Effect of Tagetes minuta on the plant parasitic southern root-knot nematode, Meloidogyne incognita, sugarbeet cyst nematode, Heterodera schachtii and nontarget aquatic macroinvertebrates
by Anuj Kumar

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology
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Abstract:
Marigolds have pesticidal properties that can be alternatives to methyl bromide and other pesticides in some systems. The hypothesis that marigold root tissue can protect sugarbeets against root-knot nematodes in sequential cropping systems was tested. Marigolds were grown in sand substrate and a 50:50 ratio of sand:MSU soil mix. Above ground marigold tissues were cut at soil level and removed 5.5 months postgermination. Sugarbeets were planted in pots of both substrates containing intact marigold roots and in pots without marigold roots. Second stage juvenile (J2) southern root-knot nematodes, Meloidogyne incognita Kofoid and White, were used to inoculate (300 J2s per 5 week old sugarbeet seedlings). The root gall index was highest (4.19) for sand without marigold roots. Presence of roots of Mexican marigold, Tagetes minuta and MSU soil mix, separately, significantly reduced populations of M. incognita indicated by root-gall index. Integration of these two amendments further reduced nematode populations. SandrMSU mix with marigold roots had the lowest gall index (1.69).

No studies of marigolds effect on cyst nematodes have been reported for any host plant. The hypothesis that root exudates of T. minuta, residual after roots are still left in soil, are effective against the sugarbeet cyst nematode, Heterodera schachtii (Schmidtt) was tested. Above ground marigold tissues were removed 2.5 months post germination. Sugarbeets were planted in pots of both substrates containing intact marigold roots and in pots without marigold roots. Cysts were placed in mesh-lined frames (10 cysts/frame, 6 frames/pot). Marigold roots significantly reduced the number of juveniles infecting the sugarbeets. Mean p,i. was 5.95 J2s and 0.67 eggs/cc soil. Ssand 100% had the lowest mean p.f. with 1.52 J2s and 0.16 eggs/cc soil and sand:MSU mix with marigold roots had a significantly different p.f. with 4.25 J2s and 0.48 eggs/cc soil. .

Environmental effects of marigold compounds are unknown. I hypothesized that if marigold root extracts used as a pesticide, migrate through soil into nearby streams/ rivers . or are accidentally applied to wetlands, the .,compounds would be detrimental to aquatic, nontarget macroinvertebrates. Test organisms Gammarus lacustris Sars (Amphipoda), Lepidostoma pluviale (Milne)(Trichoptera), Drunella grandis (McDunnough) (Ephemeroptera), Baetis tricaudatis (Dodds) (Ephemeroptera), Rhithrogena morrisoni (Banks) (Ephemeroptera) and Hydropsyche cockerelli (Banks) (Trichoptera) were used for the bioassays. To obtain root extract, T minuta was grown in a greenhouse and extracted at 10 months. On harvest day, extracts were made and formulated with a commercial combination of petroleum solvents/surfactants. Bioassays were conducted in a cold water bath apparatus using three 21-liter glass aquaria slightly submerged within three 38-liter aquaria. Mortality was attributed to the presence of inert, surfactant/petroleum-based solvent. Of the macroinvertebrates tested, R. morrisoni was the most sensitive to inert materials (no LC50 as mortality progressed rapidly in inert at 0.01 ppm) and D. grandis was the least sensitive (LC50 for 0.01 ppm inert=6.91ppm). Marigold root extracts had a toxic effect at the highest concentration (4ppm) on all macroinvertebrates tested.

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Anuj Kumar

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

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No studies of marigolds effect on cyst nematodes have been reported for any host plant. The hypothesis that root exudates of *T. minuta,* residual after roots are still left in soil, are effective against the sugarbeet cyst nematode, *Heterodera schachtii* (Schmidtt) was tested. Above ground marigold tissues were removed 2.5 months post germination. Sugarbeets were planted in pots of both substrates containing intact marigold roots and in pots without marigold roots. Cysts were placed in mesh-lined frames (10 cysts/frame, 6 frames/pot). Marigold roots significantly reduced the number of juveniles infecting the sugarbeets. Mean p.i. was 5.95 J2s and 0.67 eggs/cc soil. Ssand 100% had the lowest mean p.f. with 1.52 J2s and 0.16 eggs/cc soil and sand:MSU mix with marigold roots had a significantly different p.f. with 4.25 J2s and 0.48 eggs/cc soil.

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INTRODUCTION

Theories are necessarily transient; they last only to be replaced by new ones more consistent with accumulated facts. With the rise in documented environmental hazards from synthetic pesticides, there has been a trend towards using eco-friendly biopesticides. Until a few years ago, pesticides produced from plants were theorized to be "safe" (not toxic to the environment). However some of these plant derived products might be detrimental to some degree to the environment. Marigold is one such plant which has shown promise as a pesticide while presumably being safe. There have been various references about the efficacy of marigolds in pest management of insects and nematodes, as outlined in my Literature Review (Davide 1979, Green et al. 1993). Worldwide, major crops such as carrots, sugarbeets, tomatoes and apples are susceptible to infection by various pathogens including nematodes (Whitehead, G.A. 1998). Nematodes are prevalent especially in intensive cropping systems and have evolved meticulate survival mechanisms (Weischer and Steudel 1972). Nematodes cause approximately $100 billion in crop damage worldwide annually (Weischer and Steudel 1972). Many toxic chemicals formulated as pesticides known to be harmful to the environment are used to control these nematodes (Ijani and Mmbaga 1988). Historically, botanicals, especially marigolds have been shown to be effective in reducing some nematode populations, as outlined in my Literature Review (Miller and Ahrens 1969). Pest resistant crop cultivars have been developed for some crops (Cai et al. 1997). For other crops, commercially competitive

resistant varieties have not been developed and here, marigolds could be an effective component in Integrated Pest Management strategies.

Currently nematicides are used to control nematodes in production of irrigated sugarbeets and other irrigated vegetables. Of these nematicides, methyl bromide (Civerolo et al. 1993), has been scheduled for 100% phase out by the EPA by 2005. Among other commonly used pesticides, 1,3 dichloropropane, aldicarb, terbufos and carbofuran, the latter three have been identified as high risk under FQPA by the EPA.

It is well documented in the literature that using marigolds as a sequential crop or intercrop reduces populations of some nematode genera including Root-knot nematode (*Meloidogyne* spp.) (Oduor-Owino and Waudo 1994, Powers et al. 1993). The cyst nematodes (*Globodera* and *Heterodera* spp.) and the Root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites. The cyst forms when the body of female is filled with eggs, while her body wall becomes leathery and forms a tough cover. There are no data reported in the literature on the effect of marigolds on any cyst nematode. A root-knot gall on the other hand is formed by the response of the host plant's roots to the infection of the nematodes which causes the giant cells. Although there are many reference to the usage of marigolds for reducing root-knot nematodes (Ijani and Mmbaga 1988) , no studies have been reported in the literature on the effect of the roots of *T. minuta* alone left in the soil against the southern root-knot nematode, *Meloidogyne incognita* (Kofoid and White) and no nematicidal marigold studies have been reported with sugarbeets as a host.

Organic amendments in soil such as animal by-products, wastes and plant extracts have been shown to decrease populations of several various nematodes including spiral

nematodes, reniform nematodes and root-knot nematodes, etc. (Whitehead 1998). Research shows that organic ammendment in soil stimulates microbial activity resulting in increased enzymatic actions and accumulation of specific end products which may be nematicidal (Rodriguez-Kabana et al. 1987). Most efficacious of these ammendments are those with low C:N ratios and a mucopolysaccharide, chitin has been shown to particularly effective in reducing nematode populations (Rodriguez-Kabana 1986). Singh and Sitaramaiah (1966) discovered that organic amendments such as oil cakes of neem or margosa at 1.8 ton/ ha reduced the populations of the Javanese root-knot nematode, *Meloidogyne javanica* (Treub) Chitwood on okra and the tomato crop that followed it and improved crop growth.

While there have been studies of the effectiveness of the Mexican marigold against nematodes, there have been no specific studies of its interactions with either the southern root-knot nematode or sugarbeets. Sugarbeets *Beta vulgaris* L. are an economically important crop in Montana and are the primary source of sugar in temperate climates and are a major source of profit for farmers in the USA, Russia, Germany and France (Weischer and Steudel 1972). Although *H. schachtii* is of major economic importance in nearly every sugarbeets growing area, this cyst nematode can attack over 200 plant species in 23 different plant families (Whitehead 1998).

There are no reports existing on the efficacy of marigolds on the cyst nematodes, except for Uhlenbroek and Bijloo (1958, 1959), who discussed nematicidal activity of an extract from whole plants of *T. patula* against larvae of the potato cyst nematode, *Heterodera rostochiensis* Woll., and no activity against its cysts. These authors did not

include the data. Our paper (Kumar et al. in review) on which a part of this thesis is based will be the first reported data of nematicidal properties of marigolds on cyst nematodes.

However, it is possible that if marigolds are used as a pesticidal intercrop or as a plant extract, some of the compounds may seep into the ground water and migrate into nearby rivers and streams. Even though Philogene et al.(1985), had observed that a phototoxin extracted from marigolds, alpha-terthienyl, was shown to be extremely insecticidal against mosquitoes and did not affect non-target organisms like the ostracod, caddisfly and snail (*Physa* sp.), the interaction of root extracts with fauna in these aquatic ecosystems should be examined before marigolds can be used as an alternative pesticide.

The effect of marigolds on the environment must be studied in greater detail before further steps are taken to utilize them as an alternative pesticide. If extracts of *T.minuta* are as mosquito larvicides or for control of schistomiasis in aquatic ecosystems, or the plant is used as a pesticidal intercrop for control of plant parasitic nematodes, it is logical to examine the effects of marigold root extracts on common nontarget aquatic macroinvertebrates.

HYPOTHESES TESTED

The following hypotheses were tested:

1. Exudates from roots of the Mexican marigold, *Tagetes minuta* have compounds which reduce infection of sugarbeet by the southern root-knot nematode, *Meloidogyne incognita.*

2. *T. minuta*, used as a sequential crop with root tissue remaining, will significantly reduce the populations of the sugarbeet cyst nematode, *Heterodera schachtii.* Morever, this nematicidal effect will be persistant in the soil after the root tissue has been removed.

3. Compounds from the *T. minuta* root extract, if used as a pesticidal agent will adversely affect non-target aquatic macroinvertebrates.

LITERATURE REVIEW

Insecticidal Properties of Marigolds

It has long been known that marigolds, *Tagetes minuta, Tagetes erecta,* and *Tagetes patula* can be used as insecticides (Philogene et al. 1985; Perich et al.1994; Weaver et al.1994, 1998), fungicides (Ackerman et al. in review and Welty and Prestbye 1993) and nematicides (Miller and Ahrens, 1969; Davide 1979). *Tagetes minuta* has insecticidal components in its leaves, flowers and roots (Weaver et al. 1994). Because of the broad efficacy of marigolds against soil pests including insects, nematodes, and fungi, marigold compounds have potential as methyl bromide alternatives (Civerolo et al. 1993).

Nematicidal Properties of Marigolds

In the intensive production system of various irrigated crops, pesticides are widely used to control nematodes (Johnson 1985). Of these nematicides, methyl bromide (Civerolo et al. 1993), has been scheduled for 100% phase out by the EPA by 2005. 1,3 dichloropropane, aldicarb and terbufos have been recognised by the EPA to be high risk pesticides to the environment.

It is well documented in the literature that marigolds have a nematicidal effect. Nematicidal properties of Mexican marigold, *Tagetes minuta* for Javanese root-knot nematode, *Meloidogyne javanica* (Treub) Chitwood, have been determined by planting them along with tomatoes and testing their growth rates and fruit yield in a nematode-infested area (Oduor-Owino and Waudo 1994). The French marigold, *Tagetes patula* L.,

has also been reported to be effective in controlling nematode populations (Edwards et al. 1994, Reulo 1983). Miller and Ahrens (1969) reported suppression of meadow lesion nematode, *Pratylenchus penetrans* (Cobb) Chitwood and Oteifa, populations and improved growth of tobacco after the French marigold, *Tagetes patula,* was grown in the same soil. Five months after inoculation, there were 31 fold less nematodes per 100 gm soil in marigold plots than in pigweed plots; 3 years after inoculation, there were three fold less nematodes per 100 gm soil in marigold plots than in pigweed plots.

In Tanzania, *T. minuta* and the African marigold, *Tagetes erecta,* reduced populations of northern root-knot nematode, *Meloidogyne hapla* Chitwood, by a statistically significant (4.5 fold) decrease (number of galls per root weight) compared to untreated controls and improved plant growth and fruit yield (Ijani and Mmbaga 1988). Compared to these two marigold species, ethylene dibromide gave the most effective nematode control but had the poorest shoot height and fruit yields (Ijani and Mmbaga 1988). Populations of apple root-lesion nematode, *Pratylenchus pratensis* (deMan) Filipjev (which Merwin and Stiles [1989] called *Pratylenchus penetrans* Filipjev), were reduced 96% in the apple orchard plots where *T.patula* was grown as a cover crop (Merwin and Stiles 1989). Flower, leaf, and stem portions of Mexican marigold reduce populations of root-knot nematodes in tomatoes and eggplants (Siddiqui and Alam 1987).

Tagetes erecta, intercropped with tomato, showed control of southern root-knot nematode, *Meloidogyne incognita* (Kofoid and White) (statistically lower gall index [3.18 on a scale of 1-5; P-value < 0.05] for the marigold plot compared to controls [gall index $=3.82$], but no increase in fruit yield (Davide 1979). Marigolds effectively reduced

populations of *M. javanica* (Treub) Chitwood, when used in rotation with carrots (Huang 1984). In Tanzania, *Tagetes* spp. were found to decrease root galls in tomato while also increasing the fruit yield compared to ethylene dibromide, a common nematicide, which decreased the fruit yield but gave the most effective root gall reduction (Ijani and Mmbagat 1988). However, in a field test comparing variations between a cucurbit monocropped and a cucurbit intercropped with *T.patula*, it was found that there were no consistent differences in densities of various plant parasitic nematode genera such as *Meloidogyne* spp., *Tylenchus* spp., and *Aphelenchoides* spp. between the two treatments (Powers et al. 1993).

No experimentally documented reports exist on the efficacy of marigolds on cyst nematodes. Uhlenbroek and Bijloo (1958, 1959) reported high nematicidal activity of an extract from whole plants of *T. patula* against larvae of the potato cyst nematode, *Globodera rostochiensis* Woll. and no activity against its cysts, but did not include the data.

Chemistry of Marigolds

Marigold roots contain alpha-terthienyl, a light sensitive compound, which reportedly was nematicidal in vitro (Uhlenbroek and Bijloo 1958). The hairy roots of *T.patula* induced by infection with *Agrobacterium rhizogenes* (Riker, Banfield, Wright, Keitt and Sagen 1930) Conn 1942, depending on the isolate, caused a 12.7 fold increase in alpha-terthienyl production. Several fractions of the extract of these crushed roots of *T. patula,* which had been inoculated by *A. rhizogenes* had high nematicidal activity. For the fraction containing alpha-terthienyl, nematicidal activity was 99.2% higher than that

of the lowest activity fraction. But other fractions had 92.5% and 95.2% higher activity than the lowest activity fraction for *Caenorhabditis elegans* and *P.penetrans* (Kyo et al.1990).

Economic Importance and Potential Uses

Marigolds show economic promise on several levels. In agricultural areas, marigold flowers can be harvested for use as a chicken feed coloring agent (Fletcher and Halloran 1983), while the rest of the plant can be plowed under and used as a broad spectrum pesticidal green manure. Therefore, a single crop of marigolds has both a pesticidal, green manure, and cash crop potential. Development of a commercially viable, plant-based pesticide often requires that there are related products from the same process that can be marketed in other areas such as for health purposes. For example, marigolds can be used against pests which cause diseases in humans. Whole plant extracts of *T. minuta* were the most lethal of all three species of *Tagetes* to adults and larvae of the yellow fever mosquito, *Aedes aegypti* (Perich et al.1994). Larvicidal properties of steam distillates from three *Tagetes* species against the third instar of *A. aegypti* persisted at least nine days in the aquatic environment (Green et al.1993). A cercaricidal aqueous infusion of *T. patula* protected snail hosts (*Physa occidentalis*) from the digenetic trematode cercariae, which causes schistosomiasis (Graham et al.1980). A phototoxin extracted from marigolds, alpha-terthienyl, was shown to be extremely insecticidal against mosquitoes, but did not affect nontarget organisms like the ostracod, caddisfly and snail (*Physa* sp.)(Philogene et al. 1985).

If extracts of *T. minuta* were to be used for control of aquatic pests (mosquito larvae) or vectors of disease-causing organisms (schistomiasis), or if the plant was used as a pesticidal

intercrop, the effects of marigolds on the environment and nontarget aquatic fauna must be studied in greater detail before they are widely used as an alternative pesticide.

MATERIALS AND METHODS

Evaluation of Marigold Root Exudates on the Southern Root-Knot Nematode, *Meloidogyne incognita*

Sugarbeet Culture

Sugarbeet seeds (HH88) planted in sand began germinating within eight to twelve days. Seedlings were watered daily and fertilizer (0.9 g of 10-20-10 = Nitrogen:Phosphorus:Potassium in 3.8 l of water) was applied weekly after growth of the first true leaves. Four-week old seedlings were used for this experiment.

Nematode Collection

Juvenile nematodes were collected from cucumber plants previously infected with *M.incognita*. J2 larvae were collected from roots following chopping, then milling with 20 ml tap water in a Waring blender. This solution was poured into two 1000 ml Erlenmeyer flasks (filled up 800 ml) aerated via two polyvinyl tubes (0.95 cm inner diameter) connected by a Y-valve attached to an air outlet. Two glass pipettes were placed at the ends of the tubing inside the flasks and cotton was used to cover the tops of the flasks with Parafilm surrounding the tops. This aeration system was run for 10 days and water was added daily to maintain a constant volume of 800 ml. With this method, juveniles were stimulated to emerge from the macerated tissue.

Marigold Culture

Seeds of *T.minuta* were obtained from Richters Inc., Goodwood; Ontario, Canada-LOC IAO and were originally produced in Australia. The marigold seeds were germinated

(91% germination) in a shallow tray with cells (50 X 30 X 6 cm) (diameter of cell: 4 cm) in vermiculite:perlite (50:50) in a mist chamber in the greenhouse on 20 January 1998. The seedlings were transplanted in 100% sand in 10 cm pots, one per pot, on 5 February 1998 and were again transplanted to 20 cm pots, three per pot on 27 March 1998. Some pots contained 100% sand and others a 50:50 proportion mixture of sand:MSU soil mix, composed of Aquagro 2000G^{R} (a non-ionic organic granular media wetting agent, Aquatrols Corporation of America Inc., Cherry Hill, NJ-08003) mixed 0.1% by weight with 1 part Canadian sphagnum moss, 1 part washed concrete sand and 1 part Bozeman silt loam on a volume:volume basis. Sunlight was the only light source in the greenhouse during this period ranging from an average of 9.5 hrs per day in January to 15.5 hrs per day in June. The plants were watered daily and fertilized (0.9 g of 10-20-10 = N:P:K. in 3.8 l of water) weekly. The average temperature in the greenhouse for 1998 was 24^{0}C during daytime and 18^{0}C at night +4^{0}C and average relative humidity ranged from 20-100% (depending on the watering of plants and seasonality). These marigolds were used for the intact root experiment when they were 5.2 months old.

Inoculation

Eight sugarbeets were planted in forty 20.32 cm (794.68cc) pots, twenty pots with marigold roots and twenty pots without the roots. Pots were arranged randomly on the greenhouse bench. A 4 ml aliquot was taken three times from the solution aerating the macerated root tissue wth galls in the two flasks after 10 days and a mean value of 5.4 ml was calculated to contain 300 juveniles (from the chopped roots). This amount of solution was pipetted inside a depression in the soil made near each sugarbeet plant.

Treatments

Four treatments were used in this experiment with five replicates (20.32 cm pots) in each treatment and eight sugarbeet plants in each pot: (1) Sugarbeets grown in pure sand with 300 juveniles per plant in each pot, (2) Sugarbeets growing in sand with roots of *T.minuta* with 300 juveniles per plant in each pot, (3) Sugarbeets growing in MSU soil mix and sand (50:50) with 300 juveniles per plant in each pot and (4) Sugarbeets growing in sand and MSU soil mix (50:50) with roots of *T.minuta* roots with 300 juveniles per plant in each pot. Four controls were maintained with the same treatments as above except they did not have juveniles. Thus, there were 40 pots total, 20 pots with juveniles and 20 pots without juveniles (controls).

Plants were watered daily and fertilized every week (0.9 g of 10-20-10 = N:P:K. in 3.8 l of water). In the root experiment, two destructive harvests were conducted to assess the nematode infection on the sugarbeet controls and the treatments. The photoperiod in the greenhouse for the intact roots experiment for 45 days (29 June to 14 August) was an average of 14.3 hrs per day.

Evaluation of the First Destructive Harvest (15 days post inoculation)

Four sugarbeets were uprooted, placed inside individual flasks with water and their root characteristics (No. of galls/sugarbeet root, J2s and root length) recorded (Table 1). The roots were chopped and blended in a Waring blender and aerated for 10 days for juveniles to emerge, which were later counted.

Evaluation of the Second Destructive Harvest (45 days post inoculation)

The remaining 4 sugarbeets were uprooted, placed inside individual flasks with water and their shoot (petiole length and total shoot height) and root (gall index, number of galls, J2s, root weight and gall diameter) characteristics were determined (Table 2). Gall index was defined as: 1= 0% sugarbeet roots galled; 2= 1 to 25% sugarbeet roots galled; 3= 26 to 50% sugarbeet roots galled; 4= 51 to 75% sugarbeet roots galled; 5= 76 to 100% sugarbeet roots galled. Roots were chopped up, blended and aerated for 10 days and juveniles counted (at 16X) with a dissecting microscope. (roots were maintained in individual flasks with tap water at 10^0C for 1.5 days until crushing).

Data Analysis

Analysis of variance was calculated on all the recorded data for preharvest and postharvest parameters using a computer-based statistical package, ANOVA (SAS Institute, 1988). The Student-Neuman-Keuls comparison of means of number of J2s, number of galls, gall diameter, gall index, root weight, petiole length, shoot height and number of galls per root wet weight between and within treatments and P-values were obtained.

Evaluation of Marigold Root Exudates on the Sugarbeet Cyst Nematode, *Heterodera scachtii*

Intact Roots Experiment

Sugarbeet and Marigold Culture. Sugarbeets and marigolds were reared by the same methods described in the root-knot nematode experiment under the same environmental conditions.

Cyst Collection. Cysts were collected either from field soils in which sugarbeets previously infected with the nematode had been grown or from sugarbeet stock cultures maintained in the greenhouse. This soil was suspended in a plastic container (19 l bucket), allowed to settle for 10 seconds and the water and remaining suspended soil were washed through a #35 sieve (aperture=0.05 cm^2) and #60 sieve (aperture=0.025 cm^2) (Hubbard Scientific, Chippewa Falls, WI-54729). Material retained on the #60 sieve was collected in a glass bottle. Mean number of cysts were counted in the two aliquots taken from the resulting suspension. Only dark brown, plump cysts were retained for research. Cysts which were cracked and already infected with bacteria and/or fungi or other cysts wholly solidified inside or light brown in color and empty inside were not selected for use. To estimate the initial population of viable eggs and larvae, six cysts were crushed separately using a cyst crusher and viable eggs and larvae counted.

Intact Root Treatments. Eight treatments with 5 replicates were used in this experiment with one pot per replicate. Each pot contained *T. minuta* growing in one of two kinds of soils: pure sand or sand mixed with MSU soil mix in a 50:50 proportion. Tops

(stem and leaf tissue) of these marigolds were removed at the soil level on the day before inoculation and the roots were retained intact in the soil. In each of the replicate pots (20.32 cm-794.68cc), 8 sugarbeets were planted equidistant from each other. Treatments used were: (1) sand with 60 cysts per pot (2) sand in which three *T.minuta* had grown for 2.5 months with 60 cysts per pot (3) MSU soil mix and sand (50:50) with 60 cysts per pot and (4) MSU soil mix and sand (50:50) in which three *T.minuta* plants had grown for 2.5 months with 60 cysts per pot. The remaining four treatments had the same soil treatments but no nematode cysts). Thus, there were 40 pots total.

Inoculation. Plastic photographic slide frames (5.08 cm. X 5.08 cm.) And nylon mesh were used to contain the cysts during the period of the experiment. The nylon mesh (aperture=0.02 cm) was inserted in the frame to retain ten cysts that were placed inside this mesh with a pipette. Mean population initial (p.i.) was 5.92 J2/cc and 0.67 eggs/cc soil. Six slide frames were placed vertically in the soil, all equidistant from the sugarbeet roots in the pot, with the top edge of the slide frame visible.

Plants were watered as needed and fertilized every week (0.9 gm of 10-20-10 in 3.8 l of water). Two destructive harvests were conducted to assess the nematode infection on the sugarbeet controls and the treatments. The photoperiod in the greenhouse for the intact roots experiment lasting 45 days was an average of 14.30 hrs per day.

Evaluation of First Destructive Harvest. (10 days post inoculation). Four sugarbeets were uprooted and the roots were stained using acid fuschin stain solution, heated in a microwave for 30 seconds three times and crushed with a Tissue Homogenizer (TH115 S/N-813 from OMNI International, Gainesville, VA-22065-2304). Juveniles were

counted on a counting chamber (Table 3). Slide frames were removed from pots and placed in separate flasks with tap water. After the number of unhatched cysts was counted in a dissecting microscope at 6.4 power, the frames were placed immediately back in the pots.

Evaluation of Second Destructive Harvest (45 days post inoculation). With the remaining four sugarbeets, the following preharvest criteria were evaluated: petiole length, dry weight of the aboveground parts (shoots=stem and leaves), shoot height and the number of yellowing and wilting leaves (Table 4). At the conclusion of the experiment, sugarbeets were uprooted and placed in individual glass flasks. The wet weight of their roots were recorded (Table 5). Roots were processed as in previous harvest. In addition, 7 aliquots of 8 unhatched cysts each were taken from each treatment (replicates combined). Final populations of the eggs and larvae were determined (Table 5).

Roots Removed Experiment

Sugarbeet Culture. Sugarbeets were the grown in the same tray as described for the intact root experiment, but they were used at the four month stage.

Marigold Culture. Marigolds used were grown in the same tray as for the intact root experiment, but only sand was used in the pots. Marigolds tops were removed at soil level 5.5 months after germination and roots retained in soil in the greenhouse for an additional 1.5 months. The photoperiod in the greenhouse for the dose response experiment for 45 days was average 11.0 hrs per day. Roots were then removed and soil

was mixed with sand at a similar moisture content in the following proportions (5 replicates, 1 pot per replicate, 1 sugarbeet per pot):

Treatments. (1) 100% sand in which *T. minuta* plants had grown (2) 75% sand in which *T. minuta* had grown mixed with 25% sand not exposed to *T. minuta*, (3) 50% sand in which *T. minuta* had grown mixed with 50% sand not exposed to *T. minuta*, (4) 25% soil in which *T. minuta* had grown mixed with 75% plain sand not exposed to *T. minuta* and (5) 0% soil in which *T. minuta* plants had grown mixed with 100% sand not exposed to *T. minuta*.

Cysts were collected from the soil using the same methods as described for hypothesis one. The same criteria was also used to determine viability of cysts. To estimate the initial population of viable eggs and larvae, four aliquots of six cysts each were crushed and eggs and larvae counted.

Inoculation. Slide frames containing 10 cysts were used in the same method as the intact root experiment. Mean population initial (p.i.) was 1.36 J2/cc and 0.13 eggs/cc soil. One slide frame per pot was used. Five controls were used with the same treatments as above except with no cysts. Thus 50 pots total were used in this experiment. Plants were watered as needed and fertilized every week (0.9 gm of 10-20-10=N:P:K in 3.8 l of water).

Evaluation of Destructive Harvest. (45 days postinoculation). A destructive harvest was conducted to assess the nematode infection on the sugarbeet controls and the treatments. The plants were uprooted slide frames were placed separately in petri dishes with water and taken to the lab (Table 6). The roots of the sugarbeets were crushed using

the previously described tissue homogenizer and the population of infective J2s was determined. The number of unhatched cysts was counted from the slide frames. Four cysts each were then taken from the slide frame to count the number of viable eggs and juveniles from them and estimate population final (p.f.).

Data Analysis. Analysis of variance was calculated on all the recorded data for preharvest and postharvest criteria for the intact roots and dose response experiments using a computer-based statistical package, ANOVA (SAS Institute, 1988). Mean comparison tests (Student-Neuman-Keuls) were conducted within and between treatments and P-values were obtained .

Evaluation of Toxicity of Marigold Root Extracts for Aquatic Nontarget Macroinvertebrates

Selection of Test Organisms

Six species of aquatic macroinvertebrates were selected for bioassays during the 15-mo study period (April 1997-June 1998) and were collected from a relatively unpolluted trout stream, Bridger Creek (tributary of the East Gallatin River) (sample site = R6E,T1S, Section31) and a polluted stream on the MSU campus, Campus Creek. Pollution being defined by the EPT (no. of Ephemeroptera, Plecoptera and Trichoptera) richness. *Gammarus lacustris* Sars (Amphipoda), *Lepidostoma pluviale* (Milne)(Trichoptera), *Drunella grandis* (McDunnough) (Ephemeroptera), *Baetis tricaudatis* (Dodds) (Ephemeroptera), *Rhithrogena morrisoni* (Banks) (Ephemeroptera) and *Hydropsyche*

cockerelli (Banks) (Trichoptera) were used for the bioassays. Species determinations were made by Dan Gustafson, Department of Biology, Montana State University. These six species were chosen because they: 1) were abundant, contributed to lotic communities in coldwater streams in SW Montana, were widely distributed in such streams in the western United States; 2) represented different classes and orders; 3) represented different functional feeding groups; and 4) had varying sensitivities to environmental (organic) pollution as shown by the Hilsenhoff biotic index (Hilsenhoff 1987).

Plant Extraction

Seeds of *T.minuta* were germinated in a greenhouse in the Plant Growth Center at Montana State University in September 1992. In February 1993 they were potted, three plants per pot. Roots of each plant in each pot were harvested and extracted in our lab by simultaneous steam distillation and methylene chloride extraction (Weaver et al. 1994) on 28 July 1993 using a Lickens & Nickerson distillation extractor (Kontes Scientific Glassware and Instrumentation, Vineland, NJ). The resulting extracts were stored under N_2 at -20° C in 7 ml glass vials with a Teflon^R-lined screw cap.

Selection Procedures for Individual Test Organisms

Test organisms were collected from the streams mentioned above on the morning of the start of each bioassay using a standard "D"-frame kick net and by "shuffle" kicking the stream substrate. Bioassay organisms were selected only if they swam vigorously and in a normal behavioral pattern for that species and if they met the size criteria for that species. Size criteria were: *G. lacustris* 0.9-1.2 cm; *L. pluviale* 0.4-0.6 cm, case only; *D. grandis* 0.9-1.2 cm; *B. tricaudatis* 0.4-0.6 cm (minus terminal gills); *R. morrisoni*

0.6-0.9 cm; and *H. cockerelli* 0.8-1.2 cm, without terminal gills. Size criteria were used to maximize the probability of selecting individuals of the same life stage, thereby minimizing response variability due to size. We tried as much as possible to obtain organisms of the same size (and thus, the same instar) in all our bioassays. Temperature in the stream was recorded. Macroinvertebrates were then transported to the lab in a plastic bucket filled with water directly from the stream. In the lab, assay organisms were maintained in cool water under a similar temperature range as the stream where they were collected. Selection was made in the lab with a size guide and where normal swim behavior was observed.

Bioassay Facility/Cold Water Bath Environment

The cold water bath facility for aquatic organisms consisted of three 21-liter glass aquaria slightly submerged within three 38-liter aquaria partially filled with flowing city tap water (Figure 1). The 38-liter aquaria were connected to each other with 2.54 cm plastic tubing to allow the water to flow from one 38- liter aquarium to the next, and to flow out of the system from the final 38-liter aquarium (Dunkel and Richards 1998). In the first 38-liter aquarium, an opening was at 1/4 the height from its top edge. The second one had 2.54 cm opening at 1/4 the height from top on one end and 1/2 of the height from top on other end. The third aquarium had a 2.54 cm opening at 1/2 the distance from top on both ends. This system enabled the water to flow easily from one aquarium to another. The tap water was turned on and remained running through the duration of the experiment. Aquarium thermometers were kept in the aquaria and temperatures were monitored every hour during the day time. The test chambers consisted of 125-ml Erlenmeyer flasks,

containing 100 ml of partially dechlorinated tap water, immersed in the 21-liter aquaria. The water was sufficiently dechlorinated by filling a 38-liter aquarium with city tap water and allowing the chlorine to dissipate overnight (ca. 19 hrs). Flasks were filled with 100 ml of partially dechlorinated water and kept in the 21 liter aquaria to chill over the next night (ca. 24 additional hrs). The water used in the bioassays had been dechlorinated for approximately 43 hours. After this process, from a sample, mean (n=3) total chlorine level was interpolated (based on a linear dissipation curve) between 23 and 48 hours to be 0.102 mg/l at 43 hrs. Chlorine was measured with the reagent Carboxylate salt (NJTSRN-80100131-5001) salt of N,N-Diethyl-p-Phenylenediamine (NJTSRN-80100131-5002) Potassium Iodide (7681-11-0) Sodium Phosphate dibasic (7558-79-4) from [Permachem^{R} Reagents]. Total chlorine content was measured colorimetrically (Hach DR/700 Colorimeter, P.O.Box 389, Loveland, CO-80539) at room temperature.

Bioassay Procedures

The full formulation of the root extract was made in the laboratory. This formulation consisted of two parts: a) the extract that resulted from the simultaneous steam distillation/methylene chloride extraction and b) and inert ingredients. As the extract was immiscible in water, it was mixed with inert ingredients before creating the water dilution. The inert ingredients were obtained from Agridyne Inc., Salt Lake City, Utah, U.S.A., as the inert component of Align^{R}. The inert ingredients consisted of substances such as surfactants, a stabilizer (mineral oil), and petroleum solvents (Dunkel and Richards 1998). Concentrations of inert ingredients at 8ppm, 4ppm, 2ppm, 1ppm, 0.1ppm and 0.01ppm were tested on the organisms initially in the fall of 1997. A concentration of inert

ingredient at 0.01ppm was found to be relatively non-toxic. The concentration of the inert was then maintained at 0.01 ppm for subsequent bioassays in which concentrations ranging from 0.4ppm to 8 ppm root extract of *T. minuta* were tested. Eight flasks for each treatment including the controls contained four organisms. Test organisms were added manually to each flask with a forceps and this beginning start of exposure was noted as "time zero". Observations were recorded at 0.25, 1, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72, and 96 hours, or until a significant number of organisms had died. Timings were subject to change depending on the invertebrate response. Behavioral observations varied with individual species, but included normal behavior, specific abnormal swimming movements, moribundity and death. Moribundity was defined as the inability to regain movement of the body after the flask was agitated for 10 seconds. Death was recorded after two consecutive moribundity readings.

Upon termination of each assay, the test organisms were transferred to 70% ethyl alcohol for long-term preservation. Glassware used in the assay was immersed in 25 gm of KOH dissolved in 15 ml water and mixed in 5 liters of isopropyl alcohol for 24 h and then triple-rinsed with hot and cold tap water, and deionized water with detergents (Contrad^{R} or Micron^{R}).

Measurement of Macroinvertebrate Weight and Head Capsules

Dry weights and head capsules of the macroinvertebrates were measured to determine mean organism size and associated variability. Test organisms were taken from their ethanol storage solution and washed with deionized water to remove the ethanol. They were then dried, one treatment per watch glass, in a drying oven under vacuum, at

50°C, until dry (0.5 hrs) . The organisms were then weighed individually or in groups on an electronic analytical balance (Denver Instruments AB 250D). After weighing had been completed, the organism's head capsules were measured with the use of a Leica Wild M3C stereo dissecting scope and an ocular micrometer. The organisms were put back in ethanol solution for storage after recording the measurements for body weight and head capsule width. Different key anatomical features were used for each species. For *G. lacustris* this measurement was the widest distance between the posterior border of the antennal socket and the posterior border of the photosensitive area. For *L. pluviale* this measurement was the gap between medial boundaries of the eye sockets. For *D. grandis* this measurement was the gap between the two projections (tubercles) on the frons. For *R. morrisoni* this measurement was the narrowest gap between medial ocular borders, and for *H. cockerelli*, the longest distance between the ventral margin of the frons and the dorsal depression of the vertex.

Analysis of Marigold Root Extract

The GC/MS analysis of the marigold root extract was performed on a VG 70E-HF double focusing mass spectrometer. The ion source was operated at 200°C, with an electron current of 100 microamperes and the ion acceleration potential at 6000 volts. The mass spectra were recorded at a resolution of 1200, scanning exponentially down over a mass range of 400-45 amu at a scan rate of 0.60 s per mass decade. The Hewlett Packard 5890 Series II Plus gas chromatograph was operated in the splitless mode. It was equipped with a 30 meter HP-5 capillary column with 0.25 mm i.d. and a 0.25 micron film thickness. Ultrahigh purity helium (obtained from a local distributer, Bozeman, MT,

USA) was used as the carrier gas after having been passed through a water trap, molecular sieve traps, and two oxygen traps (Alltech, Deerfield, IL, USA). Column head pressure was ramped with the oven temperature to maintain near constant and reproducible flow through the column. Initial GC oven temperature and hold time were 30°C and 4 min. respectively. Final hold time was 15 minutes. The temperature was increased 5°C per min. to 310°C. The injection port temperature was 260°C and the GC/MS interface temperature was 280°C. The injection volume was 1 microliters via a Hamilton syringe. The GC/MS was controlled by a VG data system utilizing a DEC alpha computer, a VG SIOS interface and VG software. Compound identification was made by a spectral interpretation, and library search with the National Institute of Science and Technology (NIST) database (62,235 compounds).

Statistical Analysis

Probit analyses (Finney 1952) were conducted on all mortality toxicologic data using a computer-based statistical package, Probit procedure (SAS Institute, 1988). The Chi Square probability was determined for each probit line. Statistical analyses included the calculation of probits chi square values, lethal time for mortality of 50% of the bioassayed organisms (LT_{50}) and lethal concentrations for 50% of the bioassayed individuals (LC_{50}). The body weight and head capsule measurements were statistically analyzed using SAS (1988) to obtain an assay mean and standard deviations within species between assays as well as within assays between treatments. Differences among bioassay means were tested for significance using the Student-Newman-Keuls means comparison test.

RESULTS

Effect of Root Exudates of *Tagetes minuta* on *Meloidogyne incognita*

First Destructive Harvest

A statistically higher number of J2s and galls were found in the sand treatment without marigold roots compared to the other treatments ($P < 0.0001$) (Table 1). Sand without marigold roots had the highest number of galls and J2s, followed by sand with marigold roots (two times lower). No differences in J2 and gall numbers were found between the sand and MSU soil mix without marigold roots and sand with marigold roots ($P > 0.05$). Root length was statistically different between all treatments ($P > 0.05$).

Second Destructive Harvest

Sugarbeets in sand without marigold roots had significantly higher number of galls, J2s, gall index, a higher gall diameter and galls per root weight than any other treatments ($P < 0.0001$ except for mean gall diameter where $p < 0.05$; (Table 2). Sugarbeets in sand with marigold roots had a statistically lower nematode infection than in sand without marigold roots for the same categories mentioned above). Statistical differences were observed between the gall indices with sand without marigold roots (having the highest gall index) and sand:MSU soil mix with marigold roots (the lowest gall index)(Figure 2). Sugarbeets in sand without marigold roots had the highest number of galls, gall diameter, galls/sugarbeet g root wet wt. (Figure 3) and J2s (Figure 4), followed by sand:MSU soil mix without marigold roots. Sand:MSU soil mix had the lowest number of galls (and was

also statistically different from other treatments), which also resulted in the lowest gall index. Sugarbeets in sand:MSU soil mix without marigold roots did not have statistically different gall numbers from sand with marigold roots indicating there was an effect of the organic matter in soil on nematode infection and gall formation. The number of J2s were statistically higher in the treatments without marigold roots and statistically lower in the treatments with marigold roots. Statistical difference was observed for shoot height and petiole length between some treatments.

Effect of Root Exudates of *Tagetes minuta* on *Heterodera schachtii*

First Harvest of Intact Root Experiment

The mean leaf length (from expansion of leaf at the end of petiole to leaf tip) was noted for some leaves (and hence not being reported in the tables) in some pots to be ranging from 5-8 cm. Similarly, mean root length was noted for few plants (and hence, not being reported in tables) had a range of 2.5-4 cm. For the first harvest, there were no statistical differences ($P>0.05$) for the number of J2s and unhatched cysts between treatments. The p.f. for the first harvest could not be conducted because the cysts would have to be crushed from the slide frames, which was not possible as they were needed for the second destructive harvest.

Second Harvest of Intact Root Experiment

The population initial (p.i.) obtained at the beginning of the experiment was a mean of 5.95 J2/cc soil and 0.67 eggs/cc soil (Table 5). The population final (p.f.) were significantly different between treatments and p.i.: Mean p.f. (n=56) for sand with *T. minuta* was 3.53 J2/cc and 0.38 eggs/cc soil. Mean p.f. (n=56) for sand 100% was 1.52 J2/cc and 0.16 eggs/cc soil. Mean p.f. (n=56) for sand:MSU soil mix with *T. minuta* was 4.25 J2/cc and 0.48 eggs/cc soil and mean p.f. (n=56) for sand:MSU soil mix (50:50) was 4.52 J2/cc and 0.40 eggs/cc soil. The differences in p.f.'s correlate with the differences in number of J2s and unhatched cysts between treatments. There was a statistically significant decrease (3.5 fold) in the number of J2s in sugarbeets grown in sand in the presence of marigold roots (Table 5). There also was a statistically significant decrease in number of J2s when sugarbeets had the MSU soil mix added to the sand medium and sugarbeets were grown in the same soil with marigold (Figure 5). The number of unhatched cysts observed in all treatments was inversely correlated to the number of J2s observed (Figure 6). I observed the greatest reduction of nematode numbers in the sand treatment with marigold root exudates. Significantly more unhatched cysts were observed in sand:soil mix with marigold than in sand with marigold (Table 5). Sand without marigolds had the highest J2s per gram wet wt. of the roots (Table 5, Figure 7). This treatment was statistically different from the others for the number of J2s in whole roots, unhatched cysts and number of J2s/gram root weight. There was no statistical difference in mean petiole length between treatments with cysts (Table 4). The treatments without cysts had a lower average petiole length than treatments with cysts.

Statistical difference for shoot height was not clearly differentiated between all treatments with and without cysts (Table 4). No statistical difference was observed between treatments for dry top weight. Statistically significant differences were observed between some treatments for the number of yellowing and wilting leaves. Sugarbeets grown in sand:MSU soil mix had longer shoot heights than sugarbeets grown in sand:MSU soil mix with marigold roots. In some pots, a few marigold roots were observed to be regenerating new leaves. Some cysts were observed on the roots for some plants in various treatments inoculated with the nematode, but were not counted. Also, after crushing the roots for counting, the J3s and J4s were could not be properly observed and were not counted.

Destructive Harvest of Roots Removed Experiment

The population initial (p.i.) was a mean of 1.36 J2/cc soil and 0.13 eggs/cc soil (Table 6). The population final (p.f.) was statistically different in a comparison to the p.i.'s.: Mean p.f. (n=5) for sand (100%) with *T. minuta* (0%) was 0.61 J2/cc and 0.061 eggs/cc soil. Mean p.f. (n=5) for sand (75%) with *T. minuta* (25%) was 0.67 J2/cc and 0.067 egg/cc soil. Mean p.f. (n=5) for sand (50%) with *T. minuta* (50%) was 0.70 J2/cc and 0.07 eggs/cc soil. Mean p.f. (n=5) for sand (25%) with *T. minuta* (75%) was 0.81 J2/cc and 0.08 eggs/cc soil and Mean p.f. (n=5) for sand (0%) with *T. minuta* (100%) was 0.85 J2/cc and 0.08 eggs/cc soil. The differences in the p.f.'s between treatments could be due to the statistically insignificant increments in number of unhatched cysts and a decrease in number of J2s in roots as one progressed from sand (100%) with *T. minuta* (0%) to sand (0%) with *T.minuta* (100%). No significant differences were observed for the number of J2s, J2s from unhatched cysts, root wet wt. and number of J2s per root wet

wt. between and within all the treatments. Some cysts were observed on the roots of plants in various treatments inoculated with the nematode, but were not counted.

Aquatic Macroinvertebrate Toxicity to *Tagetes minuta* Root Extract

In the studies conducted in early spring 1997 to late spring 1998 (Table 7), LC_{50}s for the full formulation of marigold root extract ranged from 0.232 to 6.91 ppm at 96 h for five species of aquatic macroinvertebrates. Both the lowest and highest LC_{50}s were obtained for *D. grandis*, with the most sensitivity observed for this species in spring and the least sensitivity observed in fall. Other differences noted in these particular bioassays were that individuals in the more sensitive sampled population of *D. grandis* (spring 1998) were 11.4 times larger in dry body weight than those of the fall 1997 sampled population. The size relationship with sensitivity to formulated marigold root extract was more predictable with the two trichopteran species, *L. pluviale* and *H. cockerelli* (Table 7). The smaller trichopteran, *L. pluviale*, was 5 times more sensitive and 5.7 times smaller than the larger trichopteran, *H. cockerelli*. With *G. lacustris,* only a few test subjects in inert 0.01ppm and marigold extract at 1ppm succumbed to the formulation in spring 1998 (and none of the methylene chloride subjects) and so it was not possible to run an LC_{50} on the spring 1998 data and obtain a full set of fiducial limits. Repetitions of the LC_{50} determinations within species, within season were not significantly different, for example, in the bioassays initiated 8 and 22 September 1997 for *G. lacustris* (Table 7). Although the ppm of the inert used in these two assays was different (0.1ppm on 8 Sept 1997 and

0.01 on 22 Sept 1997), the LC_{50} did not show the small differences in toxicity that were apparent in the LT_{50} values (Table 8). For example, the LT_{50} for 4ppm full formulation (0.1ppm inert) was 6.5 hrs as compared to 16.2 hrs for that of 4ppm full formulation (0.01ppm inert). In our preliminary assay, *R. morrisoni*, was extremely sensitive. We, therefore, were not able to obtain LC_{50} data and conducted the assays for this organism with only two treatments.

Development of a formulation began with finding a material that would allow the marigold extract in methylene chloride to mix with the water environment of the macroinvertebrates. It was established that methylene chloride at concentrations far exceeding those in the assays would not affect *G. lacustris* (Table 7). We conducted a set of 3 assays (Table 8) on 27 Aug. 1997, 1 Sept. 1998, 8 Sept 1998) to establish the level of commercial inert ingredients from another plant extract pesticide (Align^{R}) that would be effective. The 27 August 1997 assay established that these inert ingredients are strong toxins. In the 1 Sept. 1997 assay, the marigold root extract was mixed with inert material 15 hours before testing. The marigold extract at 4ppm full formulation (0.1 ppm inert) had only a small effect (LT_{50} =147 hr), whereas the 0.1ppm inert ingredients prepared immediately before testing had a toxic effect (LT_{50}=11 hr). Concentrations used in other studies (4ppm) with related organisms (Dunkel and Richards 1998) and the 4 lower concentrations that we used (the lowest being 0.1 ppm) resulted in LT_{50}s of less than 12 hours (Table 8) and 100% mortality within the 96 hour assay. On 8 September 1997, we found that the 0.01 ppm inert treatment was not toxic to *G. lacustris*, and proceeded to use this formulation with the root extract to test other macroinvertebrate species. All other

species tested had LT_{50}s that were less than 96 hours when placed in an environment with 0.01 ppm inert ingredients (Table 8).

Formulated root extract of *T. minuta* caused mortality to the organisms tested, most of which might be explained by the action of the inert. For example, with *D. grandis* the addition of root extract of *T. minuta* to the formulation (0.4 to 4.0 ppm), did not substantially change the LT_{50s} (Table 8).

Chemical Composition of Root Extract of *T. minuta*

Compounds that matched with the NIST library at a purity of over 900 were: azulene, naphthalene, cyclohexene, 2,2':5',2"-terthiophene, heptadecane, hexatriacontane, octadecane, nonadecane, 2,2'-bithiophene, palmitic acid, 2-norpinene, 2-beta-farnesene, benzo(a)phenazine, pentadecanal.

Macroinvertebrate Response to Assay Environment (Controls) and Residual Chlorine

All tested organisms except *R. morrisoni* exhibited no mortality response to the control environment (partially dechlorinated water). The mean total chlorine in water passively dechlorinated for 23 h was 0.330 mg/l and for passively dechlorinated for 48 h was 0.046 mg/l. Water used in our experiments was placed in an aquarium for 24 h for passive aeration and then placed in the bioassay flasks for approximately 19 h to chill overnight in the cold water bath system of aquaria.

Macroinvertebrate Toxicity of Inert Ingredients in Formulation

Inert ingredients were found to be the most toxic compounds in the formulation. LT_{50}'s of the inert concentrations 0.01ppm to 4ppm ranged from 1.50 to 42.54 hrs in the ephemeropterans tested, *B. tricaudatis, R. morrisoni* and *D. grandi. R. morrisoni* was the