid, exhibited severe symptoms and did not appear to be capable of purposeful behaviors such as grooming and trophallaxis.

In the experiment where termites, contaminated with AI via exposure to treated sand, were placed with uncontaminated nestmates, the latter acquired significant amounts of imidacloprid, of which greater than 90% of the radiolabel was internal. This indicates that the recipient ingested the radiolabel from grooming either the cuticle or the regurgitated gut fluid from around the mouth, or some of both. Because most of the donor's radiolabel is internal, for the recipient to get the amount of material that we observed, it probably imbibed most of it from fluid around the mouth of the donor. This exchange is not considered trophallaxis in a strict sense, since the donors did not appear to be able to engage in a behavior in which they actively regurgitated fluids to a nestmate.

When soldiers and workers were placed with donors that had been topically applied with AI, only untreated workers were observed to groom the donors and even soldiers. These workers acquired most of the radiolabel internally, and exhibited symptoms. On the other hand, soldiers were observed to contact nestmates but not groom them, and they acquired much less radiolabel and did not exhibit symptoms. Since, in topically applied AI all of the material is available on the cuticle, then the only ways the AI can be transferred are by simple contact and by grooming. Simple contact would deposit the radiolabel on the cuticle of the recipient where the AI then might be internalized via permeation through the cuticle or the recipient grooming itself. Therefore, since soldiers did not groom, the amount of radiolabel on and in the soldiers was obtained probably by simple contact with the donor. Grooming and subsequent ingestion of the AI from the cuticle of the donor is the only feasible explanation for the large amounts of radiolabel that two of the workers obtained during the time of the experiment. Therefore, grooming would appear to be an important behavior in the transfer of AI from one termite to another.

Once internalized, the termite metabolizes imidacloprid to a number of different compounds of which six have been identified. The olefin, which is a major intermediate metabolite, and the 5-OH are acutely toxic to workers at concentrations of greater than 50 ppm, but at a level of about 10-fold less than the parent. The other three compounds we tested did not exhibit any toxicity to termites. Chloronicotinic acid was not tested, but based on its polarity, is probably not toxic.

Termites that survived the 48 hr of continuous exposure to sand containing 50 ppm [<sup>14</sup>C] imidacloprid, and then were removed and placed on a clean surface appeared to recover their normal responses to disturbance after a period of time. Analyses of these termites showed that most of the internal radiolabel is present in a single, broad, polar peak that migrates not far from the origin and is composed of a mixture of non-toxic metabolites. These data suggest also that one of the major endpoints of metabolism of imidacloprid in termites maybe the chloronicotinic acid, but this still needs to be confirmed.

Properties of a termiticide have important implications in the mechanisms of acquisition, biotransformation and transfer processes, which in turn impact the ability of a compound to control termites and protect structures from infestation. We have shown that termites acquire significant amounts of imidacloprid from treated sand,

and that untreated nestmates subsequently acquired this AI from treated nestmates via grooming. We also showed that termites metabolize imidacloprid to less toxic and non-toxic compounds, both types of which were excreted onto the substrate. Termites, once removed from contact with the treatment, recovered from imidacloprid intoxication, and this recovery appears to be due to detoxification of imidacloprid via metabolism and excretion. Knowing acquisition, metabolism, and transfer mechanisms of imidacloprid in termites provides data to develop models that describe the termite-termiticide interaction at the colony level and to enhance the performance of imidacloprid as a termiticide.

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