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Original Article

Lethal dose and horizontal transfer of bistrifluron, a benzoylphenylurea, in workers of the Formosan subterranean termite (Isoptera: Rhinotermitidae)

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The lethal dose and horizontal transmission of bistrifluron were examined in workers of the Formosan subterranean termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) in laboratory no-choice feeding tests. The concentration of bistrifluron in baits was 5,000 ppm (wt/wt) in a series of tests. When termites were exposed to bistrifluron bait for 1 week, toxicity appeared slowly with an LT_{50} (50% lethal time) of 6.2 weeks. Much faster efficacy was observed after 2-week exposure. The amount of bistrifluron recovered from moribund termites indicated that approximately 400 ng/termite or more bistrifluron should accumulate in a single worker for insecticidal efficacy. The bistrifluron amount analyzed from various body parts of the termite body was not significanly different between immediately after 1-week exposure to bistrifluron bait and after the subsequent 2-week exposure to untreated bait. The rate of bistrifluron transferred from 20 donors to 20 recipients in 1 week was 6% of the amount of bistrifluron taken by the donors during the 1-week exposure to bistrifluron bait, and much smaller amounts of bistrifluron were transferred from donors to recipients for the subsequent 2 weeks. The bistrifluron that was originally ingested by *C. formosanus* workers appeared to partly remain in the termite body.

Keywords: bistrifluron, termite control, bait toxicant, analysis, *Coptotermes formosanus*.

Introduction

Subterranean termites are social insects and serious pests in structures, mainly in tropical and temperate regions. Their social behavior should be considered for termite management purposes and various termite management systems have been proposed and their feasibility for controlling subterranean termites has been tested.[1–3] Chemical control has been widely accepted as a long-lasting and cost-effective measure, and is related to soil treatment with liquid termiticides, dusting treatment of the foraging areas of termites, and baiting programs. Baiting techniques to suppress the activity of an entire targeted colony have been studied as an environmentally acceptable measure over the past 30 years.[4] In the mid-1990 it was shown that a benzoylphenylurea (BPU) compound, hexaflumuron, could successfully eliminate colonies of the Formosan subterranean termite *Coptotermes formosanus* Shiraki and the Eastern subterranean termite *Reticulitermes flavipes* (Koller).[5,6] Since then, many products that contain slow-acting insecticides as active ingredients have been launched onto the market.[7] To date, only BPUs have been shown to be highly effective for eliminating colonies of subterranean termites as slow-acting termiticides.[8–12] One BPU, bistrifluron, may be useful as a bait toxicant for the control of subterranean termites.[13–15] The chemical structures of hexaflumuron and bistrifluron are shown in Fig. 1.

Bait systems generally include two processes: the aggregation of foraging termites in underground monitoring stations and their unconscious intake of a slow-acting bait toxicant. Thus, the success of bait systems largely depends on how efficiently foraging termites take the bait toxicant before they die

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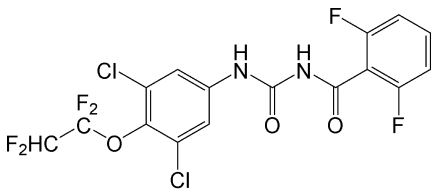
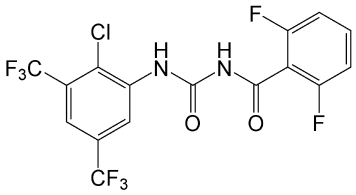


Hexaflumuron



Bistrifluron

Fig. 1. Chemical structures of hexaflumuron and bistrifluron.

and how quickly the toxicant is transferred among colonymates. Meanwhile, there is little information available about the lethal doses of existing bait toxicants, although the amount of toxicant has been determined with a few termite species when the colony was eliminated or termite activity was significantly suppressed.[5,8,16)]

We previously reported the potential of bistrifluron as a bait toxicant against *C. formosanus* and the Japanese termite *Reticulitermes speratus* (Kolbe), the most economically important species in Japan, in the laboratory;[14,15)] however, it seems difficult to conclude how effective bistrifluron would be at eliminating subterranean termite colonies without data on the lethal time and dose, and the behavior of the insecticide in an individual termite body. The kinetics of termiticides both within individual termites and among nestmates may provide insights for studies of termite behaviors and vice versa. These data may be useful for improving bait-application programs. In this study, we tried to estimate the toxicity of bistrifluron (lethal time and dose) against *C. formosanus* workers in a bioassay and chemically analyzed the amount of termiticide in termite bodies using liquid chromatography.

Materials and Methods

1. Insects

Undifferentiated larvae (workers) of *C. formosanus* were obtained from a laboratory colony. The colony was originally collected as a whole nest in Miyazaki Prefecture, Japan, in February 2000. The colony has been maintained at 26±2°C in the laboratory since then. Our unpublished studies indicate that the behaviors and responses of the colony to chemicals, including bistrifluron, were not much different during the period for which the colony has been maintained.

2. Experiment 1: No-choice feeding test with bistrifluron-treated disks for various exposure periods

A no-choice feeding test was conducted to examine the effect of the duration of exposure to bistrifluron and the amount of bistrifluron taken by termites on termiticidal efficacy. The experimental device was similar to that described by Kubota *et al.* (2006).[14)] A filter paper disk (No.1026, 33 mm in diameter, *ca.* 0.2 g) (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) was treated by pipetting 1 ml of an acetone solution of bistrifluron (96.5% technical grade, Dongbu HiTech Co., Ltd. (formerly, Dongbu Hannong Chemical Co., Ltd.), Seoul, Korea) to obtain 5,000 ppm (wt/wt) in the filter paper disk. The concentration of bistrifluron (5,000 ppm) was decided according to the commercially applicable concentration. The treated disks were dried under ambient conditions for several hours until the acetone had completely evaporated. Untreated disks were also included in the test as controls. Each disk was introduced into a small plastic cup (*ca.* 14 ml) with small entry holes (*ca.* 3 mm in diameter), and this cup was placed in a larger plastic cup (200 ml). The bottom of this larger container had several small holes made in the base and was covered with 2–3 mm of plaster.

One hundred termite workers were released into each experimental unit, and the units were then kept at 25±2°C for 12 weeks in an incubation chamber with a damp cotton pad over the bottom so that termites could take up water through the plaster covering the bottom of each experimental unit. The termites were allowed to feed on the treated filter papers for 3 days, 1 week and 2 weeks. The treated filter papers were removed after the respective time periods and replaced with untreated filter paper, and the termites were allowed to feed on the untreated filter paper for the rest of the experiment. The mortality rates of termites were recorded at 2, 4, 6, 9 and 12 weeks after test initiation (including the exposure periods, Fig. 2). Moribund or slow-moving individuals were counted as surviving termites as long as they could stand and walk by themselves.

The effect of the exposure period on termite mortality was statistically analyzed by the Dunnett test (α=0.05) at 2, 4, 6, 9 and 12 weeks. Mortality data were transformed to the arc sine of the square root for statistical analyses.[17)] The 50% lethal time (LT_{50}) was also calculated for each exposure period by the probit method.[18)]

3. Experiment 2: Lethal dose of bistrifluron

One hundred termites were released into the above-described experimental device so that they were exposed to a 5,000 ppm (wt/wt) bistrifluron-treated filter paper disk. The devices were kept at 25±2°C for the given test periods in an incubation chamber with a damp cotton pad over the bottom. The termites were allowed to feed on the treated filter papers for 1 and 2 weeks. The treated filter papers were removed after the respective time periods and replaced with untreated filter paper, and the termites were allowed to feed on the untreated

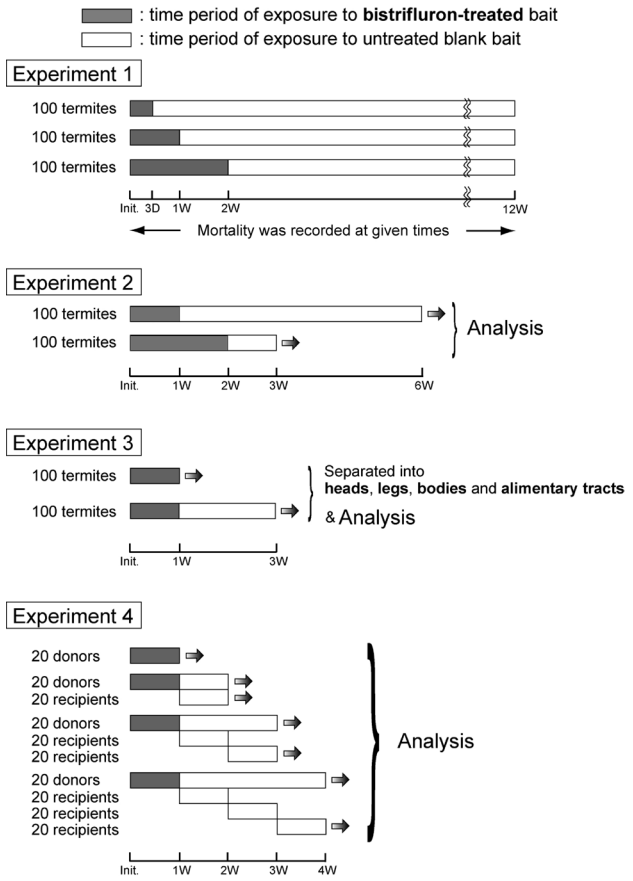


Fig. 2. Schematic illustrations of the series of no-choice feeding tests. In Experiment 1, 5 replications were made; In Experiment 2, 10 termites from 5 replications (2 termites from each replication) were analyzed; In Experiment 3, 10 termites were separated into heads, legs and remaining bodies and alimentary tracts were extracted from other 10 termites. The same parts were collectively analyzed and 4 replications were made; In Experiment 4, 10 termites were collectively analyzed and 4 replications were made.

filter paper for the rest of the experiment (Fig. 2). Five replications were tested for each exposure period. Ten moribund termites were collected from the experimental devices 3 weeks after test initiation in the 1-week exposure test and 6 weeks after test initiation in the 2-week exposure tests, respectively (Fig. 2). Each collection was close to the LT_{50}. These termites were randomly selected from five devices (two termites from each replication), and the amount of bistrifluron was analyzed separately by the method described below.

The amounts of bistrifluron recovered from individual termites were statistically compared between 3 weeks after test initiation in the 1-week exposure test and 6 weeks after test initiation in the 2-week exposure tests by a *t* test (α=0.05).

4. *Experiment 3: Recovery of bistrifluron from various body parts of termites*

Test termites were prepared in the same manner as described above. Four devices, the same as in the above-described experiments, were prepared and incubated at 25±2°C for 1 week. Twenty termites were selected from each device right after 1-week exposure (Fig. 2). Four other devices were prepared similarly and incubated at 25±2°C for 3 weeks while the treated disks were replaced by untreated disks 1 week after test initiation (Fig. 2). Twenty termites were selected from each device 3 weeks (2 weeks after 1-week exposure) after the initiation of the test. Ten of the 20 collected termites were dissected into body parts, such as heads, legs and remaining body parts, under a stereoscopic microscope. The same parts were collectively analyzed by the method described below. Alimentary tracts were extracted from the other 10 termites and analyzed similarly.

The amounts of bistrifluron recovered from each body part were statistically compared between 1 and 3 weeks by a *t* test (α=0.05).

5. *Experiment 4: Loss of bistrifluron through trophallaxis between bistrifluron-fed and unfed termites*

Nile Blue A (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was added to acetone solutions of bistrifluron at 0.5 g/l to produce stained disks to mark termites that had fed on bistrifluron-treated disks in blue, making it easy to recognize marked termites as donors.[19] Twenty termites were released into the above-described device and exposed to a 5,000 ppm (wt/wt) bistrifluron-treated filter paper disk (blue-stained). Sixteen devices were prepared and the termites were exposed to the stained disks for 1 week. Four devices were randomly chosen immediately after 1-week exposure and 10 termites were collected from each of the four test devices, and then analyzed by the method described below (Fig. 2). In the remaining 12 devices, the treated disks were replaced by untreated disks and 20 untreated termites (recipients) were released into the devices immediately after 1-week exposure of donors to treated disks (Fig. 2). Four of the 12 devices were disassembled weekly and 10 blue-marked donors and 10 unmarked recipients (white) were separately examined by chemical analysis (Fig. 2). Recipients were replaced by new untreated termites in the remaining devices at every collection. Each device was incubated at 25±2°C for the given periods in the same manner as described above.

The amounts of bistrifluron from donors at 1, 2, 3 and 4 weeks after test initiation were statistically analyzed by the Tukey-Kramer test (α=0.05) and those of recipients at 2, 3 and 4 weeks after test initiation were statistically analyzed similarly.

6. *Chemical analysis*

The termites were chemically analyzed by HPLC immediately after they were collected. They were kept chilled in a vial on ice until they were crushed and homogenized in a mortar with a pestle after being rinsed with HPLC-grade acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan). A preliminary chem-

Table 1. Effect of exposure period on the mortality of *Coptotermes formosanus* workers fed on 5,000 ppm bistrifluron-treated filter paper disks

Exposure period to bistrifluron-treated filter paper	Mortality (%) over time (week)[a]				
	2	4	6	9	12
0 day[b]	4.8±1.8	9.0±1.7	14.4±2.9	24.6±5.6	34.8±5.1
3 days	4.2±0.7	7.4±0.5	16.0±4.9	36.0±16.4	54.2±19.2
1 week	3.6±0.9	10.8±1.9	37.2±8.8*	88.6±7.0*	100.0±0.0*
2 weeks	19.8±15.3	74.6±11.2*	100.0±0.0*	100.0±0.0*	100.0±0.0*

[a] The values represent mean±S.D. The symbol * denotes siginificant difference from '0 day' data by the Dunnett test ($P<0.05$). Time includes the exposure periods. [b] Untreated control.

ical assay demonstrated no peak of bistrifluron in HPLC analysis with termites that had been exposed to an untreated filter paper disk. Homogenized termites were washed into a flask with acetonitrile and then subjected to ultrasonication (42 kHz±6%) for more than 1 hr in an ultrasonic device (BRANSON 3510, Branson Ultrasonic Corporation, Dunbary, CT, USA) to extract bistrifluron. The acetonitrile suspension was filtered with a PTFE filter of 0.45 μm (DISMIC-13HP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). HPLC analysis was performed using a Shimadzu LC-10Avp (Shimadzu Corporation, Kyoto, Japan), with a column of SUMIPAX ODS A-217 (4.6 mm in internal diameter, length 150 mm, Sumika Chemical Analysis Service, Ltd., Tokyo, Japan). Flow rate, injection volume and wavelength were 0.5 ml/min, 20 μl and 254 nm, respectively. The mobile phase was acetonitrile/water=80/20. Analytical-grade 2,4,6-trichloroaniline (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as an internal standard. HPLC-grade acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan) was used as a solvent. When a given amount of bistrifluron was mixed well with homogenized termites in solvent, 100% bistrifluron was recovered by the above-described procedure. At least equivalent amounts to 1.0 ng/termites of bistrifluron could be detected when a termite was analyzed individually. The amount of bistrifluron recovered from solvent that was used to clean termites that had been exposed to 5,000 ppm bistrifluron-treated disks for 1 or 2 weeks was much smaller than 5% of that recovered from whole bodies.

Results

1. *Experiment 1: Effect of exposure period on termite mortality in the no-choice feeding test*

When *C. formosanus* workers were exposed to 5,000 ppm bistrifluron-treated filter paper disks for 3 days, there was no significant difference in the mortality increase compared to the untreated control for 12-week test periods (Table 1, $P<0.05$, Dunnett test); however, 1-week exposure resulted in a significant increase in mortality at 6 weeks compared to the untreated control, and the mortality reached 100% at 12 weeks (Table 1, $P<0.05$, Dunnett test). The data gave an LT_{50} of 6.2 weeks (Table 2). Prolonged exposure for 2 weeks produced a significant increase in mortality at 4 weeks with 100% mortality at 6 weeks (Table 1, $P<0.05$, Dunnett test). The data gave an LT_{50} of 2.9 weeks (Table 2).

2. *Experiment 2: Lethal dose of bistrifluron*

The amounts of bistrifluron recovered from individual moribund termites were 397.7±57.7 ng/termite at 6 weeks and 492.0±50.1 ng/termite at 3 weeks when *C. formosanus* workers were initially exposed to 5,000 ppm bistrifluron bait for 1 week and 2 weeks, respectively (Table 2).

3. *Experiment 3: Recovery of bistrifluron from various body parts of termites*

As shown in Table 3, the amounts of bistrifluron recovered from heads, legs and other body parts of termites were 90.5, 4.5 and 559.1 ng/termite, respectively, just after *C. formosanus*

Table 2. Amounts of bistrifluron in moribund termites with LT_{50} values when *Coptotermes formosanus* workers were exposed to 5,000 ppm bistrifluron-treated filter paper disks for various periods

Exposure periods to filter paper	LT_{50} (week)[a]	95% CL	Bistrifluron amount recovered from individual termites (ng/termite) at ≈LT_{50}[b]
0 day[c]	>12.0	—	—
3 days	>12.0	—	—
1 week	6.2	5.0–8.3	397.7±57.7
2 weeks	2.9	2.8–3.1	492.0±50.1

[a] 50% lethal time. [b] At 6 weeks for the 1-week exposure and at 3 weeks for the 2-weeks exposure. There was no significant difference between the amounts of bistrifluron recovered from individual termites with 1-week exposure and 2-week exposure by *t* test ($P<0.05$). The values represent mean±S.D. [c] Untreated control.

Table 3. Amounts of bistrifluron recovered from each body part of *Coptotermes formosanus* workers exposed to 5,000 ppm bistrifluron-treated filter paper disks for 1 week[a)]

Exposure period to bistrifluron	Body part	Bistrifluron amount (ng/termite) at given times	
		Immediately after 1-week exposure	2 weeks after 1-week exposure
1 week	Head	90.5±10.3	95.8±4.8
	Legs	4.9±1.8	6.4±0.7
	Body	559.1±57.0	385.7±12.2
	Alimentary tract	60.8±6.3	48.8±4.3
	Whole body[b)]	654.4±63.1	487.9±14.0

[a)] There was no significant difference between the amounts of bistrifluron at 1 and 3 weeks for any body part by *t* test ($P<0.05$). The values represent mean±S.D. [b)] Summation of the amounts in the head, legs and body.

workers were exposed to 5,000 ppm bistrifluron bait for 1 week. After subsequent 2-week exposure to untreated bait following 1-week exposure to treated bait, these amounts were 95.8, 6.4 and 385.7 ng/termite, respectively (Table 3). There was no significant difference in the amount of bistrifluron between the post-exposure periods for any of the body parts (Table 3). The amounts of bistrifluron recovered from alimentary tracts were 60.8 and 48.8 ng/termite, respectively. These levels were equivalent to one tenth of that from a whole termite body (Table 3).

4. Experiment 4: Loss of bistrifluron through trophallaxis between bistrifluron-fed and unfed termites

The amount of bistrifluron detected in donors was 546.0 ng/termite just after exposure for 1 week, and this significantly decreased to 250–300 ng/termite after contact with recipients for 1 week (Table 4); however, the loss of bistrifluron from donors was not significant during the 1–3 weeks of contact with recipients (Table 4). The amount of bistrifluron recovered from the recipients was 32.6 ng/termite after 1 week of contact with donors, and the amounts were very small after additional periods of contact for 1 and 2 weeks (2.2 and 1.6 ng/termite, respectively), as indicated in Table 4.

Discussion

An amount of bistrifluron equivalent to that taken by termites in 1-week exposure to 5,000 ppm bistrifluron bait seems to be required for a lethal effect on *C. formosanus* workers under the current experimental conditions (Table 1). The amount of bistrifluron that was detected from moribund termites at the LT_{50} value of 6 weeks was 397.7 ng/termite (Table 2). Foraging populations of *C. formosanus* colonies sometimes number in the several millions and by simple arithmetic *ca.* 400 mg or more bistrifluron is needed to eliminate 1,000,000 foragers of a single *C. formosanus* colony. Hexaflumuron, which is in the same chemical group as bistrifluron, has been shown to be effective for eliminating whole colonies of termites after several 100 to 1,000 mg of hexaflumuron were consumed by each colony;[5,8,16)] our estimate roughly corresponds to these findings. In addition, bistrifluron shows faster action than hexaflumuron in *C. formosanus* workers,[14)] and more successful colony elimination is expected with bistrifluron bait.

When termites were exposed to 5,000 ppm bistrifluron bait for 2 weeks, the LT_{50} was much smaller than that with 1-week exposure: *ca.* 3 weeks (Table 2). This result corresponds to the fact that bistrifluron is slow acting but faster than hexaflumuron, as reported by Kubota *et al.* (2006).[14)] The amount of bistrifluron recovered from moribund termites at 3 weeks was 492.0 ng/termite. There was no significant difference in the amount of bistrifluron detected from moribund termites between 6 weeks with 1-week exposure and 3 weeks with 2-week exposure (Table 2, $P<0.05$, *t* test). These results mean that the termites took no or very little bistrifluron individually during the 2nd week of exposure. The drastic decrease in the uptake of bistrifluron, or even no uptake, in the 2nd week may be explained by the insecticidal effect of bistrifluron on termite feeding activities. An alternative explanation is that elimination increased as more of the compound was taken up, and that body titers reached a steady state concentration although the uptake might not have changed; however, since feeding intensity by visual check also drastically decreased after 1-week exposure to bistrifluron bait (quantitative data not available), the decrease in the uptake of bistrifluron in the 2nd week seemed to be caused by the insecticidal effect of bistrifluron. Although the cause of the large difference in LT_{50}s between the two exposure periods remains unexplained, the results of the current study seem to indicate that the uptake

Table 4. Changes in the amount of bistrifluron regularly recovered from *Coptotermes formosanus* worker donors and recipients after 1-week exposure to 5,000 ppm bistrifluron-treated filter paper disks[a)]

Termite	Bistrifluron amount (ng/termite) at given times (week)[b)]			
	1	2	3	4
Donors	546.0±41.0a	247.4±5.1b	304.6±11.4b	287.6±2.2b
Recipients	—	32.6±3.2a	2.2±1.3b	1.6±1.6b

[a)] Values represent mean±S.D., and those in the same row with different letters are significantly different by the Tukey–Kramer test ($P<0.05$). [b)] Including the exposure periods.

and accumulation of ≥400 ng bistrifluron by an individual termite could possibly provide slow-acting insecticidal efficacy.

Although we should not expect that all of the bistrifluron taken up by termites will be accumulated, there was no significant difference between the amounts of bistrifluron recovered from any body part immediately after 1-week exposure to bistrifluron bait and after the subsequent 2-week exposure to untreated bait (Table 3, $P < 0.05$, *t* test). The amount of bistrifluron recovered from alimentary tracts was *ca.* 10% of that from whole termite bodies even immediately after 1-week exposure, which suggests that bistrifluron molecules moved rapidly around the termite's internal body and existed in each body part stably (Table 3). It is also possible that a large proportion of bistrifluron is not present in a termite body in a stable form (*e.g.*, accumulated in fat bodies), but just circulates among organs in a termite body and/or among individual termites by trophallaxis. Therefore, determination of the amount transferred from treated to untreated nestmates should contribute to evaluating the stability of bistrifluron in a termite body. When termites were first exposed to 5,000 ppm bistrifluron bait for 1 week and then mixed with the same number of unexposed nestmates, 6% of the bistrifluron taken up by the exposed workers (donors) was transferred to the unexposed workers (recipients), and some bistrifluron was lost from the donors during the 1-week period of mixing (Table 4). However, subsequently bistrifluron appeared to remain unchanged inside the termite body because there was no significant difference in the bistrifluron amounts in donors at 2, 3 and 4 weeks and very little bistrifluron was detected in recipients at 2 and 3 weeks (Table 4).

The present results support the notion that once a large amount of bistrifluron is taken up by a *C. formosanus* worker, it stably exists in the termite body for several weeks, and the lethal dose of bistrifluron was estimated to be *ca.* 400 ng/termite. The present results also indicate that a small portion of bistrifluron taken up by foragers is transferred to their nest mates. High stability in a termite body and low transferability to nest mates indicate that termiticidal efficacy of bistrifluron seems rather due to the uptake of a sufficient amount of bistrifluron by each forager than subsequent horizontal transfer to other nestmates. Unfortunately, there is no evidence regarding how much bistrifluron can be transferred among colonymates in the field to compare the results further; however, it is possible that some proportion of bistrifluron is transferred to nestmates by frequent trophallaxis within a shorter period of time than 1 week. For example, the largest proportion of hexaflumuron taken up by *Reticulitermes hesperus* Banks in California was transferred from donors to recipients after exposure to hexaflumuron bait for a day.[20] In addition, the materials transferred from donors to recipients seem to be retransferred to other nestmates by cascade events.[21] Further studies are needed to clarify these points. Transfer of bistrifluron from donors to recipients seems to be due to trophallaxis, but it is unclear whether an exchange of materials in their alimentary tract or uptake of bistrifluron sticking to their body surface through allogrooming was the major route of bistrifluron transfer. The bistrifluron amounts recovered from alimentary tracts were around 10% while bistriflruon recovered from the body surface of worker termites exposed to treated baits was in a very small amount (much less than 5%). Such information will be helpful to simulate the efficacy of bistrifluron bait. By examining the rate at which foraging termites take up bistrifluron and transfer it to their nestmates, we should be able to obtain important information about subterranean termite control through bait application.

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