Hexaflumuron Efficiency and Impact on Subterranean Termite (*Reticulitermes* spp.) (Isoptera: Rhinotermitidae) Gut Protozoa

By

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Abstract of Thesis Presented to the Graduate School of Virginia Polytechnic and State University in Partial Fulfillment of the Requirements for the Degree of Masters of Science

Hexaflumuron Efficiency and Impact on Subterranean Termite (*Reticulitermes* spp.) (Isoptera: Rhinotermitidae) Gut Protozoa.

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The effect of competing food sources on termite consumption of hexaflumuron bait and subsequent mortality was examined. Also, the effect of hexaflumuron on the termite gut fauna was evaluated to determine if hexaflumuron could kill termites via a secondary mode of action.

Hexaflumuron consumption in no-choice and choice tests was evaluated at 2d and 5d. Total diet consumption was not different between the treatment groups. Hexaflumuron consumption was reduced by a factor of 3 in the presence of a competing control diet and reduced by a factor of 57 in the presence of an inulin diet. In the choice test, termites preferred inulin over hexaflumuron.

Termite mortality after hexaflumuron consumption was quantified at 5d, 15d, 20d, 25d, and 35d in three treatment groups. Mean mortality for termites fed only hexaflumuron diet was significantly higher than termites fed only control diet. Mean mortality for termites given a choice was no different than mortality for termites fed only hexaflumuron or control diet. LT_{50}s for termites fed control diet, hexaflumuron diet, or both diets in the choice test, were 24.4d, 18.7d, and 20.6d, respectively. The choice test LT_{50} did not differ from the LT_{50} of either no-choice test. Termites fed only hexaflumuron diet had an earlier LT_{50} than termites fed only control diet.

The effect of hexaflumuron on gut fauna survival was evaluated at 5d and 15d. No significant differences were found in total numbers of protozoa in termites fed either

hexaflumuron or control diet. *Pyrsonympha* was the only protozoa significantly reduced by hexaflumuron consumption.

This is dedicated to my parents, Marlene and Louis Perrott, whose love and support have always been my greatest source of encouragement.

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Table of Contents

ABSTRACT .... **II**

ACKNOWLEDGEMENTS .... **V**

LIST OF TABLES .... **VIII**

LIST OF FIGURES .... **IX**

CHAPTER ONE: INTRODUTION **1**

CHAPTER TWO: TERMITE PEST MANAGEMENT **5**

- INTRODUCTION 5
- SUBTERRANEAN TERMITE CONTROL METHODS 9
- LIQUID TERMITICIDES 10
- LIMITATIONS TO LIQUID TERMITICIDES 11
- BAIT SYSTEMS 12
- PROBLEMS WITH BAIT SYSTEMS 14
- INSECT GROWTH REGULATORS 15
- SUMMARY 17

CHAPTER THREE: THE EFFECT OF COMPETING FOOD SOURCES ON RETICULITERMES SPP. (ISOPTERA: RHINOTERMITIDAE) CONSUMPTION OF HEXAFLUMURON-TREATED BAIT. **19**

- INTRODUCTION 19
- METHODS AND MATERIALS 21
- RESULTS 33
- DISCUSSION 42

CHAPTER FOUR: THE EFFECT OF COMPETING FOOD SOURCES ON HEXAFLUMURON CONSUMPTION AND SUBSEQUENT MORTALITY OF RETICULITERMES FLAVIPES (ISOPTERA: RHINOTERMITIDAE) **46**

- METHODS AND MATERIALS 48
- RESULTS 53
- DISCUSSION 59

CHAPTER FIVE: THE EFFECT OF HEXAFLUMURON ON 3 GENERA OF PROTOZOA IN THE EASTERN SUBTERRANEAN TERMITE (RETICULITERMES FLAVIPES (KOLLAR) (ISOPTERA: RHINOTERMITIDAE)) HINDGUT. **62**

- INTRODUCTION 62
- METHODS AND MATERIALS 65
- RESULTS 72
- DISCUSSION 80

CHAPTER SIX: SUMMARY **83**

APPENDIX A **87**

LIST OF REFERENCES **89**

VITA **106**

List of Tables

TABLE 3-1. TO DETERMINE TERMITE HEALTH, MEAN PERCENT MORTALITY AT 2 AND 5 DAYS FOR THE SUBTERRANEAN TERMITE POPULATION FED HEXAFLUMURON DIETS, INULIN DIETS, CONTROL DIETS, OR A COMBINATION OF TWO DIETS. 36

TABLE 3-2. MEAN TOTAL CONSUMPTION (MG) DETERMINED BY DIET WEIGHT AT 2 AND 5 DAYS FOR THE SUBTERRANEAN TERMITE POPULATION FED HEXAFLUMURON DIETS, INULIN DIETS, CONTROL DIETS, OR A COMBINATION OF TWO DIETS. CONSUMPTION WAS NOT CORRECTED BY TERMITE MORTALITY. 37

TABLE 3-3. MEAN CONSUMPTION (MG) OF HEXAFLUMURON DIET DETERMINED BY DIET WEIGHT AT 2 AND 5 DAYS FOR THE SUBTERRANEAN TERMITE POPULATION IN NO-CHOICE AND CHOICE TESTS. CONSUMPTION WAS NOT CORRECTED BY TERMITE MORTALITY........ 38

TABLE 3-4. MEAN CONSUMPTION (MG) OF EACH DIET IN THE CHOICE TESTS DETERMINED BY DIET WEIGHT AT 2 AND 5 DAYS FOR THE SUBTERRANEAN TERMITE POPULATION IN CHOICE TESTS: HEXAFLUMURON AND CONTROL DIETS; INULIN AND CONTROL DIETS; HEXAFLUMURON AND INULIN DIETS. CONSUMPTION WAS NOT CORRECTED BY TERMITE MORTALITY. 39

TABLE 3-5. MEAN CONSUMPTION (μG) OF HEXAFLUMURON AT 2 AND 5 DAYS BY A SINGLE SUBTERRANEAN TERMITE IN NO-CHOICE AND CHOICE TESTS. MEAN CONSUMPTION WAS DETERMINED BY THE DPMS COUNTED IN SINGLE TERMITE SAMPLES. CONTROL CONSUMPTION WAS NOT MEASURED. 40

TABLE 3-6. MEAN CONSUMPTION (μG) AT 2 AND 5 DAYS OF EACH DIET IN THE DUAL LABEL CHOICE TEST BY A SINGLE SUBTERRANEAN TERMITE FED ^{14}C-HEXAFLUMURON AND ^{3}H-INULIN DIETS. MEAN CONSUMPTION DETERMINED BY DPMS COUNTED IN SINGLE TERMITE SAMPLES........ 41

TABLE 4-1. THE MEAN TOTAL CONSUMPTION (MG) BY TREATMENT FOR 100 SUBTERRANEAN TERMITES FED HEXAFLUMURON DIETS. THIS DETERMINED THE EQUIVALENT CONSUMPTION OF DIETS. CONSUMPTION WAS NOT CORRECTED BY TERMITE MORTALITY........ 56

TABLE 4-2. MEAN CONSUMPTION (MG) OF EACH DIET BY 100 SUBTERRANEAN TERMITES FED HEXAFLUMURON AND CONTROL DIETS AT DIFFERENT TIME PERIODS. THIS DETERMINED EQUIVALENT CONSUMPTION OF EACH DIET IN THE CHOICE TEST. CONSUMPTION WAS NOT CORRECTED BY TERMITE MORTALITY. 57

TABLE 4-3. MEAN MORTALITY OVER A PERIOD OF 35 DAYS FOR 100 SUBTERRANEAN TERMITES FED ONLY HEXAFLUMURON DIETS, ONLY CONTROL DIETS, AND A........ 58

COMBINATION OF HEXAFLUMURON AND CONTROL DIETS. 58

TABLE 5-1. MEAN PERCENT MORTALITY AT 5 AND 15 DAYS FOR 100 SUBTERRANEAN TERMITES FED HEXAFLUMURON DIETS OR CONTROL DIETS. THIS DETERMINED TERMITE HEALTH. 74

TABLE 5-2. MEAN TOTAL CONSUMPTION (MG) AT 5 AND 15 DAYS FOR 100 SUBTERRANEAN TERMITES FED HEXAFLUMURON DIETS OR CONTROL DIETS. THIS DETERMINED EQUIVALENT CONSUMPTION OF DIETS. 75

TABLE 5-3. MEAN NUMBER OF TOTAL PROTOZOA PRESENT AT 5 DAYS IN A SAMPLE FOR SUBTERRANEAN TERMITES FED CONTROL DIETS........ 76

TABLE 5-4. MEAN NUMBER OF EACH SPECIFIC GENERA OF PROTOZOA (*TRICHONYMPHA, PYRSONYMPHA, DINENYMPHA)* PRESENT AT 5 DAYS IN A SAMPLE FOR SUBTERRANEAN TERMITES FED CONTROL DIETS.. 77

TABLE 5-5. MEAN TOTAL NUMBER OF PROTOZOA PRESENT AT 5 AND 15 DAYS IN A SAMPLE FOR SUBTERRANEAN TERMITES FED HEXAFLUMURON DIETS OR CONTROL DIETS. 78

TABLE 5-6. MEAN NUMBER OF THE SPECIFIC GENERA OF PROTOZOA (*TRICHONYMPHA, PYRSONYMPHA, DINENYMPHA)* PRESENT AT 5 AND 15 DAYS IN A SAMPLE FOR SUBTERRANEAN TERMITES FED HEXAFLUMURON OR CONTROL DIETS. 79

TABLE A-1. SCINTILLATION VIALS FILLED WITH VARIOUS COMBINATIONS OF ^{14}C AND ^{3}H TO BE COUNTED FOR THE CORRECTION CURVE. 88

List of Figures

FIGURE 3-1. THE NO-CHOICE SUBTERRANEAN TERMITE ARENA **24**

FIGURE 3-2. THE CHOICE SUBTERRANEAN TERMITE ARENA **25**

FIGURE 3-3. SPILLOVER CURVE FOR ^{14}C IN THE PRESENCE OF ^{3}H-SAMPLES. **31**

FIGURE 3-4. SPILLOVER CURVE FOR ^{3}H IN THE PRESENCE OF ^{14}C-SAMPLES. **32**

FIGURE 4-1. THE NO-CHOICE SUBTERRANEAN TERMITE ARENA **50**

FIGURE 4-2. THE CHOICE SUBTERRANEAN TERMITE ARENA **51**

FIGURE 4-3. SIGMOIDAL DOSE RESPONSE (VARIABLE SLOPE) CURVE. **55**

FIGURE 5-1. THE NO-CHOICE SUBTERRANEAN TERMITE ARENA **68**

FIGURE 5-2. THREE OF THE LARGEST AND MOST ABUNDANT GENERA OF PROTOZOA FOUND IN THE SUBTERRANEAN TERMITE, *RETICULITERMES FLAVIPES* (KOLLAR), HINDGUT **70**

FIGURE 5-3. CENTER GRID ON BRIGHTLINE HEMACYTOMETER **71**

Chapter One:
Introduction

Subterranean termites are one of the world's most economically important pests because they damage structural wood (Hickin 1971). The pest control industry spends approximately two billion dollars a year to suppress termite infestations and/or try to avoid their damage to structures (Su and Scheffrahn 1990b). Worldwide there are 15 species of subterranean termites that are considered to be economically important pests (Hickin 1971). The two most economically important species in Virginia are the Eastern Subterranean termite (*Reticulitermes flavipes* (Kollar)) and the Dark Southern Subterranean termite (*Reticulitermes virginicus* (Banks)).

Subterranean termites are very successful because they are social insects. Social insects live in large family groups and work together for the good of the colony. The worker termite caste is the most numerous and is responsible for all of the labor in the colony. Worker termite tasks include taking care of the brood, maintaining and repairing the nest, and foraging for food (Krishna 1969). Other castes, such as soldiers and functional reproductives, are entirely dependant on the worker termite for food (Grassé 1939; Noirot and Noirot-Timothee 1969). Worker termites forage for food, bring it back to the colony, and feed all other nestmates. Therefore, worker termites are the caste that damages structural wood.

Efforts to control subterranean termite infestations inside structures have depended heavily on liquid termiticide applications. These applications consist of trenching around the perimeter of a structure and/or drilling holes at given intervals into the foundation block and slabs (Rambo 1985). Liquid termiticides are applied around the foundation at a rate of 4 gallons per 10 linear feet, per foot of depth to the footer (Rambo 1985). In general, liquid applications are intended to serve as a defensive barrier between the termites in the soil and the structure above ground. Repellent products, such as Demon TC® (A.I. Cypermethrin), Prelude® (permethrin), and Torpedo® (permethrin) work to repel worker termites from tunneling toward the foundation (Su and Scheffrahn 1990a). Other liquid termiticides, such as Termidor® (fipronil) or Premise® (imidacloprid), are nonrepellent and work to kill termites upon contact (Kard 1998, 2000; Gahlhoff and Koehler 2001; Thorne and Breisch 2001). Even at sublethal doses, these

nonrepellent termiticides increase mortality by causing termites to become sluggish and cease all grooming and tunneling behaviors (Boucias et al. 1996). Evidence indicates that termites exposed to sublethal doses of nonrepellent termiticides can even transfer the toxicant to other nestmates (Clement 1998; Thorne and Breisch 2001).

Although liquid termiticides have been shown to repel or suppress termite pest populations, they have several limitations. First, these chemicals may or may not affect termite populations located away from treated areas (Su and Scheffrahn 1988a, 1990a; Su et al. 1993b; Forschler 1994). For example, large populations of termites may persist in the soil surrounding a building, even though records indicate termiticide treatments were applied (Grace et al. 1989). Secondly, termites exposed to the non-repellent termiticides may die before returning to the nest to spread the toxicant to other nestmates. Furthermore, if the termiticide is not uniformly applied, foraging termites can evade repellent termiticides in the soil and gain access to structures. Finally, the active ingredients in liquid termiticides degrade over time in the soil and eventually have to be reapplied (Mauldin et al. 1987).

Although liquid termiticides are still the primary method of subterranean termite control, new methodologies have been developed to try to overcome some of their limitations. The most significant of these new methodologies has been the termite bait systems. Bait systems take a more direct approach to termite control than liquid applications by targeting the termites themselves. Bait systems consist of multiple in-ground stations containing untreated wood monitors. Stations are placed in the soil around the perimeter of the house. A pest control operator must periodically check all stations and determine whether monitors are infested. If termites are found inside, the monitors are replaced with a bait consisting of a toxicant formulated into a cellulose matrix.

To date, all commercial baits are formulated to be slow acting. The first bait system was developed in 1990 by Dow AgroSciences: the Sentricon® Termite Elimination System. The slow acting ingredient in this product is hexaflumuron [N-(((3,5-dichloro-4-(1,1,2,2-tetrafluroethoxy)phenyl)-amino)carbonyl)-2,6-diflurobenzamide]. Hexaflumuron, a benzophenylurea, is an insect growth regulator, which functions as a chitin synthesis inhibitor. Another bait system, Exterra® (Syntex), contains a different chitin synthesis inhibitor, diflubenzuron, as its active ingredient. The

third most widely sold product is the Firstline System® (FMC), which contains the stomach toxicant, sulfluramid. All of these bait systems are formulated to allow worker termites to consume the toxicant from the station and live long enough to return to the colony and feed it to other nestmates. Therefore, bait systems work at the colony level to either suppress foraging termites, or eliminate the colony altogether (Dow AgroSciences 1999).

Like liquid termiticides, bait systems also have their limitations. Perhaps the most significant challenge to termite bait system performance in the field is the presence of competing food resources. When bait stations are installed in the ground, termites are already feeding on a structure or some natural food source in the area. If natural food sources are more attractive to termites than a bait station, foragers may not be recruited to the bait station. If termites are already infesting a structure, they will not leave the structure to feed on a bait station. Because it is impossible to eliminate all alternative food sources in the environment, these food sources will always be competition for bait stations. Therefore, with so many competing food sources available, there are many obstacles for bait stations to overcome to become a successful method of termite control. Do competing food sources decrease termite consumption at the bait stations? If so, is termite mortality due to bait consumption also decreased because of competing food sources? There are many unanswered questions regarding the effects of competing food sources on bait efficacy.

The bait systems also face other challenges. For example, the number of termites that feed on the bait matrix determines how quickly the toxicant can be spread to other individuals in the colony. Also, because a termite colony is made up of many long-lived individuals and has overlapping generations, individual termites molt at different rates. Baits formulated with chitin synthesis inhibitors would be more effective against young nymphs, the termites still actively molting, than against older nymphs, which molt less frequently. Because a portion of the population is made up of these infrequently molting termites, it is not well understood how chitin synthesis inhibitors can eliminate an entire termite colony. Is it possible that bait systems overcome this challenge with a second mode of action? Because the termite hindgut is lined with chitin, it is possible that hexaflumuron exposure could disrupt the chitin lining of the hindgut and the gut fauna

living there. Chitin synthesis inhibitors have never been tested against the termite gut fauna. Do chitin synthesis inhibitors have any direct effect on the termite gut fauna?

The following studies were conducted to evaluate several functional aspects of one such bait system, the Sentricon System®, and its active ingredient, hexaflumuron. The first objective of my research was to evaluate the effect of competing food sources on subterranean termite consumption of hexaflumuron bait and subsequent termite mortality. My second objective was to investigate the possibility that hexaflumuron negatively impacts the subterranean termite gut protozoa.

Chapter Two:
Termite Pest Management

Introduction

Termites are beneficial insects in nature because they break down fallen wood and recycle nutrients back to the soil. However, these same insects can become pests when they attack the structural wood in businesses and homes. Wood has been utilized as a component of human structures since the birth of civilization, and the invasion by subterranean termites into structures has been a problem for centuries. Subterranean termites are considered one of the most economically important pests in the world. In the United States, the pest control industry profits billions of dollars annually by controlling infesting termite populations. Furthermore, subterranean termites account for approximately 80% of the total amount spent on termite control annually (Su 1991).

There are over 15 species of subterranean termites worldwide that are considered to be economically important pests (Hickin 1971). However, the most destructive termite species in the United States are: the Formosan subterranean termite (*Coptotermes formosanus* Shiraki), the Eastern Subterranean termite (*Reticulitermes flavipes* (Kollar), the Western Subterranean termite (*Reticulitermes hesperus* Banks), the Dark Southern Subterranean termite (*Reticulitermes virginicus* (Banks), and the Light Southern Subterranean termite (*Reticulitermes hageni* Banks).

Subterranean termites have adapted to live in many different geographic regions, such as the tropical, sub-tropical, and temperate regions of the world (Von Hagen 1942; Hickin 1971). *Reticulitermes*, for example, are distributed widely in North America, as they are found throughout southeastern United States, north along the Atlantic coast into Maine, and west along the southern shores of the Great Lakes (Weesner 1970). *Reticulitermes* have even been found in 28 municipalities in southern Ontario (Cutten 1988).

Subterranean termites are very successful insects. Their success stems from the fact that they are cryptobiotic, meaning their nests and foraging activities are concealed beneath the soil, within wood, and inside mudtubes (reviewed in Thorne et al. 1999). Because it is hard to gain access to them, subterranean termites have very few predators. Furthermore,

their cryptobiotic nature makes controlling them very difficult. Subterranean termite success is also due to their long-lived nature (Kofoid 1946). For example, the queen termite can live 15 years or more (Hickin 1971). Termite colonies are, therefore, made up of many overlapping generations at any given time. Another reason for termite success is that they can utilize cellulose as a food source. Because most animals cannot digest cellulose and do not eat wood, termites have little competition for their food. Finally, like all termites, subterranean termites are social insects. They live in large colonies, where all individuals work together for the good of the colony. Subterranean colonies can range from about 0.2- 5 million individuals (Grace et al. 1989) and have been estimated to establish up to 13-14 colonies per acre (Aventis 2003).

Subterranean termite colony nests have a complex infrastructure and are segregated into different castes: primary reproductives, secondary reproductives, soldiers, and workers. Each caste has different responsibilities. For instance, after a termite colony becomes mature, usually between 5 and 10 years (Gold and Jones 2000), one thousand or more winged termites (Nutting 1970) swarm annually from the parent nest and attempt to found new colonies (Kofoid 1946). After swarming, the alates lose their wings, and surviving males and females pair for mating. These pairs become the king and queen termites, or primary reproductives, of the new colony. The king and queen termites mate periodically for the rest of their lives, producing eggs as often as every minute (Hickin 1971). Eventually, egg production is aided by secondary reproductives, which also have mature sex organs. If the king and queen termite should die, the secondary reproductives become the replacement reproductives and continue laying eggs for the colony (Hickin 1971). Because more than one secondary reproductive may produce eggs, their egg-laying capacity may surpass that of the primary queen (Long et al. 2003). The soldier caste is developed to protect the colony from invading insects, mainly ants (Kofoid 1946). Soldiers respond to any nest disturbances and can be found blocking openings in the walls of the nest or foraging tubes when a colony is expanding into new territory (Noirot and Noirot-Timothee 1969). Finally, the worker caste makes up the majority of the colony and is responsible for most tasks, including taking care of the brood, maintaining and repairing the nest, foraging for food, and feeding the rest of the colony (Krishna 1969). Other castes are completely dependent on the worker termites

for food, because the soldiers' enlarged mandibles are adapted for defense, and colony reproductives are too preoccupied with egg production to forage (Grassé 1939; Borror et al. 1989). Because worker termites have to forage for food and feed the rest of the colony, it is the worker caste that damages structural wood.

When worker termites forage for food, they form extensive underground galleries or exploration tunnels. Foraging begins with one exploration tunnel that branches from the nest and eventually fans out radially into many smaller tunnels (Campora and Grace 2001). Foraging is nonrandom, because the exploration area is divided evenly to minimize redundancy (Robson et al. 1995; Reinhard et al. 1997; Campora and Grace 2001). Eventually, termite tunneling is increased in sites where food has been discovered and is decreased in areas where no food has been found (Campora and Grace 2001). Termites have been found foraging at considerable distances from the nest (79-100m) (King and Spink 1969; Grace et al. 1989; Su et al. 1993a), and it has been estimated that *R. flavipes* foraging galleries can extend over an area of 1091m^2 (Grace et al. 1989).

When foraging for food, worker termite feeding behaviors and food preferences vary widely (reviewed in Waller 1991). Consumption rates vary among different colonies of subterranean termites, as some colonies are more voracious than others (Su and La Fage 1984a; Lenz 1985). Factors within the colony itself may allow for some of the variation, such as colony age and size (Wood 1978; Su and La Fage 1987); reproductive status (Wood 1978); population density (Esenther 1980; Lenz and Williams 1980; Lenz and Barrett 1984); termite vigor (Su and La Fage 1984b; Lenz 1985); and previous feeding history (Wood 1978). The condition of the food source may also influence feeding by subterranean termites. Such conditions include whether the wood has been damaged by conspecifics (Delaplane and La Fage 1989a); the moisture level of the wood (Delaplane and La Fage 1989b); the size of the food source itself (Waller 1988; Lenz 1994; Cornelius and Osbrink 2001); food placement (Oi et al. 1996); and the presence of other available feeding materials in the vicinity (Smythe and Carter 1970; Behr et al. 1972; Howard and Haverty 1979b; Su and Tamashiro 1986).

After foraging for food and feeding from an available food source, workers are able to transfer cellulose from one colony member to another via trophallaxis (Wheeler 1918). Stomodeal trophallaxis, or mouth to mouth feeding, occurs when a worker termite

transfers food into the mouth of another termite (McMahan 1969). After returning to the nest from foraging, workers immediately transfer food to one or several nestmates (Suarez and Thorne 2000b). The recipient touches the head of the donor with its antennae and stimulates the donor to produce a droplet of food on its mouth, which the recipient then imbibes (McMahan 1969). This action allows for the transfer of viscous liquid or solid wood fragments and saliva (McMahan 1969). A second type of trophallaxis, or proctodeal trophallaxis, is more important to immature termites. This type of trophallaxis involves anus to mouth transfer. With each molt, termites shed their exoskeletons and the chitin linings of their foregut and hindgut. The microorganisms living in the hindgut are also shed with each molt (Krishna 1969). It is necessary for immature termites that have recently molted to feed on exudates from the anus of another termite (Grassé and Noirot 1945) to regain the hindgut microorganisms lost during the molt.

Although termites feed on cellulose materials, they are unable to digest cellulose on their own. Instead, termites rely on hindgut symbiotic protozoa as an indirect means of digesting nutrients (Imms 1920; Honigberg 1970). Although termites themselves are able to break down starch into glucose units (Breznak and Brune 1994), the majority of termite nutrition and energy sources (acetate, propionate, and organic acids) come from microbial metabolism (Odelson and Breznak 1983).

Because protozoa provide nutrition for termites, a large quantity and variety of protozoa are found in the gut. In fact, the termite gut has been estimated to house over 30,000 protozoa (Mauldin et al. 1981), which make up one-third of the total body weight of the termite (Katzin and Kirby 1939). This hindgut fauna includes a number of different species of protozoa. For example, up to fourteen species in eight different genera have been described as components of the hindgut in *R. flavipes* (Yamin 1979). Some of the largest and most abundant of these protozoa in the hindgut of *R. flavipes* are in the genera: *Trichonympha*, *Pyrsonympha*, and *Dinenympha.* Members of the protozoa order Oxymonadida, *Pyrsonympha* and *Dinenympha*, are found only in *Reticulitermes* (Koidzumi 1921), whereas *Trichonympha,* which is in the order Hypermastigoes, is widely distributed among the lower termites (Honingberg 1970).

Because termites depend heavily on symbiotic protozoa to digest wood for them, there is a correlation between death of one or more components of the gut fauna and death of the termite (Eutick et al. 1978; Cleveland 1923; Cleveland 1924; Cleveland 1925b). Wood preservatives and wood based toxins negatively affect protozoa in the termite gut (Speck et al. 1971). Furthermore, it has been shown that feeding on certain woods causes termites to loose their gut fauna, because of chemical components of the wood (Mannesmann 1972; Mauldin et al. 1981; Cook and Gold 2000). Kanai et al. (1988) found that tripropylisocyanurate, a stomach poison, kills symbiotic protozoa in *Coptotermes formosanus* and *R. flavipes*. While, Waller (1996) found that antibiotics, such as ampicillin, tetracycline, and urea, suppress the numbers of gut protozoa, Maudlin and Rich (1980) found that antibiotics kill the gut protozoa and subsequently starved the termite to death. These results indicate that a complete and normally functioning gut fauna is essential to subterranean termite survival.

Subterranean Termite Control Methods

Subterranean termites can cause extensive damage when they forage into wooden structures, and considerable effort has been put into methods of control. Today, two very different approaches are used for the control of subterranean termite infestations: the application of liquid termiticides and the use of termite bait systems.

Liquid termiticides are applied to the soil beneath a structure and are intended to provide a defensive barrier between the termites in the soil and the structure above. Liquid termiticides are applied by drenching or injecting the soil along the outside and inside of foundations where feasible, around supporting piers, chimney bases, and pipes, under filled porches and terraces, and under driveways (Rambo 1985). If necessary, the concrete slab is drilled so the soil underneath may also be treated. Liquid termiticides are applied around the foundation at a rate of 4 gallons per 10 linear feet, per foot of depth to the footer (Rambo 1985).

The bait systems have a more direct approach to termite control. The baits target the termites themselves and are designed for colony elimination. Bait systems do not utilize a permanent liquid barrier in the soil. Instead, they are used to monitor for termite

infestations with multiple in-ground stations containing pieces of untreated wood. A pest control operator places these in-ground stations around the perimeter of the foundation, and the stations are checked periodically (monthly, bimonthly, or quarterly). When a termite infestation is discovered, a toxicant formulated in a cellulose matrix is inserted into the bait station. Termites foraging at the station carry the toxic materials back to the colony. Thus, the bait systems are designed as a stand alone treatment and are normally not combined with conventional liquid applications.

Although liquid termiticides are still the primary method of subterranean termite control, bait systems are becoming more widely used. Both approaches differ greatly in terms of their methodology, yet their ultimate goal is the same: protecting structures from termite invasion.

Liquid Termiticides

The use of chemical applications as a means of eliminating subterranean termites from structures was suggested as early as the late 1800s but the actual evaluation of potential chemicals did not get underway until the 1940s (Aventis 2003). At this time, several compounds became available for use against subterranean termites, including calcium cyanide, sodium cyanide, and carbon disulfide. Termite control using liquid termiticides was greatly improved with the discovery of more effective chemicals. For instance, the chlorinated hydrocarbons (i.e. chlordane, heptachlor, endrin, aldrin, and dieldrin) were found to be very effective against termites and were relatively stable in the environment. Chlorinated hydrocarbons were used as a liquid barrier around structures for several decades. However, their use as termiticides was cancelled by the EPA by 1988 due to their environmental persistence and/or high volatility.

Over the next 30 years, organophosphate and pyrethroid chemicals came into common use as liquid termiticides. Organophosphates, like chlorpyrifos (Dursban TC®), were less persistent in the environment and were widely accepted, although they were more toxic to vertebrates than chlorinated hydrocarbons. However, the organophosphate termiticides were also banned by the EPA in 2000.

Pyrethroid termiticides are some of the most widely used liquid termiticide treatments today. These chemicals have a relatively long residual life, are effective at low concentrations, and have low acute mammalian toxicity. Pyrethroids are repellent compounds by nature and are used to repel foraging workers away from a treated structure (Su et al. 1982). Pyrethroids products in use today are Tribute® (A.I. fenvalerate), Demon TC® (Cypermethrin), Dragnet® (permethrin), Prelude® (permethrin), Prevail® (cypermethrin), Talstar® (bifenthrin), and Torpedo® (permethrin).

Non-repellent termiticides are not detectable by termites. As termites tunnel into treated soil, they contact the insecticide and are either killed quickly or pick up a sublethal dose and become intoxicated. The intoxicated termites forget to feed or groom themselves and, therefore, can die due to indirect effects of exposure. Products in use today include Premise® (imidacloprid) and Termidor® (fipronil). Imidacloprid is in the insecticide class of chloroniotinyls, whereas fipronil is a pyrazole insecticide.

Limitations to Liquid Termiticides

Although liquid termiticides have been shown to suppress termite pest populations, they have several limitations. For example, liquid applications are limited by the type of soil found near the structure. Sandy soils accept termiticide solutions readily, but depending on the particle size of the sand, the termiticide may not spread evenly beneath the soil to give a continuous barrier (Rambo 1985). The behavior of liquid termiticides in clay soils also varies with its consistency. Many clay soils are too compacted to receive termiticides and must be rodded at narrow intervals to provide termiticide overlap beneath the soil surface (Rambo 1985). Likewise, loam soils also vary in degree of compaction. Because organic matter interferes with the distribution of the termiticide in the soil, all organic matter present reduces the chances of achieving a continuous barrier in the soil (Rambo 1985). If termiticides are not uniformly applied, foraging termites can evade repellent termiticides in the soil and still gain access to structures. Uniform distribution with non-repellent termiticides is less of a problem but still hinders efficacy.

Liquid applications may or may not affect termite populations located away from treated areas (Su and Scheffrahn 1988a, 1990a; Su et al. 1993b; Forschler 1994). For example, buildings that have been treated with liquid termiticides may still have large populations of termites persisting in the surrounding soil (Grace et al. 1989). Termites exposed to non-repellent termiticides may die before returning to the nest and transferring the toxicant to nestmates. Large portions of a colony may never be exposed to a toxicant if foragers are not able to return to the nest. Finally, the active ingredients in liquid termiticides degrade in the soil over time and eventually have to be reapplied (Mauldin et al. 1987).

Bait Systems

Although liquid applications are the primary method of termite control in the pest management industry, bait systems are widely used today, especially in situations where liquid treatments have failed (Kistner and Sbragia 2001). Bait systems can be used effectively to control termite colonies in areas where they pose structural damage problems with limited accessibility (Kistner, Sbragia 2001). Many studies have demonstrated that bait systems can eliminate entire colonies of subterranean termites (Esenther and Gray 1968; Su et al. 1982; Su 1994; Chambers and Benson 1995; DeMark et al. 1995; Su et al. 1995b; Grace et al. 1996; Su and Scheffrahn 1996a; Su et al. 1997; Atkinson et al. 1998; Haagsma and Bean 1998; Tsunoda et al. 1998; Peters and Fitzgerald 1999; Prabhakaran 2001), whereas liquid applications have been shown to not affect large portions of the termite population around a structure (Su and Scheffrahn 1988a). Colony elimination occurs because the slow acting nature of the ingested toxicant allows foragers time to return to the colony and transfer the toxic material to unexposed nestmates before killing the carrier (Beard 1974; Sheets et al. 2000). Because bait systems work to eliminate colonies, they can provide long term protection to structures (Chambers and Benson 1995; Grace and Su 2001).

The first bait system, the Sentricon® Termite Elimination System, was developed in 1990 by Dow AgroSciences and was registered for use in 1994. Sentricon® is a stand alone treatment that is not to be used in combination with liquid termiticides.

Furthermore, Sentricon® is a monthly monitoring system and when termite infestations are found, the wood monitor is removed and the Recruit II® bait is inserted into the station. Now the most widely used bait system, the Recruit II® bait utilizes a chitin synthesis inhibitor, hexaflumuron [N-(((3,5-dichloro-4-(1,1,2,2-tetrafluroethoxy)phenyl)-amino)carbonyl)-2,6-diflurobenzamide], as its active ingredient. Hexaflumuron has been used to control a variety of agricultural pests, but it is also useful against subterranean termites (Su and Scheffrahn 1993; Su 1994; Chambers and Benson 1995; Su 1995). It is lethal at low concentrations and has no associated feeding deterrence (Robertson and Su 1995). Sheets et al. (2000) determined that the rate at which workers eliminate hexaflumuron from the body is slower than the rate they take up food. This delay causes a build-up of the toxicant to occur in the termite body.

Since the introduction of the Sentricon® System, other bait systems have been designed and marketed. Another chitin synthesis inhibitor, diflubenzuron, is the active ingredient in the Exterra® Termite Interception and Bait System developed by Ensyntax. Like hexaflumuron, diflubenzuron, was first introduced as an insecticide for other insects, such as the gypsy moth and various caterpillar species. Due to its success in controlling these insects, diflubenzuron was evaluated for efficacy against subterranean termites (Doppelreiter and Lorioth 1981). The Exterra® System is a monthly or quarterly monitoring system. The diflubenzuron bait, Requium®, is inserted into the stations if termites are found feeding on the monitors. The design of the Exterra® bait station allows for Requium® to be added to the station, without removing the wood monitors so foraging termites are not disturbed. The Exterra® System is also designed as a stand alone treatment. Although Exterra® seems to be structured similar to that of Sentricon®, diflubenzuron has not attained as high a level of success as hexaflumuron. Su and Scheffrahn (1993) determined that *C. formosanus* were deterred from feeding on baits with diflubenzuron concentrations >2ppm and had very low rates of consumption at concentrations of 500 and 1,000ppm diflubenzuron. Furthermore, mortality was lower for termites feeding on diflubenzuron treated wood than that for termites feeding on wood treated with hexaflumuron.

A third widely used bait system was created by FMC in 1996, the Firstline® Termite Bait System. This system contains the slow acting stomach toxicant sulfluramid. Unlike the hexaflumuron and diflubenzuron, sulfluramid works by disrupting energy metabolism in

termites (Valles and Koehler 1997). The Firstline® System consists of an in-ground station with up to four wooden monitors inside. The stations must be checked every three months for signs of infesting termites. If termites are found, the infested wooden monitors are replaced with a sulfluramid bait. Like diflubenzuron, sulfluramid deters feeding at higher concentrations and is usable only at a small range of concentrations (4-10ppm for *C. formosanus* and 18-30ppm for *R. flavipes;* Su and Scheffrahn1991). Firstline® cannot be used as a stand alone treatment and must be used in combination with liquid applications.

Problems with Bait Systems

Bait systems are a useful method of subterranean termite control, but they also have limitations. Chitin synthesis inhibitors, like hexaflumuron and diflubenzuron, are only effective on actively molting individuals. *Reticulitermes* colonies, for example, contain many generations of individuals, each molting at different rates (Noirot and Pasteels 1987). It is possible that bait systems using these active ingredients are not effective in eliminating those members of the colony that are not molting regularly. Environmental conditions, such as temperature, can also affect bait efficacy, as temperature can interfere with subterranean termite feeding and molting rates (Smythe and Williams 1972; Delaplane et al. 1991; Sponsler and Appel 1991). Van den Meracker et al. (2002) found that temperatures below 20°C slow development and increase the time between molts. Therefore, the effects of baiting take longer to appear in cold temperatures.

Bait stations are a relatively small presence within the termite environment, so foraging termites may not locate the stations for some time or may never find them. Because termites are cryptobiotic, pest control technicians cannot predict where colonies are located and must speculate where to install the stations (Potter et al. 2001). If foraging termites locate the stations, they must then consume the bait and take it back to the colony. If the colony is large (e.g. 2,000,000 individuals), it may take a significant amount of time for enough bait to be disbursed to kill the colony. However, if the bait is not continuously consumed, populations may recover after baiting and survive to cause additional structural damage (Su et al. 1995a).

However, the most significant hindrance to termite bait performance is perhaps the presence of competing food resources. The number of termites that feed on a bait matrix determines how quickly the toxicant can be spread to other individuals in the colony. In a natural environment, other food sources may compete with the baits, and foraging termites may be divided among a number of food sources at any one time.

Insect Growth Regulators

Esenther and Gray (1968) were the first to suggest using a slow acting toxicant for termite control. Many slow acting toxicants have been impregnated into wooden bait blocks and tested against subterranean termites, such as mirex (Esenther and Beal 1974, 1978; Paton and Miller 1980) hydramethylnon (Su et al. 1982), avermectin B_1 (Su et al. 1987), A-9248 (diiodomethyl para-toyl sulfone) (Su and Scheffrahn 1988b) sulfluramid (Su and Scheffrahn 1991), hexaflumuron (Su 1994) and diflubenzuron (Su and Scheffrahn 1993).

Although many of these chemicals successfully reduced termite populations, special emphasis has been placed on the use of IGRs as the slow acting toxicant in bait systems. In addition to not killing foraging termites on contact and allowing sufficient time for the termites to return to the colony and feed unexposed nestmates, IGRs are also detrimental to the colony dynamics. Because IGRs induce presoldier and intercaste production in termites (Lüscher 1969; Howard and Haverty 1979a; Jones 1984; Su et al. 1985; Su and Scheffrahn 1989), the ratio of caste members inside a termite colony is disrupted and the production of foraging workers is reduced (Hrdý and Křeček 1972; Haverty 1977). With more soldier termites, the colony has more dependent mouths to feed and fewer worker termites to find food. Therefore, IGRs, such as hexaflumuron, were the first toxicants to be marketed in bait systems.

IGRs work by three different methods: as juvenile hormone analogues (JHA), as precocenes, and as chitin synthesis inhibitors (Ware 1994). Juvenile hormones (JHs) are hormones in the insect’s body direct normal growth, development, and maturation of insects. JHAs can mimic these hormones and disrupt insect development or their emergence as adults (Ware 1994). Precocenes affect the corpus allatum, the gland that

secrete hormones, primarily JH. These hormones are important insect hormones because they control reproduction, molting, and metamorphosis (Ware 1994). Chitin synthesis inhibitors (CSIs) disrupt the insect molting process, which ultimately causes death in immature insects because they die in the process of shedding their exoskeleton.

It is the CSIs that are used as many termiticides, especially in the bait systems. CSIs are benzoylphenyl ureas (BPUs) insecticides, and inhibit normal chitin formation in larvae (Ishaaya 1990) by causing abnormal deposits of endocuticle to accumulate (Mulder and Gijswijt 1973). BPUs have also been shown to suppress fecundity and exhibit ovicidal toxicity in some species of insects due to the chitin component of their eggshells (Ascher and Nemny 1974, 1976; Sarasua and Santiago-Alvarez 1983; Muzzarelli 1986; Marco et al. 1998).

The use of CSIs in termite control is very favorable because they target only a few selective organisms and are not broad spectrum insecticides. The selectivity is due to many factors, including their toxicity only to immature arthropods and not vertebrates, to the fact that they must be ingested for activation, and to their slow activation (Granett 1987; Horowitz et al.1992). The selectivity of CSIs allows for safe use around mammals and non-target organisms, for low rate of accumulation in the soil, and for low biomagnification in the environment (Verloop and Ferrell 1977).

Using CSIs in bait stations as a means to control subterranean termite infestations has become increasingly important (reviewed in Su and Scheffrahn 1990c). Evidence indicates that CSIs provide an effective method for termite control because of their slow acting properties, their ability to increase presoldier production within the termite colony, their capacity to cause gut defaunation and subsequent starvation of termites, and their nonrepellent nature (Haverty and Howard 1979; Haverty et al. 1989; Howard 1983; Howard and Haverty 1978, 1979a, b; Jones 1984; Su et al. 1985; Su and Scheffrahn 1989).

CSIs, such as hexaflumuron, were considered for use in bait systems because of their success in controlling agricultural pests. Hexaflumuron was found to be effective against many species of agricultural crop pests, such as army beetworm (*Spodoptera exugua)* (Belda and Guerrero 1992), the spiny bollworm (*Earias insulana* (Boisd) (Horowitz et al. 1992), the sugar beet weevil (*Aubeonymus mariaefranciscae* Roudier)

(Marco et al. 1998; Perez- Farubism et al. 1998), and a number of pests on apples and peaches (Komblas et al. 1989). Furthermore, hexaflumuron has been observed to have some ovicidal activity (Komblas and Hunter 1986; Horowitz et al. 1992) and affect fecundity and egg hatchability in different insect species (Asher et al. 1986; Horowitz et al. 1992; Marco and Viñuela 1994). Because hexaflumuron is useful against agricultural pests and the fact that it acts more quickly than other CSIs (Sbragia et al. 1983), Dow AgroSciences began researching hexaflumuron for use in controlling subterranean termites.

Summary

Subterranean termites are extremely successful insects, and it has been difficult to find effective and accurate methods of control. For years liquid termiticides have been used as the primary means of termite control. However, termite foragers can tunnel through gaps in repellent termiticides if they are not applied evenly. Furthermore, nonrepellent liquid termiticides work primarily against those termites that have entered the treated soil. Often the colony remains unaffected and worker termites continue to forage in other areas of the soil. However, one of the biggest problems for liquid termiticides is that eventually the chemicals lose their residual qualities in the soil and have to be reapplied.

Although bait systems overcome some of the problems of liquid applications, they still have limitations. For example, the risk factor of termite infestations for structures whose only defense is a bait system is unknown. Because the majority of bait stations are not used in combination with liquid treatments, foraging termites still have access to the structure. In addition, it is possible for colonies only partially suppressed from baiting to recover and reinfest the structure (Su et al. 1995b).

It is important for termite control methods to continuously be researched and attempt to reflect newer, more advanced technologies. Thus, my research focuses on the efficiency of one type of termite control: hexaflumuron from the Sentricon® termite bait system. By understanding the extent that secondary food sources affect termite consumption of hexaflumuron bait and subsequent termite mortality, it is possible to

improve bait conditions. If, for example, termites should show a preference for some food source over hexaflumuron bait, this preferred food source could be incorporated into the bait matrix to increase palatability. Only with the continual refinement of termite management methods can subterranean termites be more efficiently controlled.

Chapter Three:
The Effect of Competing Food Sources on *Reticulitermes* spp. (Isoptera: Rhinotermitidae) Consumption of Hexaflumuron-Treated Bait.

Introduction

Understanding the nutritional ecology of subterranean termites is imperative to grasping termite colony dynamics. The division of labor within the nest and the evolution of the worker termite caste were based on the necessity of the colony to forage away from the nest for food (Abe 1987; Noirot and Pasteels 1987; Myles 1999). Worker termites are responsible for foraging for food and feeding the rest of the dependent colony (Krishna 1969). Because termite colonies are very large (e.g. 100,000 to 1,000,000) and not all workers forage at the same time, the worker caste has evolved to be the most numerous in order to meet the nutritional needs of the entire colony.

When foraging for food, termite feeding behaviors and food preferences vary widely. Most subterranean termites eat dead wood and decaying wood litter (Wood 1978), but they may also attack live trees and paper (Waller and La Fage 1987). However, termite feeding can vary due to colony factors, environmental factors, or food source factors (reviewed in Lenz 1994 and Waller 1991). Natural variation of consumption rates occurs among different colonies of subterranean termites, because some colonies consume diets at a faster rate than others (Su and La Fage 1984a; Lenz 1985). Also, caste proportions within a colony can influence feeding rates. An increase in soldier proportions within the colony has been shown to increase worker consumption rates (Becker 1962; Su and La Fage 1987). Furthermore, environmental factors like temperature (Smythe and Williams 1972; Haverty and Nutting 1974), humidity (Collins 1969), and season influence consumption because termites eat at different rates during the different seasons of the year (Su and La Fage 1984a; Lenz 1985; Cornelius and Osbrink 2001). Finally, termite feeding and consumption rates vary in response to conditions of the food supply. Wood density, wood diameter and particle size, and nutritional content, such as nitrogen levels, determine how quickly the food source is consumed (Behr et al. 1972; La Fage and Nutting 1978; Wood 1978).

A general understanding of subterranean termite colony feeding and consumption rates is imperative for the success of certain termite control methods. For example, the basis of termite bait systems requires that foraging termites feed from a toxic cellulose matrix. Therefore, the bait systems are completely dependent on termites locating the bait stations while tunneling, accepting the bait as a food source, and recruiting other workers to also feed on the bait. One such bait system, the Sentricon® Termite Elimination System, was developed in 1990 by Dow AgroSciences. Now the most widely used bait system, Sentricon® utilizes hexaflumuron as its active ingredient. Hexaflumuron [N-(((3,5-dichloro-4-(1,1,2,2-tetrafluroethoxy)phenyl)- amino)carbonyl)-2,6-diflurobenzamide] is a slow acting chitin synthesis inhibitor (Su et al. 1987; Nakagawa et al. 1992). Because the mean half life of hexaflumuron within a termite is about 9 days, foraging termites survive long enough after ingestion to transfer the toxicant to the entire colony (Sheets et al. 2000). The speed of termite colony elimination is therefore based on the number of worker termites recruited to the station and how many other food sources are currently being fed on by the colony.

The most significant hindrance to termite bait system performance in the field is the presence of competing food sources. When bait stations are installed in the ground, termites usually are already feeding on a structure or some natural food source in the area. Because termites can be faithful to a given food source, even if other food sources, like a bait, are found (Heidecker and Leuthold 1984; Oi et al. 1996), a natural food source may be more attractive to termites than the bait. Furthermore, termite feeding behaviors are influenced by the types of cellulose materials found (Smythe and Carter 1970; Behr et al. 1972; Howard and Haverty 1979b; Su and Tamashiro 1986). For example, Smythe and Carter (1970) found that total consumption by *Coptotermes formosanus* was altered by the type of test (choice or no-choice) and which cellulose diets were presented (black walnut, black cherry, or redwoods). Termites are also known to prefer larger food sources over smaller ones (Waller 1988; Lenz 1994; Cornelius and Osbrink 2001) and will not leave a larger food source to consume a smaller one. Bait system efficacy may be interfered with due to so many food source options in a natural environment and so many factors influencing termite food choice.

The effect of alternative food sources on termite bait consumption has never been quantified. In this study, ^{14}C-hexaflumuron, ^{3}H-inulin, and control diets were presented alone or in combinations to quantify termite consumption of different formulated baits. The purpose of this study was to determine the effect of competing food sources on subterranean consumption of hexaflumuron bait.

Methods and Materials

Subterranean Termite Collection. Five wild populations of *Reticulitermes spp.* were collected from fallen wood in forested areas of Fairfax County (N38° 43.29' and W77° 30.93'), Montgomery County (N37° 12.46' and W80° 24.47'), Rockbridge County (N37°48.00' and W79° 25.00'), and Roanoke County (N37° 19.53' and W79° 58.53'), Virginia. Termites were harvested by placing the wood in plastic storage tubs (70L; Sterlite®; Sterlite Corporation, Townsend, MA) on top of damp, recycled paper towels (Acclaim®; Fort James Corporation, Deerfield, IL). Termites infested the moist paper towels as the wood dried out. The infested paper towels were transferred to plastic storage containers (11.3L; Rubbermaid, Wooster, Ohio) containing vermiculite (500g; moistened 150% by weight [Lenz et al. 1987]; Cherokee Vermiculite Horticultural Fine Cherokee Products, Jefferson City, TN). Termite containers were stored in complete darkness (~21°C and 97% RH) until needed for testing. Subterranean termites were tested within one month after being collected from the field.

Termite Diet Preparation. Recycled brown paper toweling (Acclaim®; Fort James Corporation, Deerfield, IL) was cut into squares (35 x 35 mm) and used as a diet substrate. Because hexaflumuron cannot be stored at temperatures above 50°C and all paper squares were dried in an oven (60ºC), the initial weight of each diet was recorded before diets were treated. Diet substrates were placed into glass Pyrex Petri dishes (100x 20mm; Corning Glass Works, Corning, NY) and dried overnight in a single wall, gravity convection, laboratory oven (60ºC; Blue M SW-17TA; Blue M Electric Company; Blue Island, IL). After 24h, each diet substrate was taken out of the oven, and they were immediately numbered in the corner with a pencil and weighed on a balance (Mettler AE163; Lab Tech, Inc.) to the nearest 0.1mg. After weighing, diets were treated with either the visual dye, as a

control, or the visual dye combined with one of the radiochemicals, as a radioactive treatment.

Visual Marker and Radiolabeled or Non-radiolabeled Chemicals. Two radiolabeled chemicals, a technical grade chemical (non-radiolabeled), and a visual marker were formulated and applied to diet substrates. Nile Blue-A (Allied Chemical Company, Morristown, NJ) was selected as the visual marker because of its long-term visibility and low associated mortality for *Reticulitermes flavipes* (Haagsma and Rust 1993; Oi and Su 1994; Su et al. 1991; King 2000; Suarez and Thorne 2000b). The purpose of the dye was to ensure that termites were feeding on the diets. Termites feeding on experimental diets were dyed a bright blue color. Radiolabels were used to quantify the effects of competing food sources on hexaflumuron consumption. Inulin-methoxy, [methoxy-^{3}H-] (lot 2978-124, specific activity 200mCi/g) was purchased from the radiosynthesis group at the DuPont Company (Wilmington, DE). Radiochemical purity of the inulin had been determined to be 98.7% by high pressure liquid chromatography. Hexaflumuron-dichlorophenyl-UL-[^{14}C] (lot F0662-54, specific activity of 21.5mCi/mmol) was obtained from the radiosynthesis group at Dow AgroSciences (Indianapolis, IN). Radiochemical purity of the hexaflumuron had been determined to be 98.7%. Nonradiolabeled technical-grade hexaflumuron (98% pure) was also obtained from chemical resource services at Dow AgroSciences (lot# 17/95; Indianapolis, IN).

Control Diets. Control diets were formulated by dissolving 11.3mg of Nile Blue-A in 20ml of acetone and applying a 150µl aliquot of dye solution to each diet square. The final Nile Blue-A concentration on the square was 0.1% (weight of Nile Blue A/weight of paper square).

^{3}H-inulin Diets. The experimental stock solution was formulated by combining 218µl of an initial inulin stock solution (0.25µCi/µl) with 3564µl of acetone for a final activity of 1.5×10^{-3} µCi. Nile Blue-A (1.95mg) was added to the inulin experimental formulation, so that the final Nile Blue-A concentration on the square was 0.1% (w/w). A 150µl aliquot of the inulin/dye solution was applied to each diet square. ^{3}H has a lower energy spectrum than ^{14}C, so approximately 9x more ^{3}H-inulin was added to the diets than ^{14}C-hexaflumuron to facilitate counting procedures.

^{14}C-hexaflumuron Diets. A hexaflumuron stock solution was formulated to achieve a final concentration of 0.5% hexaflumuron (w/w) and 0.27µCi per diet. ^{14}C-hexaflumuron (1.09µg) was dissolved in 1000µl of acetone (Stock A). Technical-grade hexaflumuron (0.0066g) was dissolved in 4ml of acetone (Stock B). A final solution (stock C) was formulated by adding 11805µl (Stock A) to 3888µl (Stock B). Nile Blue-A (0.00195g) was the added to the Stock C solution, so that the final Nile Blue-A amount applied was 0.1% (w/w). A 150µl aliquot of the Stock C hexaflumuron solution was applied to each diet square.

Sand Preparation: Play sand (Quikcrete®; Quikcrete Companies, Atlanta, GA) was washed with tap water 4 times to remove impurities. Washed sand was dried for 48h in a single wall gravity convection laboratory oven (270ºC; Thelco®; Precision PS Scientific, Chicago, IL) prior to use. Dried sand was then placed in a plastic storage bag (3.79L; Target Corporation, Minneapolis, MN) and moistened with distilled water (15% by weight of the sand). Moistened sand was then kneaded by hand in the storage bag and stored for at least 24h to ensure even distribution of moisture. Sand was stored in the plastic bag until needed for testing.

Experimental Arenas: Laboratory arenas were used to evaluate the effect of competing food sources on hexaflumuron efficacy. Subterranean termites were exposed to a hexaflumuron diet in no-choice test to determine total consumption of a single diet. Termites were also exposed to both diets simultaneously in choice tests to determine how consumption was divided between the two diets.

No-Choice Bioassay. No-choice arenas were assembled by connecting two Petri dishes with Tygon tubing (inner diameter 3.2mm, outer diameter 6.4mm; Fig. 3-1). Petri dishes were washed 4 times in tap water, and Tygon tubing was soaked in tap water for 3h prior to use. The washing of arena materials was done to eliminate static electricity and to remove any impurities. One Petri dish (95 mm x 15 mm; Fisher Scientific) served as a housing chamber and was filled with moistened sand from the storage bag (~45g). A second Petri dish (60 mm x 15 mm; Fisher Scientific) served as a diet chamber and contained one of the diets. Termites placed inside the treatment no-choice arenas could forage only on the ^{14}C-hexaflumuron diet. Likewise, termites placed inside the control no-choice arenas could forage only on the nonradioactive control diet.

Figure 3-1. The No-choice subterranean termite arena. Termites were placed in the larger housing chamber where they could forage through a Tygon tube into a smaller diet chamber. The diet chamber contained dyed and/or hexaflumuron-treated paper towels.

Choice Bioassay Set-up. Choice arenas were similar to the no-choice arenas with the exception that the housing chambers were attached to two diet chambers, each with a separate diet (Fig. 3-2). Termites placed inside choice arenas could simultaneously forage on two diets held in the different chambers. Choice bioassays were set up for each of the following combinations: ^{14}C-hexaflumuron and control diet, ^{3}H-inulin and control diet, and ^{14}C-hexaflumuron and ^{3}H-inulin diet.

Bioassay Design. Bioassays were arranged in a 5 (treatment) x 2 (day) factorial experiment. The experiment was arranged as a randomized complete block design (RCBD), blocked by termite field population to account for within treatment variability due to differential feeding between colonies. Ten bioassay arenas were set up for each of the five treatment groups: no-choice control diet, no-choice hexaflumuron diet, choice hexaflumuron/control diets, choice inulin/control diets, and choice hexaflumuron/inulin diets. Each treatment group was further subdivided into two test periods: 2d and 5d. Each test day within a treatment group was replicated five times for a total of 50 arenas with each replicate containing termites from a different field population. Once the data from an arena had been recorded on a particular test day, the arena was removed from the test.

Figure 3-2. The Choice subterranean termite arena. Termites were placed in the larger housing chamber where they could forage through two different Tygon tubes into either of the smaller diet chambers. One diet chamber contained dyed paper towels as the control, and the second diet chamber contained dyed hexaflumuron-treated paper towels.

Prior to testing, 100 worker termites (at least 3rd instar) were aspirated out of the storage containers and transferred into the housing chamber of one experimental arena. Termites were allowed to acclimate for 72h and forage on untreated paper towels in the diet chamber(s). After the acclimation period, paper towels were removed, and all clinging termites were gently tapped back into diet chamber. Experimental diets were then placed into the test chambers. Diets were secured in the Petri dishes to minimize radioactive contamination of the diet chamber or for uniformity. All diets were placed on top of a glass coverslip (Rect. No.1 22 x 30cm; Corning Labware and Equipment) in the bottom of the diet chamber and held in place by placing 1/3 of a standard paper clip on top of the diet. A magnet (19.05mm Diameter; ProMag®, Marietta, OH) was placed underneath the bottom of the Petri dish holding the diet between the paper clip and the magnet. Three pieces of masking tape were put on the outside of the arena to seal the housing chamber lid.

Humidity Chamber. All bioassay arenas were placed inside humidity chambers, which were constructed by pouring play sand into the bottom of plastic storage containers (11.3L; Rubbermaid, Wooster, OH) to a depth of 4.5 cm. Tap water was poured over the sand to the point of saturation. Any standing water was absorbed with paper towels. A sheet of aluminum foil (Super Foil™; Atlantic Paper & Foil Corporation, Hauppauge, NY) was laid down to cover the saturated sand. Arenas containing termites were placed inside plastic storage bags (3.8L; Target Corporation, Minneapolis, MN) and set on top of the aluminum foil in the chamber. The top of the plastic bag was rolled down to allow for air circulation. Plastic bags were used to separate the arenas from each other and to catch escaped termites. Escaped termites were returned to their respective experimental arenas. Humidity chambers were closed with snap top lids and were placed in total darkness in a cabinet (~21°C and 97% RH) for the duration of the experiment.

Recording Mortality. Termite mortality was recorded to ensure that the test insects were vigorous (Sheets et al. 2000), and that mortality had no influence on consumption. Termites that did not seem to exhibit proper molting were considered moribund and were subtracted from the survivor count (Su and Scheffrahn 1993). The number of termites surviving at 2d and 5d was counted and subtracted from the total number of termites.

Analysis of Diet Consumption. After termite mortality was recorded for each bioassay, the partially consumed diets (radioactive and nonradioactive) were removed and dried for 24h as previously described and weighed to determine total consumption. Post-test weights of partially consumed diets were subtracted from the initial diet weights to determine total consumption by treatment. Initial diet weights were the weights taken before paper towels were treated with dye and/or hexaflumuron, as the weight of the isotope, hexaflumuron, and/or inulin was determined to be miniscule.

Measuring Radioactivity. After mortality was recorded (2d and 5d), 20 surviving termites were randomly selected from each radioactive treatment arena for consumption analysis. Random selection was not limited to dyed termites, as white, nonfeeding termites were also selected. Termites were thoroughly rinsed with distilled water three times to remove any radioactivity residing on the outside of their bodies. Termites were then placed on paper towels to remove excess water. Each rinsed termite

was then placed into a separate microcentrifuge tube (1.5ml; Fisher Brand), and the body was homogenized in 200µl of distilled water. The active homogenate was transferred into a glass scintillation vial (20ml; Kimble Glass, Vineland, NJ). Microcentrifuge tubes were rinsed three times with 200µl of distilled water per rinse. Each rinse was added to the homogenate. Eight ml of scintillation cocktail (Scintiverse® BD; Fisher Scientific, Fair Lawn, N J) was then added to each vial. The amount of radioactivity contained within each sample was quantified by using a Beckman Coulter ™ LS 6500 scintillation counter. Because these samples contained very low dpm counts, sample vials containing only a single isotope (from no-choice tests) were counted for 10 minutes. However, sample vials that potentially could contain dual isotopes (from choice-tests) were counted for 20 minutes to improve the accuracy of the counts.

Background Measurements. After mortality was counted in the no-choice control arenas, 2 termites were selected at random from each control arena. These termites were rinsed, homogenized, and prepared for scintillation counting as previously described. Control termites were counted in the scintillation counter for 10 minutes. These counts were made for each termite population at both 2d and 5d and served as the background radioactivity measurements. Background measurements also accounted for any quenching that occurred due to the termite body occluding light transmission. Before the test data was analyzed, background counts for that replication at 2d or 5d was subtracted from the count of each spectral peak for each sample (Traniello et al. 1985; Suarez and Thorne 2000a).

Spillover Correction. In theory, most isotopes have a unique energy signature, so that when using two isotopes together in a scintillation vial, they can be separated by a scintillation counter. Because ^{14}C (E_{mean} = 0.05 MEV, E_{max} = 0.156 MEV) and ^{3}H (E_{mean} = 0.005 MEV, E_{max} = 0.018 MEV) have very similar energy emissions (Wang et al. 1975), there is a region of energy emission overlap. Therefore, a Beckman Coulter ™ LS 6500 scintillation counter cannot accurately distinguish one isotope from another and creates a spillover effect. Spillover occurs when a portion of the decays per minute (dpm) from one isotope are actually reported as dpm from the other isotope and vice versa. Due to this inaccuracy, a spillover must be corrected to improve accuracy.

All experimental data (no-choice and choice tests) were corrected for spillover. Because all samples were run in dual label windows to keep the procedure consistent (as opposed to running some samples a single label window for each respective isotope), both no-choice and choice tests had to be corrected for spillover.

Spillover Correction for One Isotope Present. Background counts were subtracted from all experimental dpm readings. In no-choice tests ("hexaflumuron only" and "control only") and choice-tests with one radioactive diet ("hexaflumuron + control" and "inulin + control"), the termites were exposed to only one isotope. Therefore, if dpm counts appeared for an isotope to which termites had not been exposed, these counts were subtracted off and added back to the correct isotope column.

Spillover Correction for Two Isotopes Present. In the choice test, "hexaflumuron + inulin," however, termites could feed from two different radiolabeled diets simultaneously. Therefore, one termite could potentially contain both isotopes, and experimental data would have to be corrected for spillover by a correction curve. For the purpose of correcting these dually radiolabeled termites, spillover correction curves were created by plotting the dpm versus spillover for a series of isotope standards. For each isotope, therefore, it was necessary to extract radioactivity from the diets used in the experiment, make stock solutions with known amounts of each isotope from the extractions, and create standards containing various concentrations of isotope.

Diet Extraction. A small square (~1cm^2) was cut from each of twelve ^{14}C-hexaflumuron diets and twelve ^{3}H-inulin diets. A 500µl aliquot of acetone was placed into 12 microcentrifuge tubes (1.5ml; Fisher Brand), and one cut ^{14}C-diet square was placed into each tube. Although acetone is not an ideal solvent because it increases quench, it was used as the solvent for extraction due to the low solubility of hexaflumuron in water. Centrifuge tubes were then sonicated (Solid State/Ultrasonic FS-28; Fisher Scientific) for 30 minutes, and spun in a micro-centrifuge (Model 59A; Fisher Scientific) for 5 minutes. Next, the supernatant was removed and all diet extractions for ^{14}C were pooled together as a hexaflumuron solution in one glass scintillation vial for creating the standards (20ml; Kimble Glass, Vineland, NJ). This process was repeated for the ^{3}H-inulin diets to create an inulin solution. Inulin is soluble in water, so the inulin diets were extracted in a 500µl aliquot of distilled water, not acetone.

The two radioisotope solutions had to be diluted so that they contained the desired specific activity for each isotope (for ^{3}H-stock: 34dpm/µl and for ^{14}C-stock: 4dpm/µl). Therefore, the solutions for each isotope were diluted with distilled water. For ^{14}C-hexaflumuron, 18µl of solution was mixed with 15ml of distilled water in a 20ml scintillation vial. After vials were diluted, 8ml of cocktail was added to the vial, and the vial was placed into the scintillation counter. The dilution was repeated until the desired specific activity of the solution was achieved. For ^{3}H-inulin, 13µl of solution was mixed with 12ml of water in another 20ml scintillation vial until the desired specific activity was reached for this isotope, as described previously.

Creation of Radioactive Standards. Radioactive standards were created from the diluted solutions for each isotope. Because dually-labeled termites contained different amounts of radioactivity, the range of each isotope in experimental termites was determined. Radioactive standards for each isotope were created separately so that they corresponded to the range of each isotope found in the dual-labeled termites. Ten standards were made for each isotope, and each standard contained a different volume (i.e. amount) of the respective isotope. Ten glass scintillation vials (20ml) were filled with increasing volumes (with increasing radioactivity) of diluted ^{3}H-inulin (0µl, 3µl, 25µl, 50 µl, 75µl, 100µl, 125µl, 150µl, 175µl, and 200µl). Ten glass scintillation vials were also filled with increasing volumes of diluted ^{14}C-hexaflumuron (0µl, 25µl, 75µl, 150 µl, 225µl, 300µl, 375µl, 450µl, 525µl, and 600µl). These 20 vials were placed in the scintillation counter and counted to determine the amount of spillover associated with each volume for each isotope. The standards were counted in the ^{14}C, ^{3}H-dual label window for 20 minutes to increase accuracy of the counts. Background dpm counts were subtracted from the radioactive standards.

Correction Curves. Correction curves were created by plotting the dpm versus spillover for each of the isotope radioactive standards. The curves were used to correct each experimental sample for spillover in order to make the experimental dpm (amount of consumption) for each single termite more accurate.

The dpm for standards containing only ^{3}H and ^{14}C were plotted separately. The actual ^{3}H-counts and the spillover ^{14}C counts were plotted as two separate lines on the

same graph (Fig. 3-3). The same was done for vials containing only ^{14}C (Fig. 3-4). These two single isotope graphs were used for spillover correction.

For the "hexaflumuron + inulin" choice test, the data were divided into three categories: where ^{3}H was the dominant isotope, where ^{14}C was the dominant isotope, and samples where both isotopes were high. When ^{3}H was the dominate isotope, the correction curve shown in Fig. (4-3) was used to correct for spillover. The ^{3}H-dpm count for each experimental vial was located on the graph along the ^{3}H-standard line. By running down the y-axis to the intersection of the spillover line (^{14}C line), it was possible to determine how many dpms of spillover corresponded with each experimental ^{3}H-count. These dpms were then subtracted from the ^{14}C-dpm column and added back to the ^{3}H-dpm column. When ^{14}C was the dominate isotope, the correction curve shown in Fig. (3-4) was used to correct for spillover as described for ^{3}H. In the case where both isotopes had high dpm counts, both correction curves shown in Fig. 3-3 and 3-4 were used. Both isotopes were corrected simultaneously, by correcting spillover for the isotope with the highest dpm and then correcting for spillover for the second isotope.

Statistical Analysis. The mean percentage of termite mortality within treatment groups (5 levels) was compared using GLM analysis of variance (ANOVA) for 2d and 5d separately. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

The analysis of population diet consumption (mg) determined how much of the diets were consumed by 100 subterranean termites. The mean total diet consumption (mg) of diets in the five treatments was compared using GLM ANOVA for 2d and 5d separately. Total consumption at each day was modeled as a function of population (blocked) and treatment. This analysis was blocked by termite field population to account for within treatment variability due to differential feeding between colonies. . Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999). The mean consumption of hexaflumuron diet in the no-choice test was compared with the amount of hexaflumuron diet consumed in the "hexaflumuron + control" choice test or the "hexaflumuron + inulin" choice test using the Student's *t*-test. Mean consumption of inulin diet in the "inulin + control" choice test was compared with the amount of inulin

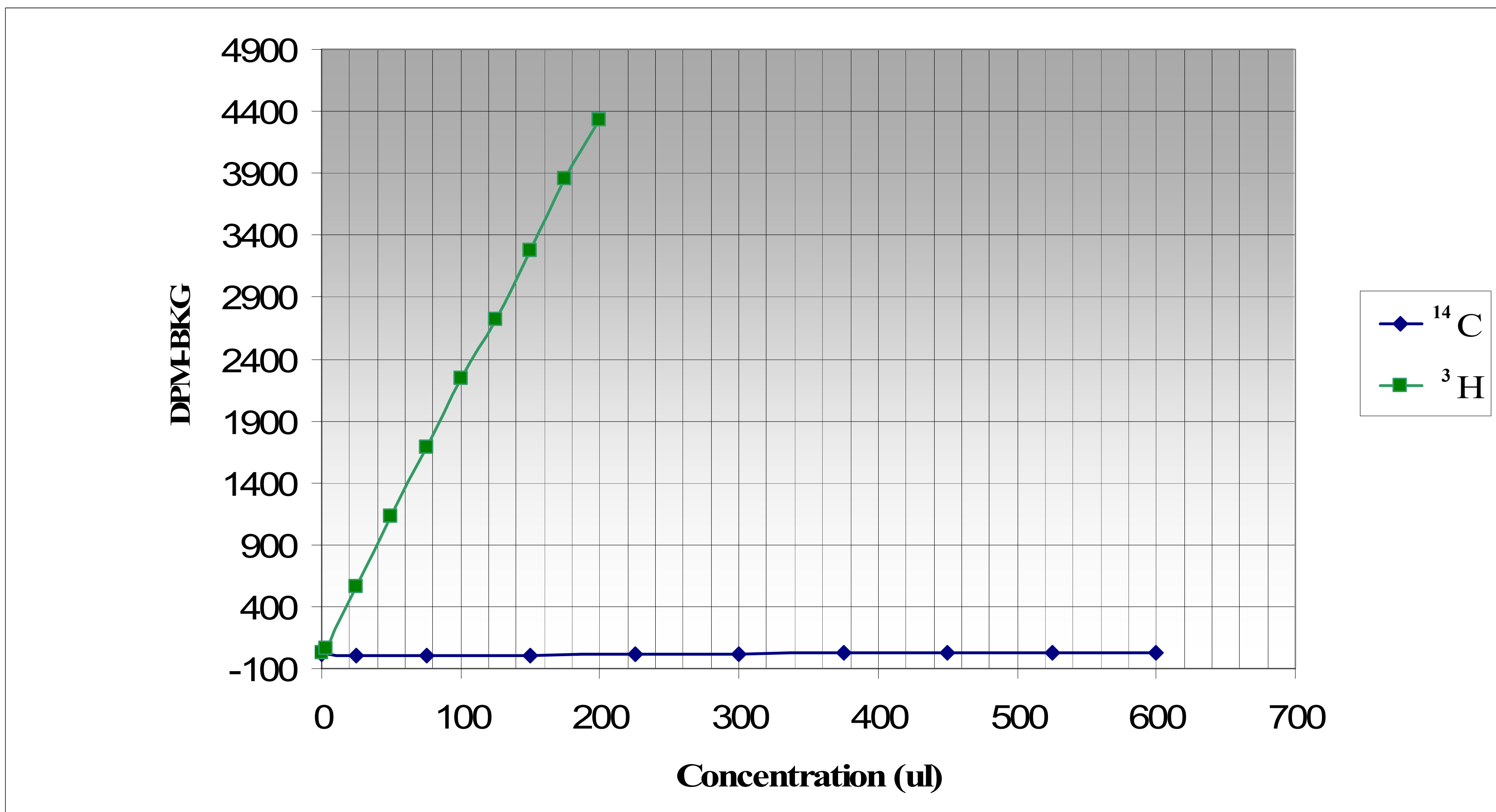

Figure 3-3. Spillover Curve for ^{14}C in the presence of ^{3}H-Samples.

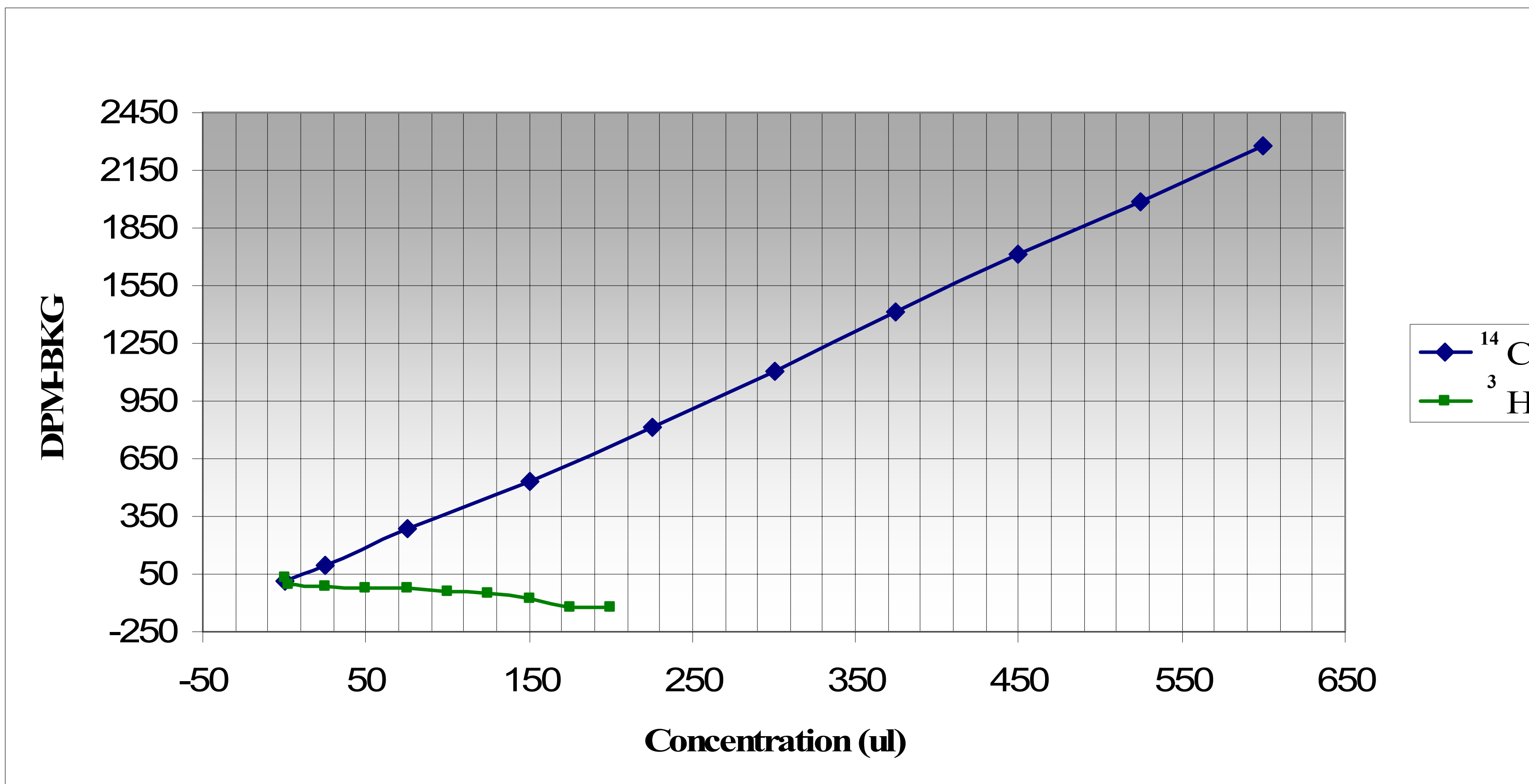

Figure 3-4. Spillover Curve for ^{3}H in the presence of ^{14}C-Samples.

diet consumed in the "hexaflumuron + inulin" choice test using GLM ANOVA for 2d and 5d separately. Mean consumption in each choice test at each day was modeled as a function of population (blocked) and treatment. For each comparison, values of $\alpha \leq 0.10$ were used to indicate significance (SAS Institute 1999). Finally, mean consumption of the two diets within each of the three choice tests was analyzed to determine whether termites preferred one diet over another. Due to the binomial nature of termite consumption of the two diets in the choice test, diet consumption of both diets was analyzed using a Student's *t*-test for $\mu = 0.50$. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

Radioactive diet analyses were used to determine how much a single termite consumed of hexaflumuron diet in the presence of a competing food source. Mean consumption of hexaflumuron diet in the no-choice test was compared with hexaflumuron consumption in the individual choice tests using GLM ANOVA with Least Squares adjustment for multiple comparisons of mean values for 2d and 5d separately. For each analysis, mean consumption was modeled as a function of population (blocked), treatment, time, and the interaction between treatment and time. Mean consumption of inulin diet in the "hexaflumuron + inulin" choice test as compared to the "inulin + control" choice test was compared using GLM ANOVA with Least Squares adjustment for multiple comparisons of mean values for 2d and 5d separately. For each day, mean consumption was modeled as a function of population (blocked), treatment, time, and the interaction between treatment and time. Finally, mean consumption of the two diets within the radioactive choice test "hexaflumuron + inulin" was analyzed to determine whether termites preferred one diet over another. Diet consumption of both diets was analyzed using a Student's *t*-test for $\mu = 0.50$. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

Results

Mortality. In general, subterranean termite mortality was very low (< 3% by 5d). At 2d termite mortality was not significantly different between any of the treatment

groups ($\alpha = 0.05$; $P = 0.598$; Table 3-1). Likewise, at 5d mortality between treatments was not significantly different ($\alpha = 0.05$; $P = 0.974$).

Diet Consumption Based on Diet Weight. At 2d the mean total consumption (mg) of diets by the subterranean termite population was not significantly different for any of the arena tests, either no-choice or choice ($\alpha = 0.05$; $P = 0.714$; Table 3-2). Overall, termites ate the same amount (~3mg) of hexaflumuron diet, control diet, and combined diets (hexaflumuron, control, and inulin combinations). Although at 5d, termites ate considerably more diet (~11mg), consumption of diets in all five treatment groups were still not significantly different ($\alpha = 0.05$; $P = 0.148$).

Mean termite population consumption of hexaflumuron in the presence of competing food sources versus that of hexaflumuron consumption in the no-choice test is listed in Table 3-3. At 2d mean consumption by 100 termites fed only hexaflumuron diets in a no-choice test was 3.0mg. When the hexaflumuron diet was offered with the control diet in the choice test, consumption of the hexaflumuron diet was reduced to 1.6mg. However, this reduction was not significant ($\alpha = 0.10$; $P = 0.170$). When inulin diet was competing with the hexaflumuron diet in a choice test, termite consumption of hexaflumuron was significantly reduced to 1.0mg ($\alpha = 0.1$; $P = 0.068$). At 5d, in the no-choice test, consumption of hexaflumuron was 11.4mg. However, in the choice test when hexaflumuron diets were competing with control diets, termite consumption of hexaflumuron was significantly reduced to 3.2mg ($\alpha = 0.1$; $P = 0.080$). Interestingly, in the choice test where inulin diets were competing with the hexaflumuron diets, termite consumption of hexaflumuron was reduced significantly to only 0.2mg ($\alpha = 0.10$; $P = 0.045$).

Finally, the amount of each diet consumed within the three choice tests was compared to see if the termite populations preferred one diet over another. At 2d in the choice test, termites showed no feeding preferences for either diet offered in any of the choice tests (Table 3-4). At 5d in the choice test, the consumption of the hexaflumuron diet was not significantly different than consumption of the control diet ($\alpha = 0.05$; $P = 0.841$) and consumption of the inulin diet was not significantly different than that of the control diet ($\alpha = 0.05$; $P = 0.333$). However, in the choice test where hexaflumuron was

in competition with the inulin diet, termites consumed significantly less of the hexaflumuron diet than the inulin ($\alpha = 0.05$; $P < 0.0001$).

Diet Consumption Based on Radiolabeled ^{14}C- hexaflumuron. When comparing the average termite's consumption of hexaflumuron between the no-choice and a "hexaflumuron + control" choice test, the interaction treatment*day was not significant ($F = 0.862$, $df = 1$, $P = 0.862$). Also, the effect of day on hexaflumuron consumption was not significant ($F = 1.27$, $df = 1$, $P = 0.402$). However, the effect of treatment on hexaflumuron consumption was significant ($F = 7.76$, $df = 1$, $P = 0.017$). At 2d hexaflumuron consumption was significantly reduced from 38.9µg in the no-choice test to only 14.5µg when the competing with the control diet ($\alpha = 0.05$; $P = 0.090$; Table 3-5). Similarly, at 5d hexaflumuron consumption was significantly reduced from 56.9µg in the no-choice test to only 19.3µg in the choice test ($\alpha = 0.05$; $P = 0.058$).

When comparing mean termite consumption of hexaflumuron between the no-choice and the "hexaflumuron + inulin" choice test, the interaction treatment*day was not significant ($F = 1.14$, $df = 1$, $P = 0.306$). Also the effect of day on hexaflumuron consumption was not significant ($F = 0.11$, $df = 1$, $P = 0.748$). However, the effect of treatment on hexaflumuron consumption was highly significant ($F = 48.89$, $df = 1$, $P < 0.0001$). At 2d hexaflumuron consumption was significantly reduced from 38.9µg in the no-choice test to only 7.3µg when competing with the inulin diet ($\alpha = 0.05$; $P = 0.001$). Similarly, at 5d hexaflumuron consumption was significantly reduced from 56.9µg in the no-choice test to 2.3µg in the choice test ($\alpha = 0.05$; $P < 0.0001$).

Finally, the amount of each diet consumed within the choice test "hexaflumuron + inulin" was compared to see whether single termites preferred one diet over another. At 2d termite consumption of the inulin diet (12.6µg) was not significantly different than consumption of the hexaflumuron diet (7.3µg; $\alpha = 0.05$; $P = 0.285$; Table 3-6). However, by 5d termite consumption indicated a significant preference for inulin ($\alpha = 0.05$; $P = 0.002$). On average individual termites consumed 19.4µg of inulin diet and only 2.3µg of hexaflumuron diet.

Table 3-1. To determine termite health, mean percent mortality at 2 and 5 days for the subterranean termite population fed hexaflumuron diets, inulin diets, control diets, or a combination of two diets.

Time Period (day)	Treatment Group	n	Mean Percent Mortality (Mean ± SEM)	*F*-statistic	*P*-value
2	Control Only	5	0.01 ± 0.01[1]	0.710	0.598[1]
	Hexaflumuron Only	5	0.01 ± 0.01		
	Hexaflumuron + Control	5	0.02 ± 0.01		
	Inulin + Control	5	0.02 ± 0.01		
	Hexaflumuron + Inulin	5	0.03 ± 0.02		
5	Control Only	5	0.02 ± 0.01	0.120	0.974
	Hexaflumuron Only	5	0.03 ± 0.02		
	Hexaflumuron + Control	5	0.02 ± 0.01		
	Inulin + Control	5	0.03 ± 0.02		
	Hexaflumuron + Inulin	5	0.02 ± 0.01		

[1]GLM ANOVA (SAS Institute 1999). Significance was indicated at $\alpha = 0.05$ as applied to the percent mortality means for each time period.

Table 3-2. Mean total consumption (mg) determined by diet weight at 2 and 5 days for the subterranean termite population fed hexaflumuron diets, inulin diets, control diets, or a combination of two diets. Consumption was not corrected by termite mortality.

Time Period (day)	Treatment	n	Total Mean Consumption (mg) (Mean ± SEM)	*F*-statistic	*P*-value
2	Control Only	5	3.2 ± 0.3[1]	0.53	0.714[1]
	Hexaflumuron Only	5	3.0 ± 0.7		
	Hexaflumuron + Control	5	3.9 ± 0.9		
	Inulin + Control	5	2.7 ± 0.6		
	Hexaflumuron + Inulin	5	3.1 ± 0.4		
5	Control Only	5	13.6 ± 2.1	1.97	0.148
	Hexaflumuron Only	5	11.4 ± 3.9		
	Hexaflumuron + Control	5	9.6 ± 3.1		
	Inulin + Control	5	11.3 ± 3.2		
	Hexaflumuron + Inulin	5	6.2 ± 1.3		

[1]GLM ANOVA (SAS Institute 1999). Significance was indicated at $\alpha = 0.05$ as applied to the total consumption means at each time period.

Table 3-3. Mean consumption (mg) of hexaflumuron diet determined by diet weight at 2 and 5 days for the subterranean termite population in no-choice and choice tests. Consumption was not corrected by termite mortality.

Time Period (day)	Test	Diet	n	Mean Hexaflumuron Consumption (mg) (Mean ± SEM)	*t*-statistic	*P*-value
2	No-Choice	Hexaflumuron	5	3.0 ± 0.7 a[1]	2.80	0.170[1]
	Choice	Hexaflumuron	5	1.6 ± 0.9 a		
	Choice	*Control*[2]	*5*	*2.2 ± 0.8*		
	No-Choice	Hexaflumuron	5	3.0 ± 0.7 a	6.18	0.068
	Choice	Hexaflumuron	5	1.0 ± 0.8 b		
	Choice	*Inulin*	*5*	*2.0 ± 0.6*		
5	No-Choice	Hexaflumuron	5	11.4 ± 3.9 a	5.44	0.080
	Choice	Hexaflumuron	5	3.2 ± 1.4 b		
	Choice	*Control*	*5*	*6.4 ± 3.8*		
	No-Choice	Hexaflumuron	5	11.4 ± 3.9 a	8.32	0.045
	Choice	Hexaflumuron	5	0.2 ± 0.1 b		
	Choice	*Inulin*	*5*	*6.0 ± 1.3*		

[1]Student's *t*-test (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.10$ as applied to the hexaflumuron consumption means for each choice test at each time period.
[2]Italics denotes consumption of competing diet. However, consumption of competing diet in choice test was not involved in analysis.

Table 3-4. Mean consumption (mg) of each diet in the choice tests determined by diet weight at 2 and 5 days for the subterranean termite population in choice tests: hexaflumuron and control diets; inulin and control diets; hexaflumuron and inulin diets. Consumption was not corrected by termite mortality.

Time Period (day)	Choice Test	Diet	n	Diet Consumption (mg) (Mean ± SEM)	*t*-statistic	*P*-value
2	Hexaflumuron + Control	Hexaflumuron	5	1.6 ± 0.9 a[1]	-0.877	0.43[1]
		Control	5	2.2 ± 0.8 b		
	Inulin + Control	Inulin	5	1.3 ± 0.3 a	0.646	0.553
		Control	5	1.4 ± 0.7 a		
	Hexaflumuron + Inulin	Hexaflumuron	5	1.0 ± 0.8 a	-1.223	0.288
		Inulin	5	2.0 ± 0.6 a		
5	Hexaflumuron + Control	Hexaflumuron	5	3.2 ± 1.4 a	-0.215	0.841
		Control	5	6.4 ± 3.8 a		
	Inulin + Control	Inulin	5	8.9 ± 3.7 a	1.099	0.333
		Control	5	2.3 ± 1.3 a		
	Hexaflumuron + Inulin	Hexaflumuron	5	0.2 ± 0.1 a	-20.647	<0.0001
		Inulin	5	6.0 ± 1.3 b		

[1]Student's *t*-test for $\mu = 0.5$ (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$ as applied to the diet consumption means for each choice test at each time period.

Table 3-5. Mean consumption (µg) of hexaflumuron at 2 and 5 days by a single subterranean termite in no-choice and choice tests. Mean consumption was determined by the dpms counted in single termite samples. Control consumption was not measured.

Time Period (day)	Test	Treatment	n	Total Hexaflumuron Consumption (µg) (Mean ± SEM)	Total Hexaflumuron Consumption (µg) (LS MEAN)	*P*-value
2	No-Choice	^{14}C-Hexaflumuron	5	38.9 ± 2.9[2]	3.31 a[1]	0.090[1]
	Choice (Control)[3]	^{14}C-Hexaflumuron	5	14.5 ± 1.7	2.16 b	
	No-Choice	^{14}C-Hexaflumuron	5	38.9 ± 2.9	3.31 a	0.001
	Choice (^{3}H-Inulin)[4]	^{14}C-Hexaflumuron	5	7.3 ± 1.2	1.36 b	
5	No-Choice	^{14}C-Hexaflumuron	5	56.9 ± 4.3	3.77 a	0.058
	Choice (Control)	^{14}C-Hexaflumuron	5	19.3 ± 1.7	2.47 b	
	No-Choice	^{14}C-Hexaflumuron	5	56.9 ± 4.3	3.77 a	<0.0001
	Choice (^{3}H-Inulin)	^{14}C-Hexaflumuron	5	2.3 ± 0.1	1.17 b	

[1]Least Squares Method (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.10$ as applied to the hexaflumuron consumption means for each choice test at each time period.
ANOVA with a 2 factor (time and treatment) randomized block design treatment source of variation was not significant for time period*treatment group ($F = 0.03$; df = 1; $P = 0.862$) (SAS Institute 1999). ANOVA with a 2 factor (time and treatment) randomized block design treatment source of variation was not significant for time period ($F = 0.76$; df = 1; $P = 0.402$) (SAS Institute 1999). ANOVA with a 2 factor (time and treatment) randomized block design treatment source of variation was significant for treatment group ($F = 7.76$; df = 1; $P = 0.017$) (SAS Institute 1999).
[2]Denotes the data means from which the Least Squares means were calculated.
[3]Denotes a choice test where control diet competed with hexaflumuron diet.
[4]Denotes a choice test where inulin diet competed with hexaflumuron diet.

Table 3-6. Mean consumption (µg) at 2 and 5 days of each diet in the dual label choice test by a single subterranean termite fed ^{14}C-hexaflumuron and ^{3}H-inulin diets. Mean consumption determined by dpms counted in single termite samples.

Time Period (day)	Diet	n	Diet Consumption (µg) (Mean ± SEM)	*t*-Statistic	*P*-Value
2	^{3}H-Inulin	5	12.6 ± 1.0 a[1]	-1.232	0.285[1]
	^{14}C-Hexaflumuron	5	7.3 ± 1.2 a		
5	^{3}H-Inulin	5	19.4 ± 1.2 a	-7.256	0.002
	^{14}C-Hexaflumuron	5	2.3 ± 0.1 b		

[1]Student's *t*-test, for µ= 0.5 (SAS Institute 1999). Means followed by different letters are significantly different at α = 0.05 as applied to the diet consumption means at each time period.

Discussion

Su and La Fage (1984a) found that wood consumption rates by termite colonies with a low percentage of mortality over time (39.74-78.48mg/g/day; milligrams of wood per gram of termite per day) were significantly greater than the consumption rate of termites with a high percentage of mortality over time (23.80-44.67mg/g/day). In this experiment, termites in all treatment groups appeared healthy and had little associated mortality at the end of the 5d test period. Therefore, the relative health of the termite populations did not affect the consumption of the diets. The low mortality in this experiment was in part due to test duration. Sheets et al. (2000) demonstrated that the LT_{50}s for termites consuming hexaflumuron baits was around 26.6d. They also established that the onset of toxicity for termites feeding on hexaflumuron did not occur until around 15d. Thus, the termites would not have been expected to show signs of hexaflumuron poisoning during the 5d time frame of this experiment. Furthermore, because mortality was not significantly different between termites in all treatment groups, and the treatment groups consisted of both radioactive and nonradioactive diets, it can be concluded that there was little, if any, termite mortality associated with radioisotope exposure during the 5d time frame.

Although termite vigor can be one of the major influences on consumption rates in the laboratory, influences on termite feeding behavior are far more complex in the field. Many factors contribute to the variation in termite feeding behavior. Previous feeding history of the colony affects food choice in subterranean termites (McMahan 1966; Heidecker and Leuthold 1984; Wood 1978). For example, a termite colony may become preconditioned to prefer a food source which the colony has previously consumed, rather than consuming a food source normally preferred by termites of the same species. Termites are also known to prefer wood that has been previously damaged by conspecific consumption and often prefer wood with high moisture content (Delaplane and La Fage 1989a, b). Finally, feeding behavior can vary with colony age and size (Wood 1978). For instance, foraging distance is limited in smaller colonies, because fewer workers are available to look for food. Therefore, smaller colonies may feed on available, but not preferred, food sources. Because so many factors influence termite

feeding and food preferences, controlled lab studies are one of the few methods to test hypotheses on termite feeding preferences or food source palatability.

In this laboratory experiment, termites were exposed to only 1 or 2 food sources at a time. It was found that 100 termites consumed approximately equal amounts of total diet in choice and no-choice tests. These results indicated that hexaflumuron at the 0.5% concentration (w/w; Sentricon® % A.I.), when presented in the no-choice test to termites, was found to be equally as palatable as the untreated food source and caused no feeding deterrence. Other studies have also determined that hexaflumuron was very palatable to subterranean termites at concentrations $<$ 2% (w/w) (Su and Scheffrahn 1993, 1996c; King and Karr 2000).

Although termites ate approximately the same total amount of diet in all the test arenas, termites can only eat a certain amount of diet. When their consumption was divided between two equally palatable food sources, termites ate less of each diet. Thus, hexaflumuron consumption was reduced in the presence of a competing food source. Termites, which are generalist feeders, consumed portions of both diets in the choice tests because hexaflumuron, inulin, and the untreated control diet were very palatable. Yet, the palatability of the diets resulted in smaller portions of each diet being consumed.

In the binomial choice analyses, termites significantly preferred to consume inulin. This preference had not been anticipated but indicated that hexaflumuron consumption could be considerably influenced (reduced) by the particular type of food resource competing with it for termite consumption.

The ability to quantify consumption of cellulose and to follow food flow in a termite colony is imperative to the study of termite nutritional ecology. Radiolabels have been successfully used to study trophallaxis in social insects (Oertel 1953; Wilson and Eisner 1957; Alibert 1959; Crossley 1963; Gösswald 1963; Afzal 1983, 1984; Traniello et al. 1985; Rosengaus et al. 1986; Suarez and Thorne 2000b); foraging patterns (Kloft and Hölldobler 1964; Kloft et al. 1965; Easey 1981); and feeding behaviors of termites (Gösswald 1962; McMahan 1962, 1963, 1966). Radiolabels have also been used successfully to study the metabolism of bait system toxicants within termites. Sheets et al. (2000) used radiolabels to determine the rate of uptake, clearance, insect-to-insect transfer, and metabolism of ^{14}C-hexaflumuron in *R. flavipes*. Therefore, the use of

radiolabels spiked on cellulose diets can be used to quantify termite consumption of a bait toxicant and how much of a diet a single termite has consumed.

In this experiment, only those diets that were radioactive, not the control diets, could be used to determine consumption in individual termites. Therefore, comparisons using the control diets could not be quantified, such as total termite consumption of all diets. It was determined that competing food sources reduced the amount of ^{14}C-hexaflumuron in individual termites. The average termite sampled in a ^{14}C-hexaflumuron choice test contained less of the ^{14}C-radiolabel than a termite sampled from a no-choice test. Termite consumption in the dual labeled choice test, ^{14}C-hexaflumuron and ^{3}H-inulin, further indicated that when given a choice, termites consumed considerably more inulin. This result supported the finding in the population consumption analysis, which determined that termites preferred inulin over hexaflumuron.

Because the population consumption analysis and the radioactive individual termite analysis resulted in similar conclusions, it seems that once food preferences were established in the arenas by the foraging population, individual termite consumption patterns did not vary much from the population behavior. For example, when the population was feeding from both diets in the choice test arenas, individual termites also fed from both diets in choice arenas and did not limit themselves to only one diet. Furthermore, the inulin diet was shown to be preferred both by individual termites and by the population as a whole.

In a natural environment, termites feed at multiple locations along their foraging path and do not limit feeding to one food source at a time (Grace and Su 2001). Therefore, foragers may locate several suitable food sources at any given time. As determined in the choice test experiments, consumption of more than one food source at any time results in portions of both diets being consumed but less consumption of any one diet. Because a single termite has a limited consumption capacity and cannot consume more than this amount, the total amount a termite population could potentially consume in a given period is also limited. As the termite population locates more acceptable food sources by foraging to new locations, consumption of each established food source would be reduced. If a termite population had established a bait station as a suitable food source, the amount of total consumption of the toxic bait would be

dependant on how many workers were recruited to the site, but more importantly how many other food sources the colony was already consuming.

The type of food sources available to termites also affects consumption. It is known that termites can consume less acceptable food sources until more desirable food sources are located (Smythe and Carter 1970). In this experiment, termites sustained themselves in the no-choice test on the hexaflumuron diet. However, when hexaflumuron was presented in the presence of inulin, termites showed a strong preference for the inulin and consumption of the hexaflumuron was reduced.

Inulin is a widespread β-linked carbohydrate in nature and is present in more than 30,000 plant species (Inulin Plaza 2003). This carbohydrate belongs to the group of compounds called fructans, meaning it contains one or more fructosyl-fructose linkages. Inulin is a dietary fiber and is often used as a food additive in human foods. Because of its water solubility, inulin can bind and change surface textures, thereby improving the flavor of many foods (Inulin Plaza 2003).

The termite preference of inulin warrants further investigation, as it has not been tested against other food sources termites have been shown to prefer. Perhaps, bait efficacy can be improved if a compound, like inulin, is added to the cellulose bait matrix to make the food source more attractive to termites than other food sources they may encounter. Furthermore, it is necessary to evaluate the effect of competing food sources on hexaflumuron bait efficacy in a field experiment. There are many more food sources competing with bait stations in a natural environment and many other factors that may influence termite feeding behaviors.

Chapter Four:

The Effect of Competing Food Sources on Hexaflumuron Consumption and Subsequent Mortality of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae).

Introduction

Several methodologies exist to control subterranean termites, but recently an emphasis has been placed on the use of bait systems in situations where termites pose structural problems. One advantage to using bait systems is that they can be placed in areas with limited accessibility (Kistner and Sbragia 2001). Another advantage is that these systems eliminate the need for applying large amounts of liquid termiticide to the soil. Instead, baits work by providing foraging termites with a food source. Termites forage into a bait station and consume a cellulose matrix infused with a toxicant. The slow acting nature of the bait toxicant allows enough time for termites to return to the colony and transfer the toxicant to unexposed termites by trophallaxis (Beard 1974; Sheets et al. 2000). The toxicant then begins to kill the colony, and if foragers continue to feed on the toxic bait, the colony soon decreases to below a critical mass, at which point it collapses (Su and Scheffrahn 1996a).

The Sentricon® Termite Elimination System is a bait system developed by Dow AgroSciences in 1990. Sentricon® was designed to be a colony elimination system (Esenther and Gray 1968; Su et al. 1982; Su 1994; DeMark et al. 1995, Su et al. 1995b; Grace et al. 1996; Su and Scheffrahn 1996a; Su et al. 1997; Atkinson et al. 1998; Haagsma and Bean 1998; Tsunoda et al. 1998; Peters and Fitzgerald 1999; Prabhakaran 2001). The active ingredient in the Sentricon® system is hexaflumuron [N-((3,5-dichloro-4-(1,1,2,2-tetrafluroethoxy) phenyl) -amino) carbonyl)-2,6-diflurobenzamide]. Hexaflumuron is an effective termiticide, because it works as a slow acting chitin synthesis inhibitor (Su et al. 1987; Nakagawa et al. 1992). Laboratory studies have determined the LT_{50} of subterranean termites exposed to the Sentricon® bait (0.5% hexaflumuron) is approximately 26.6 days (Sheets et al. 2000). Because the mean half life of hexaflumuron within a termite is ~9 days, foraging termites survive long enough after ingestion to transfer the toxicant to other

members of the colony (Sheets et al. 2000). Finally, hexaflumuron is resistant to the termite detoxification processes (Sheets et al. 2000). Thus, in the laboratory, hexaflumuron has proven to be an effective termiticide.

Although hexaflumuron has the necessary characteristics for termite colony elimination, Sentricon®, like all commercial bait systems, faces a number of difficulties in the field. Bait stations take up a relatively small space in the environment, so foraging termites may not locate the stations for many months. Ostaff and Gray (1975) found that only 16.8% of the bait stations were attacked by termites the first year after installation. Also, termites tunnel more extensively in areas where a food has been previously discovered but tunnel less in areas where food has not been found (Campora and Grace 2001). Therefore, termites may not find bait stations in areas that were unsuccessfully explored before the stations were installed. Furthermore, pest management technicians cannot predict where colonies are located underground and must speculate where bait stations should be placed to the best advantage (Potter et al. 2001). Once foraging termites locate the stations they must consume the bait and take it back to the colony. If the colony is large (e.g. 2,000,000 individuals), it may take a significant amount of time for enough bait to be disseminated throughout the colony to kill enough workers to cause colony collapse. Moreover, a chitin synthesis inhibitor is only effective on actively molting termites. *Reticulitermes* colonies contain many generations of individuals, each molting at different rates (Noirot and Pasteels 1987). It is possible that hexaflumuron is not as effective in eliminating those members of the colony that are not regularly molting.

Perhaps the most significant hindrance to termite bait system performance in the field is the presence of competing food resources. When bait stations are installed in the ground, termites are usually already feeding on a structure or some other food source in the area. Termites can be faithful to a food source they have already located, even if other food sources are later found (Wood 1978; Heidecker and Leuthold 1984; Oi et al. 1996). If the natural food source is more attractive to the termites than the bait, foragers may not be recruited to the bait stations. Subterranean termites are also known to prefer larger food sources over smaller ones (Waller 1988; Lenz 1994; Cornelius and Osbrink 2001) and will not leave one to consume another.

Because it is impossible to eliminate all alternative food resources in the environment, these food sources will always compete with bait stations for termite foragers. If bait consumption was reduced, it would be expected that termite mortality would also decline. Yet the effect of competing food resources on the efficacy of termite baits has never been quantified. The purpose of this study was to determine whether the presence competing food sources would reduce mortality in subterranean termites exposed to hexaflumuron bait.

Methods and Materials

Subterranean Termite Collection. Five wild populations of *R. flavipes* were collected from wood billets in Harrison County, Mississippi (N30° 24.44' and W89° 4.21'). Termites were maintained in plastic rearing containers (984 ml; Rubbermaid, Wooster, OH) containing moistened corrugated cardboard. Rearing chambers were stored in complete darkness at room temperature (~21°C and 97% RH) until termites were needed for testing. Subterranean termites were tested 4 days after being collected from the field.

Termite Diet Preparation. Recycled brown paper toweling (Acclaim®; Fort James Corporation, Deerfield, IL) was cut into squares (35 x 35 mm) and used as a diet substrate. Because hexaflumuron cannot be stored at temperatures above 50°C and all diet paper squares were dried in an oven (60ºC), the initial weight of each diet was recorded before diets were treated. Diet substrates were dried overnight in a single wall, gravity convection, laboratory oven (60ºC; Blue M SW-17TA; Blue M Electric Company; Blue Island, IL). After 24h each diet substrate was removed from the oven, and they were immediately numbered in the corner with a pencil and weighed on a balance (Mettler AE163; Lab Tech, Inc.) to the nearest 0.1mg.

After weighing, diets were treated with either a visual dye, as a control, or visual dye combined with hexaflumuron, as a treatment. The purpose of the dye was to ensure that feeding occurred. Nile Blue-A was selected as the visual marker because of its long-term visibility and low associated mortality for *R. flavipes* (Haagsma and Rust 1993; Oi and Su 1994; Su et al. 1991; King 2000; Suarez and Thorne 2000b). Termites feeding on experimental diets were dyed a bright blue color.

The control diets were formulated by dissolving 11.3mg of Nile Blue-A (Allied Chemical Company, Morristown, NJ) in 20ml of acetone. A 150µl aliquot of dye solution was applied to each control diet square, so that the final Nile Blue-A amount applied was 0.1% (weight of Nile Blue A/ weight of paper square).

Treated diets were formulated in 20ml acetone by dissolving 11.3mg of Nile Blue-A (Allied Chemical Company, Morristown, NJ), so that the final Nile Blue-A concentration was 0.1% (w/w), and 58mg hexaflumuron (98% pure technical-grade; Dow AgroSciences, Indianapolis, IN), so that the final hexaflumuron concentration was 0.5% (w/w). A 150µl aliquot of the hexaflumuron/dye solution was applied to each treated diet square.

Sand Preparation: Play sand (Quikcrete®; Quikcrete Companies, Atlanta, GA) was washed with tap water 4 times to remove impurities. Prior to use, washed sand was dried for 48h in a single wall gravity convection laboratory oven (270ºC; Thelco®; Precision PS Scientific; Chicago, IL). Dried sand was then placed in a plastic storage bag (3.79L; Target Corporation, Minneapolis, MN) and moistened with distilled water (15% by weight of the sand). Moistened sand was then kneaded by hand in the storage bag and stored for at least 24h to ensure even distribution of moisture. Sand was stored in the plastic bag until needed for testing.

Experimental Arenas: Laboratory arenas were used to evaluate the effect of competing food sources on hexaflumuron efficacy. Subterranean termites were exposed to a hexaflumuron diet in no-choice test to determine total consumption of a single diet. Termites were also exposed to both diets simultaneously in choice tests to determine how consumption was divided between the two diets.

No-Choice Bioassay. No-choice arenas were assembled by connecting two Petri dishes with Tygon tubing (inner diameter 3.2mm, outer diameter. 6.4mm; Fig. 4-1). Petri dishes were washed 4 times with tap water, and Tygon tubing was soaked in tap water for 3h prior to use. The washing of arena materials was done to eliminate static electricity and any impurities. One Petri dish (95 mm x 15 mm; Fisher Scientific) served as a housing chamber and was filled with moistened sand from the storage bag (~45g). A second Petri dish (60 mm x 15 mm; Fisher Scientific) served as a diet chamber and contained one of the diets. Termites placed inside the treatment no-choice arenas could forage toward the diet chamber containing only the hexaflumuron diet. Likewise,

Figure 4-1. The No-choice subterranean termite arena. Termites were placed in the larger housing chamber where they could forage through a Tygon tube into a diet chamber. The diet chamber contained dyed and/or hexaflumuron treated paper towels.

termites placed inside the untreated no-choice arenas could forage only on the control diet.

Choice Bioassay. Choice arenas were designed similarly to the no-choice arenas with exception that the housing chambers were attached to two diet chambers, each with a separate diet (Fig. 4-2). One diet chamber contained paper towels treated with hexaflumuron and dye. The second chamber contained paper towels treated with dye alone. Termites placed inside choice arenas could simultaneously forage on both the hexaflumuron and control diets.

Humidity Chamber. All bioassay arenas were placed inside humidity chambers. The chambers were constructed by pouring play sand into the bottom of plastic storage containers (11.3L; Rubbermaid, Wooster, OH), so that sand was 4.5 cm deep. Tap water was poured over the sand to the point of saturation. Any standing water was absorbed with paper towels. A sheet of aluminum foil (Super Foil^{TM}; Atlantic Paper & Foil Corporation, Hauppauge, NY) was laid down to cover the saturated sand. Arenas containing termites were placed inside plastic storage bags (3.8L; Target Corporation, Minneapolis, MN) and set on top of the aluminum foil in the chamber. The top of the

Figure 4-2. The Choice subterranean termite arena. Termites were placed in the larger housing chamber where they could forage through either Tygon tube into a diet chamber. One diet chamber contained dyed paper towels as the control, and the second diet chamber contained dyed hexaflumuron treated paper towels.

plastic bag was rolled down to allow for air circulation. Plastic bags were used to separate the arenas from each other and to catch any escaped termites. Escaped termites were returned to their respective experimental arena(s). Humidity chambers were closed with snap top lids and placed in total darkness in a cabinet (~21°C and 97% RH) for the duration of the experiment.

Bioassay Design. Prior to testing, 100 worker termites (at least 3rd instar) were aspirated out of storage containers and transferred into the housing chamber of one experimental arena. Termites were allowed to acclimate for 72h and forage toward an untreated paper towel in the diet chamber(s). After the acclimation period, paper towels were removed, and all termites clinging to the paper towels were gently tapped back into the diet chamber. Experimental diets were then placed into the diet chambers. If the diets were depleted before the conclusion of the test, they were replaced. Three pieces of masking tape were put on the outside of the arena to secure the housing chamber lid.

Bioassays were arranged in a 5 (day) x 3 (treatment) factorial experiment. The experiment was arranged as a randomized complete block design (RCBD), blocked by termite field population to account for within treatment variability due to population effects. Fifteen bioassay arenas were set up for each treatment group: hexaflumuron only, control only, and the choice test (hexaflumuron and control diets). Each treatment group was further subdivided into five test periods: 5d, 15d, 20d, 25d, and 35d. Each treatment group was replicated 5 times for each test day for a total of 75 arenas, and each replicate contained termites from a different field population. Replicates were eliminated after counting.

Recording Mortality. Termite mortality was recorded for each test period day. Termites that did not exhibit proper molting were considered moribund and were subtracted from the survivor count (Su and Scheffrahn 1993). On recording days, the number of live termites was counted and subtracted from the initial number of termites to determine the percentage of termite mortality.

Analysis of Diet Consumption. The effect of bait competition on mortality was determined by quantifying the amount of hexaflumuron and control diet consumed by the termites. After each test day, the partially consumed diets were removed, dried for 24h as previously described and weighed to determine total consumption. Post-test weights of partially consumed diets were subtracted from the initial diet weight to determine total consumption by treatment. The initial diet weights were the weights taken before paper towels were treated with dye and/or hexaflumuron, as the weight of the treatment was determined to be miniscule.

Statistical Analysis. Differences in total consumption (mg) of paper towel diets in each treatment group were evaluated using a GLM two-way analysis of variance (ANOVA) with test period (5 levels) and treatment group (3 levels) as main effects. Consumption was modeled as a function of treatment, time, and the interaction between treatment and time. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute1999).

For the choice tests, mean consumption of the two diets, hexaflumuron and control, was compared using the Student's *t*-test to determine whether termites

preferentially consumed one diet over another. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute1999).

Mean termite mortality was analyzed using planned comparisons to determine overall differences in termite mortality between the treatment groups for the entire test period (Sokal and Rohlf 1981). Mortality was modeled as a function of population (blocked), treatment, time, and the interaction between treatment and time. This analysis was blocked by termite field population to account for within treatment variability due to differential feeding between colonies. Planned comparisons evaluated differences in termite mortality between two treatment means at a time (hexaflumuron only and choice test; hexaflumuron only and control only; control only and choice test). Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute1999).

The LT_{50}s for termites in the three treatment groups were determined by sigmoidal dose response (variable slope) with a line fit to the mortality data using non-linear regression (GraphPad Prism 2002). LT_{50}s were compared between the treatment groups using ANOVA to determine differences in time to mortality. Values of $\alpha \leq 0.1$ were used to indicate significance.

Results

Diet Consumption. When comparing mean total consumption (mg), the interaction treatment*day was not significant between termites in either the no-choice test or the choice test ($F = 0.19$, df = 8, $P = 0.991$). The effect of time on total termite consumption was significant ($F = 9.65$, df = 4, $P < 0.0001$). The longer termites were held in arenas, the more diet they consumed. Therefore, the longer termites were held in the arenas, the more diet they consumed. The effect of treatment on total termite consumption was not significant ($F = 0.59$, df = 2, $P = 0.560$; Table 4-1). Overall, termites ate the same amount of hexaflumuron diet, control diet, or the combined amount of hexaflumuron and control diets in the choice test at each time period (~10mg at 5d, ~27mg at 15d, ~40mg at 20d, ~40mg at 25d, and ~ 50mg at 35d).

A comparison of mean diet consumption within the choice test determined that termites accepted both diets equally (Table 4-2). Student *t*-tests indicated that

consumption of the hexaflumuron and control diet was not significantly different at 5d (5.5mg to 3.3mg, t = -0.82, P = 0.435), 15d (21.9mg to 7.1mg, t = -1.55, P = 0.160), 20d (18.4mg to 29.5mg, t = 0.51, P = 0.626), 25d (22.5mg to 9.6mg, t = -0.84, P = 0.427), and 35d (10.3mg to 35.6mg, t = 1.17, P = 0.277).

Mortality. Planned comparisons determined there were significant differences in termite mortality between the treatment groups. Mean termite mortality was significantly greater for termites fed only the hexaflumuron diets (14.6% by 5d, 37.4% by 15d, 63.8% by 20d, 84.8% by 25d, and 92.8% by 35d; Table 4-3) compared with those fed only the control diets (7.0% by 5d, 32.2% by 15d, 39.8% by 20d, 50.4% by 25d, 77.4% by 35d; F = 4.55, df = 1, P = 0.039). The percentage of termite mortality in the choice test (8.6% by 5d, 21.2% by 15d, 55.6% by 20d, 73.2% by 25d, 82.2% by 35d) was not significantly less than mortality for termites fed only the hexaflumuron diets (F = 1.85, df = 1, P = 0.181). Finally, termite mortality was not significantly different for termites fed only the control diets compared with termites given a choice of diets (F = 0.648, df = 1, P = 0.405).

A nonlinear regression model using a 4-parameter logistic equation provided a good fit to the data for the three treatment groups: $Y = EC_{min} + [(EC_{max}-EC_{min}/ (1 + 10^{(LogEC50-x)-H})]$. H is a parameter that controls the slope of the curve. EC_{min} refers to the y-value at the bottom plateau. EC_{max} is the y-value at the top plateau. When entering a specific mortality, the top must be held constant at 100%. *Log EC50* is the x-value when the response is halfway between the EC_{min} and EC_{max}. The nonlinear regression fit a line through the hexaflumuron only bioassay data (df = 2; slope ± SEM, 4.8 ± 0.42; 95% CI, 3.01-6.64; R^2, 0.99), through the control only bioassay data (df = 2; slope ± SEM, 2.6 ± 0.60; 95% CI, -0.02-5.16; R^2, 0.97), and through the choice bioassay data (df = 3; slope ± SEM, 4.0 ± 1.16; 95% CI, -0.99-8.98; R^2, 0.96; Fig. 4-3).

From Fig. 4-3, it was determined that the relative order of the LT_{50}s for termites in the control only bioassay, hexaflumuron only bioassay, and choice bioassay was 24.46d (df = 2; SEM, 0.0009; 95% CI, 17.14-34.89), 18.7d (df = 2; SEM, 0.0009; 95% CI, 17.03-20.48), and 20.60d (df = 2; SEM, 0.0009; 95% CI, 14.48-29.31), respectively. The LT_{50} for termites fed in the choice test was not significantly different than the LT_{50} in either no-choice test, yet the LT_{50} for termites eating only hexaflumuron diets was

significantly earlier than the LT_{50} for termites fed only control diets ($\alpha = 0.1$; MSE = 0.003, $F = 3.98$; $P = 0.079$).

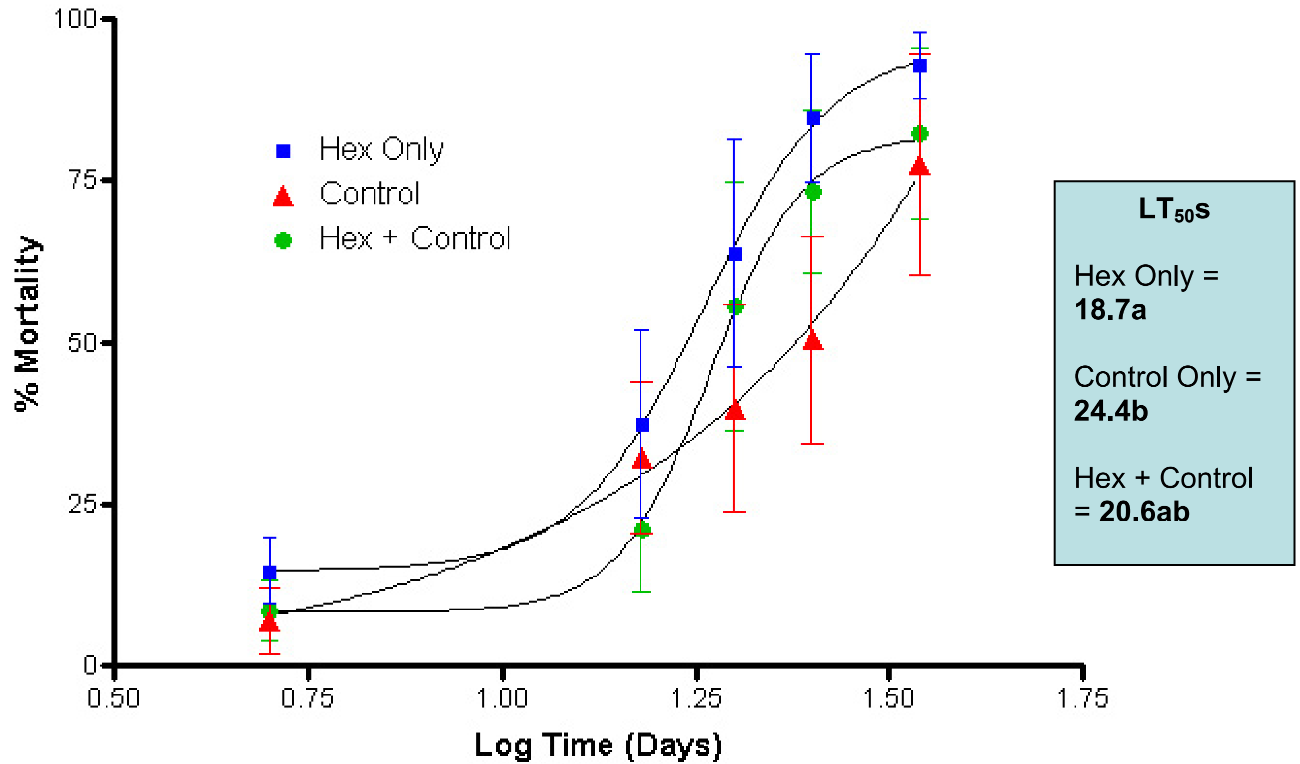

Figure 4-3. Sigmoidal Dose Response (Variable Slope) Curve. Mortality over time (transformed by logarithm) for 100 subterranean termites fed hexaflumuron diets (no-choice), control diets (no-choice), or a combination of both diets (choice tests).

Table 4-1. The mean total consumption (mg) by treatment for 100 subterranean termites fed hexaflumuron diets. This determined the equivalent consumption of diets. Consumption was not corrected by termite mortality.

Time Period (day)	Treatment Group	n	Total Consumption (mg) (Mean ± SEM)	*F*- Statistic	*P*-Value
5	Hexaflumuron Only	5	9.5 ± 2.0[1]	0.59	0.560[1]
	Control Only	5	12.5 ± 1.7		
	Hexaflumuron + Control	5	8.8 ± 1.2		
15	Hexaflumuron Only	5	21.7 ± 5.9		
	Control Only	5	33.8 ± 7.9		
	Hexaflumuron + Control	5	29.0 ± 5.5		
20	Hexaflumuron Only	5	34.7 ± 9.6		
	Control Only	5	44.6 ± 10.7		
	Hexaflumuron + Control	5	48.0 ± 14.6		
25	Hexaflumuron Only	5	37.0 ± 16.4		
	Control Only	5	41.1 ± 8.6		
	Hexaflumuron + Control	5	32.1 ± 11.4		
35	Hexaflumuron Only	5	47.9 ± 15.7		
	Control Only	5	51.2 ± 22.0		
	Hexaflumuron + Control	5	46.0 ± 18.3		

[1]GLM 2-way ANOVA (SAS Institute 1999). Values of $\alpha \leq 0.05$ were used to indicate significance

Table 4-2. Mean consumption (mg) of each diet by 100 subterranean termites fed hexaflumuron and control diets at different time periods. This determined equivalent consumption of each diet in the choice test. Consumption was not corrected by termite mortality.

Time Period (day)	Diet	n	Diet Consumption (mg) (Mean ± SEM)	*t*-statistic	*P*-value
5	Hexaflumuron	5	5.5 ± 2.3 a[1]	-0.82	0.435[1]
	Control	5	3.3 ± 1.3 a		
15	Hexaflumuron	5	21.9 ± 7.7 a	-1.55	0.160
	Control	5	7.1 ± 5.6 a		
20	Hexaflumuron	5	18.4 ± 13.2 a	0.51	0.626
	Control	5	29.5 ± 17.3 a		
25	Hexaflumuron	5	22.5 ± 12.1 a	-0.84	0.427
	Control	5	9.6 ± 9.5 a		
35	Hexaflumuron	5	10.3 ± 9.1 a	1.17	0.277
	Control	5	35.6 ± 19.7 a		

[1]Student's *t*-test (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$ as applied to the diet consumption means at each of the five time periods.

Table 4-3. Mean mortality over a period of 35 days for 100 subterranean termites fed only hexaflumuron diets, only control diets, and a combination of hexaflumuron and control diets.

Time Period (day)	Treatment	n	Percent Termite Mortality (Mean ± SEM)	Percent Mortality Over All Test Days (Mean ± SEM)
5	Hexaflumuron Only	5	14.6 ± 5.1[1]	58.6 ± 14.6 a[2,3]
15		5	37.4 ± 14.7	
20		5	63.8 ± 17.7	
25		5	84.8 ± 9.9	
35		5	92.8 ± 5.2	
5	Control Only	5	7.0 ± 5.1	41.3 ± 11.5 b
15		5	32.2 ± 11.6	
20		5	39.8 ± 16.1	
25		5	50.4 ± 16.0	
35		5	77.4 ± 17.1	
5	Hexaflumuron + Control	5	8.6 ± 4.7	48.1 ± 14.3 a, b
15		5	21.2 ± 9.6	
20		5	55.6 ± 19.1	
25		5	73.2 ± 12.6	
35		5	82.2 ± 13.2	

[1]Percent mortality means obtained for each time period from which the overall means were calculated.
[2]Planned Comparison, GLM ANOVA (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$.
[3]Comparison of "Hexaflumuron Only" with "Control Only" ($F = 4.55$; $P = 0.39$). Comparison of "Hexaflumuron Only" with "Hexaflumuron + Control" ($F = 1.85$; $P = 0.181$). Comparison of "Control Only" with "Hexaflumuron + Control" ($F = 0.648$; $P = 0.426$).

Discussion

In a natural environment, termite feeding behaviors are more complex than choosing between two food sources in a choice test arena. In the field, there are many food resources available to termite colonies at any one time. Grace and Su (2001) found that termites feed at multiple locations along their foraging path and do not limit feeding to one food source at a time. Moreover, in the field different termite colonies naturally vary in terms of their consumption rates and behaviors because of different environmental and colony influences (Su and La Fage 1984a; Lenz 1985; Waller 1991). For example, consumption rates can differ depending on the temperature in the termite's environment (Smythe and Williams 1972; Haverty and Nutting 1974), whether the located food source has been damaged previously by conspecifics (Delaplane and La Fage 1989a), or by the previous feeding history and food preferences of the colony itself (Wood 1978). These influences are not factors in laboratory experiments.

In this experiment, termites fed hexaflumuron diets, control diets, or a combination of both diets in the choice test consumed equal amounts of diet in the arenas. Thus, hexaflumuron bait at the 0.5% concentration (w/w; Sentricon® % A.I.) was as equally palatable to termites as a regular, untreated food source. Other laboratory studies have also determined that hexaflumuron has no feeding deterrence at concentrations <2% (w/w) (Su and Scheffrahn 1993, 1996c; King and Karr 2000). This lack of a feeding deterrence is further supported by field tests, which have demonstrated bait palatability and subsequent colony elimination using a hexaflumuron bait matrix at 0.5% (Su 1994; Su et al. 1997; Atkinson et al. 1998; Haagsma and Bean 1998; Rust et al. 1998; Ryder et al. 1998; Peters and Fitzgerald 1999; Prabhakaran 2001). The fact that termites in this experiment consumed approximately the same amount of diet regardless of treatment indicates that differences in mortality were not the result of differential bait consumption.

Termite mortality was due to an effect of the treatment. Termites fed hexaflumuron diets in the no-choice test died significantly faster than termites fed only control diets. However, termites in the choice test were not found to be significantly different than that of either no-choice test. Therefore, these results are inconclusive and it cannot be stated whether competing food sources affect or do not affect consumption of

hexaflumuron bait. These inconclusive results are most likely due to problems with the bioassay itself, as control groups sometimes had high associated mortality. It is thought mortality could have been associated with the small size of the housing arena. A larger bioassay arena would have allowed for more moistened sand in the termite environment, given the termites more room to forage, and diluted the buildup of waste within the arena. Further testing would be necessary to conclude the effect of competing food sources on bait efficacy.

Although competing food sources have the potential to reduce hexaflumuron consumption, hexaflumuron is still an effective bait because of its slow acting nature. Because hexaflumuron has a mean half life of 9d before it is cleared from the body, termites have time to spread the toxicant to unexposed nestmates (Su et al. 1987). Hexaflumuron is not easily metabolized by termites (Sheets et al. 2000). Termites that are actively feeding on hexaflumuron have a continual increase of the toxicant in their bodies, because termites consume hexaflumuron more quickly than it is metabolized (Sheets et al. 2000). Therefore, unaltered hexaflumuron can still be transferred to other termites by trophallaxis (Sheets et al. 2000), by feces, as nestmates may consume toxic feces (Sheets et al. 2000), or by cannibalism of a toxic individual, as termites can consume dead or injured termites especially when in low nitrogen level environments (Cook and Scott 1933; Moore 1969). The rate of termite trophallaxis occurs over a period of hours as opposed to minutes (Beard 1974; Rosengaus et al. 1986; Suarez and Thorne 2000b), so the trophallaxic exchange of hexaflumuron occurs rapidly for the first 8 hours after termites return to the colony from a bait station (Sheets et al. 2000). Furthermore, should a colony member ingest toxic fecal material, this termite consumes a dose of active hexaflumuron (Sheets et al. 2000). Hexaflumuron can be dispersed throughout the termite population and cause death to termites that did not feed on the hexaflumuron directly. If competing food sources were found to reduce hexaflumuron consumption, the slow acting nature of hexaflumuron could compensate for any decrease in bait consumption.

In addition to further laboratory testing, a field evaluation of the effect of competing food sources on hexaflumuron bait efficacy also has cause for investigation.

There are many more food source options in a natural environment that could compete with the bait stations for termite consumption.

Chapter Five:

The Effect of Hexaflumuron on 3 Genera of Protozoa in the Eastern Subterranean Termite (*Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae)) Hindgut.

Introduction

Wood is made up of three major structural components: cellulose (28-50%), hemicelluloses (20%-30%), and lignin (18-30%) (Breznak and Brune 1994). Because cellulose makes up the majority of wood, it is believed that lignin and hemicelluloses form a covalently linked matrix around the cellulose microfibrials (Kirk and Farrell 1987; Jeffries 1990). These covalent linkages prevent the digestion of wood by most animals, because they lack a complete cellulytic system (Jeffries 1990; Martin 1991). However, in nature the breakdown of wood can be carried out by microorganisms, such as bacteria, fungi, and protozoa. For example, protozoa can take up wood particles by endocytosis and store them in food vacuoles, where the particles are fermented into acetate, CO_2, and H_2 (Breznak and Brune 1994). The actual breakdown of wood inside the food vacuole is aided by enzymes, such as carboxymethylcellulases, glucanases, and ß-glucosidases (Odelson and Breznak 1985a, b; Yamin 1978, 1980, 1981; Yamin and Trager 1979; Martin 1991). Specifically, these enzymes aid in removing lignin and disrupting/cleaving the cellulose microfibrials (Martin 1991).

Like most animals, termites are incapable of digesting wood on their own. For this reason termite species, including all subterranean termites, that ingest cellulose as a food source, house symbiotic protozoa in their gut as an indirect means of digesting nutrients (Imms 1920; Cleveland 1923, 1924, 1925a; Honigberg 1970). Although termites themselves are able to break down starch into glucose units (Breznak and Brune 1994), the majority of termite nutrition and energy sources (acetate, propionate, and other organic acids) come from microbial metabolism (Odelson and Breznak 1983). Furthermore, bacteria and protozoa in the hindgut are needed for the conversion of acetate into lipids (Mauldin 1982). Although there is evidence that termites can produce some cellulase components in the foregut and midgut, only the protozoa packed hindgut

is capable of breaking down purified cellulose (Hungate 1938). Because termites must depend heavily on symbiotic protozoa to digest wood for them, there is a correlation between death of one or more components of the gut fauna and death of the termite (Eutick et al. 1978; Cleveland 1923, 1924, 1925b).

There are many hypotheses as to why termites in spite of evolutionary advantages are still dependent on protozoa for digestion (reviewed in Breznak and Brune 1994). One theory is that the amount of cellulose hydrolysis occurring in the termite foregut and midgut is not substantial enough to carry out most metabolic processes. Secondly, the termite gut, being a long tube with minimal invaginations, has limited surface area. Perhaps the protozoa's bodies increase the surface area inside the gut, which may increase retention time of food particles. For example, it is known that *Reticulitermes flavipes* can hold food in the gut as long as 26h (Odelson and Breznak unpublished data cited in Breznak and Brune 1994).

Because protozoa provide nutrition for termites, a large quantity and variety of protozoa are found in the termite gut. The termite gut has been estimated to house over 30,000 protozoa (Mauldin et al. 1981), so that the protozoa residing in the hindgut make up to one-third of the total body weight of the termite (Katzin and Kirby 1939). Numbers of protozoa in the hindgut remain relatively constant in termites of the same caste (Lund 1930; Dropkin 1944; Mannesmann 1970). However, newly molted individuals or termites developing wingpads in preparation for the final molt have reduced numbers of protozoa present in their gut (Katzin and Kirby 1939). Up to fourteen species in eight different genera of protozoa have been described as components of the hindgut in one species of subterranean termite, *Reticulitermes flavipes* (Yamin 1979). The largest and most abundant protozoa found in the gut in *R. flavipes* are in the genera: *Trichonympha, Pyrsonympha,* and *Dinenympha.* The role of *Pyrsonympha* and *Dinenympha* in the nutrition regime of subterranean termites is relatively unknown. However, it is known that members of the genus *Trichonympha* are crucial for termite survival, even if other members of the gut fauna are present (Cleveland 1925a; Maudlin et al. 1981). The genus *Trichonympha* occurs widely in the hindgut of both subterranean termites and cockroaches (Yamin 1979), whereas protozoa in the genera *Dinenympha* and *Pyrsonympha* are found only in the subterranean termite genus *Reticulitermes*.

Different termites may have variation in protozoa species composition because not all protozoa in the hindgut contribute equally to the survival of the termite host (Cleveland 1925b, 1926). Members of the genera, *Trichonympha*, *Prysonympha*, and *Dinenympha* are always present in healthy termite workers (Buhse et al. 1975; Grosovsky and Margulis 1982). *Trichonympha agilis* is thought to be key in cellulose digestion and is usually the first protozoa lost when a termite is starved (Grosovsky and Margulis 1982). It is believed that *Dinenympha* and *Pyrsonympha* may have a similar role in digestion, for termite populations that lack one protozoa often lack the other (Grosovsky and Margulis 1982). They are usually found in the hindgut together and may have a minor role in the breakdown of cellulose (Grosovsky and Margulis 1982).

Chemical compounds introduced into the termite gut can reduce protozoa populations. For example, feeding on certain woods can cause termites to lose their gut fauna due to the chemical components of the wood (Mannesmann 1972; Mauldin et al. 1981; Cook and Gold 2000). Wood preservatives and wood based toxins have also been determined to negatively affect protozoa in the termite gut (Speck et al. 1971). Furthermore, antibiotics, such as ampicillin and tetracycline, have been shown to decrease numbers of protozoa present in the gut (Waller 1996). Mauldin and Rich (1980) discovered that another antibiotic chlortetracycline eliminated protozoa and subsequently killed termites. Studies have also demonstrated that exposure to certain pesticides, such as the stomach poison tripropylisocyanurate, causes protozoa in the hindgut to die before the termite itself is affected and eventually dies (Kanai et al. 1988).

Because the hindgut is lined with chitin (Chapman 1998) and some protozoa adhere to the surface of this cuticular layer (Breznak and Pankratz 1977), chitin can be a selective target for many pest control agents. For example, chitinases, enzymes that cleave chitin by hydrolysis, can be used as biopesticides (Kramer and Muthukrishnan 1997). Chitinases can be introduced into an insect and cause perforations to occur in the peritrophic membrane (Kramer and Muthukrishnan 1997), which could increase oxygen permeability. An increase in oxygen could disrupt the anaerobic environment needed for cellulase activity. Therefore, it has been suggested that pesticides that contain chitin synthesis inhibitors (CSIs) might also affect the gut protozoa. Similarly, CSIs are an effective means for insect control because they interfere with the molting process.

However, no studies have been conducted to determine whether CSIs can also act as a protozoacide. It is possible that CSIs may disrupt the chitin hindgut lining enough to cause a decline in the number of hindgut protozoa. Therefore, CSIs may reduce termite fitness by reducing hindgut protozoa.

One such CSI, hexaflumuron [N-((3,5-dichloro-4-(1,1,2,2-tetrafluroethoxy) phenyl)-amino)carbonyl)-2,6-diflurobenzamide], is the formulated active ingredient in the Sentricon® Termite Elimination Baiting System. Hexaflumuron has never been tested for activity on the termite gut fauna. In this study, laboratory studies were conducted to investigate the effect of hexaflumuron on the survival of 3 genera of large protozoa found in the *R. flavipes* hindgut: *Trichonympha*, *Dinenympha*, *and Pyrsonympha*.

Methods and Materials

Subterranean Termite Collection. Five wild populations of *R. flavipes* were collected from fallen wood in forested areas of Montgomery County (N37° 12.46' and W80° 24.47') and Fairfax County (N38° 43.29'and W77° 30.93'), Virginia. Termites were harvested by placing the wood in plastic storage tubs (70L; Sterlite®; Sterlite Corporation, Townsend, MA) on top of damp, recycled paper towels (Acclaim®; Fort James Corporation, Deerfield, IL). Termites infested the moist paper towels as the wood dried out. Infested paper towels were then transferred to plastic storage containers (11.3L; Rubbermaid; Wooster, Ohio) containing vermiculite (500g; moistened 150% by weight [Lenz et al. 1987]); Cherokee Vermiculite Horticultural Fine Cherokee Products; Jefferson City, TN). Termite containers were stored in complete darkness (~21°C and 97% RH) until termites were needed for testing. Subterranean termites were tested within one month after being collected from the field.

Termite Diet Preparation. Recycled brown paper toweling (Acclaim®; Fort James Corporation, Deerfield, IL) was used as a diet substrate. Brown paper towels were cut into squares (35 x 35 mm). Because hexaflumuron cannot be stored at temperatures above 50°C and all paper squares were dried in an oven (60ºC), the initial weight of each diet was recorded before diets were treated. Diet substrates were dried overnight in a single wall, gravity convection, laboratory oven (60ºC; Blue M SW-17TA; Blue M Electric Company;

Blue Island, IL). After 24h each diet substrate was removed from the oven, and they were immediately numbered in the corner with a pencil and weighed on a balance (Mettler AE163; Lab Tech, Inc.) to the nearest 0.1mg.

After weighing, diets were treated with either a visual dye, as a control, or visual dye combined with hexaflumuron, as a treatment. The purpose of the dye was to insure that termites were feeding and to insure that only feeding termites were chosen for protozoa analysis. Nile Blue-A was selected as the visual marker because of its long-term visibility and low associated mortality for *R. flavipes* (Haagsma and Rust 1993; Oi and Su 1994; Su et al.1991a; King 2000; Suarez and Thorne 2000b). Termites feeding on experimental diets were dyed a bright blue color.

The control diet was formulated by dissolving 11.3mg of Nile Blue-A (Allied Chemical Company, Morristown, NJ) in 20ml of acetone, so that the final Nile Blue-A amount applied was 0.1% (weight of Nile Blue A/ weight of paper square). A 150µl aliquot of dye solution was applied to each control diet square.

Treated diets were formulated in 20ml of acetone by dissolving 58mg hexaflumuron (98% pure technical-grade; Dow AgroSciences; Indianapolis, IN), so that the final hexaflumuron concentration was 0.5% (w/w), and 11.3mg of Nile Blue-A (Allied Chemical Company, Morristown, NJ), so that the final Nile Blue-A concentration was 0.1% (w/w). A 150µl aliquot of the hexaflumuron/dye solution was applied to each treated diet square.

Sand Preparation: Play sand (Quikcrete®; Quikcrete Companies, Atlanta, GA) was washed with tap water 4 times to remove impurities. Washed sand was dried for ~48h in a single wall gravity convection laboratory oven (270ºC; Thelco®; Precision PS Scientific; Chicago, IL) prior to use. Dried sand was then placed in a plastic storage bag (3.79L; Target Corporation, Minneapolis, MN) and moistened with distilled water (15% by weight of the sand). Moistened sand was then kneaded by hand in the storage bag and stored for at least 24h to ensure even distribution of moisture. Sand was stored in the plastic bag until needed for testing.

Experimental Arenas: Laboratory arenas were used to evaluate the effect of hexaflumuron on protozoa mortality. Subterranean termites were exposed to hexaflumuron or control diets in no-choice bioassays. No-choice arenas were assembled

by connecting two Petri dishes with Tygon tubing (inner diameter 3.2mm; outer diameter 6.4mm; Fig. 5-1). Petri dishes were washed 4 times with tap water, and Tygon tubing was soaked in tap water for ~3h prior to use. The washing of arena materials was done to eliminate static electricity and any impurities. One Petri dish (95 mm x 15 mm; Fisher Scientific) served as a housing chamber and was filled with moistened sand from the storage bag (~45g). A second Petri dish (60 mm x 15 mm; Fisher Scientific) served as a diet chamber and contained one of the diets. Termites placed inside the treatment arenas could forage only on the hexaflumuron diet. Likewise, termites placed inside the control arenas could forage only on the untreated diet.

Prior to testing, 100 worker termites (at least 3rd instar) were aspirated out of the storage containers and transferred into the housing chamber of one experimental arena. Termites were allowed to acclimate for 72h and forage on an untreated paper towel diet in the diet chamber. After the acclimation period, paper towels were removed, and all termites clinging to the paper towels were gently tapped back into diet chamber. Experimental diets were then placed into the test chambers. Three pieces of masking tape were put on the outside of the arena to seal the housing chamber lid.

Humidity Chamber. All arenas were placed inside humidity chambers. The chambers were constructed by pouring play sand into the bottom of plastic storage containers (11.3L; Rubbermaid; Wooster, OH), so that sand was 4.5 cm deep. Tap water was poured over the sand to the point of saturation. Any standing water was absorbed with paper towels. A sheet of aluminum foil (Super Foil^{TM}; Atlantic Paper & Foil Corporation, Hauppauge, NY) was laid down to cover the saturated sand. Arenas containing termites were placed inside plastic storage bags (3.8L; Target Corporation, Minneapolis, MN) and set on top of the aluminum foil in the chamber. The top of the plastic bag was rolled down to allow for air circulation. Plastic bags were used to separate the arenas from each other and to catch any escaped termites. Escaped termites were returned to their respective experimental arena. Humidity chambers were closed with snap top lids and placed in total darkness in a cabinet (~21°C and 97% RH) for the duration of the experiment.

Figure 5-1. The No-choice subterranean termite arena. Termites were placed in the larger housing chamber where they could forage through a Tygon tube into a diet chamber. The diet chamber contained dyed and/or hexaflumuron treated paper towels.

Bioassay Design. Bioassays were arranged in a 2 (day) x 2 (treatment) factorial experiment. The experiment was arranged as a randomized complete block design (RCBD), blocked by termite field population to account for within treatment variability due to population effects. A total of ten bioassay arenas were set up for each no-choice treatment: hexaflumuron only and control only. Each treatment group was further subdivided into two test periods: 5d and 15d. Each test day within a treatment group was replicated five times with termites from a different field populations. Once the data from an arena had been recorded, the arena was removed from the test.

Recording Termite Mortality. Termite mortality was recorded to ensure that termite mortality was not a factor in protozoa health. Termites that did not exhibit proper molting were considered moribund and were subtracted from the survivor count (Su and Scheffrahn 1993). The number of termites surviving at 5d and 15d was counted and subtracted from the initial number of termites.

Analysis of Diet Consumption. The amount of hexaflumuron diet and control diet consumed by the termites was determined. It was important to insure that consumption of both diets by termites occurred so that correct conclusions could be drawn on the effects of hexaflumuron on gut protozoa and termite mortality.

After termite mortality was recorded for each bioassay, partially consumed diets were dried for 24h as previously described and weighed to determine consumption. Post-test diet weights were subtracted from the initial diets weight to determine total termite consumption. Initial diet weights were the weights taken before paper towels were treated with dye and/or hexaflumuron, as the weight of the treatment was determined to be miniscule.

Dissections. Termite gut dissections followed the protocol designed by Belitz and Waller (1998). Termites were immobilized on a blue ice-pack (21.6 x 5.3 x 10.2 cm; Rubbermaid; Wooster, OH). The anterior portion of the termite was held with a pair of forceps, while a second pair of forceps pinched the tip of the abdomen, and the complete gut was pulled out of the body.

Protozoan Analysis. A 60µl aliquot of solution (0.6% NaCl in distilled water mixed with a trace amount of Neutral Red dye) was placed in a microcentrifuge tube (1.5ml; Fisher Brand). The guts from three termites were added to the centrifuge tube and macerated together. The glass cover slip was placed over a brightline hemacytometer (0.100mm deep; Hausser Scientific), and using a Pasteur pipette, both chambers of the hemacytometer were filled (without overflowing) by capillary action. The protozoa were observed at 400X with a Nikon YS2 phase contrast microscope.

The gut fauna of subterranean termites has been shown to vary between different termite colonies (Smythe and Williams 1972). Because natural variation rather than the treatment could account for some of the quantitative differences in protozoa, numbers of protozoa for termites fed the control diets were quantified. Termites from the 5 different colonies were fed a control diet for 5d to determine the average numbers of protozoa in three genera (*Trichonympha*, *Pyrsonympha*, and *Dinenympha)* present (Fig. 5-2). Numbers of protozoa were counted and only those protozoa found in the center square in each hemacytometer grid were quantified (Fig. 5-3). Secondly, the number of *Trichonympha, Pyrsonympha,* and *Dinenympha* found in both treatment groups at 5d and 15d were counted on the hemacytometer. Thirty termite hindguts were analyzed from each arena (10 sets of three hindguts were homogenized together.

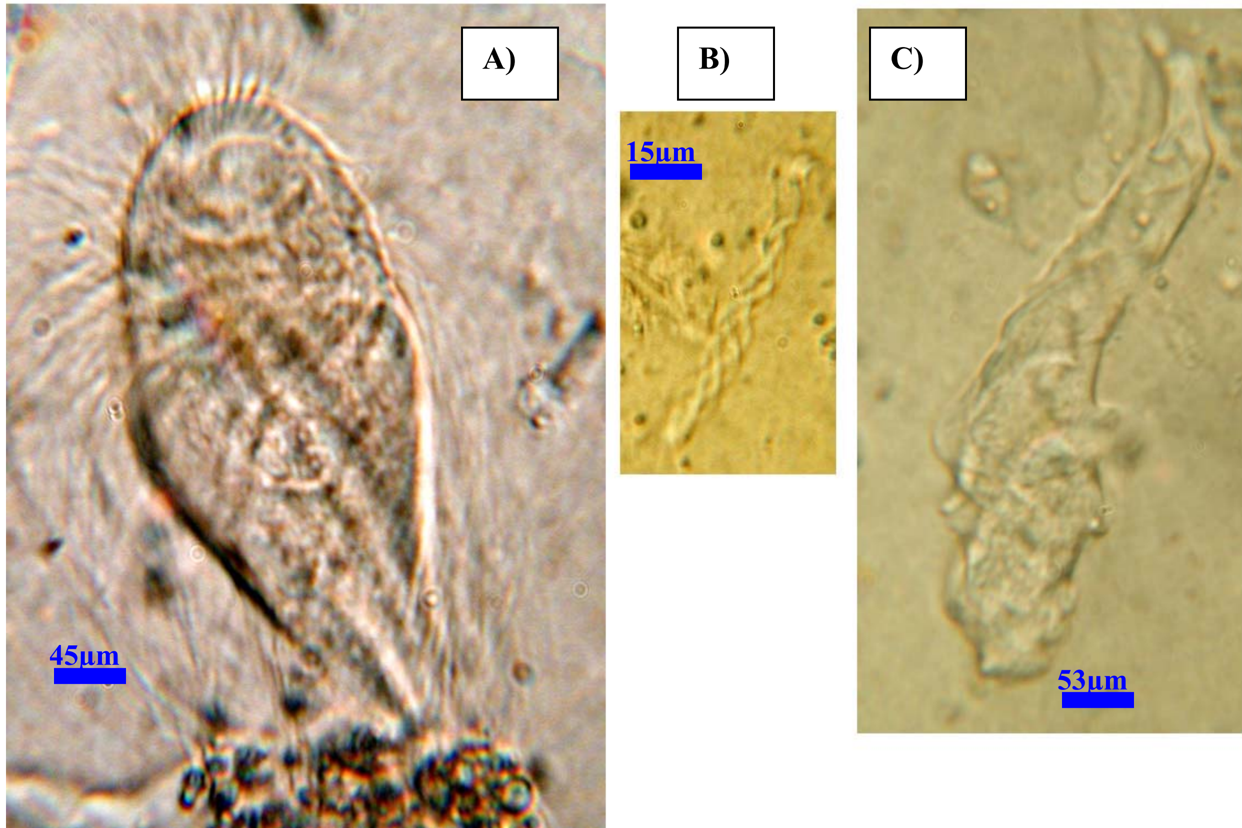

Figure 5-2. Three of the largest and most abundant genera of protozoa found in the subterranean termite, *Reticulitermes flavipes* (Kollar), hindgut: (A). *Trichonympha,* (B). *Dinenympha,* (C). *Pyrsonympha.* These measurements are estimations.

Statistical Analysis. The mean percentage of termite mortality and mean consumption (mg) of paper towel diet at 5d and 15d were compared using the Student's *t*-test. Total numbers of protozoa occurring naturally within control populations were compared using GLM ANOVA, and the means were separated using Fishers' Least Significant Differences (LSD) test for 5d and 15d separately. For each test day, total number of protozoa was modeled as a function of population. Mean number of protozoa found within the three genera (*Trichonympha, Prysonympha, Dinenympha*) within control populations also were compared using GLM ANOVA, and means were separated using Fishers' LSD test for 5d and 15d separately. For each test day, mean numbers of protozoa were modeled as a function of population. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

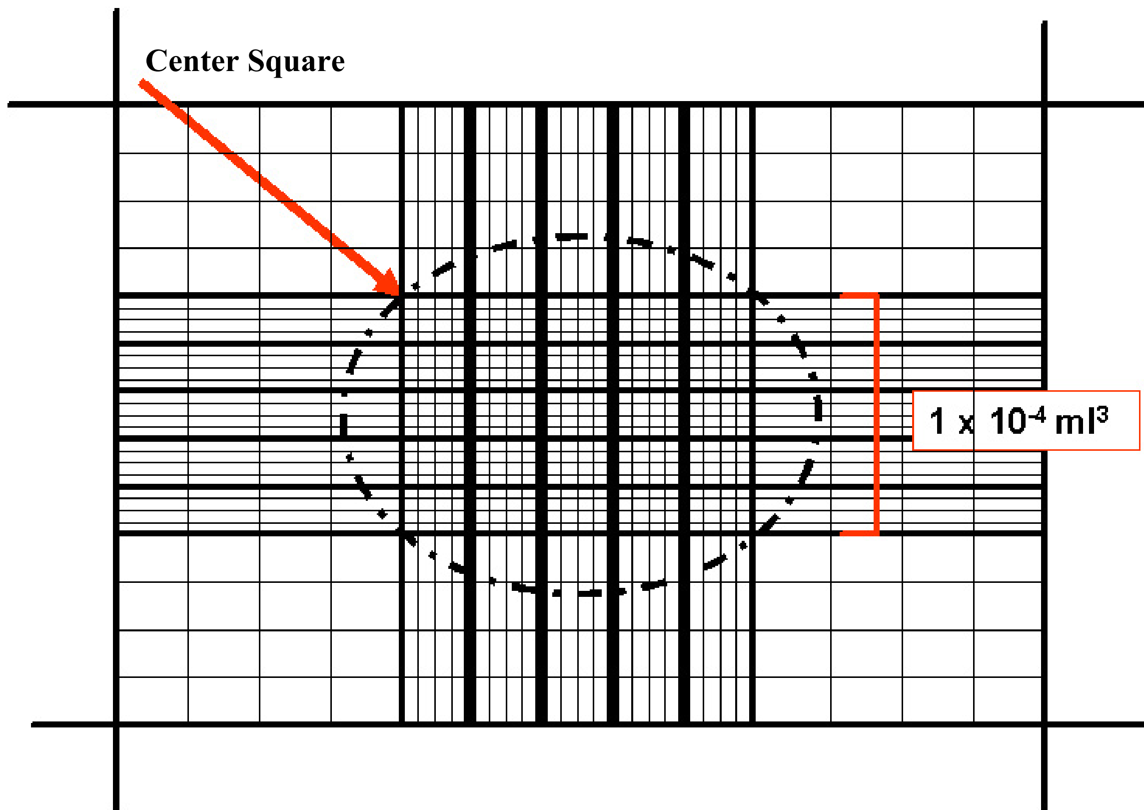

Figure 5-3. Center grid on Brightline hemacytometer. An aliquot of termite gut homogenate was placed on a Brightline hemacytometer. The number of protozoa found within the center square (circled area) were counted. The center square has a volume of 1x 10^{-4}ml^3.

Total numbers of protozoa in each treatment were compared using a GLM split-plot (in time) ANOVA, and the means were separated using Fishers' LSD test. Total numbers of protozoa were modeled as a function of treatment, time, the interaction between treatment and time. The interaction between time, population, and treatment was used as the error term. Values of $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

The number of protozoa (*Trichonympha, Prysonympha, Dinenympha)* in each treatment (hexaflumuron and control) were compared using GLM ANOVA. The numbers of each protozoa genera were modeled as a function of population (blocked), treatment, and population by treatment interaction. Separate analyses were run for 5d and 15d. Values of P $\alpha \leq 0.05$ were used to indicate significance (SAS Institute 1999).

Results

Mortality. Overall subterranean termite mortality was very low (Table 5-1) and there were no significant differences between the treatment groups at 5d ($P = 0.172$). No mortality was observed in termites feeding on control diets at 5d, and those feeding on hexaflumuron diets had an average of 1% mortality. At 15d there was also no significant differences between the treatment groups ($P = 0.954$). Termites feeding on control diets had an average of 7% mortality, and termites feeding on hexaflumuron diets had an average of 6% mortality.

Diet Consumption. Mean consumption (mg) of diets treated with hexaflumuron (Table 5-2) was almost identical to that of control diet. At 5d mean consumption of control diets was 9.8mg and mean consumption of hexaflumuron diets was 8.2mg ($P = 0.123$). At 15d mean consumption of control diets was 30.2mg and mean consumption of treated diets was 36.2mg ($P = 0.469$).

Protozoa Analysis. Termites fed only control diets were used to quantify the amount of natural variation occurring in numbers and types of protozoa between different subterranean termite populations. Mean total numbers of protozoa at 5d within the five control groups were significantly different between populations ($P < 0.001$; Table 5-3). Population 1 had the fewest numbers of protozoa, while population 2 had the greatest numbers of protozoa. The numbers of protozoa in termites from populations 3, 4, and 5 were not significantly different. Likewise, the mean numbers of the specific genera of protozoa varied between populations. Population 2 had significantly more *Trichonympha* than population 3 and population 4 but was not significantly different than population 1 or population 5 ($P = 0.029$; Table 5-4). Mean numbers of *Pyrsonympha* were not found to be significantly different between populations ($P = 0.317$). Finally, mean numbers of *Dinenympha* for each termite population were found to be proportionally similar to the overall numbers of protozoa. Population 1 had the fewest number of *Dinenympha*, population 2 had the greatest number, and populations 3, 4, and 5 were not significantly different ($P < 0.001$).

When evaluating the effects of hexaflumuron on the total number of protozoa present, the interaction between time and treatment was found to be not significant ($F = 0.74$; $P = 0.407$). The main effect of time (day) was found to be not significant ($F = 0.54$;

P = 0.476). The main effect of treatment was also found to be not significant. No differences were found in the overall numbers of protozoa between termites fed hexaflumuron or control diets (*P* = 0.734; Table 5-5).

However, there were differences between the specific genera. At 5d there was no interaction between population and treatment for any of the three genera of Protozoa (for *Trichonympha, F* = 1.80, *P* = 0.137; for *Pyrsonympha*, *F* = 1.07, *P* = 0.377; for *Dinenympha*, *F* = 1.68, *P* = 0.161), and this interaction was only significant at 15d in *Dinenympha* (*F*= 11.04, *P* < 0.0001). Termites feeding on hexaflumuron treated diets had significantly less *Trichonympha* in their hindgut after 5d as a result of treatment than termites feeding on control diet (*P* = 0.041; Table 5-6). However, at 15d termites feeding on hexaflumuron treated diets had similar numbers of *Trichonympha* to the control groups (*P* = 0.672). The hexaflumuron diet did not have a significant impact on the number of *Pyrsonympha* at 5d (*P* = 0.161). However, by 15d there were significantly fewer *Pyrsonympha* in termites feeding on the hexaflumuron diet than termite feeding on the control diet (*P* = 0.047). Termites fed the hexaflumuron diets did not have significantly fewer numbers of *Dinenympha* than those termites fed the control diets at either 5d (*P* = 0.301) or 15d (*P* = 0.766).

Table 5-1. Mean percent mortality at 5 and 15 days for 100 subterranean termites fed hexaflumuron diets or control diets. This determined termite health.

Time Period (day)	Treatment	n	Percent Mortality (Mean ± SEM)	*t*-statistic	*P*-value
5	Control	5	0.00 ± 0.00 a[1]	-1.5	0.172[1]
	Hexaflumuron	5	0.01 ± 0.01 a		
15	Control	5	0.07 ± 0.03 a	0.06	0.954
	Hexaflumuron	5	0.06 ± 0.03 a		

[1]Student's *t*-test (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$ as applied to the percent mortality means at each time period.

Table 5-2. Mean total consumption (mg) at 5 and 15 days for 100 subterranean termites fed hexaflumuron diets or control diets. This determined equivalent consumption of diets.

Time Period (day)	Treatment	n	Total Termite Consumption (mg) per 100 termites (Mean ± SEM)	Total Consumption (mg) per termite (Mean ± SEM)	*t*-statistic	*P*-value
5	Control	5	9.8 ± 0.7 a[1]	0.098 ± 0.007	1.72	0.123[1]
	Hexaflumuron	5	8.2 ± 0.6 a	0.082 ± 0.006		
15	Control	5	30.2 ± 5.7 a	0.302 ± 0.057	-0.76	0.469
	Hexaflumuron	5	36.2 ± 5.4 a	0.362 ± 0.054		

[1]Student's *t*-test (SAS Institute 1999). Means followed by different letters are significantly different at α = 0.05 05 as applied to the total consumption means at each time period.

Table 5-3. Mean number of total protozoa present at 5 days in a sample for subterranean termites fed control diets.

Population Number	n	Total Number of Protozoa Present in 2 x 10^{-4}ml^3 of Homogenate (Mean ± SEM)	F-statistic	P-value
1	10	143 ± 14 c[1]	7.95	<0.001[1]
2	10	306 ± 31 a		
3	10	236 ± 13 b		
4	10	204 ± 18 b		
5	10	220 ± 21 b		

[1]GLM ANOVA and Fishers' test of least significant differences (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$.

Table 5-4. Mean number of each specific Genera of Protozoa (*Trichonympha, Pyrsonympha, Dinenympha)* present at 5 days in a sample for subterranean termites fed control diets.

Population Number	n	Genera of Protozoa	Number of protozoa Present present in hindgut (Mean ± SEM)	*F*-statistic	*P*-value
1	10	*Trichonympha*	13 ± 1 a,b[1]	2.99	0.029[1]
2	10	*Trichonympha*	16 ± 1 a		
3	10	*Trichonympha*	11 ± 1 b		
4	10	*Trichonympha*	9 ± 1 b		
5	10	*Trichonympha*	12 ± 1 a, b		
1	10	*Pyrsonympha*	8 ± 1 a	1.22	0.317
2	10	*Pyrsonympha*	7 ± 1 a		
3	10	*Pyrsonympha*	7 ± 1 a		
4	10	*Pyrsonympha*	9 ± 1 a		
5	10	*Pyrsonympha*	10 ± 1 a		
1	10	*Dinenympha*	122 ± 12 c	8.76	<.0001
2	10	*Dinenympha*	282 ± 30 a		
3	10	*Dinenympha*	218 ± 12 b		
4	10	*Dinenympha*	185 ± 16 b		
5	10	*Dinenympha*	198 ± 20 b		

[1]GLM ANOVA and Fishers' test of least significant difference (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$ as applied to the number of protozoa present means for each genera of protozoa.

Table 5-5. Mean total number of protozoa present at 5 and 15 days in a sample for subterranean termites fed hexaflumuron diets or control diets.

Time Period (day)	Treatment	n	Total Number of Protozoa Present in 2 x $10^{-4}ml^3$ of Homogenate (Mean ± SEM)	*F*-statistic	*P*-value
5	Control	50	222 ± 11[1]	0.12	0.734[1]
	Hexaflumuron	50	196 ± 10		
15	Control	50	188 ± 9		
	Hexaflumuron	50	199 ± 13		

[1]GLM Split-plot (in time) ANOVA (SAS Institute 1999). Significance was determined at $\alpha = 0.05$.

Table 5-6. Mean number of the specific Genera of protozoa (*Trichonympha, Pyrsonympha, Dinenympha)* present at 5 and 15 days in a sample for subterranean termites fed hexaflumuron or control diets.

Time Period (day)	Treatment	n	Genera of Protozoa	Mean Number of Protozoa Present in Hindgut (Mean ± SEM)	*t*-statistic	*P*-value
5	Control	50	*Trichonympha*	12 ± 1 a[1]	8.78	0.041[1,2]
	Hexaflumuron	50	*Trichonympha*	9 ± 1 b		
	Control	50	*Pyrsonympha*	8 ± 1 a	2.95	0.161
	Hexaflumuron	50	*Pyrsonympha*	6 ± 1 a		
	Control	50	*Dinenympha*	201 ± 11 a	1.41	0.301
	Hexaflumuron	50	*Dinenympha*	180 ± 10 a		
15	Control	50	*Trichonympha*	7 ± 1 a	-0.41	0.680
	Hexaflumuron	50	*Trichonympha*	7 ± 1 a		
	Control	50	*Pyrsonympha*	8 ± 1 a	3.02	0.003
	Hexaflumuron	50	*Pyrsonympha*	5 ± 1 b		
	Control	50	*Dinenympha*	172 ± 9 a	-0.82	0.415
	Hexaflumuron	50	*Dinenympha*	186 ± 13 a		

[1]GLM ANOVA (SAS Institute 1999). Means followed by different letters are significantly different at $\alpha = 0.05$ 05 as applied to the number of protozoa present means for each genera of protozoa.
[2]df=1, 4

Discussion

All termites fed both hexaflumuron and control diets appeared healthy and had little associated mortality at the end of the 15d test period. Overall, subterranean termite mortality was very low for both treatments (<1% at 5d and <7% at15d). Consequently, hexaflumuron induced morbidity in the termite populations did not appear to be the cause for the protozoa mortality observed at 15d. Sheets et al. (2000) demonstrated that the LT_{50}s for termites consuming hexaflumuron baits termites was around 26.6d. They further established that the onset of toxicity for feeding termites does not occur until around 15d. Thus, it was not expected that the termites in this experiment would show signs of hexaflumuron poisoning during the 15d duration of this experiment. Therefore, termite morbidity can be eliminated as a cause of protozoa death. Furthermore, diet consumption of both hexaflumuron and control diets were nearly equal. Therefore, it seems probable that differential consumption of one of the two diets also had no bearing on protozoa decline.

Because the results indicated that specific protozoa did have some decline due to the hexaflumuron treatment, it was necessary to first quantify the degree of natural variation found for numbers of protozoa between different termite populations. Natural variation could mask the effect of the hexaflumuron on protozoa numbers. In a natural environment, many factors may contribute to protozoa species composition and population dynamics. Protozoa survival and ability to metabolize cellulose are dependent on conditions found in the termite colony and the environment surrounding the colony itself (Belitz and Waller 1997). For example, geographical location, season, and termite colony age have been shown to directly influence numbers of protozoa found in the termite hindgut (Lenz and Becker 1972; Grosovsky and Margulis 1982). The type of wood consumed by termites also affects protozoa composition between different termite populations (Mannesmann 1972, 1974; Mauldin et al. 1981). Cook and Gold (1998) found significant differences in the mean number of protozoa in *R. flavipes* (including *Trichonympha*, *Pyrsonympha*, and *Dinenympha*) between termites from different sites, different castes, and different individuals of the same population. Furthermore, they determined that protozoa species abundance is proportional to the total number of

protozoa present, meaning that a single species of protozoa will have greater or fewer representatives depending on how many protozoa are present.

Smythe and Williams (1972) found that the gut fauna is generally characteristic of species but variation does occur between populations. The species composition of protozoa in this experiment also varied between termites populations from different locations. By comparing numbers of protozoa found in the control groups at 5d, it was determined that total numbers of protozoa significantly differed between termites found at different sites. It was determined that the numbers of *Trichonympha* and *Dinenympha* varied between the different termite populations. However, numbers of *Pyrsonympha* between populations were not significantly different between the populations, suggesting that the reduction in *Pyrsonympha* observed in this study was the result of hexaflumuron exposure rather than variation between populations.

Other studies have concluded that exposure to CSIs can negatively impact specific protozoa in the termite hindgut. In one such treatment experiment, Waller (unpublished data, personal communication) found that termites feeding on 0.2% diflubenzuron had significantly less large protozoa (greater than 25µm in length) in their hindgut and increased mortality. Yet, she determined that although large protozoa were reduced in termites fed diflubenzuron when compared with the control, the numbers of large protozoa were still plentiful.

The results of this study were similar to Waller's (unpublished data, personal communication) study, in that no significant differences were observed for the total number of protozoa in termites in the hexaflumuron treatment compared with the control. However, the numbers of specific genera of protozoa indicated different results. The number of *Trichonympha* found in the gut of termites feeding on control diet were significantly different than termites feeding on hexaflumuron diet at 5d but not significant by 15d. Because *Trichonympha* numbers in termites fed hexaflumuron seemed to recover with time, this situation appears to be anomalous. Most likely this difference in the numbers of *Trichonympha* is due to natural variation. It appears that hexaflumuron does not really affect *Trichonympha*. The lower numbers of *Prysonympha* in the termite hindgut after 5d and 15d suggested that hexaflumuron did affect the survival of this genera of protozoa. However, the *Pyrsonympha* were not completely eliminated from the

hindgut at 15d (>150 per 5000 microns of gut). Thus, it seems that any reduction of protozoa that could be attributed to the hexaflumuron diet probably had little effect on termite fitness.

Although hexaflumuron has been shown to have negative effects on subterranean termite survival (King and Karr 2000; Sheets et al. 2000), the results of this study indicate that it does not appear to greatly impact their protozoa community. Therefore, it is unlikely that hexaflumuron has a second mode of action of killing termites by negatively impacting their gut protozoa.

Chapter Six:
Summary

Subterranean termites are one of the most economically important pests in the United States. Every year billions of dollars are spent in an effort to control subterranean termites and repair their damage to structures. One reason for their success is the fact that these termites are cryptobiotic. Because subterranean termites need to maintain contact with the soil, they establish nests beneath the ground and forage within mudtubes in wood. Subterranean foragers radiate from the colony in many underground tunnels and feed on many food sources at once in the natural environment. They cause damage when foragers tunnel into wooden structures to look for food. Because subterranean termites are concealed within the wood, it is difficult to identify an infestation until it causes noticeable damage. The cryptobiotic nature of subterranean termites makes gaining access to the colonies for treatment purposes a real challenge.

Bait systems are one method of termite control, which can overcome the problems associated with the cryptobiotic nature of subterranean termites. Bait systems are placed in the ground where termites live and are designed to destroy termite colonies themselves. Worker termites find the bait stations while foraging in the soil. Because the toxicant is formulated in a cellulose matrix inside the station, termites enter the station and readily consume the bait.

Chitin synthesis inhibitors (CSIs) are commonly used as the active ingredient in the bait matrix. CSIs disrupt the insect cuticle formation during the molting process. Hexaflumuron, one such CSI, is the active ingredient of the Sentricon® Termite Elimination System. Because hexaflumuron is formulated to be a slow acting toxicant, infected individuals do not immediately show signs of poisoning. Termites can consume the bait, return to the colony, and share the toxicant with other nestmates. Hexaflumuron can be dispersed throughout the termite colony and kill termites that did not feed on the hexaflumuron directly. Bait systems are, therefore, designed to suppress or eliminate entire termite colonies.

However, bait systems have limitations, the most significant of which is the presence of competing food resources in the termite natural environment. It is impossible

to eliminate all alternative food resources in the environment. Therefore, these food sources compete with bait stations for termite foragers. Termites feed at multiple sites along their foraging path and do not limit feeding to one food source at a time. Because a single foraging termite has a limited consumption capacity and cannot consume more than this amount, the total amount a termite population could potentially consume in a given period is also limited. Total consumption of a single food source is dependent on how many workers are recruited to a site and how many other food sources the colony is already consuming. Competing food sources in the environment have the potential to greatly impact bait consumption.

This research determined that competing food sources did impact hexaflumuron bait consumption by subterranean termites. Termites consumed less hexaflumuron in the presence of competing food sources. When consumption was divided between two or more equally palatable food sources, termites ate portions of both diets. Therefore, termites consumed less of each diet in a choice test than when each diet was presented alone in no-choice tests. The type of competing food source presented with the hexaflumuron diet affected the amount hexaflumuron consumption. When a control diet competed, hexaflumuron consumption was reduced because termites ate equal portions of both diets. However, when presented with another competing food source, inulin, termites preferred the inulin diet over the hexaflumuron diet. Interestingly, inulin is a β-linked carbohydrate and is often used as a food additive in human foods. The addition of inulin to the termite diet made it more appealing than hexaflumuron diets. This is an interesting fact, because if foraging termites were to locate a food sources in the natural environment that was more appealing than the hexaflumuron bait, consumption of the bait toxicant bait would decline.

Although competing food sources were found to reduce hexaflumuron consumption, no conclusive results were made for competition effects on subsequent termite mortality. Termites fed hexaflumuron diets in the no-choice test died significantly faster than termites fed only control diets. However, termite mortality in a choice test was not found to be significantly different than that of termites fed only the control diet or only the hexaflumuron diet. Further testing would be necessary to

conclude the effect of competing food sources on bait efficacy in terms of termite mortality.

It has been considered whether bait systems, which utilize CSIs as their active ingredient, could overcome food source competition and consequential reductions in bait consumption by employing a second mode of action against the termites. Termites rely on hindgut symbiotic protozoa as an indirect means of digesting nutrients. Because the hindgut is lined with chitin and some protozoa adhere to the surface of this cuticular layer, the hindgut could be a target for CSIs. It is possible that CSIs could disrupt the hindgut lining and cause a decline in the number of protozoa. Thus, CSIs could kill termites by inhibiting proper molting and by reducing hindgut protozoa, causing the termite to starve to death.

In spite of the chitin layer present in the hindgut, this study determined that hexaflumuron did not impact the protozoa community. There were no significant differences in total numbers of protozoa found in termites fed hexaflumuron diets than fed control diets. The lower numbers of *Prysonympha* in the termite hindgut after 15d suggested that hexaflumuron did affect the population numbers of this genera of protozoa. However, the *Pyrsonympha* were not completely eliminated from the hindgut (>150 per 5000 µm of gut tissue) and numbers of *Trichonympha* and *Dinenympha* were not affected by hexaflumuron treatment. Although hexaflumuron did have an effect on the protozoa, the hexaflumuron impact was not substantial enough to kill termites by employing a second mode of action against the gut protozoa.

Because bait stations are affected by alternative food source competition and do not seem to employ a second mode of action to overcome this limitation, the best way to ensure bait efficacy is to increase the bait palatability. Although bait systems have the potential to kill entire termite colonies, the active ingredient is ineffective if the colony is not feeding on the toxic bait. Adding a water soluble food additive, like inulin, to the cellulose bait matrix would increase bait consumption due to increased food source attractiveness. This β-linked carbohydrate would be very easy to apply to the bait matrix and would increase the flavor of the bait and make it more attractive to termites. Consumption of the hexaflumuron bait would most likely increase despite the number of food sources in the area, because the bait would be more a more attractive food source.

The impact of utilizing an inulin additive to bait matrices to increase bait efficacy warrants further investigation.

Appendix A

This is supplementary information from Chapter Three, the effect of competing food sources on *Reticulitermes* spp. consumption of hexaflumuron treated bait. A series of radioactive samples with volumes of both isotopes in a single scintillation vial were also created for the formation of the Spillover Curve.

Thirty scintillation vials were labeled and filled with different volume combinations of both isotopes (Table A-1). The vials contents had the isotopes in three different combinations: ^{3}H and ^{14}C in equal allotments, ^{3}H increasing and ^{14}C decreasing, ^{3}H decreasing and ^{14}C increasing. These combination isotope vials were thought to be important to see the interaction of the two isotopes. However, upon inspection these samples were deemed not appropriate approaches for spillover correction, as the spillover effect was more easily noted when isotopes were not combined in the same vial.

Table A-1. Scintillation vials filled with various combinations of ^{14}C and ^{3}H to be counted for the correction curve.

Vial	^{14}C Stock (µl)	^{3}H Stock (µl)	Vial	^{14}C Stock (µl)	^{3}H Stock (µl)	Vial	^{14}C Stock (µl)	^{3}H Stock (µl)
1	0	0	11	0	200	21	600	0
2	25	3	12	25	175	22	525	3
3	75	25	13	75	150	23	450	25
4	150	50	14	150	125	24	375	50
5	225	75	15	225	100	25	300	75
6	300	100	16	300	75	26	225	100
7	375	125	17	375	50	27	150	125
8	450	150	18	450	25	28	75	150
9	525	175	19	525	3	29	25	175
10	600	200	20	600	0	30	0	200

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Vita

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