Laboratory and Field Studies of a Purple Dye Marker for *Reticulitermes* spp. (Isoptera: Rhinotermitidae)

by

Thomas H. Atkinson[1], Amy J. Griffin[2], and J. Edward King[2]

ABSTRACT

Laboratory data on palatability and consumption of a purple dye marker recently described by Oi (2000) composed of a blend of Neutral Red and Nile Blue A are reported. The blend deterred feeding, but a high percentage of individuals were still marked. The coloration of the blend is affected by the pH of the substrate, with more intense coloration at lower pH. In field trials using a self mark technique the blend produced a persistent mark that was rapidly dispersed throughout the foraging area of *Reticulitermes* colonies and was easily distinguishable from individuals marked with either Neutral Red or Nile Blue A alone.

INTRODUCTION

Despite many studies over the last 25 years only 2 persistent dye markers, Neutral Red (Esenther 1980) and Nile Blue A (Su *et al.* 1991), have been found that are suitable for use with *Reticulitermes* spp. in ecological studies. Recently Oi (2000) reported on a blend of Nile Blue A and Neutral Red that produces a purple dye mark in the laboratory that is persistent and distinguishable from the dye mark of either dye used alone.

Additional colors for field studies of *Reticulitermes* spp. are particularly valuable. At a particular study site, the number of dye markers that leave readily distinguishable marks limits the number of colonies that can potentially be identified. Having an additional color expands our ability to identify specific colonies at study sites to increase the number of replicates (where colony is a replicate). Additional colors also increase the resolution of trials of control methods, such as baiting, that have a colony level effect by avoiding confusion caused by overlapping dynamics of multiple colonies.

Both Nile Blue A and Neutral Red are known to deter feeding in the laboratory by *Reticulitermes flavipes* (King 2000) and in the field (Atkinson 2000). Nonetheless, both dyes mark a high proportion of individuals in laboratory tests, even in the presence of a preferred food and can successfully delineate colonies in the field when used in a self mark - capture procedure.

[1]Dow AgroSciences, 5005 Red Bluff Road, Austin, TX 78702, thatkinson@dow.com
[2]Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46268

Laboratory tests were run on the palatability of different dye formulations on different substrates. Field tests were run in Texas to validate the use of purple blends in a self mark-capture technique in which dyed matrix is provided in a foraging site and the subsequent movement of dyed individuals is used to infer foraging territories.

MATERIALS AND METHODS

Laboratory Trials

Neutral Red (92%, Sigma Chemical Co., St. Louis, MO) and Nile Blue A (87%, Aldrich Chemical Co., Milwaukee, WI) were dissolved in water such that 1.5 ml of solution applied to filter paper circles (Whatman #1, 9.0 cm diameter) would produce concentrations of 0.25% and 0.10% (wt. / wt.). These solutions in turn were applied in different ratios of pure or blended dyes, similar to the method described by Oi (2000). Oi's stock solution of Neutral Red produced a higher final concentration of dye such that our ratios are not completely comparable. Rates for both studies are summarized in Table 1. Similar procedures were followed using the LTC matrix currently used commercially in Recruit™ II, termite bait.

Dyed paper circles were allowed to dry for 24 hours and then cut into quarter pieces for feeding assays. After drying, quarter-pieces were weighed and folded in half prior to placement in feeding tests.

Eastern subterranean termites (*R. flavipes*) were collected in southern Mississippi and shipped to Dow AgroSciences laboratories in Indianapolis, IN. Termites were maintained on a mixture of wood, corrugated cardboard and moistened filter paper in the laboratory and were used in feeding tests within one month after field collection.

The test apparatus consisted of two round (5.5 cm diameter) polystyrene containers (harborage and food chambers) connected by tygon tubing (7 cm long, 2.5 mm ID) (King 2000). The harborage chamber contained a 1:1 ratio of vermiculite and white river sand covered by a ventilated lid. The substrate was moistened with distilled water to the point of saturation before termites were introduced. The food chamber contained only filter paper, with or without dye. One hundred termites were introduced into the harborage chamber. These typically began moving into the food chamber within minutes. Termite workers were held in the tests for 14-15 days in total darkness at 26° C and 80% RH.

After the studies were terminated, the number of surviving workers and visibly marked workers were recorded using white opaque collection trays. At the end of the holding period papers were removed and

---

™Trademark of Dow AgroSciences, L.L.C.

Table 1. Dye concentrations used. Concentrations used by Oi (2000) are shown for comparison.

Study	red/blue ratios (stocks)	final % red (wt./wt.)	final % blue (wt./wt.)	actual red/blue (wt./wt.)
Present study	100:0	0.250		
	75:25	0.188	0.025	7.5
	50:50	0.125	0.050	2.5
	0:100		0.100	
Oi (2000)	100:0	0.500		
	75:25	0.375	0.025	15.0
	50:50	0.250	0.050	5.0
	25:75	0.125	0.075	1.7
	0:100		0.100	

dried at 176° C for 1 hour for paper and 8 hours for wood, then cooled for 4 hours in a desiccator prior to weighing.

Separate no-choice tests were performed using filter paper and LTC matrix. Separate choice tests were performed using filter paper and LTC matrix with southern yellow pine as the choice in each pair. For each test, analysis of variance (ANOVA) with protected LSD mean separation test was performed on consumption rates and survivorship. Statistical analyses were computed with Mintab® 12.2 for Windows.

Field Trials

Standard baitube™ devices (containing approximately 20g) and without active ingredient were prepared with a blend of Neutral Red (0.125% wt. / wt.) and Nile Blue A (.05 % wt. / wt.) using the current Dow AgroSciences LTC commercial matrix.

Dyed matrix was introduced into 4 colonies of *Reticulitermes flavipes* and 1 colony of *R. virginicus* at 5 study sites in Texas. Some of these colonies were at grid sites while others were near residences. Dyed matrix was introduced during 2000-2003, depending on the site. Procedures followed those described by Atkinson (2000). Standard Sentricon® in-ground stations were used at all field sites. Stations were checked roughly every 30 days, although this varied according to season and site. Termites were introduced into baitube devices containing the dyed matrix using at the time of baiting. In all cases a baitube device containing the purple matrix was only introduced into a single station fed on by a particular colony. Other stations with termite activity were either baited with blank baitube devices (matrix

™Trademark of Dow AgroSciences, L.L.C.

Table 2. No-Choice Test. Forced feeding of eastern subterranean termites (*R. flavipes*) to blank or dyed Filter Paper (FP) after 14-day feeding exposure.

Treatment[a]	mg paper consumed/g termite/day (Mean ± SEM)[b]	No. of termite worker survivors/100 after 14 days (Mean ± SEM)[b]	% Surviving termites visibly dyed
Blank FP	21.93 ± 2.50 a	95.25 ± 2.75 a	0.0
0.10% Nile Blue FP	11.01 ± 1.45 c	67.00 ± 14.30 c	100.0
0.25% Neutral Red FP	14.42 ± 2.19 b	88.50 ± 2.90 ab	99.7
50:50 ratio, 0.1% Blue + 0.25% N. Red (purple FP)	12.61 ± 1.15 bc	72.75 ± 7.03 bc	100.0
25:75 ratio, 0.1% N. Blue + 0.25% N. Red (purple FP)	13.52 ± 1.78 b	85.25 ± 3.64 ab	98.5

[a]Each trt. replicated 4 times (100 termite workers per replicate).
[b]Within each column, means followed by the same letter are not significantly different (LSD, $p > 0.10$)

Table 3. Choice Test. Comparative consumption of southern yellow pine (SYP) and dyed filter paper (FP) by eastern subterranean termites (*R. flavipes*) after 14-day feeding exposure.

Paired Choice	mg consumed/ g termite/day (Mean ± SEM)[b]	No. of termite worker survivors/100 after 14 days (Mean ± SEM)[c]	% Surviving termites visibly dyed
SYP vs	11.43 ± 2.28 a	87.5 ± 2.18 b	0.0
Blank FP	17.94 ± 1.66 a		
SYP vs.	24.69 ± 1.63 a	82.0 ± 2.55 c	89.6
0.10% Nile Blue FP	5.47 ± 0.76 b		
SYP vs.	20.26 ± 2.73 a	86.5 ± 2.33 b	79.7
0.25% Neutral Red FP	10.07 ± 3.06 a		
SYP vs.	22.82 ± 2.42 a	89.5 ± 2.60 ab	47.6
50:50 ratio, 0.1% Blue + 0.25% N. Red (purple FP)	8.24 ± 0.29 b		
SYP vs.	19.41 ± 2.68[a]		
25:75 ratio, 0.1% N. Blue + 0.25% N. Red (purple FP)	9.68 ± 2.36[a]	93.0 ± 1.87[a]	65.3

[a]Each test replicated 5 times (100 termite workers per replicate).
[b]For each paired choice, means followed by the same letter are not significantly different (ANOVA F-test, $p > 0.05$)
[c]Within this column, means followed by the same letter are not significantly different (LSD, $p > 0.10$)

with no dye or active ingredient) or with baitube devices containing 0.5% hexaflumuron or 0.5% noviflumuron.

RESULTS

Laboratory Trials

Test 1. No-Choice Test on Dyed Filter Paper (FP)

Feeding response to purple FP was similar to that measured for 0.1% Nile Blue and 0.25% Neutral Red, with almost all termites becoming visibly dyed (Table 2). The 50:50 ratio of red:blue tended to create a more bluish-purple color while the 75:25 ratio created a more violet purple color. There was variability in coloration from the purple dyes: many termites were purple, but some also appeared red or blue. There was significant decline in consumption for purple-, red- and blue-dyed FP's when compared to blank FP, which based on previous research was not surprising (King 2000). Despite the lower consumption, all termites became visibly dyed on red, blue or purple papers.

Test 2. Choice Test between Dyed Filter Paper (FP) & Southern Yellow Pine (SYP)

Termites were given the choice to feed between untreated SYP and dyed filter paper. Once again it appeared that the feeding response to purple dyed FP's was comparable to what is observed for blue & red dyes (Table 3). It was apparent that termites prefer all red, blue and purple FP less when paired with SYP, but a substantial number of termites still became visibly dyed. The data suggest that there may be fewer visibly dyed termites with the purple dyed FP. While no direct statistical comparison is possible, generally the percentage of termites that were visibly marked was less when SYP was presented as an alternative than when they were force-fed.

Test 3. No-Choice Test on Dyed LTC

When formulating the purple blend on the LTC matrix it was observed that the initial solution produced a sample with a "sage-green" tint. The sample dye solution had a pH solution of 2.9. Neutral Red is sometimes used as a pH indicator dye (Green 1990) so another sample was acidified to a pH of 1.5 which produced a much more vivid purple color on the LTC matrix.

As was the case with filter paper, there was significantly less consumption on dyed LTC than blank LTC, yet almost all termites became visibly colored on dyed LTC (Table 4). Between purple LTC samples, the vivid purple 1.5 pH LTC created very purple termites while the "sage-green" LTC created bluish termites that were barely distinguishable from the Nile Blue A termites. The 1.5 purple pH sample was

Table 4. No-Choice Test. Forced feeding of eastern subterranean termites (*R. flavipes*) on blank or dyed LTC matrix after 12-day feeding exposure.

Treatment[a]	mg paper consumed /g termite/day (Mean ± SEM)[b]	No. of termite worker survivors/100 after 12 days (Mean ± SEM)[b]	% Surviving termites visibly dyed
Blank LTC	17.99 ± 1.45 a	92.67 ± 5.04 a	0.0
0.10% Nile Blue LTC	7.16 ± 0.39 b	73.70 ± 21.50 ab	99.5
0.50% Neutral Red LTC	6.72 ± 1.32 b	76.00 ± 15.70 ab	99.6
Purple LTC, 50:50 B:R ratio	6.58 ± 0.40 b	75.30 ± 22.20 ab	98.2
Dye pH = 1.5			
Purple LTC, 50:50 B:R ratio	7.44 ± 1.50 b	60.70 ± 14.70 b	100.0
Dye pH = 2.9			

[a]Each trt. replicated 3 times (100 termite workers per replicate).
[b]Within each column, means followed by the same letter are not significantly different (LSD, $p > 0.10$)

clearly the better choice for field-testing. The acidic level of the pH solution was very influential on resultant color of the LTC and termites. The consistency of purple color in termites was much better on the 1.5 pH purple LTC when compared to results on purple-dyed filter paper in Tests #1 and #2. This suggests that pH may have a significant effect on the color produced due to interactions with the matrix substrate.

Test 4. Choice Test between Dyed LTC & Southern Yellow Pine (October 2000)

Termites were given the choice to feed between untreated southern yellow pine (SYP) and dyed LTC. Termites significantly preferred SYP to purple LTC; however, there was enough consumption of purple LTC (Table 5) to visibly dye 91% of the termites. The same feeding response was observed for 0.5% Neutral Red LTC. Nile Blue was not a feeding deterrent in this test, but feeding on the SYP alternative was also lower than for Neutral Red or the purple blend.

Field Trials

In all cases, marked termites were distinguishable from those marked with 0.25% or 0.5% Neutral Red or those marked with 0.1% Nile Blue A. At all 5 sites, other colonies were marked with either Nile Blue A or Neutral Red or both, providing a local comparison. In general, purple-dyed termites were easily distinguished during the period when dyed matrix was available in at least one station visited by that colony. As noted in earlier work (Atkinson 2000), over time dye markers tend

Table 5. Choice Test, Comparative consumption of southern yellow pine (SYP) and dyed LTC by eastern subterranean termites (*R. flavipes*) after 15-day feeding exposure.

Paired Choice	mg consumed/ g termite/day (Mean ± SEM)[b]	No. of termite worker survivors/100 after 15 days (Mean ± SEM)[c]	% Surviving termites visibly dyed
SYP vs.	2.04 ± 2.04 a	90.5 ± 2.50	0.0
Blank LTC	15.19 ± 0.78 b		
SYP vs.	6.36 ± 1.62 a	89.3 ± 2.50	89.9
0.10% Nile Blue LTC	8.74 ± 0.61 a		
SYP vs.	17.42 ± 1.94 a	93.3 ± 1.25	82.0
0.50% Neutral Red LTC	6.13 ± 1.81 b		
SYP vs. B:R ratio	16.35 ± 1.17 a	88.3 ± 2.87	91.1
Purple LTC, 50:50	4.47 ± 0.89 b		
Dye pH = 1.5			

[a]Each test replicated 4 times (100 termite workers per replicate).
[b]For each paired choice, means followed by the same letter are not significantly different (ANOVA F-test, $p > 0.10$)
[c]Within this column, no significant differences were found between treatments (LSD, $p > 0.10$)

to disappear and fade if they are not being continuously replenished. It appeared that the blue component tended to fade most rapidly. As the purple faded after several months, it became more difficult to distinguish, especially from termites with a faint red mark. Purple, as is the case with Neutral Red and Nile Blue A, persisted longest in soldiers.

While it is assumed and probably correct that Nile Blue A and Neutral Red are not transferred by trophallaxis after the gut contents have cleared following force feeding, it is obvious that the purple blend is transferred because soldiers rapidly acquire an intense coloration. It is likely that dye is transferred to workers and smaller nymphs via trophallaxis as long as dyed matrix is available for foraging. In the case of a self-mark technique transferability is advantageous because it potentially results in a higher proportion of colony members becoming marked.

Data on consumption of dyed matrix and spread of movement of dyed termites are summarized in Table 6. In all cases dyed termites were detected inside the station containing dyed matrix by the next visit (29-45 days). In 4 out of 5 cases, dyed termites were found in other stations on the next visit as well. In the one exception (Floresville grid 1), dyed matrix was applied in early December and feeding was slow. Nonetheless, even though only a small amount of dyed matrix was consumed, marked termites were recovered from 5 additional stations by late spring. In all cases, it is probable that marked termites occurred in all

Table 6. Consumption of dyed matrix, movement of dyed termites, and persistence of dye marker in colonies of *Reticulitermes* spp. in Texas.

Site	Species	Matrix consumed (g)	No. of stations with dye	No. of stations with dyed termites[a]	Maximum distance moved (m)	Days after Dye			
						Dyed termites in release station	Dyed termites in other stations	All dyed matrix removed	Last dyed termites observed
Floresville grid 1	*R. flavipes*	5	1	5	12	32	122	122	221
Floresville grid 4	*R virginicus*	10	1	3	8	45	45	138	266
Austin grid	*R. flavipes*	10	1	2	5	35	na[b]	na[b]	na[b]
Cuero House	*R. flavipes*	14	1	4	10	29	29	61	97[c]
Austin F.L. House	*R. flavipes*	19	1	9	16	31	31	31	240[c]

[a] Other than station in which dyed matrix was originally placed.
[b] Stations monitored irregularly.
[c] Colonies eliminated.

stations at each site foraged by marked colonies. Either there was a large spatial separation from stations from which marked individuals were not found or else all other active stations at a site contained termites with a different dye marker (criteria discussed in detail by Atkinson, 2000). Visibly marked termites were still noted 4-8 months after the last exposure of foraging termites to the purple matrix.

DISCUSSION

The addition of Neutral Red, Nile Blue A or different blends clearly deterred feeding when applied to filter paper or the LTC matrix when southern yellow pine was present as an alternative. Generally a lower percentage of individuals was marked when an alternative was present than in non-choice tests. Nonetheless, a high percentage of individuals in laboratory trials still acquired a distinctive coloration.

Under field conditions the ratio of feeding preference to other available food sources is unknown, but obviously some individuals do ingest enough dye marker to become marked. In most cases, only a small number of individuals (5-25%) show clear marking under field conditions, true for any dye markers that have been used in the self marking technique.

Even so, the technique is still robust for delineating foraging territories and in this regard the purple blend performs similarly to either Neutral Red or Nile Blue A alone.

In conclusion, these results validate the use of the purple blend as an additional color for use in the self mark-capture technique for identifying colonies of *Reticulitermes* spp. in the field. Marked termites are distinguishable in the field from those marked with Nile Blue A or Neutral Red alone and sufficient numbers of termites will become marked to identify foraging sites of marked colonies

Reference

Atkinson, T.H. 2000. Use of dyed matrix in bait stations for determining foraging territories of subterranean termites (Isoptera: Rhinotermitidae: *Reticulitermes* spp. and Termitidae: *Amitermes wheeleri*). Sociobiology 36:149-167.

Esenther, G.R. 1980. Estimating the size of subterranean termite colonies by a release-recapture technique. International Research Group on Wood Preservation1-5.

Green, F.J. 1990. The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators. Aldrich Chemical Co., Milwaukee, Wisc.

King, J.E. 2000. Laboratory feeding response of *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) to dyed filter paper in no-choice and choice feeding tests. Sociobiology 36:169-179.

Oi, F.M. 2000. Purple dye marker for *Reticulitermes* spp. (Isoptera: Rhinotermitidae). Florida Entomologist 83:112-113.

Su, N.-Y., P.M. Ban, & R. H. Scheffrahn 1991. Evaluation of twelve dye markers for population studies of the Eastern and Formosan subterranean termite (Isoptera: Rhinotermitidae). Sociobiology 19:349-362.