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Abstract

The fate of the phototoxic phytochemical, α-terthienyl (α-T), which has been proposed as a novel mosquito larvicide was studied in target and non-target organisms. This study characterized the sensitivity of potential non-target organisms towards α-T in a controlled laboratory setting and assessed how the extent of exposure in the natural environment altered species sensitivity. The lethality of this compound was determined in the laboratory by uniform acute toxicity bioassays using the target mosquito larvae, *Aedes atropalpus*, and two non-target species. *Daphnia magna* represented a potentially sensitive non-target organism and *Limnephilus indivisus* (caddisfly) larvae represented a potentially insensitive non-target species. The mosquito larvae had a lethal concentration for 50% mortality (LC_{50}) value determined to be 31.97 ppb. However, *D. magna* showed an order of magnitude more sensitivity towards α-T with an LC_{50} of 1.74 ppb while *L. indivisus* larvae showed considerably less sensitivity at 64.76 ppb.

The role of temperature in acute phototoxicity was also studied. *Aedes atropalpus* was used for acute toxicity bioassays to examine the effect of temperature on lethality of α-T. Two extreme temperatures were tested, 7.5°C and 25°C. A comparison of the LC_{50}, LC_{90} values and the slopes of the regression indicated that there was no significant difference between the acute phototoxicity of α-T at the temperatures examined.

The bioaccumulation potential was investigated in the three species by toxicokinetic studies. The absorption or accumulation of ^{3}H-α-T via uptake from water into the animal's body and its subsequent elimination by whole body clearance was monitored for 96 hours in darkness. All three species exhibited two-compartment kinetics. The first compartment was rapid while the second was considerably slower. Initial rates of accumulation by the first compartment were highest for *A. atropalpus*, followed by *D. magna* and *L. indivisus*. *A. atropalpus* reached a steady state in the second compartment. The accumulation of radiolabeled (^{3}H) α-T did not plateau in the non-target species. The clearance rate constants were highest for *D. magna* and lowest for *L. indivisus*, indicating that the most sensitive species was eliminating α-T the fastest,

in contrast with previous studies. Factors such as light penetration and body size (surface to volume ratio) were most likely as important in determining α-T toxicity as excretion.

A fluorescent metabolite of α-T was identified in *Aedes atropalpus* larvae and its production was monitored for 72 hours. This is the first time a metabolite of α-T has been identified in insects. The compound, bis-thiophene carboxylic acid or BCA was identified by spiking an extract of mosquito larvae with synthetic BCA and detecting the peak by fluorescence detection following high pressure liquid chromatography. The production of BCA in the mosquito larvae remained constant from 12 to 72 hours after exposure to α-T, suggesting a steady state condition for α-T metabolism in mosquito larvae.

A 10 day field study of radiolabelled α-T (^{3}H-α-T) monitored the fate of the radioactivity in microcosms containing sediment, water and biomass collected from a nearby snow melt pool. Approximately 70% of the radiolabel was unaccounted for and it was assumed that the loss was due mostly to volatilization from the water surface. Of the 30% that was recovered, most of the radiolabel remained in the water throughout the study. In the sediment, distribution appeared to follow a biphasic pattern. On days 2 and 8 of the study, two maxima of radiolabel were observed. The biomass, consisting of *Aedes intrudens, Daphnia magna,* and *Limnephilus indivisus,* accumulated the least amount of radiolabel. The amount of radiolabel per body weight was highest in *D. magna* and lowest in the caddisfly larvae; however, the percent of the total amount was highest in the caddisfly larvae while lowest in the *Daphnia.* The proportions of radiolabel decreased with time for all three species.

The results from the above studies, combined with previous field trials, predicted mortality at the applied concentrations. This did not translate into severe impacts in the fate study under natural conditions, probably due to volatilization, presence of dissolved organic matter in the water, and rapid photodegradation of α-T. In addition, the microcosms were highly anoxic relative to the natural pools. This was most likely the result of bacterial growth due to the disturbance of the sediment.

Résumé

Le destin d'un composé phototoxique et phytochimique, α-terthienyl (α-T), proposé comme un nouveau larvicide contre les moustiques a été étudié dans des organismes non-ciblés et ciblés. Cette étude cherche à caractériser la sensibilité d'organismes non-ciblés potentiels envers α-T dans un environnement de laboratoire contrôlé et de déterminer comment l'étendue d'une exposition dans un environnement naturel change la sensitivité de l'espèce. La fatalité de ce composé a été déterminée en laboratoire par l'entremise de bioessais toxiques immédiats utilisant la larve de moustique cible, *Aedes atropalpus*, et de deux espèces non-ciblées. *Daphnia magna* représente un organisme non-ciblé potentiellement sensible et la larve *Limnephilus indivisus* (caddisfly) représente une espèce non-ciblée potentiellement insensible. La larve de moustique a une concentration fatale pour un taux de mortalité de 50% (LC_{50}) d'une valeur de 31.97 ppb. Toutefois, *D. magna* démontre un ordre de grandeur plus élevé de sensibilité envers α -T ayant une valeur LC_{50} de 1.74 ppb tandis que la larve *L. indivisus* démontre une sensibilité considérablement plus petite de 64.76 ppb.

Le role joué par la température sur la phototoxicité immediate a aussi été étudié. *Aedes atropalpus* a été utilisé au cours de bioessais toxiques immédiats dans le but d'examiner les effets de la température sur la fatalité de α-T. Les deux températures extrêmes, 7.5 °C et 25 °C, ont été étudiées. La comparaison des valeurs entre LC_{50} et LC_{90} et la pente de la régression linéaire démontre aucune différence significative entre la phototoxicité immédiate de α-T et les températures étudiées.

La bioaccumulation potentielle a été étudiée pour les trois espèces par l'entremise d'études toxicocinétiques. L'absorption ou l'accumulation de ^{3}H-α-T via l'ingestion d'eau dans le corps animal et l'élimination subséquente a été suivi pendant 96 heures dans l'obscurité. Les trois espèces ont démontré une cinétique à deux compartiments. Le premier était rapide tandis que le deuxième était considérablement plus lent. Le taux d'accumulation initial pour le premier compartiment était plus élevé pour l'espèce *A. atropalpus*, suivi par *D. magna* et *L. indivisus*. *A. atropalpus* a atteint un état constant

dans le second compartiment. L'accumulation de ^{3}H-α-T n'a pas atteint de plateau pour les espèces non-ciblées. La constante de vitesse d'évacuation était plus élevé pour *D. magna* et plus faible pour *L. indivisus*, indiquant que l'espèce la plus sensible avait un taux d'élimination de α-T plus élevé, ce qui est contradictoire avec des études antérieures. Des facteurs tel que la pénétration de la lumière et la grandeur corporelle (rapport entre la surface et le volume) sont probablement des facteurs aussi important dans la détermination de la toxicité de α-T que l'excrétion.

Un métabolite fluorescent de α-T a été identifié au sein de la larve *Aedes atropalpus* et sa production a été suivie pendant 72 heures. Ceci constitue la première fois où un métabolite de α-T a pu être identifié chez les insectes. Le composé, acide carboxilique bis-thiophène ou BCA a été identifié en l'incorporant au sein de la larve de moustique et en détectant le pic par l'entremise de détection par fluorescence après chromatographie liquide à pression élevée. La production de BCA chez la larve de moustique demeurait constante entre 12 et 72 heures après avoir été exposé à α-T, ce qui suggère une condition d'état constant pour le métabolisme de α-T au sein de la larve de moustique.

Une étude de 10 jours du composé, ^{3}H-α-T, cherchait à suivre le destin de la radioactivité chez des microcosms contenant des sédiments, de l'eau et de la biomasse. Approximativement 70% de la substance radio-isotopique n'a pu être détecté et cette perte a été attribuée en grande parti à la volatilisation possible de par la surface de l'eau. Des 30% récupérés, la plupart des radio-isotopes demeuraient dans l'eau au cours de l'étude. Dans les sédiments, la distribution semblait suivre un motif biphasique. Au cours des jours 2 et 8 de l'étude, deux maxima de radio-isotope a été observés. La biomasse, composée de *Aedes intrudens*, *Daphnia magna* et *Limnephilus indivisus*, accumulait la quantité la plus faible de radio-isotope. La quantité de radio-isotope par poids corporel était la plus élevée chez l'espèce *D. magna* et la plus faible pour la larve caddisfly; toutefois, le pourcentage de la quantité totale était la plus élevée chez la larve caddisfly et la plus faible pour *Daphnia*. Les proportions de radio-isotope diminuaient en fonction du temps pour les trois espèces.

Les résultats de la présente étude en combinaison avec des essais antérieures prédisaient la mortalité aux concentrations appliquées. Cependant, dû probablement à la volatilisation, la dissolution d'une certaine quantité de matière organique en phase aqueuse et à la photodégradation rapide de α-T, les conditions naturelles de ces études n'ont pas démontré d'impacts sévères. De plus, les microcosms étaient relativement très anoxique par rapport au bassin naturel. Ceci serait probablement le résultat de croissances bactériennes causées par la perturbation des sédiments.

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α-T	α-terthienyl
BCA	bis-thiophene carboxylic acid
BOD	biological oxygen demand
DDT	dichlorociphenyltrichloroethane
DNA	deoxyribonucleic acid
DOC	dissolved organic carbons
DOM	dissolved organic matter
dpm	disintegrations per minute
EC	emulsifiable concentrate
EC_{50}	effective concentration at which 50% percent of organisms respond
GSH	reduced glutathione
GSSH	glutathione disulphide
GST	glutathione S-transferase
^3H	tritium
HPLC	high pressure liquid chromatography
$h\nu$	a photon of light
k_e	rate constant of elimination
k_u	uptake rate constant
K_{ow}	coefficient of hydrophobicity or octanol-water partition coefficient
LC_{50}	lethal concentration at which 50% mortality occurs
LC_{90}	lethal concentration at which 90% mortality occurs
LD_{50}	lethal dose at which 50% mortality occurs
LFP	laser flash photolysis
LIOAC	laser induced optoacoustic calorimetry
MCA	mono-thiophene carboxylic acid
MFO	mixed function oxygenase system
O_2	ground state oxygen

$^1O_2(^3\Sigma)$	excited singlet state of oxygen
$O_2^{\cdot}(^1\Delta)$	superoxide anion
O.D.	optical density
PAH	polyaromatic hydrocarbons
PSMO	polysubstrate monooxygenase system
Φ	quantum yield of singlet oxygen generation
QSAR	quantitative structure activity relationship
RNA	ribonucleic acid
Sens	sensitizer
S_o	ground state sensitizer
S_1	first excited state of a sensitizer
S_n	successive higher excited states of a sensitizer
T_1	triplet state of a sensitizer
τ	lifetime or half-life of a molecule
TDS	total dissolved solids
UV	ultraviolet radiation

1.0 Introduction

1.1 Origin of α-Terthienyl

Many plants have phototoxic phytochemicals as a form of defense against damage by insects and diseases. Phototoxic phytochemicals are secondary metabolites that require light to fully express their toxic potential [Towers 1984]. Phototoxic plants are distributed worldwide in many plant families, but are most frequent in the tropics where they are exposed to the most intense sunlight throughout the year [Downum 1986].

Polyacetylenes and thiophenes are phototoxic phytochemicals commonly found throughout the Asteraceae (daisy) family of plants [Downum 1986, Towers 1984]. There are over one thousand examples of these compounds in the Asteraceae and in some fungi. Their structures may be straight chain or cyclic [Bohlmann *et al.* 1973, Lam *et al.* 1988, Towers and Arnason 1988]. In plants, the compounds may be found in the leaves, flowers, seeds, stems or roots [Towers 1984, Towers and Arnason 1988]. Sunlight increases the toxicity of the compounds. They usually have a maximum absorbance in the UV-A (320-400 nm) region [Edelson et al. 1987, Epstein 1989].

In 1947, a thiophene was isolated from the petals of the yellow African marigold, *Tagetes erecta* [Zeichmeister and Sease 1947]. This compound is a cyclic sulphur derivative from a 12-carbon polyacetylene [Arnason *et al.*, 1981*a*, Towers and Arnason 1988]. The compound was identified as 2,2':5',2"-terthiophene, more commonly known as α-terthienyl or α-T (Figure 1.1) [Arnason *et al.* 1987]. The compound has been found in many genera including *Flaveria, Eclipta, Dyssodia, Nicolletia, Chrysactina,* and *Adenophyllum* at concentrations ranging from 20 μg/g to 440 μg/g dry weight [Downum *et al.* 1985, Arnason *et al.* 1983]. α-T discourages herbivory of the leaf [Arnason *et al.* 1989], has exhibited nematocidal activity in the roots [Munakata 1979, Gommers and Bakker 1988], and is an allelochemical towards pathogens such as bacteria and fungi in the seeds [Downum 1986, Towers and Arnason 1988].

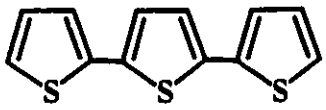


Figure 1.1. Structure of α-Terthienyl.

1.2 Photochemical Properties

Sensitizers are organic molecules that are stable in the dark [Spikes 1989]. However, when light from the visible or near UV range of the spectrum reaches the molecule it becomes excited. A photon of light is absorbed by the ground state (S_o) sensitizer promoting electronic excitation to the first excited (S_1) or successive higher excited (S_n) states (Figure 1.2). Excited states are unstable and the energy must be dissipated. The singlet state has a very short lifetime (~ 1-100 ns) making it unlikely to react with another molecule. There are several mechanisms available to transfer this energy. If a second or higher excited state is involved, the energy will first undergo non-radiative internal conversion to shift from the lowest vibrational level of the higher electronic state (for example, S_2) to the highest vibrational level of the lower electronic state (S_1). Then the excited singlet (S_1) can release its radiative energy as fluorescence and return to its ground state (S_o). S_1 can also intersystem cross (a non-radiative process) to the triplet state (T_1 or T_n) which has a longer lifetime (microseconds to seconds) due to its parallel electron spins [Grossweiner 1989, Spikes 1989]. The triplet state can also emit light upon return to the ground state as phosphorescence. Triplet excited states (T_1) have a relatively long lifetime compared to singlet excited states because of a small transition dipole connecting T_1 to the ground state, S_o. The triplet state is of particular interest since it can undergo chemical reactions with other, non-excited molecules including

photosensitization [Grossweiner 1989]. Thus, a triplet state sensitizer can initiate reactions to form products and in the process, the ground state photosensitizer is regenerated. Since the sensitizer is usually not altered in the overall reaction, it can be considered a catalyst [Spikes 1989]. The compound α-terthienyl is an excellent triplet photosensitizer since it can sensitize the formation of singlet oxygen ($^1\Delta$) from ground state oxygen ($^3\Sigma$) through a well known Type II process (Figure 1.3) [Bakker *et al.* 1979, Arnason *et al.* 1981*a, b*, Scaiano *et al.* 1987]. Type I reactions, which can form radical intermediates (Figure 1.3), may take place in the absence of oxygen but is strongly dependant on the substrate present and/or the solvent [Evans and Scaiano 1990].

Singlet oxygen is highly electrophilic, allowing it to react with electron donating substrates resulting in oxidation of the substrate [Spikes 1989]. The lifetime (τ) of singlet oxygen is strongly solvent dependent. For example, the lifetime of $^1O_2$ in H_2O is 2 μsec, while it is 700 μsec in CCl_4 [Turro 1978]. Singlet oxygen will react with free fatty acids, cholesterol and some amino acid side chains (such as histidine and methionine) *in vitro* [Spikes 1989, Foote 1987, 1976]. These biomolecules are involved in the *in vivo* mode of action of α–T as well [Hasspieler *et al.* 1990, Sen *et al.* 1990]. Quenchers *in vivo* such as β-carotene, ascorbic acid, and α-tocopherol can quench $^1O_2$ directly or compete in the photosensitization process [Foote 1987].

The rate of Type II reactions depends mostly on the concentration of oxygen and solvent since oxygen dissolves more readily in organic solvents than aqueous solutions. Thus the competition between substrate and oxygen for the triplet sensitizer determines which process will occur and in what proportions. If the rate of reaction between molecular oxygen and sensitizer is higher than the rate for substrate and sensitizer, the Type II process will dominate. If the reverse conditions are met, Type I reactions may occur (see Figure 1.3) [Foote 1976].

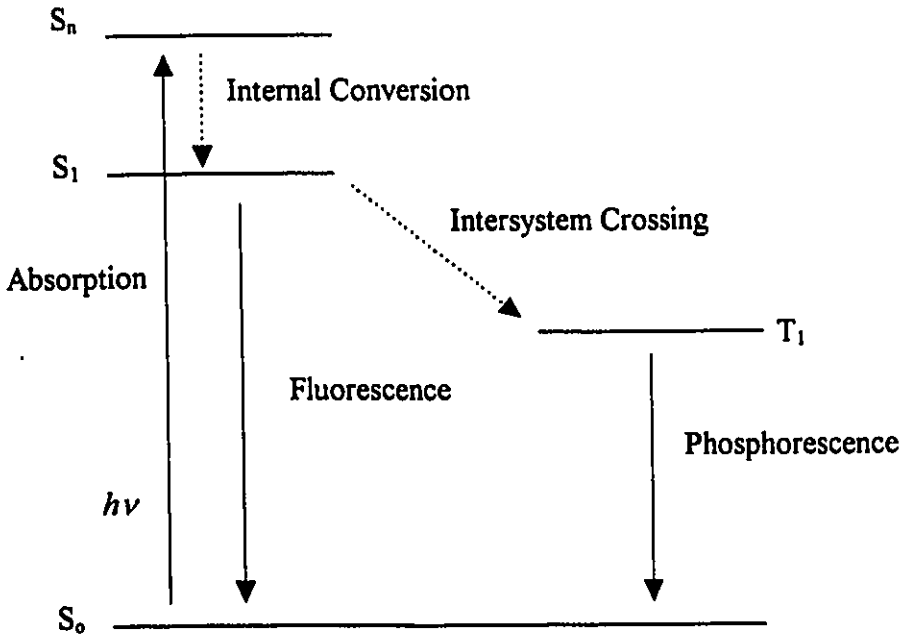


Figure 1.2. A simplified Jablonski diagram of an organic molecule when excited by a photon of light showing the possible pathways for energy transfer and emission. S_o, S_1, S_n = various singlet states; T_1 = triplet state, $h\nu$ indicates a photon of light. Dashed lines represent non-radiative energy, solid lines represent radiative energy [from Coyle 1986].

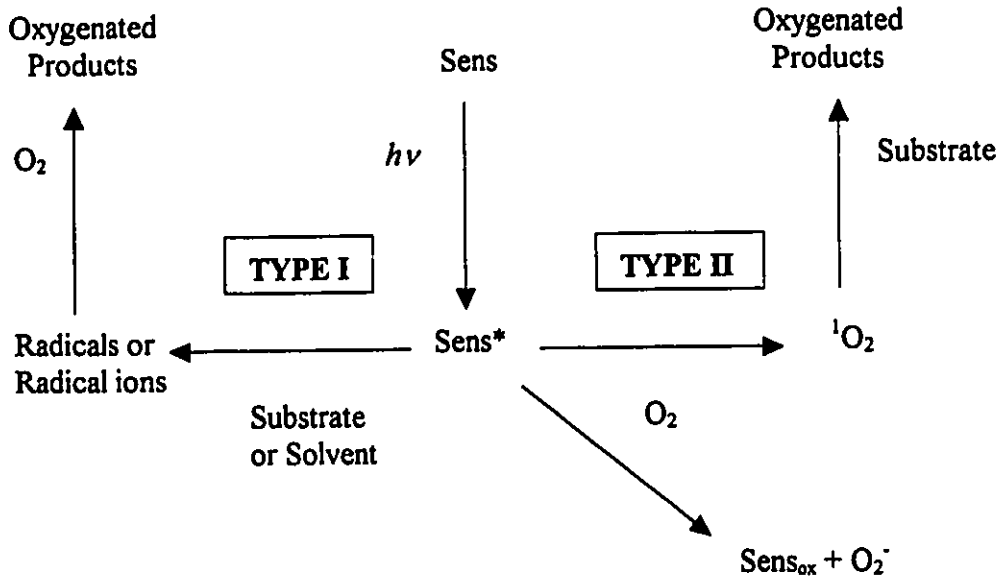


Figure 1.3. Possible mechanisms by which a triplet state sensitizer (Sen*) can oxygenate products. Sens = sensitizer, ,O_2 is ground state oxygen, $^1O_2$ is the excited singlet state of oxygen, and O_2^- is the superoxide anion [from Foote 1991]. Note that α-T is mainly a Type II photosensitizer.

Most evidence for singlet oxygen formation by photosensitization is obtained indirectly by chemical trapping and quenching studies [Foote 1976]; or directly through monitoring the $^1O_2$ luminescence which is centred at 1.27 μm using laser flash photolysis (LFP) techniques. The quantum yield of $^1O_2$ can readily be determined using the latter technique [Foote 1987].

The phototoxicity of α-T has been characterized by laser flash photolysis in the past, but recently laser induced optoacoustic calorimetry (LIOAC) has become preferable. Scaiano *et al.* [1990] studied the efficiency of singlet oxygen production by α-T excitation and found that there was 90% efficiency in polar and nonpolar solvents for intersystem crossing and the quantum yields (Φ) were in the range of 0.6 to 0.8. α-T does not show any significant phosphorescence [Scaiano *et al.* 1990]. The quantum yield of singlet oxygen formation depends on the solvent used [Kagan *et al.* 1989]. In chloroform-*d*, Φ = 0.86, which is the accepted mean value for singlet oxygen generated by α-T. This is an efficiency of 86% which indicates α-T is an excellent photooxidant [Arnason *et al.* 1989]. The efficiency is related to intersystem crossing quantum yields [D'Auria and Vantaggi 1991].

There have been many derivatives and analogs of α-T synthesized. Their quantum yield values range from 0.53 to 0.93 which indicates they are all good photosensitizers [Scaiano *et al.* 1987]. This variation is small, however the toxicity of analogs and derivatives varies widely which indicates that other factors are also important. Over 60 α-T analogs and derivatives have been prepared [Arnason *et al.* 1989]. Some derivatives are more efficient photooxidants than α-T and it has been suggested that perhaps these compounds can be considered a new class of singlet oxygen sensitizers [D'Auria and Vantaggi 1991]. When derivatives are monosubstituted with small functional groups such as CH_3, the compounds are highly active, however di-substitution and bulky halogens (heavy atoms) reduce activity [Evans *et al.* 1986, MacEachern *et al.* 1988, Marles *et al.* 1991*b*].

The biological activity of α-T depends on its physico-chemical properties [Marles *et al.* 1991*a*]. While the lifetime of singlet oxygen is relatively long (μs), the lifetime of

singlet α-T is very short (<1 ns); therefore α-T must be close to the site of action for singlet oxygen to have any effect [Evans *et al.* 1986, Marles *et al.* 1991*b*; Gorman and Rogers 1989]. High quantum yields of triplet formation and singlet oxygen generation characterize thiophene derivatives. This seems to explain the phototoxicity of α-T [Reyftmann *et al.* 1985, Scaiano *et al.* 1989]. However, biological activity varies much more than does singlet oxygen yield [Scaiano *et al.* 1987].

Hydrophobicity is a property that is generally measured as the partition coefficient of a compound between octanol and water. Octanol is used because it behaves like lipids giving a good laboratory estimate of hydrophobic behavior [Farrington 1989]. This property of a compound is very important for passive transport of a biologically active compound like α-T, from its source to the target. Hydrophobicity regulates the interaction of bioactive compounds with their bioreceptors and is more positively correlated with phototoxicity than quantum yield of singlet oxygen. In a quantitative structure activity relationship (QSAR) study the combination of hydrophobicity and singlet oxygen yield were found to account for over 80% of the variation in phototoxicity to mosquito larvae of 14 α-T derivatives and analogs. The coefficient of hydrophobicity (K_{ow}) of α-T is 5.70 [Marles *et al.* 1991*b*]. This indicates that both receptor-mediated and membrane oxidation mechanisms are important. [Marles *et al.* 1991*a*].

1.3 Mode of Action at the Cellular and Tissue Level

Phototoxic plants produce photosensitizers which can have two modes of action, photogenotoxicity and photooxidation. Phototoxins can penetrate cells and react with the organelles, including nuclei [Towers 1984]. Photogenotoxins interact with DNA or RNA causing chromosomal aberrations that can cause mutations or have lethal effects. [Aucoin *et al.* 1992, Song and Tapley 1979]. Photooxidants damage cell membranes by producing highly reactive singlet oxygen that oxidizes its biomolecular components. This leads to a total breakdown of the membrane and cell lysis.[Towers and Arnason 1988, Downum 1986, Towers 1984, MacRae *et al.* 1980].

For photodynamic action to occur, three criteria are required: a biological system, radiation, and a compound that absorbs the radiation in the system. The radiation, or light energy, of a particular wavelength is absorbed by the sensitizer and through a series of reactions, chemically alters other molecules, substrates, in the system [Spikes 1988].

There are many organic molecules in living organisms that are affected by photosensitization. To a degree, all the major cellular components are affected. Both reaction types (I and II) are involved, depending on the sensitizer, biomolecule, oxygen concentration, and reaction conditions [Spikes 1989]. Singlet oxygen, free radical formation and the superoxide anion all can cause extensive damage [Lee and Rogers 1987, Spikes 1988, 1989]. DNA and RNA are susceptible to both Type I and Type II photosensitization [Spikes 1989]. Proteins (including enzymes) can be affected by either Type I or Type II photosensitization. The overall conformation is affected by cross-linking. Five of the twenty essential amino acids can be degraded by photosensitization. Cysteine, histidine, methionine, tryptophan, and tyrosine can be damaged from chemical changes to their side chains. Conformation changes can denature enzymes [Spikes 1988, 1989]. Other biomolecules, such as carbohydrates and hormones are also photooxidized by Type I or II mechanisms [Spikes 1988].

Lipids are a diverse group of olefins that include free fatty acids, triglycerides, phospholipids (membranes), glycolipids, steroids (including cholesterol) and all derivatives. Only unsaturated lipids (those containing double bonds) are susceptible to photosensitization [Spikes 1988]. Most reactions are Type II, but Type I can also occur. [Spikes 1989]. Most biological effects involving lipids results in membrane damage by photooxidation [Foote 1976].

α-Terthienyl is known to accumulate in the lumen of the Malpighian tubules and in the midgut epithelium of mosquito larvae (*Culex tarsalis*) [Hasspieler *et al.* 1988]. In the tobacco hornworm, *Manduca sexta*, the columnar cells lining the anterior and middle regions of the midgut are punctured and appear 'pock-marked' due to damage of the cell membrane. This releases the contents of the gut into the hemocoel [Sen *et al.* 1990]. The intracellular target of α-T by photosensitization are lysosomal membranes [Sasaki *et al.* 1993]. The photooxidation by α-T in insect tissues results in oxidative stress. This is

manifested by lipid peroxidation and the oxidation of reduced glutathione (GSH) to glutathione disulphide (GSSH) [Hasspieler *et al.* 1990].

1.4 Insect Defenses Against Photooxidation by α-Terthienyl

There are two types of defense mechanisms against damage by α-T, enzymatic and non-enzymatic. Detoxification enzyme systems can biotransform or metabolize a lipophilic xenobiotic into a more hydrophilic compound that can be excreted [Sipes and Gandolfi 1991]. The cytochrome P-450 system (also known as the polysubstrate monooxygenase system (PSMO) or the mixed function oxygenase (MFO) system) is the primary enzyme system that transforms lipophilic toxins to more hydrophilic compounds by adding a hydroxyl moiety to the compound. A secondary enzyme system involves conjugation of glutathione (GSH) to xenobiotics by glutathione transferase [Hasspieler *et al.* 1990, O'Brian 1988]. The detoxification enzymes reduce the hydrophobicity of the toxicant and enable organisms to excrete a more hydrophilic toxin rather than allow it to cause cellular damage or to bioaccumulate in the organism's body [Sipes and Gandolfi 1991]. Certain insects that are phototoxin tolerant such as *Ostrinia nubilalis, Anaitis plagiata,* and *Heliothis virescens* are resistant to the phototoxic effects of α-T due to the elevated levels of such detoxification enzymes [Iyengar *et al.* 1990, Aucoin *et al.* 1991, Feng *et al.* 1993, Fields *et al.* 1991, Arnason *et al.* 1989].

Certain compounds can prevent the detrimental effects of α-T by quenching free radical formation and singlet oxygen generation. These compounds are called antioxidants and when ingested, they can provide a non-enzymatic mode of defense against α-T. Three compounds in particular are effective. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), and β-carotene are all potent quenchers of singlet oxygen and prevent lipid peroxidation by α-T [Halliwell and Gutteridge 1985, Hasspieler *et al.* 1990]. The effectiveness of the three antioxidants has been shown *in vitro* and *in vivo* using Lepidopteran larvae that are normally sensitive to Type II phototoxins [Aucoin *et al.* 1990, McRae *et al.* 1985,].

1.5 Uses of α-Terthienyl

Although α-T has effects on a broad range of organisms at various doses [Arnason *et al.* 1992], it has been of particular interest as a phototoxic larvicidal agent due to its phototoxic effects at very low concentration on mosquito larvae, blackfly larvae, and certain herbivorous insects [Arnason *et al.* 1981*b*, Philogene *et al.* 1985, Iyengar *et al.* 1987]. The toxicity of α-T to mosquito larvae varies according to the species. LC_{50} values can range from 32 ppb for 4th instar *Aedes atropalpus* larvae and 20 ppb for late 4th instar (4 ppb for late 3rd instar) *Aedes aegypti* to 16 ppb for a 3rd instar malathion resistant strain of *Culex tarsalis* and 12 ppb for a sensitive strain of *Cx. tarsalis* [Arnason *et al.* 1981*b*, Hasspieler *et al.* 1988, Marles *et al.* 1991*b*]. The malaria vector *Anopheles stephensi* is also very sensitive. The LC_{50} for 3rd instar larvae is 14 ppb [Hasspieler *et al.* 1988]. α-T is also highly phototoxic to blackfly larvae (*Simulium verecundum*) with an LC_{50} of 28 ppb [Philogene *et al.* 1985]. Blackfly larvae, however, occur in streams, not stagnant ponds like mosquitoes. By applying α-T to a fast moving stream, there is a potential to impact a large number and variety of invertebrates before degradation. This non-selective drift was shown to impact on non-target aquatic invertebrates in the field [Dosdall *et al.* 1991] reducing α-T's usefulness as a blackfly control agent. The main potential for α-T as a larvicide is in the control of mosquito larvae, particularly in areas where mosquitoes are disease vectors and their control is urgently needed.

1.6 Phototoxic botanical larvicides in the context of conventional control agents

Due to the urgent need to control disease vectors such as the malaria mosquito, there has been a history of pesticide use against these insects. Initially, the synthetic organochlorine, DDT, was developed to eradicate mosquitoes. Eventually, however, DDT was found to persist in the environment on a global scale and due to its lipophilic nature, it readily bioaccumulated. DDT is no longer used except for limited use in certain parts of Asia and Africa [Zaranyika *et al.* 1994, Menzer 1991. Kruus *et al.* 1991].

Commercial compounds today are mostly organophosphates and synthetic pyrethroids. These compounds do not persist in the environment like the organochlorines, however, they are still somewhat lipophilic and some of them produce metabolites that are more toxic than the parent compound as well as being environmentally persistent (such as fenithrothion) [NRCC 1975, 1984]. The organophosphates are effective, but some non-target organisms, especially mayfly naiads, are affected adversely at mosquito larvicidal concentrations [Mulla *et al.* 1970, 1973, 1982, Mulla and Darwazeh 1976, Samnotra and Kumar 1980, Helson and Surgeoner 1983]. The organophosphorus insecticides used as mosquito larvicides (permethrin, fenitrothion, carbaryl, and carbofuran) are acetylcholinesterase inhibitors. Their toxicity depends on exposure time as well as concentration [Parsons and Surgeoner 1991*a*, *b*].

Pyrethroids are synthetic analogues of botanical pyrethrins. They are biodegradable and unlikely to bioaccumulate [Menzer 1991]. They are also effective mosquito larvicides (cypermethrin is used commercially) [Helson 1992, Menzer 1991]. However, non-target aquatic species are extremely sensitive (amphipods, anostracans, cladocerans and insects are wiped out 80-100% while copepods, ostracods and hydracarinids are affected to a lesser extent) [Helson and Surgeoner 1986]. As well, many target insects have become resistant to pyrethroids. Since the organochlorine, organophosphate and the pyrethroid insecticides are all neurotoxins, they have similar modes of action. This property promotes cross resistance to pesticides with neurotoxic effects even if the organism has never been exposed to a particular compound [Narahashi 1983, Miller *et al.* 1983].

In the context of conventional control, α-T has several advantages. It has comparable or better efficacy to conventional control agents and provides an alternative as these materials become ineffective due to resistance. The non-neurotoxic mode of action of the phototoxic material reduces the risk of cross resistance which is common with conventional insecticides. For example, α-T is equally effective against malathion resistant and sensitive strains of *Culex tarsalis* (Hasspieler *et al.* 1988).

This material has a half-life in sunlight of approximately 6 hours and its photo-degradation leads to a dozen or more simple aliphatic degradation products with little or no phototoxicity [Arnason *et al. In Press*].

α-Terthienyl is not mutagenic to mammals and does not exhibit phytotoxicity at larvicidal concentrations [Campbell 1982, Arnason *et al.* 1987, MacRae *et al.* 1980, Tuveson *et al.* 1986]. It does however exhibit phototoxicity towards human skin when exposed to high doses [Chan *et al.* 1975]. α-T has a measurable toxicity to mammals (intra-peritoneally administered, lethal dose at which 50% mortality occurs (LD_{50}) = 110 mg/kg for rats) but the ready to use formulation is not toxic at the highest dose tested (5 mL/kg). Toxicokinetics indicated that rats can metabolize and excrete all the α-T 4 days after treatment [Marles *et al. In press*].

Another advantage of α-T is the inexpensive source (powdered *Tagetes* roots). This makes α-T very appealing as an inexpensive and effective larvicide for developing countries to combat deadly diseases such as malaria by controlling their vector, the mosquito [Arnason *et al.* 1981*b*].

1.7 Research Approach and Objectives

The basic hypothesis of toxicology and this thesis is that risk is a function of hazard and exposure [Scala 1991]. The compound, α-terthienyl has a potentially toxic mode of action to cells of different origin due to its phototoxic action and therefore may be a hazardous substance. However due to its potentially rapid clearance and metabolism from organisms, its rapid photodegradation and the requirement of sunlight to activate the phototoxic mode of action, many non-targets *in situ* may be protected from α-T's toxic effects. This would reduce the exposure component of the equation and the overall risk of α-T may be quite low as a result. In order to determine the risk to natural populations, the hazard, which is an inherent property of the compound, must be known, and the exposure must both be assessed.

The aim of this project was to characterize the sensitivity of potential non-target organisms towards α-T in a controlled laboratory situation to compare with established data at a field site. To accomplish this, initially acute toxicity bioassays were used to determine the lethality of α-T under standard conditions. This quantifies the potential susceptibility of selected species to the phototoxic effects of α-T. The lethality of this compound is a hazardous property. Comparison with actual field data gives information on how environmental factors (primarily light *in situ*) alter the species sensitivity. The second part of the study examined the exposure component of the risk equation through a study of the fate of labeled compound in organisms. To characterize the ability of the organisms to physiologically manage exposure to this phototoxin, toxicokinetics experiments were also undertaken. This was investigated through studies of uptake and elimination of α-T by the two non-target species and comparing their sensitivity to that of the target species mosquito larvae. The presence of an α-T metabolite in the target mosquito was also investigated. To further assess the fate of α-T in a more natural environment, artificial ecosystems were placed into the natural mosquito habitats.

The toxicity of α-terthienyl is predicted to be modified by *in situ* environmental factors, particularly light, but not temperature. These factors will alter species selectivity.

In terms of exposure, target and non-target organisms are predicted to be exposed to high tissue levels of α-T due to its high partition coefficient. Because invertebrates encounter plant allelochemicals like α-T in their environment, clearance and metabolism is predicted to be efficient. Fate of α-T will also be modified by environmental factors *in situ.*

The objectives of this project can be can be summarized as follows.

1. To determine the accurate LC_{50} values of α-T for the target, *Aedes atropalpus*, and the non-target species, *Daphnia magna* and *Limnephilus indivisus* under laboratory conditions to compare with actual *in situ* field data.

2. Determine the uptake and elimination rates in the selected species under controlled conditions in the laboratory.

3. Determine if exposure can lead to a detectable α-T metabolite.

4. Determine the fate of α-T in semi-natural field trials using artificial ecosystems for comparison with laboratory studies to determine how environmental conditions modify fate data.

Chapter 2 : Toxicity and Toxicokinetics of α-T in the target and non-target organisms.

2.1 Introduction

The effectiveness of α-T towards mosquito larvae is well known. Many studies have evaluated α-T and its derivatives as an alternative larvicide for mosquitoes [Philogene *et al.* 1985, Hasspieler *et al.* 1988, 1990, Arnason *et al.* 1981*b*, 1986, 1988, Dosdall *et al.* 1992, Marles *et al.* 1992]. There is much less information about the effects on non-target organisms, however. Philogene *et al.* [1986] used caddisfly larvae (age unknown and unidentified species but presumably *Limnephilus indivisus* since they were collected at the same field site) in outdoor field trials under sunlight and determined effective concentration for 50% control (EC_{50}) to be 1.32 kg/hectare. The same study found *Aedes intrudens* to have an EC_{50} of 0.046 kg/hectare. They also tested *Daphnia magna* indoors with 15 minutes of UV exposure and obtained an EC_{50} of 0.044 kg/hectare. Because the experimental methods were different, it is difficult to make definitive comparisons. However, it can be stated that *Aedes intrudens* and *Daphnia magna* are sensitive to the phototoxin while the caddisfly larvae are relatively insensitive. Bennet *et al.* [1986] also observed the phototoxicity of α-T to *Daphnia magna* but exposed them to UV light for only 1 hour after incubating the animals in the dark for 1 hour. They found the lethal concentration for 50% mortality (LC_{50}) at 24 hours to be 1.3 ppb. The present study provides a more uniform procedure to compare the acute toxicity and LC_{50} values of α-T in *Aedes atropalpus* Coquillett, *Limnephilus indivisus* Walker and *Daphnia magna* Straus.

Some aspects of the toxicokinetics of α-T have been studied previously. Hasspieler *et al.* [1988] studied the toxicokinetics of elimination in 4th instar *Aedes aegypti* (mosquito) larvae over 72 hours. Radiolabelled (^3H) α-T was measured in the bodies of the larvae. They found that sensitivity towards α-T was inversely related to the rate constant of elimination. The kinetics were triphasic, indicating three compartments

and the clearance profile was characterized by the sum of three exponential equations expressed as:

$$C = C_{o1}e^{(-k_{e1}t)} + C_{o2}e^{(-k_{e2}t)} + C_{o3}e^{(-k_{e3}t)} \quad (1)$$

where C is the internal level of radiolabel at time t, C_o is the initial level of radiolabel at time zero, and k_e is the elimination rate constant. Marles *et al.* [*In press*] studied the toxicokinetics in rats over a 4 day (72 hour) period by measuring the amount of radiolabelled (^{14}C) α-T in the urine and feces after a single administered dose. By day 4, all the ^{14}C administered had been excreted. No rate constants were calculated. Iyengar *et al.* (1987) investigated the toxicokinetics of α-T in three lepidopteran larvae, *Manduca sexta, Ostrinia nubilalis* and *Heliothis virescens* over a 96 hour period. Oral or topical administration of radiolabelled (^{3}H) α-T indicated that toxicity was inversely related to the rate of elimination of ingested α-T in the feces.

This study examines the toxicity and toxicokinetics - uptake and elimination - of α-T in the target mosquito larva (*Aedes atropalpus* Coquillett, Diptera: Culicidae) and two non-target organisms, *Daphnia magna* Straus (Crustacea: Daphniidae) and *Limnephilus indivisus* Walker larvae (Trichoptera: Limnephilidae). These non-target animals were selected because both co-exist with mosquito larvae in the shallow ponds. *Daphnia magna* was chosen to represent a sensitive non-target organism because they are broadly distributed in freshwater bodies throughout a wide range of habitats. They are also an important link in many aquatic food chains. This invertebrate is commonly used in toxicology tests because they are easy to maintain in a culture, they are generally sensitive to a broad range of aquatic contaminants, they are easy to handle and sample and their parthenogenic reproductive strategy reduces variation among individuals resulting in a more uniform response. *D. magna* in particular, is also one of the largest species of the daphnids [Environment Canada 1992]. *Limnephilus indivisus* was selected to represent an insensitive animal. They are commonly found in temporary ponds and other species of caddisfly larvae are found globally throughout various aquatic habitats [Wiggins 1973, 1977]. Caddisfly larvae are not commonly used for toxicology testing, probably because

of the difficulties involved in maintaining a culture and handling them. In this study, however, they were ideal because they have a larval case made of decomposing plant materials that is impermeable to light, thus partially protecting the body of the larvae physically from phototoxic effects.

2.2 Materials and Methods

2.2.1 Source of *Aedes atropalpus*

The *Aedes atropalpus* culture was maintained in a 25°C incubator with a 16:8 L:D photoperiod and 40 S/m^2. This is a species local to the Ottawa Valley and was originally obtained from field-caught specimens. The eggs were collected, dried, and stored at room temperature. Larvae used for experiments were hatched from this state of diapause simultaneously for uniform age and size. This reduced variability and removed the need for selecting neonates. The larvae were fed daily with commercial Tetra Min staple food for tropical fish. The flakes were ground by hand and sprinkled into the water tray containing the larvae. The larvae were raised in plastic trays filled with approximately 2 L of dechlorinated water. Air was gently bubbled into the containers to prevent a film from forming on the water surface. This was important since the mosquitoes obtained their oxygen from the air, not dissolved in the water [Clements 1963]. After 5-7 days, the larvae began to pupate. The pupae had to be transferred to a smaller container within an adult cage within 24 hours of pupation since this stage did not last more than 48 hours. The adults were fed a concentrated sucrose solution. For 7-10 days they laid eggs in a petri dish filled with dechlorinated water. Every day the dish was removed, the water was siphoned off with a pipette, and the eggs were dried. At the end of the 10 day period, egg

production dropped and the females required a blood meal to lay a second clutch of eggs. At this point the adults were killed either by removing the sucrose solution or placing the adult cage in the freezer. This avoided the need for a blood meal and made room for the next generation of mosquitoes.

2.2.2 Source of *Limnephilus indivisus*

The caddisfly larvae were collected at a site 50 km south of Ottawa, Ontario in the region of South Mountain, Ontario. The larvae were picked by hand from a temporary snow-melt pool located in a mixed forest. Due the temporary nature of the pool and the lifecyle of the insect, caddisflies could only be collected in April and May. The larvae were transported in 5 gallon plastic buckets with a large amount of leaf litter and branches from the pond to minimize their contact and reduce the cannibalism. They were used in the experiments within 24 hours of capture, thus collection was frequently in smaller amounts to ensure their health.

The species of caddisfly larvae were identified at the Royal Ontario Museum (ROM) in Toronto, Ontario, by two caddisfly specialists. Dr. Glenn B.Wiggins, Curator of the Department of Entomology and Invertebrate Zoology, identified preserved specimens of late instar larvae and Dr. Pat McCullogh, researcher at the Department of Entomology and Invertebrate Zoology, identified preserved specimens of adults. The adults were emerged in the laboratory in a well aerated container at 15^{o}C from larvae caught at the field site.

2.2.3 Source of *Daphnia magna*

The *Daphnia magna* culture was a clonal colony of parthenogenic females obtained from a culture maintained by Dr. Robert Peters, Department of Biology, McGill University. Daphnids were obtained via Dr. David Currie, Department of Biology, University of Ottawa and the colony was started on March 10, 1994. The daphnids were kept in 2 large glass cylinders in dechlorinated tap water at room temperature. They were fed every week with a green algae (*Chlorella vulgaris*) suspension in dechlorinated tap water. 500 mL were added per cylinder per week. The green algae was grown by sunlight in a 15 gallon aquarium containing a large goldfish and an air stone. The goldfish was necessary for two reasons. First, by constantly swimming around, it mixed the water and kept the algae suspended in the water column, otherwise they eventually sank to the bottom of the tank. Second, nitrogenous waste produced by the goldfish provided nutrients to the algae, encouraging growth.

2.2.4 Solution of α-T in acetone

A modified Grignard Wurz reaction was used to synthesize α-T. This procedure was previously described in MacEachern *et al.* [1988]. The α-T was provided by Dr. P. Morand, University of Ottawa. A quantity of α-T was weighed and dissolved in an appropriate volume of acetone to produce a 1 mg/mL solution (A). An aliquot of solution A was diluted in acetone to produce a 0.1 mg/mL solution (B). A 0.01 mL aliquot of solution A was diluted in acetone to produce 0.01 mg/mL solution (C).

2.2.5 Emulsifiable Concentrate Solution of ^{3}H-α-T

Tritiated (^{3}H) α-T was prepared by Dr. N. Werstiuk, McMaster University, using a high temperature dilute acid procedure. EC stock solution contained: 25.30 mg ^{3}H-α-T, 20.85 mg Atlox 3403F (Atchemix, Brantford, Ontario), and 22.37 mg Atlox 3404F (Atchemix, Brantford, Ontario), dissolved in 100 µL o-xylene. Atlox 3403F and 3404F are alkyl aryl sulfonate mixtures and dissolved in 40 µL o-xylene as has been previously described in Dosdall *et al.* [1991]. Stock 1 dissolved in 2.43 mL distilled water was used to prepare EC Stock 2 solution. The concentration of ^{3}H-α-T was 10 mg/mL or 10 ppm. Solutions were stored at -20°C in darkness.

A control solution was made by the exact same procedure except ^{3}H-α-T was omitted.

2.2.6 Acute Toxicity Bioassays

Procedures were developed according to the World Health Organization guidelines for mosquito larvae susceptibility and resistance to insecticides [World Health Organization 1963]. The bioassay procedure for mosquito larvae was described by Aucoin *et al.* [1992]. All the bioassays were based on this procedure, with modifications to optimize conditions towards each species (see below). α-Terthienyl was dissolved in acetone, as described in the Materials section. A pretest was performed for all three species to determine the range of concentrations to be used. The concentration ranges and volumes used were described for all three species in Table 2.1. Acetone was added to equalize the volumes. The organisms were exposed to ultraviolet (UV) light for 4 hours, then kept in darkness for a further 20 hours. All bioassays were done at 25°C, except one bioassay involving *Aedes atropalpus* was carried out at 7.5°C.

The UV light source consisted of 4 bulbs of 20W blacklight-blue (BLB) lamps (Westinghouse F20T12) at a height of 20 cm providing 5 W/m^2. The radiation emitted was in the 300-400 nm range with a peak at approximately 350 nm, which was ideal since α-T absorbs maximally in the UV-A range of 320-400 nm.

Finney's Probit Analysis was used to determine the LC_{50}, LC_{90}, and fiducial limits for a 24 hour bioassay. Normality was determined using the Wilks-Shapiro test and Levene's test was used to determine homoschedastisicity. Analysis of variance (ANOVA) and Scheffe's multiple comparison test was used to determine if the LC_{50}'s were significantly different. Analysis of covariance (ANCOVA) was performed to test for any significant differences among the three bioassay regressions. All statistical analyses are carried out on SAS (SAS Institute Inc. 1985).

Aedes atropalpus

20 larvae (early 4th instar) were placed into 250 mL dechlorinated tap water in a 500 mL capacity glass container. Each concentration was repeated in triplicate, giving a total of 60 larvae per concentration. The concentrations were selected in a geometric range: 8, 16, 25, 32, 48, 64, 90, 128 ppb. This bioassay was repeated at 7.5°C to test for the possibility of temperature effects on the LC_{50}.

Limnephilus indivisus

1 larva (2nd instar) was placed into 5 mL dechlorinated tap water in a 10 mL capacity glass vial. Each concentration had 60 replicates (larvae per concentration). The concentrations selected were: 8, 16, 32, 64, 128, 256, 400, 1000 ppb.

Daphnia magna

20 neonates (less than 24 hours old) were placed into 75 mL dechlorinated tap water in a 100 mL capacity glass jar. Each concentration was done in triplicate, resulting

in 60 daphnids per concentration. The chosen concentration range was: 0.5, 1, 2, 3, 4, 8, 10, 16 ppb.

Table 2.1. Procedure for preparation of concentrations used for acute toxicity bioassays.

Species	Stock α-T solution, (mg/mL)	Final test [α-T] (ppb)	α-T stock added (μL)	acetone volume added (μL)
Aedes atropalpus				
	-	0	0	80
	0.1	8	20	60
	0.1	16	40	40
	0.1	25	62.5	17.5
	0.1	32	80	0
	1	48	12	688
	1	64	16	64
	1	90	22.5	57.5
	1	128	32	48
Limnephilus indivisus				
	-	0	0	20
	0.01	8	4	16
	0.01	16	8	12
	0.01	32	16	4
	0.1	64	3.2	16.8
	0.1	128	6.4	13.6
	0.1	256	12.8	7.2
	0.1	400	20	0
	1	1000	5	15
Daphnia magna				
	-	0	0	300
	0.01	0.5	3.75	296
	0.01	1	7.5	292
	0.01	2	15	285
	0.01	3	22.5	278
	0.01	4	30	270
	0.01	8	60	240
	0.01	10	75	225
	0.01	16	120	180

2.2.7 Uptake of ^{3}H-α-T

Aedes atropalpus

This procedure was adapted from Hasspieler *et al.* [1988] and performed at 26°C. Five Pyrex glass containers (17 x 17 x 5 cm) were filled with 990 mL of dechlorinated water. Early 4th instar larvae (n = 250) were placed into each container. Each dish was aerated with a small tube using an aquarium air pump. This was to ensure proper mixing of the formulation and to keep the water surface free of film. The larvae were not fed for the duration of the experiment to keep the water from getting cloudy and ensure one mode of uptake. The larvae were added in 10 mL of water, producing a final volume of 1 L. Each container was fitted with a 'hood' consisting of a wire frame covered with black plastic to minimize exposure to light since the lights in the incubator could not be turned off. One container had only the mosquitoes in the 10 mL of water added and was considered a blank. One container had 10 µL of formulation solution added and was considered the control to monitor for any formulation effects, and three containers had 10 µL of ^{3}H-α-T EC Stock 2 (specific activity = 2.08 µCi/mg, 4.62 x 10^{5} dpm) added to each. This gave a solution of 100 ppb in each treated container. Ten larvae were sampled at 0, 10, 20, 30, 40, 50, 60 minutes, and 6, 12, 24, 48, 72, 96 hours. Water (1 mL) was sampled at 5, 15, 25, 35, 45, 55, 65 minutes and 6, 12, 24, 48, 72, 96 hours. To maintain a concentration at 100 ppb, the water was changed every 24 hours and fresh ^{3}H-α-T EC Stock 2 and control solution was added.

The larvae were rinsed with distilled water, weighed, squashed between two filter papers and dried in an oven at 45°C for 3 hours. They were then placed into 20 mL boroscillicate glass scintillation vials and dissolved with 0.5 mL Protosol (0.5 molar solution, New England Nuclear, Dupont, Boston, MA) tissue solubilizer for 24 hours. Glacial acetic acid (20 µL) was added to reduce chemiluminescence. Scintillation cocktail

(Cytoscint, ICN Biomedicals Canada Ltd.) (15 mL) was added and the solution was mixed and allowed to sit for 24 hours. The radioactivity was counted as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

The 1 mL aliquot of water was placed into a 20 mL boroscillicate glass scintillation vial. Glacial acetic acid (20 μL) was added to each vial to minimize chemiluminescence. Then 15 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) was added and the solution was mixed on a Vortex mixer. The vials were allowed to sit at room temperature for 24 hours in darkness. The radioactivity was then counted as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

To determine the mass balance, the containers were rinsed out with dechlorinated tap water and dried. Then the inside of every container was wiped with filter paper soaked in 95% ethanol. The paper was air-dried, placed into a 7 mL glass scintillation vial, filled with 5 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) and counted for 10 minutes on the Scintillation Analyzer. This residue was the amount of ^{3}H-α-T adsorbed onto the glass. The following calculations were then made:

total ^{3}H-α-T recovered = amount in larva + amount in water + amount on glass (2)

% recovery = total ^{3}H-α-T recovered / amount ^{3}H-α-T added (3)

Limnephilus indivisus

A 100 ppb solution of ^{3}H-α-T was prepared. A Hamilton syringe was used to deliver 20 μL ^{3}H-α-T EC Stock 2 (461.76 dpm/mL) into a 2 L volumetric flask which was filled to 2 L with dechlorinated water and stored in a dark incubator at 15°C until use.

Mature (4th or 5th) caddisfly larvae (*Limnephilus indivisus*) were collected from the field site in a large bucket and acclimatized in a dark incubator at 15°C for 24 hours.

Each larva had to be placed in individual vials because of their cannibalistic nature. Boroscilicate glass vials (20 mL capacity) without lids were used. Aliquots (10 mL) of 100 ppb ^{3}H-α-T solution were pipetted, using a volumetric pipette, into each vial. There were 30 replicates of ^{3}H-α-T treated vials. Aliquots (10 mL) of formulation control solution were pipetted using a volumetric pipette, into each vial. There were 10 replicates of controls. One larva was placed into each vial with 10 mL solution. Due to the limited seasonal supply of caddisfly larvae and their cannibalistic nature, sampling times were reduced. Samples were taken at 1 minute, 0.5, 1, 8, 17, 48, 72, and 96 hours. In between sampling, the larvae in the vials were placed in a dark incubator at 15°C.

At the appropriate time interval, 1 mL of water was sampled from each vial. The water samples were processed the same way they were for the experiment with *Aedes atropalpus*.

The larva was removed from the vial and rinsed with distilled water. The larva was separated from its case gently and both the case and larva were rinsed again. Each was placed separately into a weighing tray and weighed on a Mettler analytical balance. The larva and case were placed into respective 20 mL boroscillicate glass scintillation vials with enough acetone to cover them. Each was homogenized using a Polytron homogenizer. The acetone was evaporated off at room temperature. The larva was solubilized in 0.5 mL Protosol (tissue solubilizer, 0.5 molar solution, New England Nuclear, Dupont, Boston, MA) and the vials were left at room temperature in darkness for 24 hours to digest. The case was allowed to soak in 0.5 mL of methanol for 24 hours in the dark. After 24 hours, 20μL glacial acetic acid was added to both the larval and case vials to minimize chemiluminescence. Scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) (15 mL) was added to each vial and mixed on a Vortex mixer. The vials were allowed to sit at room temperature for another 24 hours in darkness. The

radioactivity was then counted for 10 minutes per sample as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

To determine the mass balance, the vials were rinsed out with dechlorinated tap water and dried. Then the inside of every vial was wiped with filter paper soaked in 95% ethanol. The paper was air-dried, placed into a 7 mL glass scintillation vial, filled with 5 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) and counted for 10 minutes on the Scintillation Analyzer. This residue was the amount of ^{3}H-α-T remaining on the glass. The same calculations were performed on the data as for *Aedes atropalpus* (equations 2 and 3).

Daphnia magna

A 115.5 ppb solution of ^{3}H-α-T was prepared (531.02 dpm/mL). A Hamilton syringe was used to deliver 23 μL ^{3}H-α-T EC Stock 2 into a 2 L volumetric flask and filled to volume with dechlorinated water. The solution was stored in dark incubator at 15°C.

Glass jars without lids (100 mL capacity) were used. There were 6 jars per trial and each jar was filled with 65 mL of 115.5 ppb ^{3}H-α-T solution. Twenty *Daphnia* were counted out into 10 mL dechlorinated water for each jar. The addition of the daphnids and water diluted the solution to a concentration of 100 ppb ^{3}H-α-T. The experiment was carried out at 15°C in the dark. Samples were taken at 0, 0.5, 1, 3, 6, 17, 27, 50, 73, and 94 hours. The solution was changed every 24 hours to maintain a 100 ppb concentration. When a time trial was sampled, 1 mL of water was taken from each vial and placed into a 20 mL scintillation vial; all the *Daphnia* were removed and rinsed with distilled water; the remaining water was discarded and the jars were rinsed with dechlorinated water and dried.

The water sample was treated according the procedure described for the mosquito experiment. The *Daphnia* were placed into a weighing tray, weighed, placed in a 20 mL glass scintillation vial and enough acetone was added to cover them (about 1 mL). The daphnids were crushed with a hand-held Teflon homogenizer in the vial. The acetone was evaporated off and 0.5 mL Protosol tissue solubilizer (0.5 molar solution, New England Nuclear, Dupont, Boston, MA) was added to liquefy the organisms. After 24 hours, 20 μL glacial acetic acid was added and 15 mL scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.). The solution was mixed and left at room temperature for 24 hours. The radioactivity was then counted for 10 minutes per sample as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

To determine the mass balance, the jars were rinsed out with dechlorinated water and dried. Then the inside of each jar was wiped with filter paper soaked in 95% ethanol. The paper was air-dried, placed into a 7 mL glass scintillation vial, filled with 5 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) and counted for 10 minutes on the Scintillation Analyzer. This residue is the amount of ^{3}H-α-T remaining on the glass. The calculations performed on the data were the same as those for *Aedes atropalpus* (equations 2 and 3).

2.2.8 Elimination of ^{3}H-α-T

Limnephilus indivisus

A Pyrex container (17 x 17 x 5 cm) was filled with 1 L of dechlorinated tap water. EC Stock 2 solution (10 μL, 4.62 x 10^{5} dpm) was added to give a concentration of 100 ppb ^{3}H-α-T. Twenty caddisfly larvae were added for each time trial. Similarly, another Pyrex container was filled with 1 L dechlorinated tap water and 10 μL of control solution

was added. Twenty larvae were added (four time trials at 5 larvae each). Both containers were placed into a dark incubator at 15°C for 30 minutes. After 30 minutes, the larvae were transferred to another container with 1 L dechlorinated tap water for 1 minute to halt the uptake of ^{3}H-α-T. The initial time trial of 0 minutes was immediately rinsed again, the case was removed from the larva gently and both were weighed separately. Other trials were rinsed with a wash bottle containing water and each larva was placed into a separate glass vial with 15 mL of dechlorinated tap water. The vials were placed back into the dark 15°C incubator. Samples were taken at 0.5, 1, 3, 6, 12, 24, 48, and 72 hours. The water in each vial was changed after 3, 6, 12 hours and every 12 hours after that.

The larvae and cases were weighed independently, but were then pooled 5 larvae per vial and 5 cases per vial to give 4 trials plus 1 control. Enough acetone was added to cover the larvae and cases. This allowed the vials to be stored temporarily without degrading the larvae or case materials. Acetone also aided with homogenization when using the polytron. The samples were homogenized until no large pieces remained. The acetone was evaporated off using a sample concentrator. The vials were immersed into wells containing water heated from 50 - 70°C. Once dry, 2 mL Protosol tissue solubilizer (0.5 molar solution, New England Nuclear, Dupont, Boston, MA) was added to each larval vial because the larvae would not dissolve in 0.5 mL. As a result, 0.5 mL aliquots of the dissolved larvae were placed into 20 mL borosillicate glass vials and 20 μL glacial acetic acid was added to each vial to minimize chemiluminescence. Scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) (15 mL) was added to each vial and mixed on a Vortex mixer. The vials were allowed to sit at room temperature for 24 hours in darkness. The radioactivity was then counted as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

Daphnia magna

A 115.5 ppb solution of ^{3}H-α-T was prepared. A Hamilton syringe was used to deliver 23 μL ^{3}H-α-T EC Stock 2 (531.02 dpm/mL) into a 2 L volumetric flask which was filled to 2L with dechlorinated water. The solution was stored in a dark incubator at 15°C.

Glass jars (100 mL capacity) without lids are used. There were 6 jars per trial and each jar was filled with 65 mL of 115.5 ppb ^{3}H-α-T solution. Twenty *Daphnia* were counted out into 10 mL dechlorinated water for each jar. The addition of the daphnids and water diluted the solution to a concentration of 100 ppb ^{3}H-α-T. The *Daphnia* in the jars were placed into a dark incubator at 15°C for 30 minutes. After 30 minutes the daphnids were removed from the solution and briefly placed into another jar containing 75 mL of dechlorinated water to rinse them. Each group of 20 organisms were placed into their respective jars for a set amount of time. The times sampled were 0, 0.5, 1, 3, 6, 12, 18, 24, 48, 72, and 96 hours. The water was changed only every 24 hours, unlike the frequent changes for the caddisfly larvae due to the sensitivity of the *Daphnia*. When a time trial was sampled, 1 mL of water was taken from each vial and placed into a 20 mL scintillation vial and all the *Daphnia* were removed and rinsed with distilled water. The remaining water was discarded and the jars were rinsed with dechlorinated water and dried.

The water sample was treated according the procedure initially described for the *Aedes atropalpus* uptake experiment. The *Daphnia* were put into a weighing tray, weighed, placed in a 20 mL capacity glass scintillation vial and enough acetone was added to cover them (about 1 mL). The daphnids were crushed with a hand-held Teflon homogenizer in the vial. The acetone was evaporated off and 0.5 mL Protosol tissue solubilizer (0.5 molar solution, New England Nuclear, Dupont, Boston, MA) was added to liquefy the organisms. After 24 hours, 20 μL glacial acetic acid and 15 mL scintillation

cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) were added. The solution was mixed and left at room temperature for 24 hours. The radioactivity was then counted for 10 minutes per sample as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

To determine the mass balance, the jars were rinsed out with dechlorinated water and dried. Then the inside of each jar was wiped with filter paper soaked in 95% ethanol. The paper was air-dried, placed into a 7 mL glass scintillation vial, filled with 5 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) and counted for 10 minutes on the Scintillation Analyzer. This residue was the amount of ^{3}H-α-T remaining on the glass. Calculations were performed as for the mosquito larvae (equations 2 and 3).

2.3 Results

2.3.1 Toxicity Tests

The acute toxicity bioassays were described by a linear regression of the log transformation of the mortality/survival ratio plotted against the log concentration of α-T. The probit line describing the toxicity of α-T to 4th instar *Aedes atropalpus* larvae at 25°C is illustrated in Figure 2.1 with 95% confidence limits (C.L.). The probit equation was $y = 2.69x - 4.06$, where y was log mortality/survival and x was log concentration. The LC_{50} and LC_{90} values were 31.97 ppb (95% C.L. 29.02, 35.17) and 72.85 ppb (95% C.L. 51.88, 177.80), respectively. The slope of the log response was 2.69 ± 0.58 ppb^{-1} (standard error). The probit line describing the toxicity of α-T to early instar *Limnephilus indivisus* larvae is shown in Figure 2.3 with 95% confidence limits (C.L.). The probit equation was $y = 1.74x - 3.18$, where y was log mortality/survival and x was log concentration. The LC_{50} and LC_{90} values were 64.76 ppb (95% C.L. 54.81, 76.17) and 236.35 ppb (95% C.L. 169.82, 354.81), respectively. The slope of the log response was

1.71 ± 0.17 ppb^{-1} (standard error). The probit line describing the toxicity of α-T to *Daphnia magna* neonates is shown in Figure 2.4 with 95% confidence limits (C.L.). The probit equation was $y = 1.92x + 0.39$, where y was log mortality/survival and x was log concentration. The LC_{50} and LC_{90} values were 1.74 ppb (95% C.L. 1.50, 2.00) and 4.99 (95% C.L. 2.51, 28.18), respectively. The slope of the log response was 1.92 ± 0.37 ppb^{-1} (standard error). No mortality was observed in the controls for the species tested. The parameters for the 24-hour acute toxicity bioassays are summarized in Table 2.2.

The slopes of the three probit lines were not significantly different ($F = 0.91$, $p = 0.4446$) and there was overlap of the confidence intervals. ANCOVA results showed that while the toxicity depended on the concentration of α-T and the species, there was no interaction effect. A simplefied ANCOVA model was fitted with slopes that were forced to be equal (no interaction term was applied). The results indicated that toxicity of α-T varied with the concentrations used and it also depended on the species. The three species, *Daphnia magna*, *Limnephilus indivisus*, and *Aedes atropalpus* (both at 25 and 7.5°C) were compared. The total residuals were not normal ($W = 0.94$, prob<W = 0.02), however, when the normality test was performed on each species, only the *Daphnia* data were not normal ($W = 0.86$, prob<W = 0.02). A plot of the residuals and the predicted values indicated that the variance was equal (homoschedastic). Since ANCOVA is robust to normality violations, the parametric test was used. The LC_{90} values differed from each other by an order of magnitude and there was no overlap of the 95% confidence limits, except a slight overlap between caddisfly larvae and mosquito larvae (25°C). The overlap was most likely due to the natural variablility of the organisms. *Daphnia magna* was an order of magnitude more sensitive (lower LC_{50} and LC_{90}) than either *Aedes* or *Limnephilus*. It was significantly different from both *Aedes* and *Limnephilus*. The difference in LC_{50} values between *Aedes* and *Limnephilus* was also statistically significant. The LC_{50} of *Limnephilus* was approximately twice as high as that of *Aedes*, and there was no overlap of the 95% confidence limits. Scheffe's Multiple Comparison test was used to compare the LC_{50}'s. The results of the ANCOVA are summarized in Table 2.3.

An acute toxicity bioassay was also done with *Aedes atropalpus* at 7.5°C. This comparison provided information on the effects of temperature on α-T acute toxicity. The

probit line describing α-T toxicity to 4th instar *Aedes atropalpus* larvae at 7.5°C is shown in Figure 2.2 with 95% confidence limits (C.L.). The probit equation was $y = 2.13x - 3.16$, where y was log mortality/survival and x was log concentration. The LC_{50} and LC_{90} values were 30.02 ppb (95% C.L. 26.45, 33.47) and 85.03 ppb (95% C.L. 74.13, 118.85), respectively. The slope of the log response was 2.13 ± 0.23 ppb^{-1} (standard error). ANCOVA was used to test for differences in the slopes of the two regressions at 7.5 and 25°C. There was no significant difference between the acute toxicity of α-T towards mosquito larvae at 7.5°C and 25°C ($F = 1.14$, $p = 0.29$). The residuals were normal ($W = 0.94$, prob < $p = 0.11$) and homoschedastic (from residuals versus predicted plot). Scheffe's multiple comparison test of the means indicated that there was no significant difference between the LC_{50}'s for the two temperatures. There was a strong overlap of the 95% confidence limits of the LC_{90}'s as well. The parameters are shown in Table 2.2 and the ANCOVA results are shown in Table 2.4.

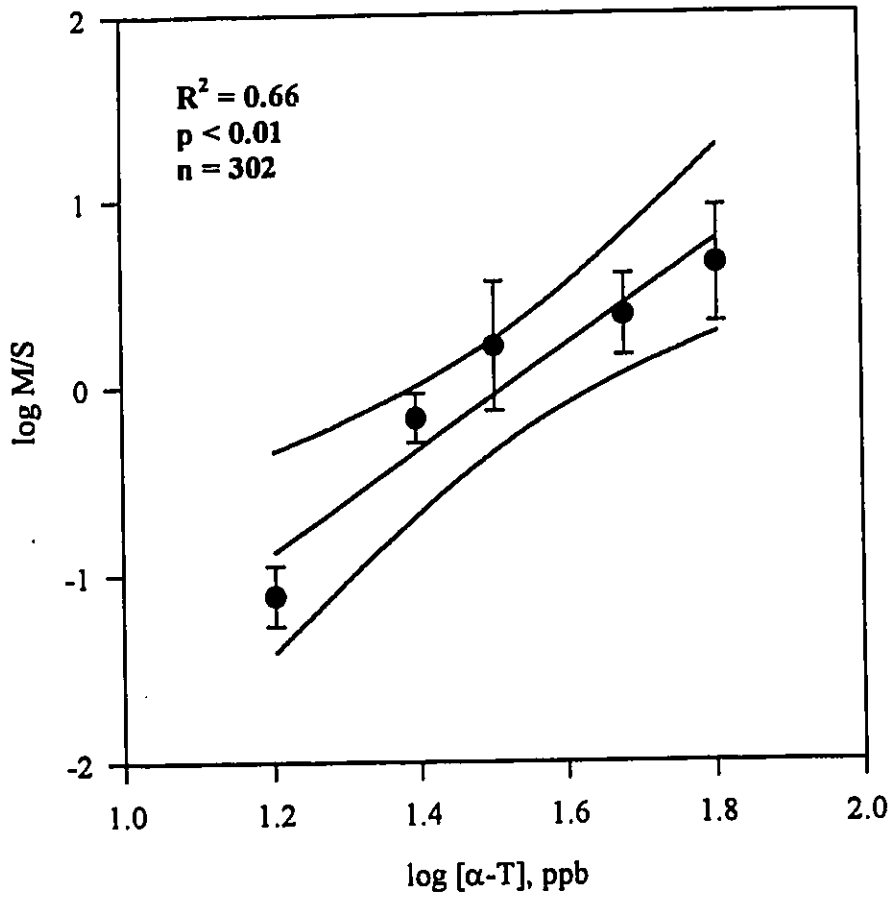


Figure 2.1. Acute toxicity of α-T towards 4th instar *Aedes atropalpus* larvae at 25°C with 4 hours UV exposure followed by 20 hours of darkness. 95% confidence limits are indicated. Error bars represent standard error. M = mortality, S = survival.

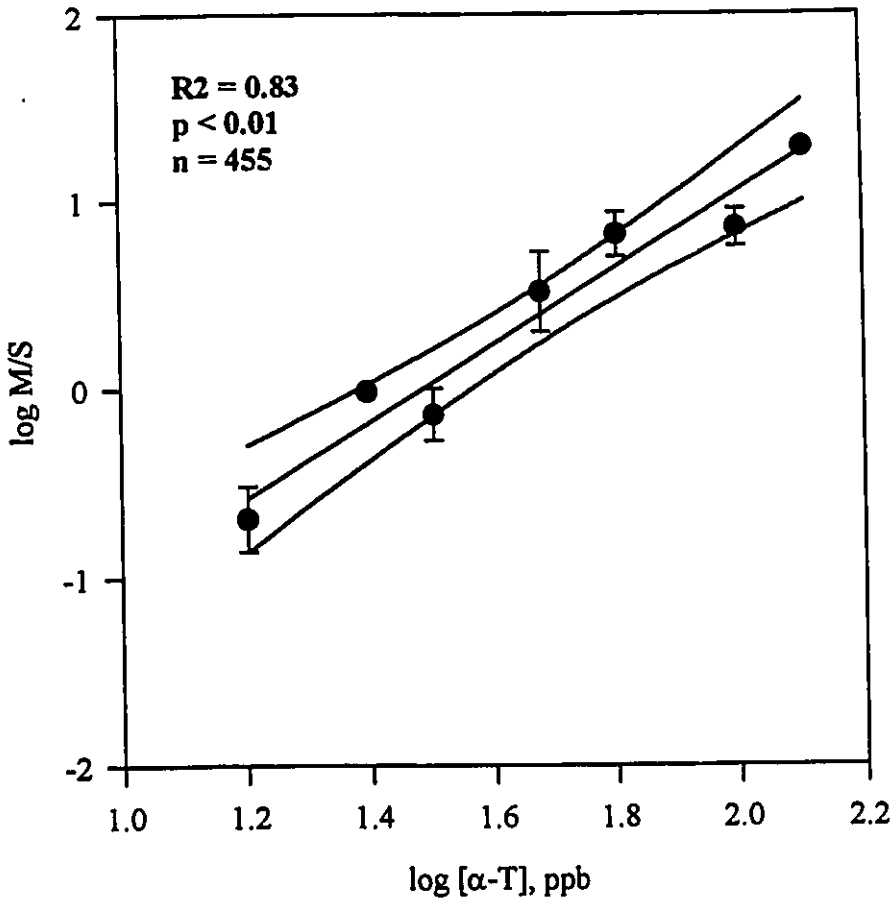


Figure 2.2. Acute toxicity of α-T towards 4th instar *Aedes atropalpus* larvae at 7.5°C with 4 hours UV exposure followed by 20 hours of darkness. 95% confidence limits are indicated. Error bars represent standard error. M = mortality, S = survival.

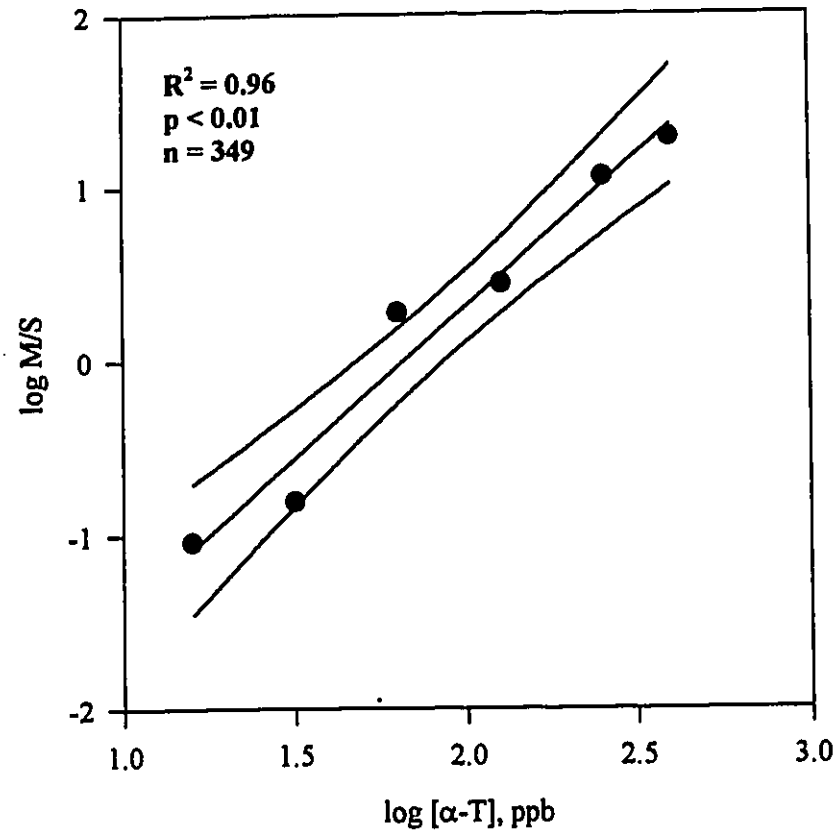


Figure 2.3. Acute toxicity of α-T towards 2nd or 3rd instar *Limnephilus indivisus* larvae with 4 hours UV exposure followed by 20 hours of darkness. 95% confidence limits are indicated. M = mortality, S = survival.

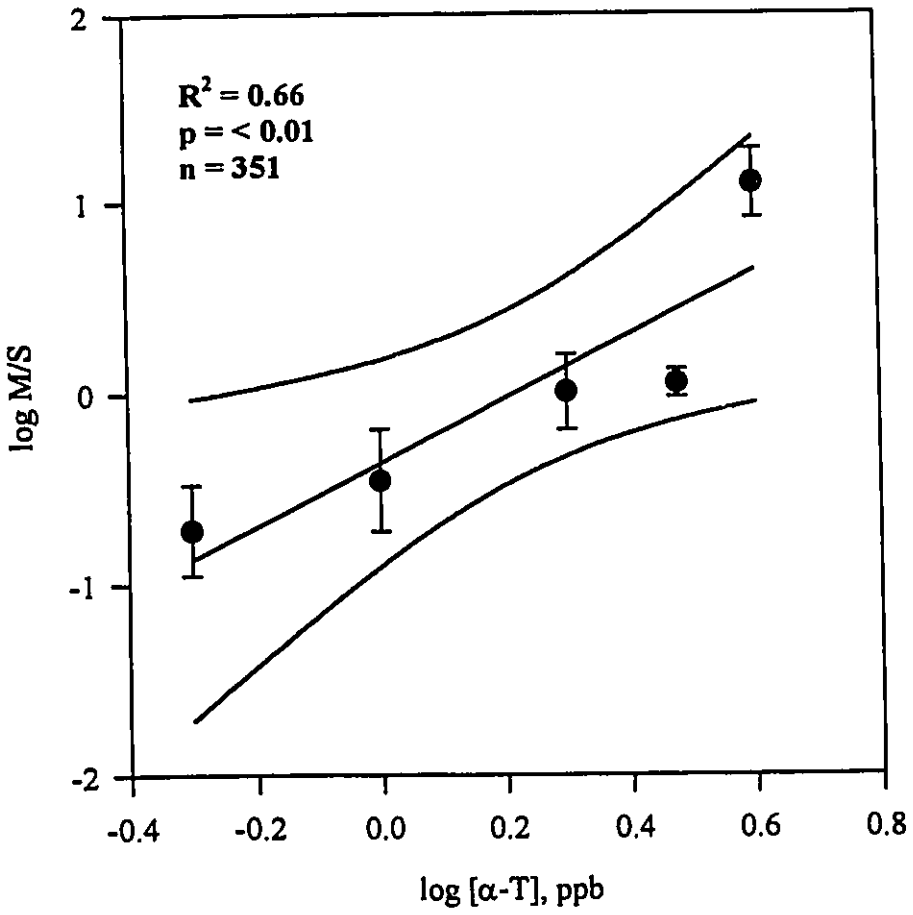


Figure 2.4. Acute toxicity of α-T towards juvenile *Daphnia magna* with 4 hours UV exposure followed by 20 hours of darkness. 95% confidence limits are indicated and error bars represent standard error. M = mortality, S = survival.

Table 2.2. Parameters for the 24-hour α-T acute toxicity bioassays for *Aedes atropalpus*, *Limnephilus indivisus*, and *Daphnia magna*.

Parameter	*Aedes atropalpus* 7.5°C	*Aedes atropalpus* 25°C	*Limnephilus indivisus* 25°C	*Daphnia magna* 25°C
LC_{50}[a]	30.02 ppb[x]	31.97 ppb[x]	64.76 ppb[y]	1.74 ppb[z]
95% C.L.[b]	26.45-33.47	29.02-35.17	54.81-76.17	1.50-2.00
LC_{90}[a]	85.03 ppb	72.85 ppb	236.35 ppb	4.99 ppb
95% C.L.[b]	74.13-118.85	51.88-177.80	169.82-354.81	2.51-28.18
Slope ± SE[c]	2.13 ± 0.23	2.69 ± 0.58	1.74 ± 0.17	1.92 ± 0.37
Probit equation	logM/S = 2.13logC - 3.16	logM/S = 2.69logC - 4.06	logM/S = 1.74logC - 3.18	logM/S = 1.92logC - 0.39

[a]Mortality assessed 24 hours after application.

[b]95% confidence limit.

[c]Slope ± standard error, ppb^{-1}

Different letters (*x*,*y*,*z*) indicate values that are significantly different from each other by Scheffe's Multiple Comparison Test.

Table 2.3. ANCOVA on 24-hour α-T acute toxicity bioassays for *Aedes atropalpus*, *Limnephilus indivisus*, and *Daphnia magna*.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
model	7	20.59	2.94	22.30	0.0001
species	3	8.49	2.83	21.45	0.0001
[α-T]	1	17.50	17.50	132.67	0.0001
species*[α-T]	3	0.36	0.12	0.91	0.4446
error	47	6.20	0.13	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

Table 2.4. ANCOVA on 24-hour α-T acute toxicity bioassays for *Aedes atropalpus* at two temperatures, 7.5°C and 25°C.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
model	3	10.56	3.52	32.99	0.0001
temperature	1	0.12	0.12	1.14	0.2936
[α-T]	1	8.40	8.40	78.78	0.0001
temp*[α-T]	1	0.11	0.11	1.06	0.3125
error	29	3.09	0.11	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

2.3.2 Uptake Kinetics

No mortality was observed during the experiments. The non-linear regression model and the corresponding parameters (rate constants and amplitude of the exponents) of the data were calculated by KaleidaGraph version 3.0.1 (Abeldeck Software 1993) using the R^2 value as a criterion . The uptake curves were fit to the model using Sigma Plot for Windows version 2.0 (Jandel Scientific Corp. 1994). The model that best described these curves was determined to be:

$$C = -C_{o1}e^{(-k_{u1}t)} - C_{o2}e^{(-k_{u2}t)} + a \qquad (4)$$

where C was the concentration of ^3H in the animal at time t, C_o was the concentration of ^3H in the water at time zero, $a = C_{o1} + C_{o2}$ assuming that no ^3H was initially in the animals at time zero, and k_u is the uptake rate constant. This is a two compartment model representing an exponential accumulation of ^3H in the body of the animals.

The accumulation of radiolabelled (^3H) α-T in early 4th instar *Aedes atropalpus* larvae is illustrated in Figure 2.5. The curve appeared biphasic, suggesting two compartments. The larvae accumulated the ^3H rapidly during the first hour after exposure. However, from 6 to 72 hours the curve appeared to plateau and the amounts of ^3H in the larvae did not significantly change (ANOVA: $F = 0.77$, $p = 0.57$). Since accumulation is the net result of uptake and concurrent elimination, it is possible that after 6 hours, the larvae reached a state of equilibrium. The rate constant of uptake was k_{u1} = 0.97 $hours^{-1}$ for the first hour. The rate constant for 6 to 72 hours was $k_{u2} = 0.11$. Most of the ^3H remained in the water. After 24 hours 65.0% of the ^3H remained in the water, 32.5% went into the larvae, and 2.4% adsorbed onto the glass container. The percent recovery of radioactivity was calculated at the 24 hour sampling time to be 80.7%.

The accumulation of ^3H-αT in mature (4th or 5th instar) *Limnephilus indivisus* larvae and their cases is shown in Figure 2.6. The larval case, which was made up of vegetative matter, also accumulated a portion of the ^3H. The uptake curve for the larval

cases was a single phase model with a rate constant $k_{uc} = 1.28 \times 10^{-2}$ hours^{-1}. The curve was almost linear. The larval uptake curve had two phases. The first phase represented the time interval from 1 minute to 1 hour and had a high rate constant of $k_{ul1} = 6.07$ hours^{-1}. The second represented the time interval 8 - 96 hours. The rate constant was much lower for this phase ($k_{ul2} = 7.02 \times 10^{-4}$ hours^{-1}), however the curve did not show a plateau. At 24 hours, 34.5% of the ^{3}H remained in the water, 28.1% went into the larval case, 34.9% was taken up by the larvae, and 2.5% adsorbed onto the glass container. The percent recovery was 89.9%.

The accumulation of ^{3}H in one week old *Daphnia magna* is illustrated in Figure 2.7. This was also a biphasic model where the first hour was characterized by the rate constant, $k_{u1} = 3.70$ hours^{-1} which described a rapid increase in the amount of ^{3}H in the animal's body. The second phase was slow and was characterized by the rate constant, k_{u2} = 1.66 x 10-2 hours^{-1}. This represented the accumulation from 3 hours to 94 hours. At 24 hours, most of the ^{3}H remained in the water (75.5%), the animals contained 24.2%, and only 0.3% adsorbed onto the glass containers. The percent recovery was 71.5%.

Analysis of Co-variance (ANCOVA) compared the slopes of the uptake regressions of the first phase. The summary of results is shown in Table 2.6. The ANCOVA model indicated that there was a significant interaction effect among the species and over time ($F = 36.96$, $p < 0.01$) , thus it can be concluded that depending on the invertebrate species, the accumulation of α-T will vary in magnitude. This is clear when comparing the data on the curves. The target organism, *Aedes atropalpus*, accumulated the highest amount of label per body weight (at 72 hours, 409.50 ± 161.80 ng α-T equivalents / mg body weight). The non-target, *Daphnia magna*, reached a maximum of 28.69 ± 2.21 ng α-T equivalents/ mg body weight at 94 hours, while the other non-target, *Limnephilus indivisus*, reached only a maximum of 5.38 ± 0.05 ng α-T equivalents / mg body weight at 96 hours.

A comparison of the uptake rate constants among species indicated that, in the first hour, *Limnephilus* had accumulated approximately 6.3 times more α-T per body weight

than the target *Aedes* and *Daphnia* had accumulated approximately 3.7 times more than *Aedes*. During the second phase of accumulation, the rates were considerably lower, however, *Aedes* continued to accumulate at a rate that was 156 times higher than *Limnephilus* and 6.6 times higher than *Daphnia*. The rate constants are summarized in Table 2.5.

2.3.3 Elimination Kinetics

No mortality was observed during the experiments. The non-linear regression model and the corresponding parameters (rate constants and amplitude of the exponents) of the data were calculated by KaleidaGraph version 3.0.1 (Abeldeck Software 1993) using the R^2 value as a criterion. The elimination curves were fit to the model using Sigma Plot for Windows version 2.0 (Jandel Scientific Corp. 1994). The model that best describes these curves was determined to be:

$$C = C_{o1}e^{(-k_{e1}t)} + C_{o2}e^{(-k_{e2}t)} \qquad (5)$$

where C was the concentration of ^3H inside the animal at time t, C_o was the internal concentration of ^3H at time zero, and k_e was the elimination rate constant. This was a two compartment model representing an exponential elimination of ^3H from the body of the animals.

Elimination of ^3H from 4th instar mosquito larvae has been demonstrated previously by Hasspieler *et al.* [1988]. These were triphasic models where the first rate constant (*Aedes aegypti*, $k_{e1} = 1.19$ hours^{-1}; *Culex tarsalis*, $k_{e1} = 2.15$ hours^{-1}) represented the first hour post-exposure, the second rate constant (*A. aegypti*, $k_{e2} = 0.22$ hours^{-1}; *Cx. tarsalis*, $k_{e2} = 0.28$ hours^{-1}) represented 2 - 12 hours past exposure, and the third rate

constant (*A. aegypti*, k_{e3} = 1.04 x 10^{-2} $hours^{-1}$; *Cx. tarsalis*, k_{e3} = 1.07 x 10^{-2} $hours^{-1}$) represented 16 - 72 hours after exposure (Hasspieler *et el.* 1988).

The elimination of label from non-target organisms was biphasic in nature. Figure 2.8 shows how mature *Limnephilus indivisus* larvae (4th - 5th instar) eliminated ^3H with a rate constant of k_{el1} = 0.11 $hours^{-1}$ representing the first 5.5 hours post-exposure. From 12 to 66 hours the second rate constant was very low (k_{el2} = 1.31 x 10^{-10} $hours^{-1}$). ^3H is also eliminated from the larval case with a biphasic decay. The initial rate constant represented the first hour after exposure is k_{ec1} = 0.26 $hours^{-1}$. The second phase represented 3.5 to 66 hours and had a rate constant that was also low (k_{ec2} = 4.50 x 10^{-11} $hours^{-1}$).

The elimination of ^3H by *Daphnia magna* was also biphasic. This relationship is illustrated in Figure 2.9. The first phase was characterized by the rate constant k_{e1} = 1.35 $hours^{-1}$, representing the first three hours after exposure. The second phase described the elimination from 5.5 to 96 hours post exposure and it had a rate constant of k_{e2} = 1.40 x 10^{-2} $hours^{-1}$.

ANCOVA on the clearance of ^3H by *Daphnia magna* and *Limnephilus indivisus* indicated that there is a significant interaction effect ($F = 29.46$, $p < 0.01$) between the species and over time for the first phase. This was reflected in the fact that the ratio of rate constants were different for the two species. The ANCOVA results are summarized in Table 2.7. Both non-target organisms were exposed to the same concentrations of ^3H-α-T for 30 minutes, however, the amount of ^3H per mg body weight initially was very different between species. The *Limnephilus* larvae contained approximately 12 times less ^3H per mg body weight than the *Daphnia*. This difference in values corresponded to the 30 minute uptake interval for each species. The initial rate constant of *Daphnia magna* was very similar to the rate constant in the first hour for *Aedes aegypti* and *Culex tarsalis* was only twice as high. In contrast, the initial rate constant of *Limnephilus indivisus* was much more similar to the second rate constant of the two mosquito species studied by Hasspieler *et al.* [1988]. They were only twice as fast as the caddisfly larvae rates. The

difference between the initial rates of *Daphnia* and *Limnephilus* was an order of magnitude or ten times. The rate constants representing the second phase of elimination in the non-targets corresponded to the third phase in the two mosquito species. The rate constant for *Daphnia* was essentially the same as for the target mosquito species while the caddisfly larvae rate constant was much lower (8 orders of magnitude) and was essentially zero. The values for the larval cases was similar to the caddisfly larvae values and was one order of magnitude smaller than those. These values are summarized in Table 2.5.

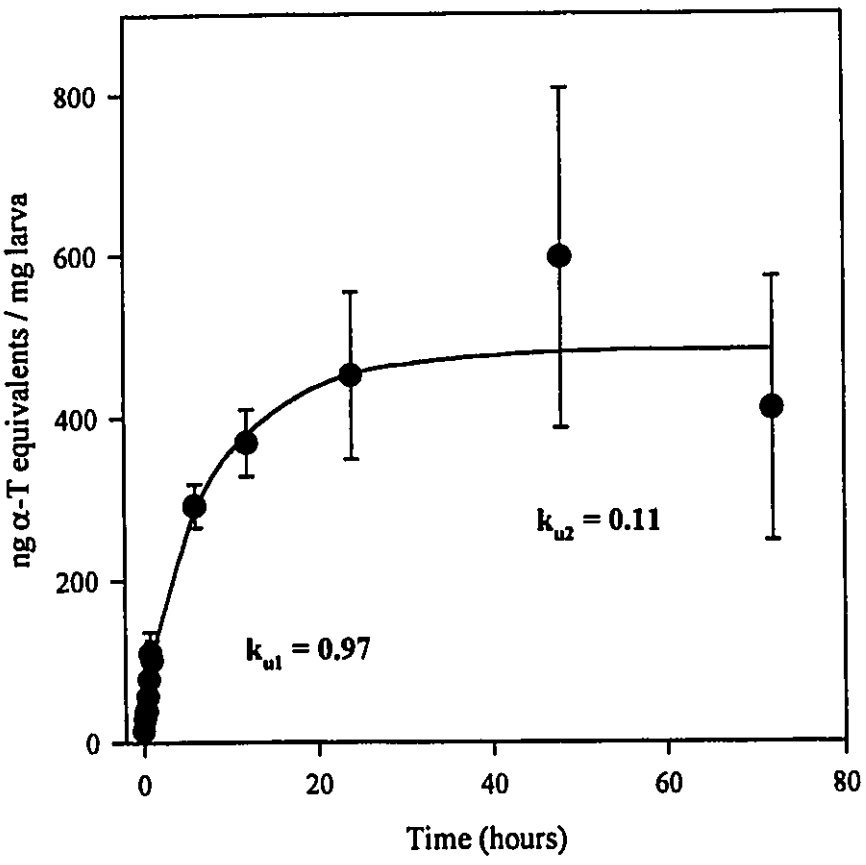


Figure 2.5. Changes of ^{3}H-α-T concentration in early 4th instar *Aedes atropalpus* larvae exposed to water containing 100 ppb ^{3}H-α-T for 72 hours. Error bars represent standard errors.

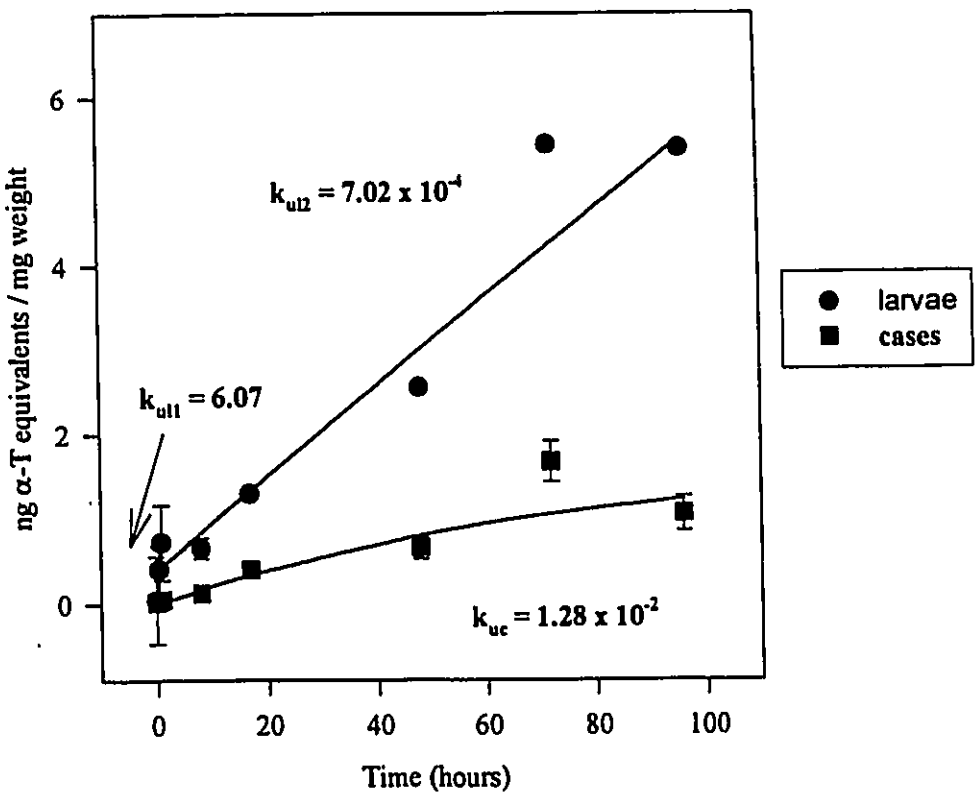


Figure 2.6. Changes of ^{3}H-α-T concentration in 4th or 5th instar *Limnephilus indivisus* larvae exposed to water containing 100 ppb ^{3}H-α-T for 96 hours. Error bars represent standard errors.

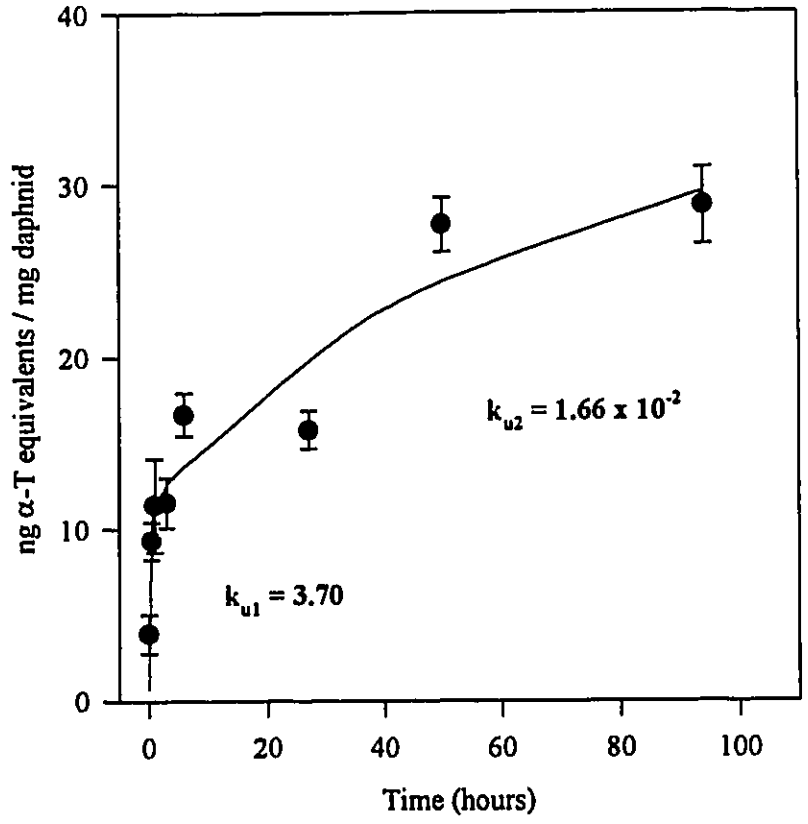


Figure 2.7. Changes of ^{3}H-α-T concentration in 1 week old *Daphnia magna* exposed to water containing 100 ppb ^{3}H-α-T for 94 hours. Error bars represent standard errors.

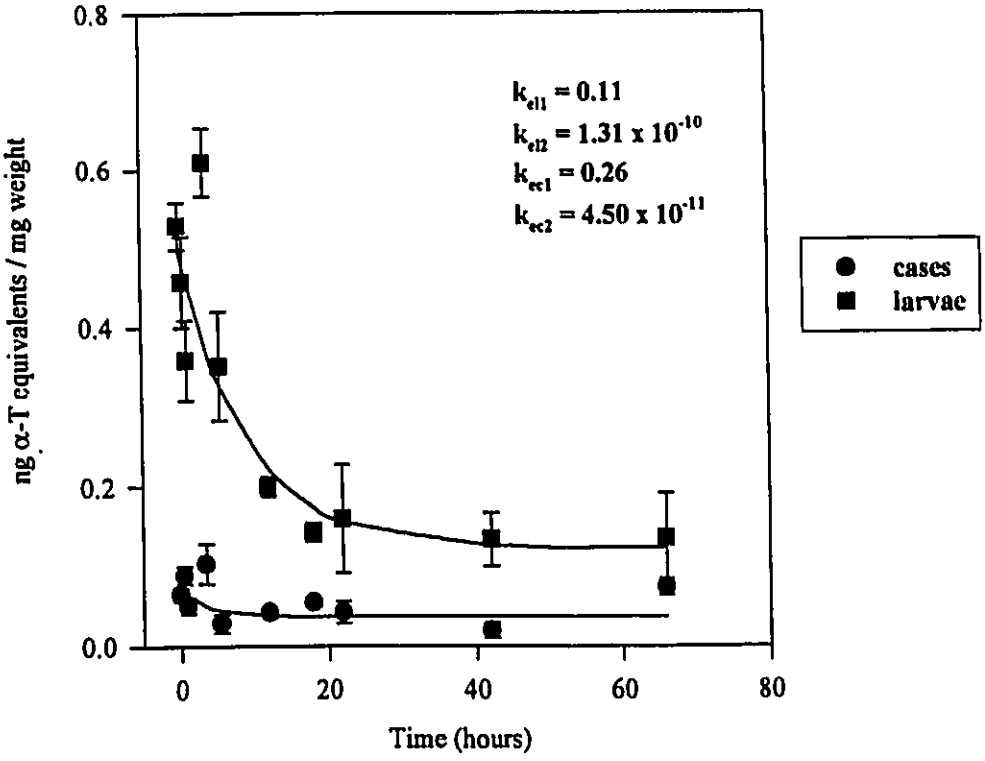


Figure 2.8. ^{3}H-α-T elimination from the case and carcass by 4th or 5th instar *Limnephilus indivisus* larvae in clean water after 30 minutes of exposure to 100 ppb ^{3}H-α-T. Error bars represent standard errors.

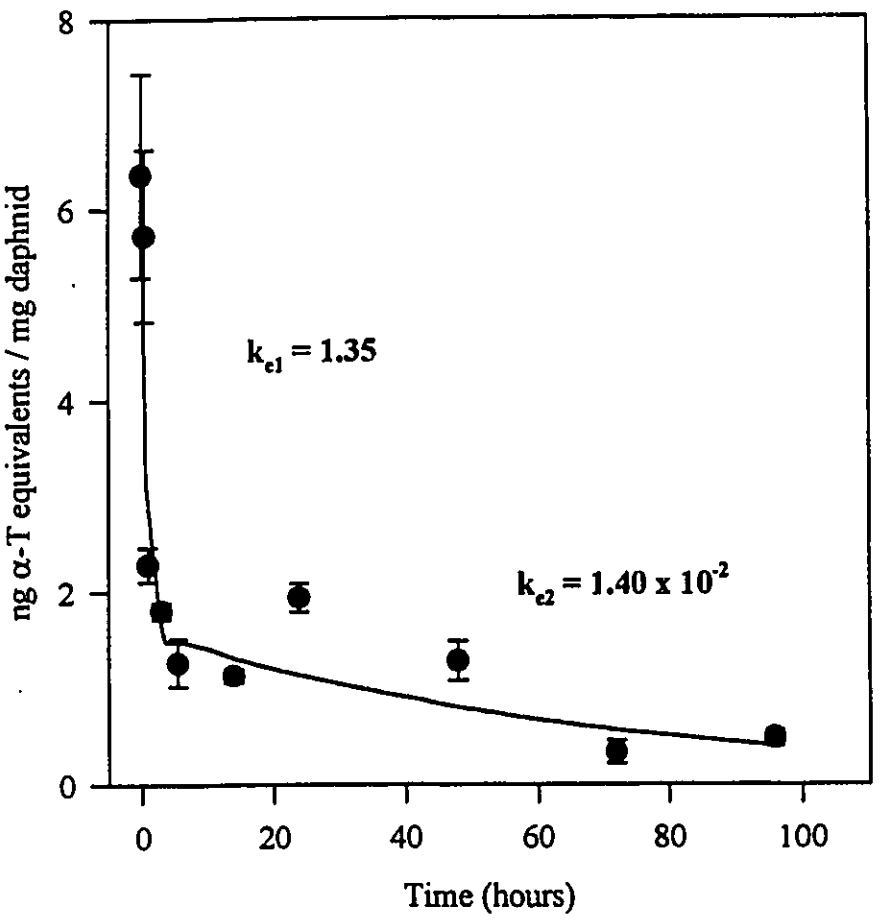


Figure 2.9. ^{3}H-α-T elimination by 1 week old *Daphnia magna* in clean water after 30 minutes of exposure to 100 ppb ^{3}H-α-T. Error bars represent standard errors.

Table 2.5. Kinetic parameters on the accumulation and clearance of ^{3}H-α-T in the test organisms.

Species	Uptake rate constants		Elimination rate constants	
	k_{u1}[a]	k_{u2}[a]	k_{e1}[a]	k_{e2}[a]
Daphnia magna	3.70	1.66×10^{-2}	1.35	1.40×10^{-2}
Limnephilus indivisus	(L)[d] 6.07 (C)[e] 1.28×10^{-2}	(L) 7.02×10^{-4}	(L) 0.11 (C) 1.26	(L) 1.31×10^{-10} (C) 4.50×10^{-11}
Aedes atropalpus	0.97	0.11	-	-
Aedes aegypti	-	-	(i) 1.19[b,c] (ii) 0.22[b,c]	1.04×10^{-2} [b,f]
Culex tarsalis	-	-	(i) 2.15[b,c] (ii) 0.28[b,c]	1.07×10^{-2} [b,f]

[a] $hour^{-1}$

[b] values obtained from Hasspieler *et al.* [1988]

[c] (i) was calculated from the first hour post treatment and considered as k_{e1}, and (ii) was calculated between 2 and 12 hours post treatment and considered to be k_{e2}.

[d] L =larvae

[e] C = cases surrounding larvae

[f] these values were considered to be k_{e3} by Hasspieler *et al.* [1988]

Table 2.6. ANCOVA on the accumulation of ^{3}H-α-T by *Aedes atropalpus, Daphnia magna* and *Limnephilus indivisus*.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
model	5	663.46	132.69	735.32	0.0001
species	2	479.53	239.77	1328.67	0.0001
time	1	84.05	84.05	465.74	0.0001
species*time	2	13.34	6.67	36.96	0.0001
error	311	56.12	0.18	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

Table 2.7. ANCOVA on the clearance of ^{3}H-α-T by *Daphnia magna* and *Limnephilus indivisus*.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
model	3	14.12	4.71	84.55	0.0001
species	1	7.62	7.62	136.84	0.0001
time	1	3.51	3.51	63.07	0.0001
species*time	1	1.64	1.64	29.46	0.0001
error	77	4.29	0.06	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

2.4 Discussion

One of the most basic and fundamental correlations in toxicology encompasses the characteristics of exposure and the resulting spectrum of effects. Acute toxicity bioassays are dose-response relationships that are described by two characteristics. The lethal concentration at which 50% (or 90%) of the test animals die (LC_{50}, or LC_{90}), and the slope of the probit regression. The term LC_{50} is used rather than the lethal dose (LD_{50}) because the animals are exposed to α-T by the water they are living in. The amount of α-T describes the concentration in the water, not the concentration in the animal's body [Klaassen and Eaton 1991]. The bioassays in this study have shown that the probit slopes are not significantly different from one another. Biologically, the slope represents the change in toxicity per unit change in α-T concentration. Acute toxicity bioassays do not provide enough information to draw any conclusions about reasons for toxicity differences. However, obvious physiological differences between species may explain the results. A narrowly defined concentration range suggests a simple rapidly saturable site for toxicity. Both non-target species, *Limnephilus indivisus* and *Daphnia magna*, had comparable slopes to *Aedes atropalpus*. The values of the LC_{50}'s and corresponding LC_{90}'s for the species are the same magnitude for mosquito larvae and the daphids, however, the LC_{90} for the caddisfly larvae is an order of magnitude higher than its LC_{50}'s. At larvicidal concentrations, under current lab conditions (the LC_{90} values for mosquito larvae), *Daphnia magna* would be wiped out. *Limnephilus indivisus* would be affected, but the populations would not be eliminated. This would seem to indicate that the most sensitive organism is the non-target *Daphnia magna* and the least sensitive organism is the non-target *Limnephilus indivisus*. Under field conditions the separation between target mosquitoes and non-target caddisfly is even greater [Philogene *et al.* 1986].

Daphnia magna is an aquatic invertebrate, however, unlike *Aedes atropalpus* and *Limnephilus indivisus*, it is not an insect. Daphnids are micro-crustaceans. They have a transparent carapace that allows light to penetrate readily into the organism. This makes them much more susceptible to phototoxins since light can pass through their bodies,

easily activating any α-T present inside. Both the mosquito and caddisfly larvae are more opaque. *Daphnia* (particularly neonates) are also much smaller in size than either of the other species. Thus they have a much higher surface to volume ratio. A much smaller amount of α-T can overwhelm this animal's body by ingestion, absorption through the carapace or absorption through the gills. This is reflected by the high mortality at very low concentrations.

Since the caddisfly larvae have an opaque case surrounding their soft bodies and they have a larger surface to volume ratio than either daphnids or mosquitoes, it was expected that they would be much less sensitive than the target mosquito. The acute toxicity bioassays confirmed that their response to α-T exposure was less than mosquitoes, however, they were much more sensitive than expected, based on previous field trials [Philogene *et al.* 1986]. This may be explained by their method of respiration. Caddisfly larvae have a case made of vegetative materials held together by silk that surround three quarters of their bodies. This limits light penetration into the larva's body, thus limiting the phototoxic effects of α-T. Water is actively circulated within the case to ventilate the gills, thus increasing exposure to the treated water and increasing oxygenation [Wiggins 1977]. In contrast, mosquitoes breathe air through a tracheal tube opening into the terminal pair of spiracles on the 8th abdominal segment [Clements 1963]. They do not filter oxygen from the water, thus exposure is more passive. Oxygen is necessary for photosensitization [Arnason *et al.* 1981*a*], however, α-T has shown activity in the absence of UV-A light towards mosquito larvae (*Aedes atropalpus*) [Marles *et al.* 1991*b*].

Temperature often affects susceptibility to pesticides. The toxicity can be affected in two ways: Toxicity may increase with increasing temperature, or it may decrease with increasing temperature. Generally, organophosphorus insecticides are positively correlated with temperature whereas most pyrethroids have a negative correlation with temperature [Grafius 1986, Turnbull and Harris 1986, Heimbach and Baloch 1994]. Temperature in aquatic environments is recognized as a critical factor since it affects respiration rates, chemical absorption, detoxification and excretion by organisms [Cairns *et al.* 1975, Howe *et al.* 1994]. The susceptibility of mosquito larvae to organophosphorus

insecticides increases with higher temperatures [Helson and Surgeoner 1983]. In contrast, the acute toxicity of mosquito larvae to α-T was not expected to be affected by temperature since the primary mode of action of the toxin is light mediated. Light mediated reactions such as photosensitization are temperature independent [Turro 1978]. This was confirmed by the bioassays done at 25°C and 7.5°C. There was no significant difference between the LC_{50}, LC_{90}, or the slopes. This lack of temperature effect is useful for mosquito larvae control since the larvae (particularly in Canada) develop in waters during the spring when temperatures vary widely [Helson and Surgeoner 1983].

Another important factor in explaining the toxicity results is that the animals used in the bioassays differed in age among species. The target organisms were mature larvae to demonstrate the sensitivity of even older larvae to the phototoxin. For pesticide regulation this is preferable. In contrast, the non-target organisms were very young which is the preferred stage for regulatory evaluators since it represents the most sensitive stage of the non-target and represents a 'worst case' scenario. The daphnids were neonates (under 24 hours old) and the caddisfly larvae were the first instar available after the pond thawed in April (2nd instar). This species of caddisfly overwinter as 1st instar larvae in diapause contained in a gelatinous matrix over the water [Wiggins 1973].

The ultimate toxicity is strongly influenced by toxicokinetics. The mathematical models describe the time course of disposition of α-T. Although disposition encompasses absorption, distribution, biotransformation, and excretion, this study only monitors the absorption or accumulation of α-T via uptake from water into the animal's body and the elimination of α-T from the body by whole body clearance. The uptake experiments were designed to maintain a constant concentration of α-T in the water over the duration of the experiment. This continued exposure complicates the uptake model since uptake and elimination are occurring at the same time. In this case, α-T will accumulate in the animals until a steady state is reached. The elimination experiments were a single exposure design to monitor the animal's ability to clear α-T from their body. Clearance describes the overall efficiency of removal of α-T. It is the most important index of an organism's ability to remove a toxin from their body [Klaasen and Rozman 1991].

The uptake of α-T by all three animals was biphasic. α-T was undoubtedly absorbed into the body via the gut and the body wall or respiratory gills. The accumulation of α-T is not a simple process, since it is the net result of uptake and elimination. The accumulation in the mosquito larvae showed the development of a steady state after 6 hours of exposure. This was probably due to the induction of detoxification enzymes that metabolize the lipophilic α-T to a more hydrophilic metabolite which can then be exreted more readily. Metabolites of α-T have been observed in the Lepidopteran larvae, *Manduca sexta* [Iyengar *et al.* 1990] and their presence have been suggested in mosquito larvae [Hasspieler *et al.* 1988]. The metabolism of α-T in mosquitoes will be discussed in more detail in Chapter 3.

The accumulation curves for *D. magna* and *L. indivisus* do not reach steady state. It is possible that steady state would be reached at a later point, however continuing the experiment beyond 96 hours would not be realistic or feasable without feeding. These animals may still have detoxification enzymes present and active, however they do not have the capacity to control the amount of α-T in their bodies at the concentrations they were exposed to. The presence of any such enzymes in caddisfly larvae is likely but uninvestigated. A recent study found that *Daphnia magna* expresses several P450 enzymes, particularly steroid hydroxyiases, that are differentially affected by exposure to xenobiotics [Baldwin and LeBlanc 1994]. As well, the initial rate constants of uptake are indicators of sensitivity. Caddisfly larvae had the most rapid initial rate and mosquito larvae had the slowest, although the total amount of α-T per mg body weight was highest for mosquitoes and lowest for caddisflies.

The elimination of α-T by all three organisms was biphasic. The biexponential decline of a toxin such as α-T is considered an open two-compartment system [Klassen and Rozman 1991]. Compartments are functional units that may be anatomical in nature or representing various states of a compound such as the parent and metabolite. They are not necessarily anatomical structures [Janku 1971 *in* Hasspieler *et al.* 1988]. The first compartment represents the main compartment involved in exchange of α-T with the environment. The process of elimination from this compartment was rapid as indicated by

the rate constants and as shown on Figures 2.8 and 2.9. The second compartment was slower to clear the α-T. It exchanged α-T only with the first compartment, not the environment. Most of the α-T absorbed by the animals passed directly through the first compartment and was cleared rapidly, however, a smaller portion was transferred to the second compartment. The slower phase of elimination may represent the induction of detoxification enzymes since the rate of elimination was much slower than initially. Since exposure was limited to 30 minutes, the animals were not saturated with the toxin, giving their detoxification systems the chance to effectively remove α-T from their bodies. Iyengar *et al.* [1987, 1990] and Hasspieler *et al.* [1988] found that the rate constant of elimination was inversely related to α-T sensitivity for Lepidoptera and mosquito larvae. In contrast, this study found that the rate constant of elimination was directly related to α-T sensitivity among aquatic species, but the proportional differences were unequal. Species differences especially with regards to light penetration, size (surface to volume ratio), and metabolic rates are the most likely reasons for the differing results from previous studies.

Chapter 3 : Identification of an α-Terthienyl Metabolite in *Aedes atropalpus.*

3.1 Introduction

Insects have the ability to defend themselves against a wide variety of insecticides. Insects do this by converting these lipophilic toxins into more hydrophilic compounds that can be removed by the normal excretion mechanisms. They can also develop insecticide resistance by enhancing their metabolic detoxification capability against these lipophilic foreign materials [Wilkinson 1983]. There are two stages of detoxification processes for xenobiotics. They are known as Phase I and Phase II enzyme reactions [Sipes and Gandolfi 1991]. The Phase I system is the main biotransformation pathway that adds polar functional groups to the lipophilic xenobiotic. The main oxidative enzyme system in insects are the poly-substrate monooxygenases (PSMO's), also known as mixed-function oxidases (MFO's), or the Cytochrome P-450 system [Wilkinson 1983, Sipes and Gandolfi 1991]. The Phase II reactions for biotransformation are biosynthetic and are primarily mediated by glutathione S-transferase (GST). It requires a cofactor, glutathione (GSH), which is a tripeptide of glycine, glutamic acid, and cysteine. The substrate (xenobiotic) must be somewhat hydrophobic, it must contain an electrophilic carbon atom, and it must react nonenzymatically with GSH at some measurable rate [Sipes and Gandolfi 1991]. Usually, the GST system acts to further detoxify reactive intermediates produced by the cytochrome P-450 system [Berenbaum 1991]. GSH is also a cofactor for GSH peroxidase which reverses lipid peroxidation. However, if GSH is depleted by the transferase system first, there will not be enough available to the peroxidase and lipid peroxidation will result [Sipes and Gandolfi 1991]. The GSH peroxidase and GST activity are catalysed by the same protein in insects which suggests a close association between detoxification and antioxidant defences. Other enzymatic antioxidants are GSH reductase, superoxide

dismutase, and catalase. Their presence has been shown in lepidopteran larvae [Pritsos *et al.* 1988, Lee and Berenbaum 1989].

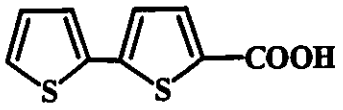
In mosquito larvae challenged with α-T, insect defences were shown to involve both the PSMO (shown in *Culex tarsalis*) and the GST (shown in *Aedes aegypti*) systems [Hasspieler *et al.* 1990, 1991].

Besides transformation by organisms, xenobiotics in the environment may be transformed by photodegradation. The degradation of α-T by photolysis in water (and methanol) produces a large number of products. Bioassays with yeast and brine shrimp have shown that the degradation products have very little or no phototoxic activity, except the bis-thiophenes which retain marginal activity. The photodegradation products that retain the ability to fluoresce are shown in Figure 3.1. These compounds are the bis-thiophene carboxylic acid (BCA) (1), mono-thiophene bi-carboxylic acid (4), and mono-thiophene carboxylic acid (5) [Arnason *et al. In Press*].

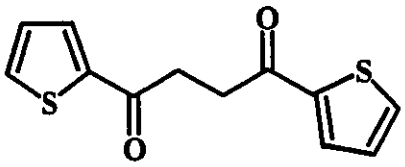
Two metabolites of α-T have been identified from metabolism studies in rats. The structures of these compounds are shown in Figure 3.1. One is a bis-thiophene di-ketone (2) and the other is the same bis-thiophene carboxylic acid (BCA) (1) found as a photodegradation product of the parent compound, α-T (3) [Marles *et al. In Press*]. No metabolite has yet been identified from any insect. Since (2) does not fluoresce, it is difficult to detect it in insects due to the extremely small amounts in question and the reduced sensitivity of detection by UV absorbance. Thus, only the BCA can be readily considered for metabolite identification in the mosquito larvae.

Fluorescence detection is an extremely sensitive technique for detecting trace amounts of these compounds. The objective of this study is to determine if exposure to α-T can lead to a detectable metabolite in the mosquito larva. This method of detection is limited to compounds that emit fluorescence when excited by light of a particular wavelength.

Figure 3.1. The structure of α-terthienyl (3) is shown with the two metabolites found in the rat. The bis-thiophene carboxylic acid or BCA (1) fluoresces while the bis-thiophene di-ketone (2) does not. BCA is also recognized as a photodegradation product, along with a mono-thiophene bi-carboxylic acid (4) and a mono-thiophene carboxylic acid (MCA) (5). Both (4) and (5) emit fluorescence [from Marles *et al. In press*, Arnason *et al In Press*].

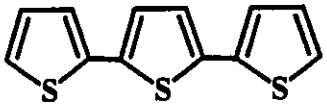


(1)



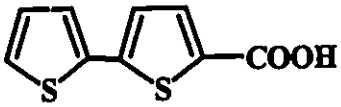
(2)

METABOLISM ↑

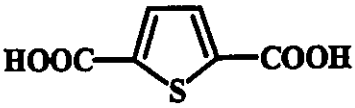


(3)

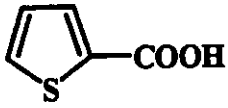
DEGRADATION ↓



(1)



(4)



(5)

3.2 Methods

The source of *Aedes atropalpus* is the same laboratory culture that was described in Chapter 2. The 4th instar larvae used for the metabolite identification were exposed to the ^{3}H-α-T by the same procedure used for the uptake experiment described in Chapter 2 that was adapted from Hasspieler *et al.* [1988]. Along with the early 4th instar larvae placed into each container for the uptake experiments, 120 additional larvae were placed into each container for metabolite analysis. At each sampling time, 20 larvae were sampled for the metabolite analysis. The times were 6, 12, 24, 48, and 72 hours after initial exposure. When the larvae were removed from the water, they were rinsed with distilled water through a 20 μm Nitex mesh sieve. The larvae were then placed into 7 mL boroscillicate glass vials with 2 mL of acetone. The samples were stored at -20°C until extraction.

The metabolite was extracted from the larvae by initially crushing them with a hand-held teflon homogenizer in the same vials. The crushed larvae were left to soak in the acetone for 48 hours in the dark at room temperature. After 48 hours, the samples were sonocated for 30 minutes then filtered through cotton wool. The samples were left to sit for another 24 hours in acetone, allowing proteins to precipitate out. An HPLC-grade membrane filter attached to a syringe was used to filter the precipitate off. The acetone was evaporated off and the extract was redissolved in 0.5 mL Omnisolv methanol.

The fluorescent metabolite, BCA, was identified with a Perkin Elmer LD40 fluorescence detector. This detector was attached to a high pressure liquid chromatography (HPLC) column (Beckman Ultrasphere C18 reverse phase; 4.6 mm x 25 cm; 5 μm pore size) and Perkin Elmer solvent pump. The samples (20 μL) were injected manually. A gradient solvent system was used to separate BCA from other compounds in the column. From 0-5 minutes, the percent of methanol increased from 20% to 70% (a solution of 1% glacial acetic acid in deionized water, pH 2.82, was used as the second solvent). From 5-10 minutes, methanol increased to 100%. For a further 10 minutes,

100% methanol was run. Then, for 10 minutes the system was flushed with 20% methanol to re-equilibrate before injecting another sample. The flow rate for this method was 1.0 mL per minute. The maximum excitation wavelength for BCA was determined by a Hewlett Packard Spectrophotometer and the maximum emission wavelength was obtained from a Perkin Elmer LS 50 Fluorimeter.

To identify a fluorescence peak as BCA, two methods were employed. First, the emission spectrum of the sample was compared to the standard. If the maximum emission wavelength of the sample was the same as the standard, using the same excitation wavelength, then the sample and the standard could potentially be the same compound. Second, a sample was spiked with a known volume of standard. For the Perkin Elmer LD40 system, a 25 µL Hamilton syringe was filled with 12.5 µL of standard compound and 12.5 µL of solvent. An air bubble in the syringe was used to mix the solution. The air bubble was released and the solution was injected into the HPLC. The ideal peak height obtained was 25% intensity. Then, using the same syringe, 12.5 µL standard and 12.5 µL of sample were mixed and injected to obtain a peak. The ideal intensity this time was 50%. Criteria used for chromatographic identity of the metabolite were: an increase of peak height of compound of interest following spiking with no peak splitting or development of shoulders, and complete resolution of the fluorescence peak of interest at reasonably long retention times. If these criteria were met, it was assumed that the sample and standard were the same compound.

Standard curves were developed for BCA in order to determine the concentrations of the compounds in each sample. A sample of pure compound (BCA) was weighed out and dissolved in an equal volume of acetone to give a concentration of 1 mg/mL. An aliquot (100 µL) of the stock solution was removed with a Hamilton syringe and in a new vial, 0.9 mL acetone was added to produce a 0.1 mg/mL (1 ppt) solution. Another aliquot (10 µL) of the stock solution was put into a separate vial and 0.99 mL of acetone was added, giving a 0.01 mg/mL (0.1 ppt) solution. Since it was very difficult to obtain 1 µL

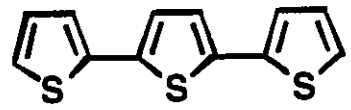
or less of the stock solution accurately, it was necessary to take aliquots of the 0.1 ppt solution. Although this produced dilution errors, it was unavoidable. An aliquot (100 μL) of the 0.1 ppt solution was taken and added to 0.9 mL acetone, giving a 0.001 mg/mL = 1 μg/mL (1 ppm). The dilution series was continued until a concentration of 1 pg/mL (1 pptr). Each solution was injected 3 times (three replicates) to obtain a fluorescence peak. Since the HPLC fluorescence detector is not interfaced with a computer, the chromatogram's peak height, representing the fluorescence intensity, was estimated with a ruler. The standard curve was described as the intensity versus concentration of BCA. If the relationship was linear, the concentration of samples could be determined using a standard curve.

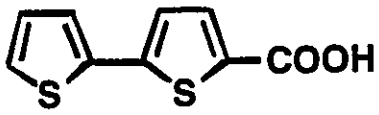
3.3 Results and Discussion

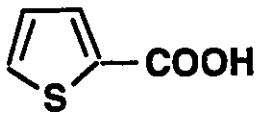
An absorbance scan of the metabolite, BCA, showed that the maximum excitation wavelength of BCA is 320 nm. A fluorescence scan indicated that the maximum emission wavelength is 400 nm (Figure 3.2). The absorbance and fluorescence scans of the parent compound, α-T and a degradation product, the mono-thiophene carboxylic acid (MCA), are shown for comparison purposes. All the peaks were normalized to an intensity of 1.0 for this comparison, thus concentrations were not considered. This was necessary since the fluorescence measurements were much more sensitive than the absorption measurements and as a result, the concentrations differed by orders of magnitude. The maximum excitation for α-T occurred at 350 nm and for the MCA, the maximum was at 250 nm. The maximum emission wavelengths occurred at 420 nm (α-T) and 320 nm (MCA). The difference between the maximal excitation and emission wavelengths was 70 nm for BCA and MCA. This difference was slightly larger (80 nm) for α-T. The difference between α-T and the breakdown products is most likely due to the increased conjugation in α-T. Fluorescence of a molecule always occurs at a lower energy than that

of the absorbed radiation or excitation energy. The lower energy is characterized by higher wavelengths that shift into the visible spectrum (400 - 780 nm) whereas absorption occurs in the ultraviolet (UV) range of the spectrum [Harris 1987, Turro 1991]. It appears that increased conjugation lowers the energy required for absorption and therefore the energy emitted in fluoresence is also proportionally lower [Turro 1991]. BCA has shown reduced phototoxicity in simple yeast bioassays when compared to α-T [Marles *et al. In press*]. MCA has also been tested for phototoxicity and was inactive in bioassays with yeast or brine shrimp [Arnason *et al. In Press*]. It is unlikely to exhibit such activity since it requires UV-B energy (200 -320 nm) for excitation. The laboratory lamps emit UV-A radiation (320 - 400 nm) and under field conditions, most high energy ultraviolet radiation from sunlight such as UV-B is filtered out by the ozone layer. The second metabolite, the bis-thiophene di-ketone (2) was not tested against brine shrimp, however it was also subjected to simple yeast bioassays and it did not show any phototoxic activity [Marles *et al. In press*]. The thiophene rings are not conjugated in (2), and the molecule does not fluoresce, thus it cannot operate by the same mode of action as its parent compound, α-T.

Figure 3.2 Absorption and fluorescence emission spectra of α-T (top), BCA (centre), and MCA (bottom) in methanol. Peak heights are normalized to an intensity of 1. The values for O.D. and fluorescence intensity have no units. The curves using triangles for symbols represent the absorption spectra and the curves using circles represent the fluorescence emission spectra.


S
S
S


S
S
COOH


S
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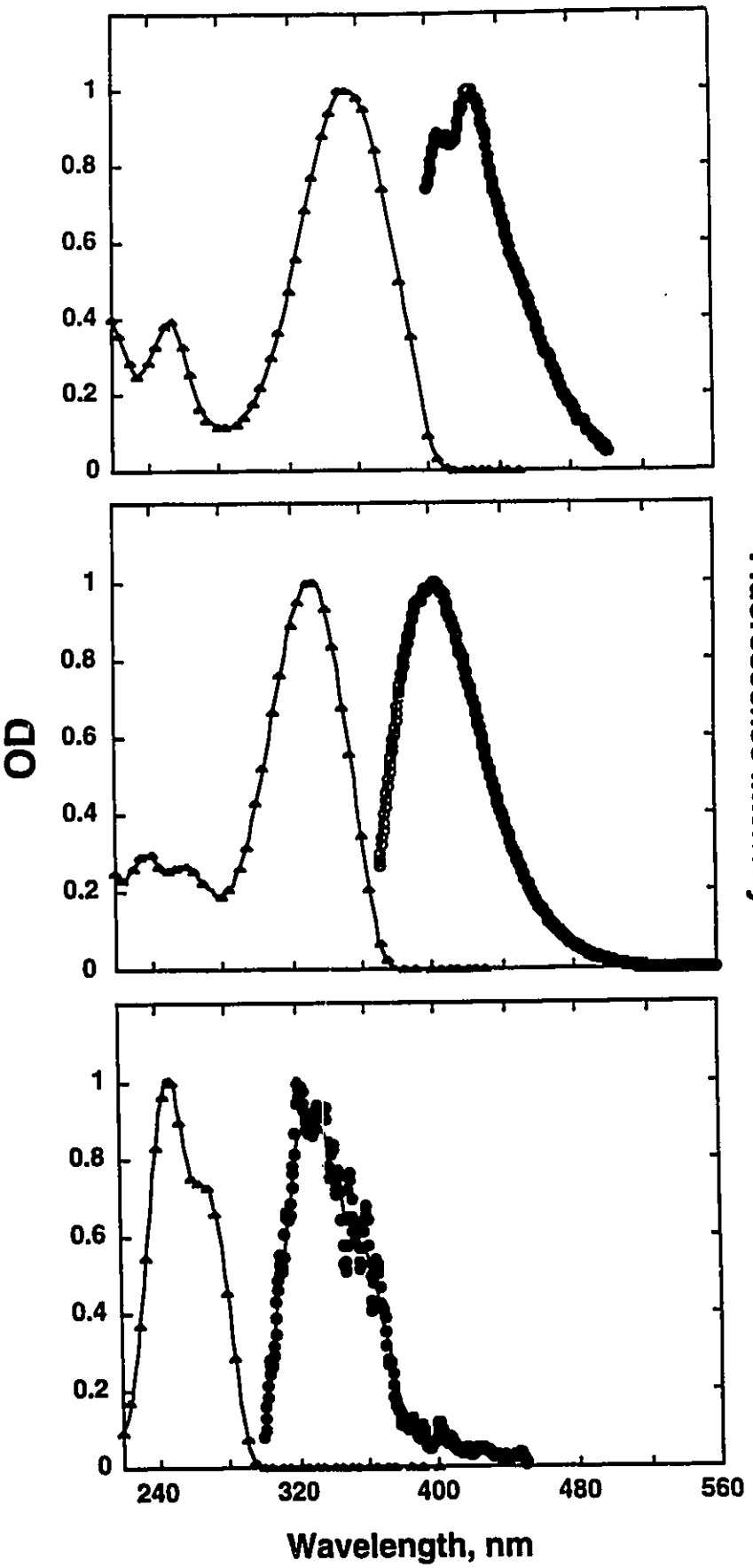

OD
Fluorescence Intensity
1
0.8
0.6
0.4
0.2
0
240
320
400
480
560
Wavelength, nm

Standard bis-thiophene carboxylic acid was synthesized and provided by Drs. M. Kobaisy and T. Durst. This synthetic compound was used to develop a reference standard curve from fluorescence intensity on the Perkin Elmer LD40 detector and HPLC. Figure 3.3 illustrates the fluorescence standard curve of BCA. This relationship shows that fluorescence intensity is directly proportional to BCA concentration when both variables are log-transformed. Although the linearity of this association correlates well with the relationship of absorbance and concentration, Beer's Law does not apply. The equation of the regression line was $\log I = 0.37 \log C + 2.29$, where I was fluorescence intensity and C was BCA concentration. The R^2 value was 0.97 ($p < 0.01$) indicating that the variance of log intensity strongly depended on the log BCA concentration. An interesting aspect of the standard curve is the range of concentrations detected. The concentrations are in the pg / mL or parts per trillion (ppt) range. This is an order of magnitude more sensitive than the range for α-T (part per billion or ppb). This extreme sensitivity was very useful for detection of BCA in small organisms such as the mosquito larva. However, it was much more difficult to obtain reliable, repeatable results since the variance in peak heights was increased. As a result the HPLC and detector system had to be kept very clean from contaminants and when possible, more replicates had to be done.

The metabolite, BCA, was identified in 4th instar *Aedes atropalpus* larvae by a dilution method using the HPLC. A sample of extract from the larvae was spiked with a known amount of pure BCA (standard). The HPLC fluorescence chromatograms are shown in Figure 3.4. The retention time for BCA was 660 seconds or 11 minutes, using the conditions described in the methods. Both the standard and the spiked peaks were sharp (narrow). There were no shoulders present and there were no other peaks that could reduce the certainty. There was a slight drift in the baseline, however that was common when a gradient was used. The spiked peak with the mosquito sample was approximately twice the intensity of the standard peak. The parent compound, α-T did not appear on the chromatogram because the solvent system used was very different from the system for α-T. α-T used an isocratic system of 72% acetonitrile (or methanol) with water. As well, BCA was more polar than α-T, thus it had a shorter retention time relative to α-T and would not be seen over the time that was measured. In addition, the

excitation and emission wavelengths were different for α-T, although they were sufficiently close to BCA that a peak would be observed at BCA's maximal wavelengths. It was not possible to confirm the metabolite structure by other analytical techniques such as gas chromatography-mass spectrometry (GC-MS) or nuclear magnetic resonance (NMR) for the samples extracted from mosquito larvae due to the extremely small amounts available. As well, the metabolites were radiolabelled since all the hydrogens in the labelled α-T were tritiated, but the amounts of metabolite present were so small that the liquid scintillation counter could not detect them. The spiking method provided identification of a peak with relative certainty since the sample corresponded to the standard in its retention time, there was a single sharp peak and the peak heights of the two chromatograms differed by 50% relative to each other. No other peaks on the chromatogram changed in height.

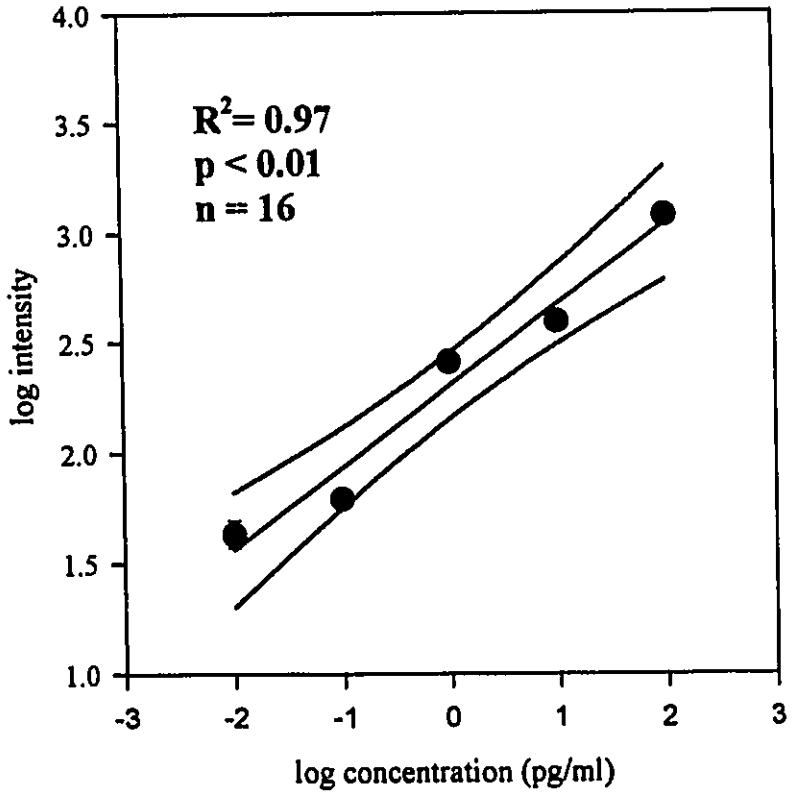
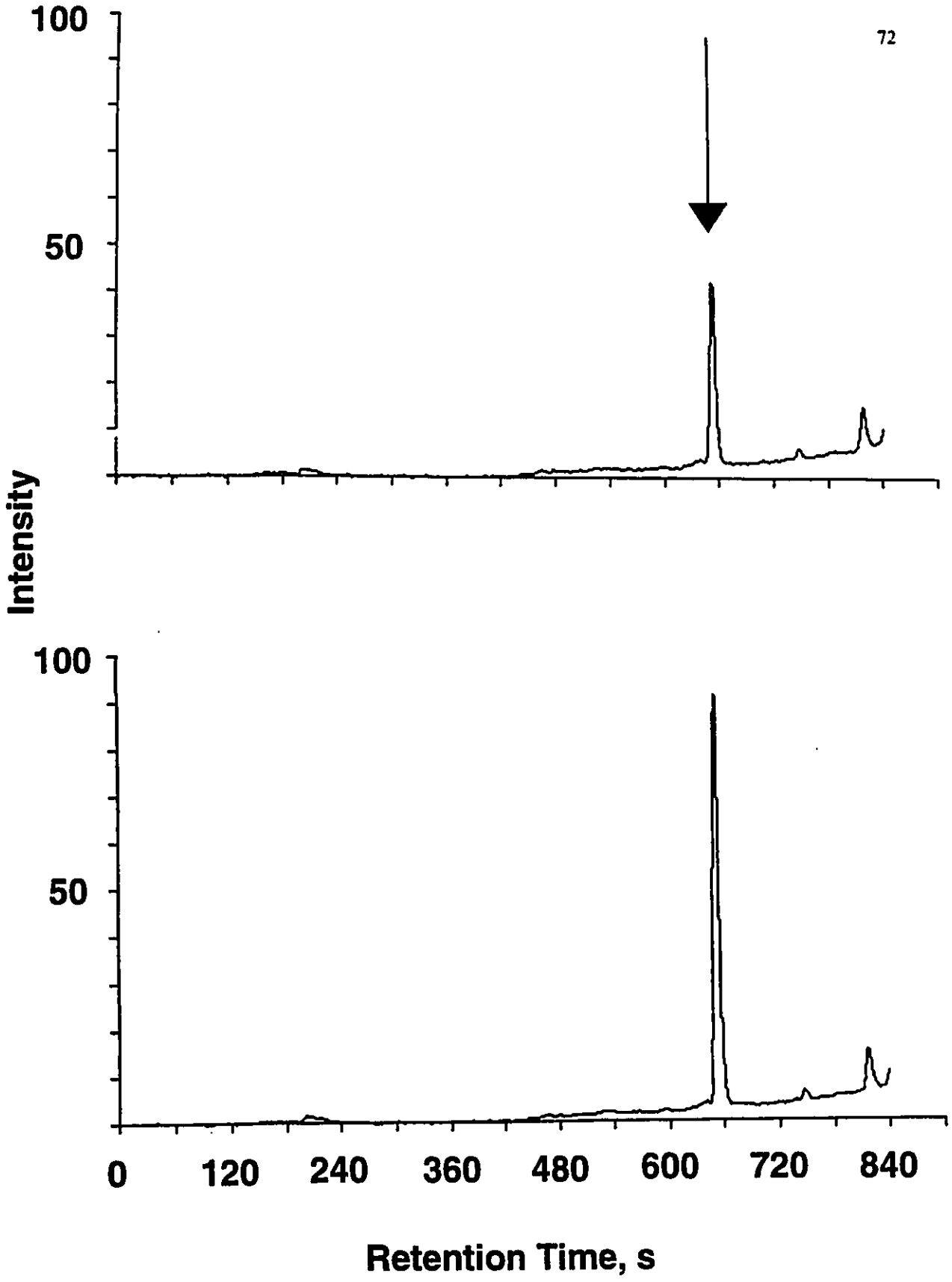


Figure 3.3. Fluorescence standard curve of BCA, a metabolite of α-T. Each point represents a mean and has standard error bars. 95% confidence intervals are shown for the regression line.

Figure 3.4 HPLC - fluorescence detector chromatogram of the α-T metabolite, BCA, identified in 4th instar *Aedes atropalpus* larvae by spiking the sample with pure, synthesized BCA. The arrow indicates the BCA peak. The top chromatogram represents a diluted solution of pure standard BCA. The bottom chromatogram shows the sample of mosquito extract spiked with pure standard BCA. The solvents system was pumped in a gradient of methanol and 1% glacial acetic acid.


100
50
Intensity
100
50
0
120
240
360
480
600
720
840
Retention Time, s

The levels of BCA in early 4th instar *Aedes atropalpus* larvae are illustrated in Figure 3.5. Samples were taken 12, 24, 48, and 72 hours after initial exposure to α-T. At 12 hours, the uptake of α-T by *A. atropalpus* larvae reached a steady state (see Figure 2.4). Although the curve for metabolite production is not a straight line, the large standard error bars imply that the values are not significantly different from each other. This was confirmed by statistical analysis. Analysis of variance (ANOVA) showed that there was no significant difference among any of the means from 12 to 72 hours ($F = 0.98$, $p = 0.45$). This indicates that the detoxification enzyme systems have reached a steady state whereby BCA is being produced constantly and then excreted. This is expected since the concentration of α-T was maintained at 100 ppb for the 72 hour experiment leading to continuous exposure for the larvae.

The induction of detoxification enzyme systems and their ability to metabolize α-T implies that eventually these insects could become resistant to the phototoxic effects of α-T. However, due to its mode of action, cross-resistance from other pesticides is not a threat. Conventional pesticides including organochlorines, organophosphates and pyrethroids have neurotoxic modes of action [Ecobichon 1991] and cross resistance to these substances in insect populations is common [Miller *et al.* 1983, Hama 1983, Matsumura 1983]. α-T is a photooxidant which does not exhibit neurotoxicity and malathion resistant insects are as sensitive to α-T as wild type populations [Hasspieler *et al.* 1990, Arnason *et al.* 1993].

In the long term, insects may develop a resistance to α-T since they already possess the enzymes required to detoxify α-T. These enzyme systems, however, could be overwhelmed by applying a larger amount of the phototoxin. In addition, synergistic compounds that inhibit the detoxification enzymes can be co-administered. Toxicity and photooxidation of membranes is enhanced by the PSMO inhibitor, piperonyl butoxide [Hasspieler *et al.* 1991, Fields *et al.* 1991] and a glutathione reductase inhibitor can also be an effective enhancer of toxicity [Arnason *et al. In Press*].

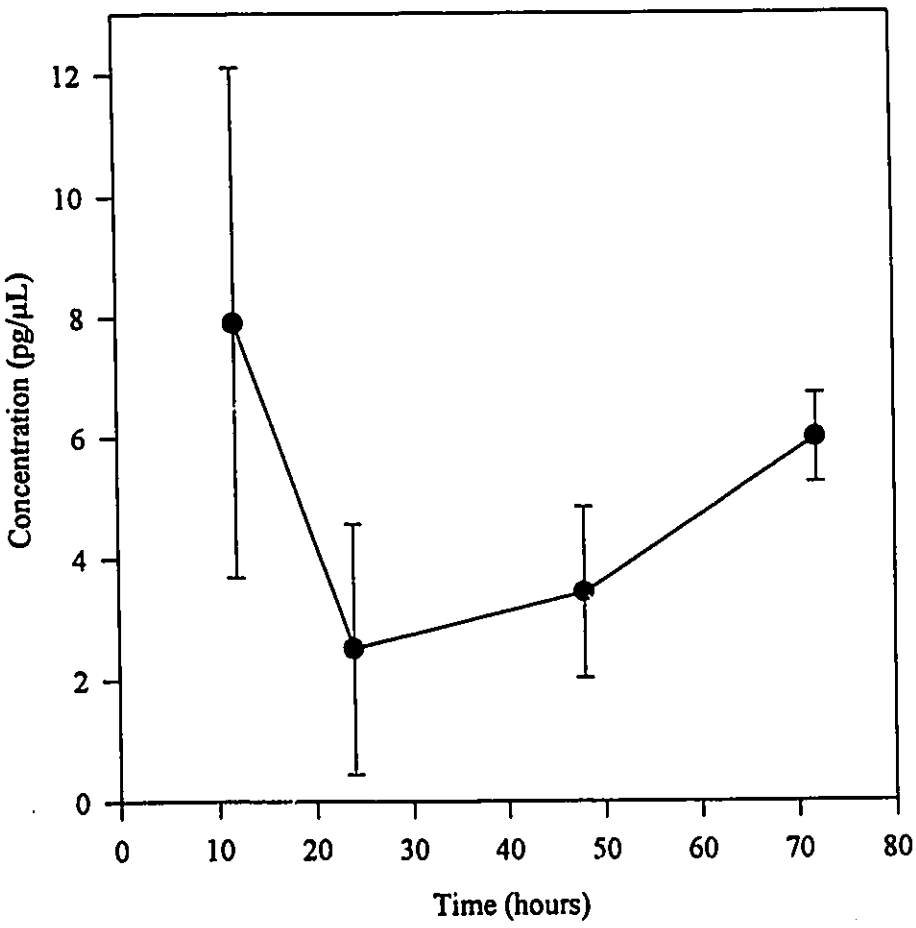


Figure 3.5. Production of BCA by early 4th instar *Aedes atropalpus* larvae, upon exposure to 100 ppb ^{3}H-α-T for 72 hours. Error bars represent standard errors.

Some insects may also be able to obtain non-enzymatic defenses from their diet to prevent oxidative stress. Some natural antioxidants are α-tocopherol (vitamin E), ascorbate (vitamin C), and β-carotene. Their effectiveness has been documented in terrestrial Lepidopteran larvae [Halliwell and Gutteridge 1985, Aucoin *et al.* 1990]. When both enzymatic and non-enzymatic defense systems are functioning, they provide effective protection from toxins such as α-T when only a small amount of α-T is administered. However, when exposed to α-T at higher, larvicidal, concentrations, these defenses are no longer effective and the insects exhibit oxidative stress [Hasspieler *et al.* 1990].

This is the first time an α-T metabolite has been identified in insects. Previous studies have shown that there are detoxification enzymes present in insects exposed to α-T. Their induction results in the rapid clearance of the phototoxin with reduced toxicity to the insect [Hasspieler *et al.* 1991, 1990, Iyengar *et al.* 1990, Feng *et al.* 1993]. Until now, however, no one has ever identified any metabolites from the insects. The ability to metabolize α-T to more hydrophilic compounds that can be excreted is, to some extent, an advantage of this botanical compound. This is an indication that although α-T is considered lipophilic, its potential to bioaccumulate may be reduced. In addition, the rapid photodegradation into reduced or non-phototoxic products prevents persistence in the environment.

Chapter 4. Fate of α-Terthienyl in an *in situ* Microcosm

4.1 Introduction

Natural snow melt pools, also known as temporary vernal pools, are basins that accumulate surface water but have no surface inlet or outlet. The water disappears from the surface in early summer (June and July) and becomes replenished only the following spring. Most surface water is obtained from melting snow and rainfall during March and April. During this short-lived pool stage, the diversity of animal species is high [Wiggins 1973, Barlocher *et al.* 1978].

Snow melt pools can be adequately modelled by microcosms. Microcosms are small ecosystems held in containers that can serve as experimental units for testing toxicity in aquatic systems. The properties of model ecosystems depend on several factors. The size of the containers determine whether they are microcosms (small glass containers or aquaria), mesocosms (larger enclosures such as limnochorrals) or macrocosms (a complete ecosystem). The isolation from the natural environment may be complete or partial. The origin of the organisms used for the study is also important. They may be laboratory cultured or collected wild species [Taub 1989, Metcalf 197?]. The present study used microcosms (by definition) since the containers were aquaria, but it was not a true model (laboratory) ecosystem since the containers were not completely isolated from the natural environment, thus not all the variables were being controlled. The organisms used for the study were collected from wild species as well. This fate study was more similar to *in situ* mesocosm studies such as enclosures in large bodies of water (for example, lakes). The aquaria were semi-submerged into the snow melt pools and were subjected to sunlight, temperature changes and rainfall. Thus, it is more appropriate to consider the setup as an *in situ* microcosm.

The *in situ* microcosm is an important tool for assessing toxicity. Single species toxicity testing does not provide information on potential indirect effects. It is not possible to predict ecosystem effects from single species effects. Not all species can be tested, although generally sensitive species are selected as the test organisms [Taub 1989, Metcalf 197?]. This type of study involves genotypic, spatial and temporal heterogeneity: since the biomass is collected from the wild, they are genetically variable; there is a spatial heterogeneity since there are different compartments within a unit and the study is temporally heterogeneous since the system is followed through time [Resh and Rosenberg 1989].

There are many requirements for standard aquatic model ecosystems. The system should contain all the trophic levels found in the natural habitat. There should be many compartments, the structures should be similar to natural conditions and it should contain all the functions of the natural systems. The test design should allow standardization, easy management, reproducibility, and predictive interpretation of direct and indirect effects. As well, the study should be reasonably cost efficient. These requirements are also the advantages of meso- or microcosms. They provide realism, replication, and repeatability [Heimbach *et al.* 1992].

The *in situ* microcosms were considered realistic with respect to the temporary vernal pools since there was no inlet or outlet for the water other than rainfall and evaporation. Material from these pools were used to setup the microcosms and the species being studied were native to the pools. Field trials including the present microcosm studies have much higher variances and less control, but they are much more realistic than a highly controlled laboratory study and provide valuable information about the fate of applied chemicals. The microcosms provide more control than field trials in snow melt pools since the number of organisms, volume of water and replication can be controlled.

The purpose of field testing is to validate risk assessment based on single species toxicity tests and evaluate the potential fate and effects of chemicals in natural ecosystems [Heimbach *et al.* 1992]. The purpose of this study is to determine the fate of α-T in semi-natural field trials using *in situ* microcosms for comparison with the laboratory studies to determine how environmental conditions modify fate data.

4.2 Materials and Methods

4.2.1 Field Site

The field site was located 50 km south of Ottawa, in the region of South Mountain, Ontario in a secondary growth beech-maple hardwood forest. The natural habitats of the mosquito larvae were snow melt pools in this forest. The pools were 4 to 8 m^2 in area by 15 to 40 cm in depth, with abundant leaf litter at the bottom. The native species of mosquito was *Aedes intrudens* that overwintered in the litter as eggs [Clements 1963]. The snow melted in late April and mosquitoes reached third or forth instars by mid-May (1993). The caddisfly larvae (*Limnephilus indivisus*) were also native to these ponds and reached third instar by mid-May since they overwintered as first instar larvae in a gelatinous matrix outside of the pond [Wiggins 1973]. *Daphnia magna* was native to the snow melt pools as well and were abundant as both adults and juveniles. They overwintered in the litter as dormant ephippial (sexually produced) eggs [Hebert 1978].

4.2.2 Emulsifiable Concentrate Solution of ^{3}H-α-T

The emulsifiable concentrate (EC) solutions were weighed out in triplicate since there were three trials. Each microcosm had 10 mg ^{3}H-α-T applied to it. The 10.02 mg

^{3}H-α-T was combined with 8.24 mg Atlox 3403F (Atchemix, Brantford, Ontario), and 8.84 mg Atlox 3404F (Atchemix, Brantford, Ontario). Atlox 3403F and 3404F are alkyl aryl sulfonate mixtures and dissolved in 40 µL o-xylene as has previously been described in Dosdall *et al.* [1991]. The EC solution was then diluted in 50 mL of distilled water and kept in a dark glass bottle at 4°C until the solution was applied. A control solution was made by the exact same procedure except ^{3}H-α-T was omitted. The 10 mg ^{3}H-α-T had a specific activity of 2.08 µCi/mg and corresponded to a total amount of radioactivity added to each microcosm as being 4.6 x 10^7 dpm. The ^{3}H-α-T was prepared by Dr. N. Werstiuk, McMaster University, using a high temperature dilute acid procedure.

4.2.3 Experimental Setup

The microcosm was made of a commercial glass aquarium with the dimensions, 30 x 60 x 30 cm. The surface area was 1800 cm^2 and the maximum volume capacity was 54 L. The outer walls of the aquarium were painted black in order to ensure that all the light entered from the surface only. This provided uniformity and a more realistic comparison to actual pond conditions. There were 3 replicates treated with ^{3}H-α-T, 1 formulation control, and 1 blank with no added chemicals. Each microcosm was partly submerged in the natural pond. There was shading by overhanging trees, but there was also some direct sunlight during the day. Sediment from the pond was placed into each aquarium to a thickness of 7 cm. The sediment consisted of a fine sand and leaf litter. A large plastic garbage bag was laid out on the sediment and 22 L of pond water, strained through a 1 mm mesh netting, was gently added to the tank. The plastic bag was delicately pulled out from one side to minimize disturbance of the sediment. The proximity of the microcosms to each other and their location in the pool is shown in Figure 4.1. Photographs of the field site are shown in Figure 4.2. Each aquarium had 4 bioassay cages added, except the blank, which had only 3 cages. The placement of the bioassay cages is shown in Figure

4.3. The bioassay cages were cylinders (12.5 x 15 cm) constructed of diamond-stamped aluminum surrounded by muslin held in place by rubber bands. Each cage contained 20 *Limnephilus indivisus* 3rd or 4th instar larvae, 20 *Aedes intrudens* late 3rd instar larvae, and 40 *Daphnia magna* adults. All three species were collected from local snow melt pools. The laboratory species, *Aedes atropalpus*, could not be used due to the risk of escape and introduction into the pond environment. Initial depth and temperature of the pond and aquaria were measured and the microcosms were allowed to equilibrate for 4 days. Standard model ecosystem studies allow 2 weeks for equilibration [Metcalf 197?], however due to the small size of the tanks and the shortness of the season, including the lifecycle of the organisms involved, the time had to be reduced. The tanks were set up May 14, 1993. On May 18, 1993, 4.6×10^7 dpm ^{3}H-α-T in EC formulation was added to each of the 3 replicates and an equal amount of EC formulation was added to the control. The 50 mL solution was gently dribbled over the water surface as evenly as possible with a pipette.

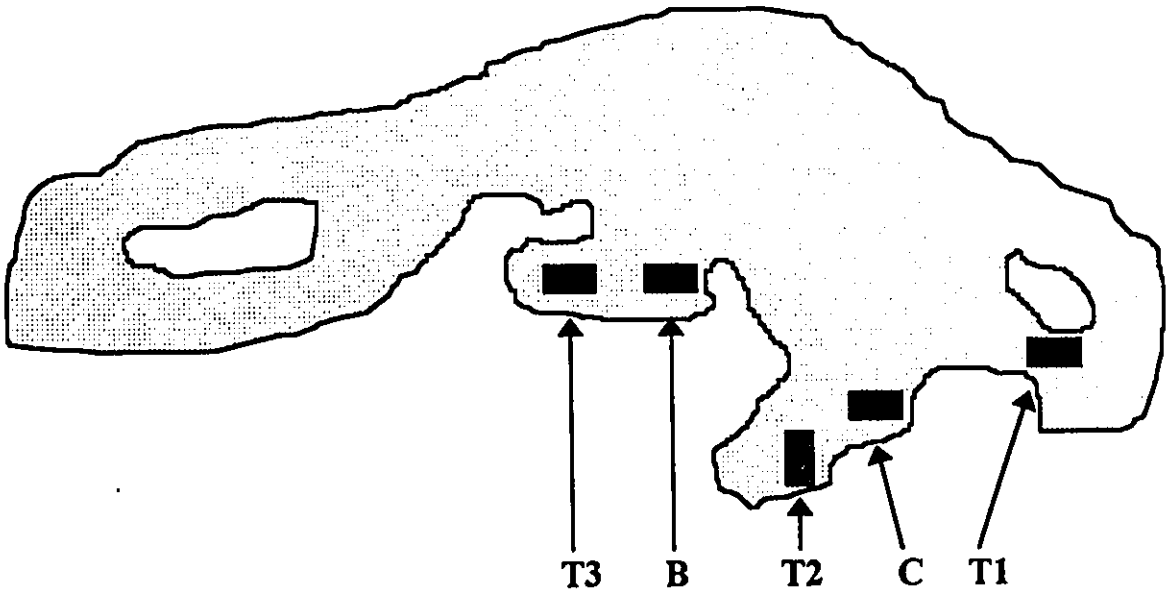


Figure 4.1. Schematic diagram of the field site. The light gray area is the natural snow melt pool, the white areas within the pool are dry land, and the dark gray rectangles represent the microcosms (aquaria). B is the blank, C is the formulation control, T1, T2, T3 are the microcosms treated with ^{3}H-α-T.

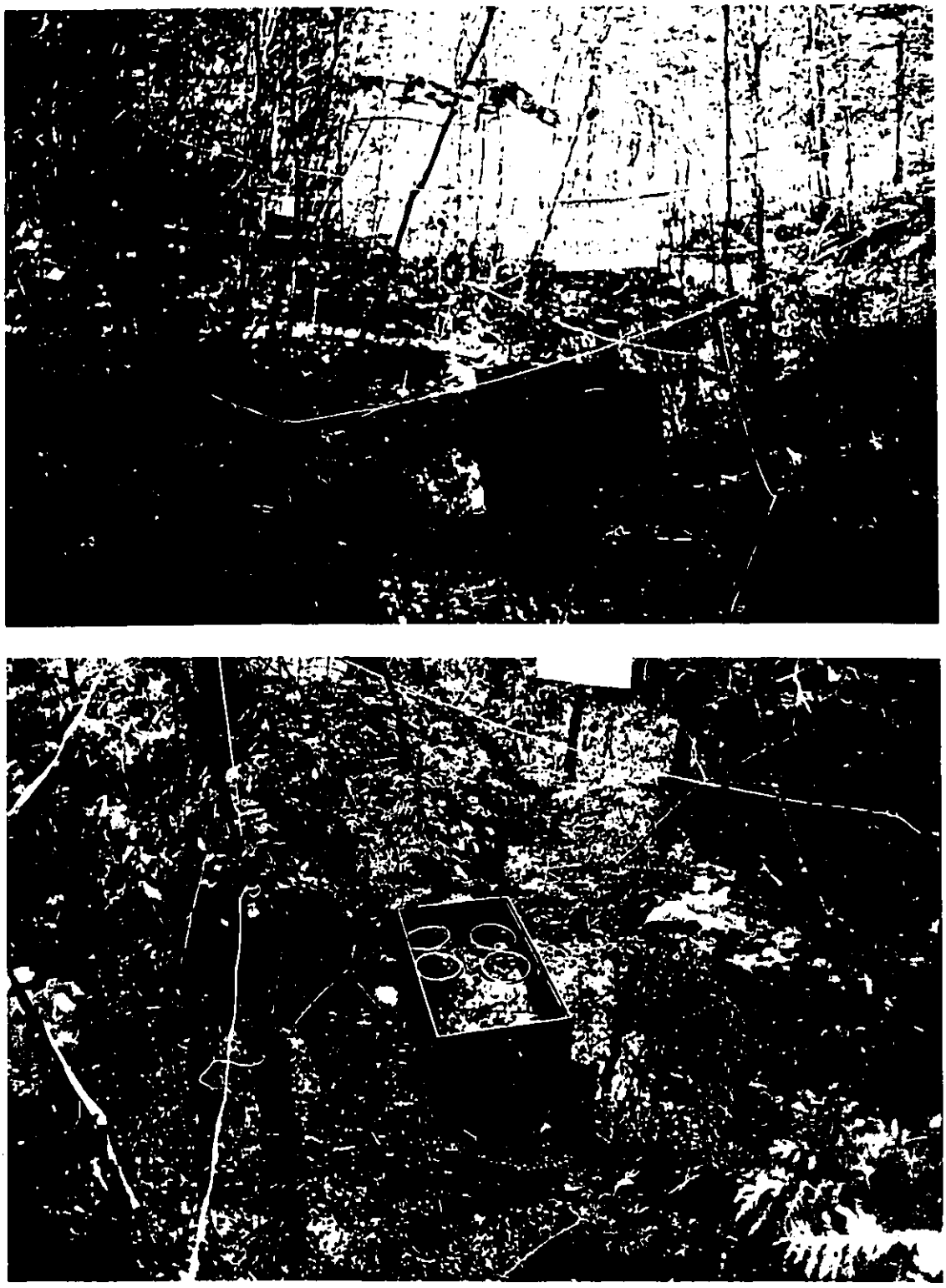


Figure 4.2. Photographs of microcosm placement in snow melt pool. The aquaria shown were not as deeply submerged as initially due to the rapid decrease in water level from the pool by the end of the fate study.

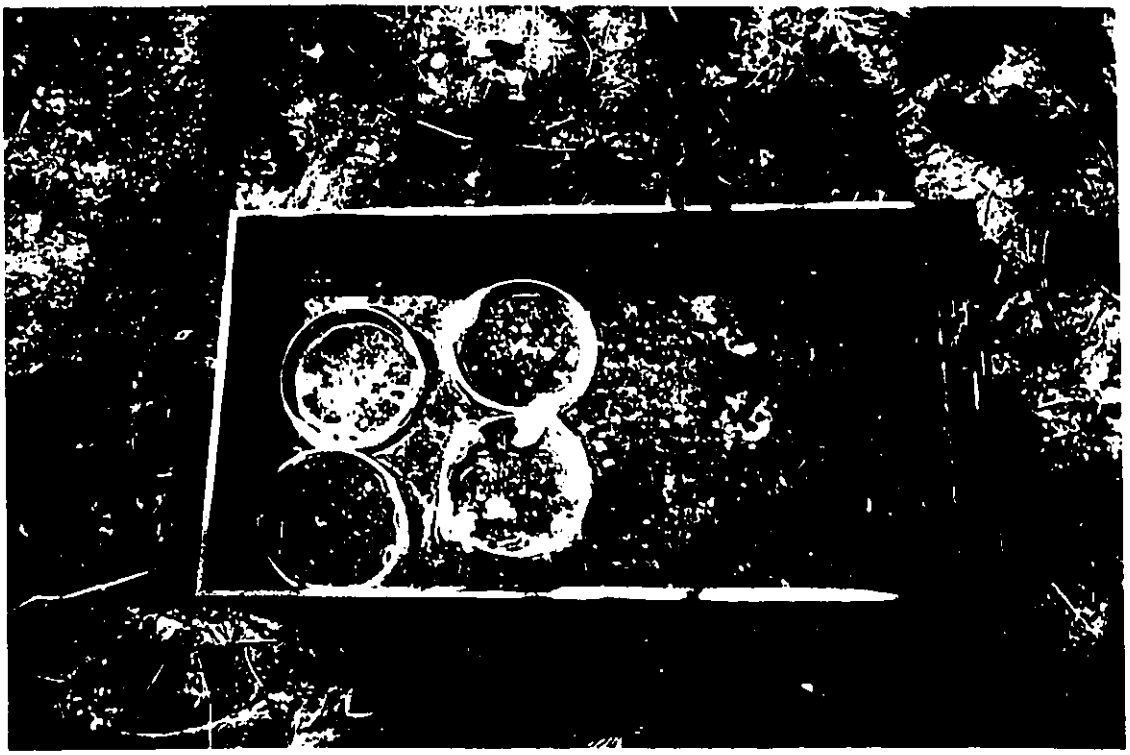


Figure 4.3. Photograph of an *in situ* microcosm treated with ^{3}H-α-T showing the placement of the bioassay cages.

Time zero was taken as the time at which the ^{3}H-α-T was added. The length of the study was only 10 days due to the short season and microcosm size restrictions. At each sampling time water depth and temperature were measured directly in the 5 aquaria and the pond. 20 mL of water was put into a glass vial and returned to the lab for pH and conductivity analysis. To determine the amount of oxygen in the water by the unmodified Winkler method, 132 mL of water were collected in a biological oxygen demand (BOD) bottle [American Public Health Association 1965, Wetzel and Likens 1979]. For analysis of ^{3}H-α-T, 50 mL of water was collected in an opaque glass container. At each sampling time, 10 *Limnephilus indivisus larvae*, 10 *Aedes intrudens* larvae, and 20 *Daphnia magna* were collected and placed into 7 mL glass scintillation vials (*L. indivisus* was placed into 20 mL vials due to their larger size) without water. The biomass samples were stored frozen at -20°C until analysis. A metallic sediment corer was used to sample the sediment by cutting through a cross section of it and removing it by suction with a minimum of disturbance to the microcosm. The sediment was put into a 500 mL plastic container that was lined with aluminum foil. All samples were kept frozen at -20°C until analysis. There were 8 sampling times over the 10 day period. The first was immediately after the addition of ^{3}H-α-T (day 0). The other times were 1, 2, 3, 4, 7, 8, 10 days after application. On day 4, 100 mL of water were sampled and on day 10, 60 mL were sampled for alkalinity to be tested in the lab. The meteorological summary of May 1993 was obtained from the Ottawa Weather Service. This provided air temperature (maximum, minimum, mean), amount of precipitation (rainfall), and the amount of sun hours.

4.2.4 Limnological Data

The following parameters were evaluated to assess the water quality in the microcosms for the duration of the fate study. These data were then compared with the pond conditions.

Temperature - The temperature was measured with a standard mercury thermometer submerged approximately 5 cm below the surface until the reading stabilized. All readings were expressed as degrees Celsius (^{o}C).

Depth - The depth was measured with a metric metal ruler and was expressed as cm. The pond was measured in more than one place and an average was taken. The same location was measured every time, however.

pH - A 20 mL sample of water was taken back to the lab and a standardized pH meter was used to determine the acidity of the water.

Conductivity - The specific conductance is a measure of the amount of total dissolved solids (TDS) in solution. Most TDS's ionized in water to some degree. The more TDS's, the greater the ionization, the lower the resistance of the solution to an electric current. Temperature also affects the degree of ionization so it must be measured at the same time [Rainwater and Thatcher 1960, Wetzel 1983]. Since conductivity was measured in the lab, the temperature did not represent field conditions, thus this parameter was only valid relatively, not absolutely. Conductance, by definition is the inverse of the resistance of a solution [Wetzel 1983]. The conductivity meter was used to measure the solutions (water from the pond and microcosms) and results were expressed in µmhos. In general, ranges categorize water hardness: <30 = extremely soft, 30 - 60 = soft, 61 - 180 = medium hard,

180> = hard [Rainwater and Thatcher 1960, Wetzel 1983]. The same 20 mL sample of water that was used for pH determination was used for specific conductance analysis.

Total Alkalinity - The total alkalinity measures the buffering capacity of the water. The ability to accept protons is determined mainly by the amount of bicarbonates and carbonates in the water, based on the equilibrium system CO_2-HCO_3-CO_3^{2-} [Wetzel and Likens 1979, Wetzel 1983]. This was determined by titrating a volume of water with a strong acid (0.1N HCl). Water was collected only twice during the course of the study. Once on day 4 (100 mL) and once on day 10 (60 mL). The samples were taken from each microcosm and the pond. The samples were separated into 2 aliquots for replication. The water was handled gently to avoid CO_2 exchange with the atmosphere since the titration measured the conversion of inorganic carbon to CO_2. The water sample was placed into a beaker with a magnetic stirring bar to slowly stir the water, and a pH electrode was inserted to measure the change in pH. Once the pH was stabilized, 100 µL 0.1N HCl was added and the pH was recorded. The addition of HCl was repeated until the pH level dropped to approximately 3.5. The pH was plotted as a function of the amount of acid added. From the curve, the inflection point was taken and the alkalinity was expressed as milliequivalents of acid combining capacity [Wetzel and Likens 1979, Wetzel 1983, American Public Health Association 1965].

Dissolved Oxygen - The oxygen content of the water was of particular importance to this study, not only as a necessity for aquatic life, but the ability of α-T to function as a photodynamic sensitizer also depends on the presence of oxygen in the water [Arnason *et al* 1981*a*]. The unmodified Winkler method is a well established accurate technique that determines the amount of dissolved oxygen in the water. This method uses a series of reactions to chemically determine the amount of oxygen by titrating liberated iodine in the water solution with a sodium thiosulphate solution (equation (1)). The assumption being

that the amount of iodine is equal to the amount of oxygen in the water sample, thus the amount of oxygen can be determined by the volume of thiosulphate used in the titration (equation (2)) [Wetzel and Likens 1979]:

$$MnCl_2 + 2NaOH + Mn(OH)_2 + 4HCl + 2KI + O_2 \longrightarrow 2NaCl + Mn(OH)_4 + 3H_2O + 2KCl + I_2 \quad (1)$$

$$4NaS_2O_3 + 2I_2 \longrightarrow 2Na_2S_4O_6 + 4NaI \quad (2)$$

therefore 1 equiv O_2 = 4 equiv $Na_2S_2O_3$

Water was collected from each aquarium and the pond by a 50 mL volumetric glass pipette which was submerged to the middle of the water column to avoid the supersaturation of the surface water. The water was gently transferred to a small biological oxygen demand (BOD) bottle with a 132 mL capacity. The bottle was filled until the water overflowed and no air bubbles were present. Separate glass pipettes were used to deliver 1 mL of manganous salt solution (100 g $MnCl_2 \cdot 4H_2O$ dissolved in 250 mL distilled water) to the sample, then 1 mL of alkaline iodide (125 g NaOH and 37.5 g KI dissolved in 250 mL distilled water) was added. Without introducing any air bubbles, a glass stopper sealed the bottle and the water solution was shaken for approximately 30 seconds. A large amount of precipitate settled to the bottom. This state was stable enough to store temporarily. Then 2 mL of concentrated HCl was added carefully below the surface to dissolve the precipitate. The stopper was replaced and the bottle was shaken well until the solution turned yellowish brown and no precipitate remained. This

colour was due to the presence of liberated free iodine which was directly proportional to the amount of oxygen which was present. The amount of iodine (and thus oxygen) was quantified by titration with sodium thiosulphate solution. An aliquot (50 mL) of the water solution was transferred to a 250 mL Erlenmeyer flask with the bottom painted white. Standardized thiosulphate solution was prepared by dissolving 24.82g $Na_2S_2O_3 \cdot 5H_2O$ in 200 mL H_2O, adding 4 g borax [$Na_2B_4O_7 \cdot 10H_2O$] and making it up to 1 L in a dark glass bottle giving a stock solution of N/10. An aliquot (125 mL) of N/10 stock was mixed with 250 mL distilled water, 3 g of borax was added and the solution was made up to 1 L. The N/80 stock had to be standardized with standard potassium dichromate ($K_2Cr_2O_7$) solution. $K_2Cr_2O_7$ was dried in an oven for 1 hour, cooled and exactly 1.225 g was dissolved into a 1 L solution with distilled water in a glass bottle (N/50 stock). In a 500 mL Erlenmeyer flask, 2.02 g KI was dissolved with 125.0 mL distilled water, 10 mL 1+9 H_2SO_4 (1 mL concentrated H_2SO_4, 9 mL water) was added, and exactly 10 mL N/50 (0.025 N) $K_2Cr_2O_7$ was also added. The solution was left in darkness for 5 minutes and diluted to about 400 mL. The solution was titrated with thiosulphate solution until it turned faint yellow, at which point a few drops of starch was added. The titration continued until the first disappearance of blue colour. The actual quantity of solution used was divided into 20. The known normality of thiosulphate solution was 0.0125 N (N/80) times this factor. This was used to determine the normality factor of the thiosulphate solution. A 10 mL burette was used for titrating the thiosulphate with the iodine solution until the colour became more faint. Then 1-2 drops of starch solution (3 g powdered commercial cornstarch was dissolved in 125 mL of distilled water by heating to 100°C then cooling to room temperature) was added to the flask and titration continued until the blue colour disappeared. The dissolved oxygen in the sample was equal to the volume of N/80 thiosulphate used times the normality factor. The units that describe the concentration of oxygen is mg per liter [Wetzel and Likens 1979, American Public Health Association 1965].

An oxygen saturation monogram was used to estimate the percent saturation of oxygen. By aligning a ruler through the amount of oxygen found in the sample, the water temperature and the altitude (Ottawa is at 600 m) a value for the percent saturation was read directly off the graph. The monogram is given in Wetzel and Likens [1979].

4.2.5 Water Analysis

Water samples were taken 0, 1, 2, 3, 4, 7, 8, 10 days after the application of ^{3}H-α-T to the microcosms. A 50 mL sample was taken from each aquarium and from the pond. The water was collected by a 50 mL volumetric pipette and stored in a 100 mL dark glass bottle. Prior to use, the bottles were stored on their side in a freezer at -20°C.

To extract the α-T, the water was thawed at room temperature, poured into a 250 mL separatory funnel. The bottle was rinsed with hexane, then methanol. The solvents were added to the funnel along with 30 mL hexane and 30 mL methanol to wash the water. The upper layer was hexane while the lower layer consisted of methanol and water. The two were separated and the methanol/water layer was returned to the separatory funnel. It was rinsed again with 30 mL of hexane, the hexane layer was collected, and the methanol/water was washed once more with 20 mL of hexane. The combined hexane or methanol fractions were evaporated using a rotary evaporator, transferred to glass scintillation vials and dried. The fractions were redissolved in 0.5 mL analytical grade (Omnisolv) methanol for liquid scintillation counting.

Liquid scintillation counting was used to measure the radioactivity. To reduce chemiluminescence, 20 mL glacial acetic acid was added. The liquid scintillation cocktail or fluor (Cytoscint, ICN Biomedicals Canada Ltd.) was added to the solution last (5 mL). The solution was mixed and left in darkness at room temperature for 24 hours. The radioactivity was counted as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer.

The efficiency of this technique was determined by spiking a sample of pond water with a known amount of ^{3}H-α-T. A beaker was filled with 500 mL of pond water (filtered through 1 mm pore size mesh) and 10 µL of ^{3}H-α-T (EC formulation, 10 mg/mL, 2.08 µCi/mg, 4.62 x 10^5 dpm) was injected onto the surface. The beaker was covered with aluminum foil and placed into a dark 15°C incubator for 24 hours. After 24 hours, the beaker was removed and the 500 mL were divided into 3 aliquots of approximately 166 mL. Each aliquot was placed into a separatory funnel and 30 mL of hexane along with 30 mL of methanol were added to remove the α-T from the water. The methanol/water fraction was washed again with 30 mL of hexane and a third time with 20 mL of hexane. The two fractions were evaporated down to volumes between 10 -20 mL (measured exactly) and a 1 mL aliquot was sampled from each fraction and prepared for liquid scintillation counting as described above (using 20 mL vials). A control was made using the same procedure with 10 µL of formulation (no ^{3}H-α-T). The following relationship was used to determine the percent efficiency of extraction:

% efficiency = amount ^{3}H-α-T recovered / amount ^{3}H-α-T added x 100% (3)

4.2.6 Sediment Analysis

The sediment samples were removed from the freezer and thawed at 15°C in a dark incubator for 24 hours. The sediment (sand, leaf litter, plant roots) was crudely cut up using a pair of scissors. The sample was placed into a homogenizing cup with enough methanol added to partially cover it. This cup was attached to a large blade Polytron homogenizer and the mixture was homogenized for 10 seconds on a setting of 6. The blade was rinsed with methanol and the slurry was poured into a metal tray lined with aluminum foil. This slurry was dried in a 60°C oven overnight or until dry. The dried

sediment was weighed, wrapped in aluminum foil and stored in the freezer until needed for extraction. To extract α-T and any breakdown products from the sediment, a portion of the dried sample was weighed exactly (~25 g) and placed into a Soxhlet thimble. Extraction was done by Soxhlet for 12 hours using a solution of hexane/acetone/glacial acetic acid (59/40/1; v/v/v). This procedure was adapted from Pierce *et al.* [1980]. After 12 hours, the extracted solution was removed and the solid sediment in the thimble was discarded. A rotary evaporator (rotovap) was used to evaporate the solution down to approximately 10 - 20 mL to remove the hexane and leave the methanol. This volume was measured exactly and a 1 mL aliquot was removed for liquid scintillation counting. To reduce chemiluminescence 20 μL (1 drop) of glacial acetic acid was added. The solution was diluted with 15 mL of scintillation cocktail, stored in darkness overnight and the radioactivity was counted as dpm on the 2000CA Tri-Carb Liquid Scintillation Analyzer.

The efficiency of this technique was assessed by spiking wet, uncontaminated sediment with ^{3}H-α-T. The sediment was weighed and put into 400 mL beaker. The sediment was covered with 250 mL dechlorinated tap water and 10 μL of ^{3}H-α-T (EC formulation, 10 mg/mL, 2.08 μCi/mg, 4.62 x 10^{5} dpm) was injected onto the water surface. The beaker was covered with aluminum foil and placed into a dark 15°C incubator for 5 days. When the beaker was removed from the incubator, the water was filtered off with a Buchner funnel. The sediment was placed onto a metal tray lined with aluminum foil which was then put into an oven at 60°C for 24 hours or until dry. The dried sample was put into polytron cup and homogenized with Soxhlet solvents (described above). The sediment was filtered through No.1 Whatmann filter paper in Buchner funnel. An aliquot of the sediment was weighed exactly (~25 g) and put into thimble. The filtrate was refiltered through No.42 Whatmann filter paper and the solvent was put into a roundbottom flask that attached to the Soxhlet apparatus. The extract was prepared for analysis by the technique described for the microcosm samples. A control was made using

the same procedure with 10 μL of formulation (no ^{3}H-α-T). The percent efficiency of extraction was calculated using equation (3).

4.2.7 Biomass Analysis

The biomass samples were removed from the freezer and thawed prior to analysis. The contents of each vial was weighed wet in a weighing tray, placed in a 20 mL glass scintillation vial and enough acetone was added to cover them (about 1 mL for the mosquito larvae and *Daphnia*). The caddisfly larvae were separated into two vials each containing 5 larvae and their cases (about 5-10 mL of acetone was used to cover them). The mosquito larvae and daphnids were crushed with a hand-held Teflon homogenizer in the vial. The caddisfly larvae and their cases were homogenized together with a Polytron Homogenizer. The acetone was evaporated off and 0.5 mL Protosol tissue solubilizer (0.5 molar solution, New England Nuclear, Dupont, Boston, MA) was added to liquefy the organisms (1 mL Protosol was used for the caddisfly larvae). After 24 hours, 20 μL glacial acetic acid and 15 mL scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) was added. The solution was mixed and left at room temperature for 24 hours. The radioactivity was then counted for 10 minutes per sample as disintegrations per minute (dpm) on a 2000CA Tri-Carb Liquid Scintillation Analyzer. No experiments were done to determine the efficiency of extraction since α-T was not extracted from the organisms. All the organisms were used entirely, rather than aliquots of them.

To determine as many components as possible for the mass balance of the fate study, the aquaria were rinsed out with dechlorinated tap water and dried. One wall of the length and one wall of the width were wiped entirely with filter paper soaked in 95% ethanol. Another paper was used to wipe to bottom of the tank. The papers were air dried, placed into 7 mL glass scintillation vials, each was filled with 5 mL of scintillation cocktail (Cytoscint, ICN Biomedicals Canada Ltd.) and counted for 10 minutes on the

Scintillation Counter. The values for the sides were doubled and added to the bottom to represent the entire aquarium. This residue was the amount of ^{3}H-α-T adsorbed onto the glass. The following calculations were then made:

total ^{3}H-α-T recovered = amount in biomass + amount in water + amount on glass (4)

% recovery = total ^{3}H-α-T recovered / amount ^{3}H-α-T added (5)

These methods did not distinguish between α-T and any breakdown products, however they provided information about the total quantities of radiolabelled compounds present and this was a reliable technique to indicate the overall fate of ^{3}H-α-T in an aquatic system.

4.2.8 Statistical Analysis

The water quality data were analyzed by one-way analysis of variance (ANOVA) to test for overall differences in the means of the treated microcosms, control microcosms, and the natural pools. The oxygen data along with the distribution of ^{3}H in the species comprising the biomass, water and sediment, were all analyzed by one-way ANOVA with a repeated measures design. The oxygen data compared treated and control microcosms with the natural pool. The distribution analysis compared the different compartments, but could not compare the different microcosms in the same test because the data did not provide enough degrees of freedom for a two-way ANOVA (repeated measures). A repeated measures design was used because the assumption of independence was violated by repeated sampling of the microcosms for the duration of the study [Zar 1984]. The repeated factor was time. All analyses were done using SAS [SAS Institute Inc. 1985].

4.3 Results

The field site chosen for the fate study was a natural mosquito breeding area. Snow melt pools were common and the mosquito larvae (and adults) were plentiful. The study dates (May 18-28, 1993) were chosen because of the late season that year. The snow did not melt until the beginning of May and as a result, aquatic insect populations did not flourish for another week. The most abundant species collected were mosquito larvae (*Aedes intrudens* Dyar), microcrustaceans (*Daphnia magna* Straus), and caddisfly larvae (*Limnephilus indivisus* Walker). Collection of other invertebrate species were sporadic, therefore, only these three species were used to represent the biomass. The microcosms were setup when the mosquito larvae reached the 3rd instar stage. The weather conditions for the duration of the fate study are summarized in Table 4.1. The data obtained from the Ottawa Weather Service was averaged over the 10 days after application of α-T. The amount of rainfall was much higher than the average rainfall due to heavy rain on the sixth day after application. There was rain at the field site on day 1, 6, and 10 but days 1 and 10 were light showers. The amount of rainfall is also illustrated in Figure A5 (Appendix) with respect to the water depth in the microcosms. The average amount of sun per day was 7.0 ± 1.3 hours. The air temperature varied on average by 10 degrees on a daily basis, with a mean value of 11.7 ± 0.5°C. The mean air temperature is also shown with respect to the water temperature in Figure A4 (Appendix).

Data was also obtained characterizing the water quality. Water samples were collected on each sampling day and analyzed for the oxygen content, alkalinity, conductivity, and pH. The water depth and temperature were measured on site. The results were averaged over the 10 day study and summarized in Table 4.2. The daily values showing the trends in the water quality are shown in Figures A1 to A5. The oxygen content trends are shown in Figure 4.4 due to the importance of oxygen as a factor for α-T toxicity. Analysis of variance (ANOVA) was performed on the overall average of the log-transformed values for conductivity, pH, water temperature, water depth and alkalinity. The residuals were not normally distributed, except for alkalinity, but the

variances were all equal (homoschedastic). ANOVA is robust to violations of the normality assumption, thus the parametric test was carried out. The pH values of the five microcosms and the snow melt pool were not significantly different from each other ($F = 0.028$, $p = 0.973$). The pH values indicated that the waters were moderately acidic. There was no significant difference in the alkalinity of the treated and control microcosms and the pool ($F = 2.089$, $p = 0.180$). The alkalinity of the water was very low, thus the values for the amount of milliequivalent acid combining capacity or meq $[CO_3^{2-}]$ per L water indicated that the concentration of CO_2 was very high. Since the pH was low (below 7), most of the inorganic carbon species in the buffering system were in the form of free CO_2. Thus these waters were poorly buffered. There was no significant difference in the water conductance of the treated and control microcosms and the pool ($F = 0.331$, $p = 0.720$) indicating that the total dissolved solids in the microcosms do not differ significantly from the natural pool. The mean specific conductance values are all below 30 μmhos which categorizes the water hardness as extremely soft (from the scales provided by Rainwater and Thatcher 1960, and Wetzel 1983). The water temperature of the microcosms and the pool were all approximately the same and they were also not significantly different from the mean air temperature ($F = 0.068$, $p = 0.934$). This is not surprising since the bodies of water were so shallow. The water depth of the 5 microcosms did not significantly differ from each other ($F = 2.662$, $p = 0.111$), however, the microcosms were significantly more shallow than the natural pool ($F = 55.22$, $p = 0.001$).

The water quality of the microcosms (both treated and untreated) was a good representative of the water quality of the natural snow melt pool when considering the parameters analyzed in this study. The only exception to this was the oxygen content of the water. The oxygen content of the water was reported as percent saturation because the solubility of oxygen in water is influenced by the water temperature (as well as air pressure and location altitude). The percent saturation was calculated from the concentration of oxygen found in the water taking into account the other influences mentioned above. The average percents over the entire study are shown in Table 4.2. Analysis of variance on these means indicated that the percent saturation of oxygen in the

natural pond was significantly different from the saturation in the microcosms. The oxygen content of the microcosms did not differ between treated and controls ($F = 31.121$, $p = 0.001$, Student-Neuman-Keuls multiple comparison test). The mean values indicate that the average oxygen content of the natural pool over the 10 day study was completely saturated while the microcosms were anoxic. Figure 4.5 illustrates the percent saturation of oxygen in the treated microcosms, the controls and the natural pool for the duration of the fate study. Initially, the pool was supersaturated, the controls were saturated - slightly supersaturated, while the treated microcosms were moderately saturated. The oxygen content of all the water bodies tested declined after the initial readings, probably due to an increase in temperature, however, oxygen content of the pool remained moderately high and returned to a supersaturated state on day 4 of the study. In contrast, the microcosms became anoxic (extremely low in oxygen content), particularly 3 and 4 days after treatment. There was a moderate increase by day 7, probably due to heavy rainfall on the 6th day of the study. At the end of 10 days, the treated microcosms remained anoxic while the controls were closer to the natural pool conditions. A one-way repeated measures ANOVA compared the oxygen content of the treated microcosms with the controls and the natural pool taking into account the time of the study. The percent data were transformed initially to proportions then the arcsine of the square root of the proportions were analyzed. The residuals were normal ($W = 0.918$, $p = 0.256$) by the Wilks-Shapiro test, and homoschedastic ($F = 909.9$, $p = 0.0001$) by Levene's test. There was a significant interaction effect of the type of enclosure (treated microcosms, controls and the natural pool) and time ($F = 12.37$, $p = 0.0001$). This indicates that depending on the enclosure, the oxygen content of the water varied over time. The ANOVA table is shown in Table 4.3.

The concentration of ^{3}H-α-T in each compartment of the microcosms is illustrated in Figure 4.5. The amount of α-T equivalents was determined per milligram of weight for the sediment and biomass and per microliter of volume for the water. Since the density of water is 1 mg per μL, the volume can be assumed to be the equivalent of the mg of weight from the other two compartments. The concentration in the biomass per mg was higher than the concentration in either the sediment or the water. Trends were analyzed non-

statistically from the time of α-T application. The concentration of ^{3}H in the water clearly declined overall. In the biomass, the concentration of ^{3}H was less obvious, but appeared to increase slightly. The sediment values peaked at Day 2, declined and peaked again at Day 7. The trends based on the concentrations of ^{3}H were useful to show the amounts of radiolabel in each compartment of the microcosm, however, the overall fate of the phototoxin could not be ascertained.

Figure 4.6 shows the fate of ^{3}H-α-T in the course of the study in the *in situ* microcosms. Most of the ^{3}H remained in the water. Although it declined by the second day after application, the water retained the highest percent of the initial amount of ^{3}H throughout the study. The distribution in the sediment appeared to follow a biphasic pattern with two maxima. The sediment contained less than 10% of the initial amount throughout the study. The proportion in the sediment peaked on Day 2. A second increase peaked on Day 8. The biomass accumulated the least amount of radiolabel as a percent of the initial amount applied. Less than 0.1% was distributed into the biomass. The maximum distributed into the organisms on Day 3 then steadily declined The compartment labeled as biomass was the sum total of the percent α-T equivalents distributed in the mosquito larvae (*Aedes intrudens*), the caddisfly larvae (*Limnephilus indivisus*) and the daphnids (*Daphnia magna*). To determine if there were any differences among the water, sediment and biomass over the 10 day fate study, a one way ANOVA with repeated measures design was performed. The percentage values were changed to proportions and an arcsine transformation of the square root of the proportions was done. The residuals were not normally distributed using the Wilks-Shapiro test ($W = 0.82$, $prob<W = 0.04$) and the variances were not equal (not homoschedastic) by Levene's test ($F = 3.84$, $p = 0.08$), however, both parameters were very close to meeting the assumptions. ANOVA is robust if these violations are not severe. The ANOVA may be more liberal with this data set, however the results of the ANOVA are highly significant and are unlikely to be affected. The ANOVA indicated that there was an interaction effect of the three compartments (water, sediment, biomass) with time. The distribution of α-T varied with time depending on the microcosm compartment. The ANOVA results are summarized on Table 4.4. The individual microcosms did not differ significantly from

each other during the 10 day fate study of ^{3}H-α-T and thus the three compartments were not affected by them ($F = 0.08$, $p = 0.93$).

The concentration of ^{3}H-α-T equivalents in the three species that comprise the biomass is shown in Figure 4.7. The amount of ^{3}H per mg of body weight was highest in *Daphnia magna* and lowest in the caddisfly larvae (*Limnephilus indivisus*). This difference in accumulation was present even on the day of treatment. This difference became less pronounced with time. The trend in the *Daphnia* was not very clear. There were three maximum concentrations, Days 2, 4, and 8, and two extremely low points (Days 7 and 10) on the curve that were lower than the amounts of ^{3}H in the mosquito larvae (*Aedes intrudens*). The mosquito larvae also had a somewhat erratic trend, however, it was much less pronounced. Generally, the concentration of label in the mosquitoes appeared constant. The concentration in the caddisfly larvae was the lowest, but the amount of label per body weight in these insects appeared to increase throughout the study.

The fate of ^{3}H-α-T in the three species is illustrated in Figure 4.8. In contrast to the concentrations in the organisms, the distribution of the compound (relative to the initial amount added to the microcosms) decreased with time for all three species. Also, the percent of label was highest in the caddisfly larvae and lowest in the *Daphnia*. In the percent values, body weight of the organisms was not taken into account. Since the caddisfly larvae were the largest species in the fate study, they accumulated the highest proportion of ^{3}H in their bodies, while the *Daphnia* and the mosquito larvae were more comparable in size. The proportion in the *Daphnia* generally appeared higher than in the mosquito larvae. The trends exhibited by the *Daphnia* were erratic and difficult to interpret, however, the overall trend appeared to decrease after Day 1. The mosquito larvae appeared to show a steady, slow declining trend. To determine if there were any differences among the *Daphnia*, mosquito or caddisfly larvae over the 10 day fate study, a one way ANOVA with repeated measures design was performed. The percentage values were changed to proportions and an arcsine transformation of the square root of the proportions was done. The residuals were normally distributed according to the Wilks-Shapiro test ($W = 0.94$, $prob<W = 0.59$), but they were not homoschedastic (Levene's

test, $F = 2.72$, $p = 0.14$). Since the results of the ANOVA were not marginal, the parametric test was used since ANOVA is robust to heterogeneity of variances. The ANOVA results are shown in Table 4.5. The interaction effect was significant indicating that depending on the species, the percentage of labelled (^3H) α-T equivalents varied in magnitude over time. The individual microcosms did not differ significantly from each other during the 10 day fate study of ^3H-α-T in the three species and thus did not affect the fate in the biomass ($F = 0.08$, $p = 0.93$).

A great deal of the ^3H was not accounted for. The percent recovery on the day of application was only 54.4%, after one day the recovery dropped to only 29.60% and after eight days it was only 28.51%. These values were calculated from the percent of initial amount of radioactivity found in the sediment, water, biomass, and residue found on the glass surface of the microcosms. The amount on the glass walls and bottom of the containers was negligible (4.6×10^{-4}%). The loss of label could be the result of volatilization from the surface of the water [Maguire 1991], complexation or adsorption to dissolved organic carbons (DOC) [Gensemer *et al.* 1994], or loss through extraction procedures. The efficiency of the extraction of ^3H from the sediment was only 61.3%, and the efficiency of extraction from the water was 74.2%. Since the entire biomass was sampled and used for analysis, there was no extraction procedure, hence no percent efficiency could be calculated.

It should be noted that no mortality was observed in the microcosms, both controls and treated replicates. Considering the amount of ^3H-α-T added to each tank (10 mg), this was not expected. The lack of lethal toxicity towards the biomass in each microcosm may be related to the very low percent recovery.

Table 4.1 Weather conditions at the field site in South Mountain, Ontario, for the duration of the fate study (May 18-28, 1993) where *in situ* microcosms were treated with ^{3}H-α-T.

parameter	average value ± standard error
maximum daily air temperature (°C)	16.8 ± 0.8
minimum daily air temperature (°C)	6.5 ± 0.7
mean daily air temperature (°C)	11.7 ± 0.5
average rainfall (mm)	2.7 ± 1.6
total rainfall (mm)	40.6
sun hours	7.0 ± 1.3

Table 4.2. Water quality data describing *in situ* microcosms with and without ^{3}H-α-T treatment and adjacent snow melt pool. Parameters were sampled 0, 1, 2, 3, 4, 7, 8, and 10 days after application of the EC formulation. Values represent the means over the entire sampling period with standard errors. Treated microcosms consisted of 3 replicates, controls consisted of 2 replicates, and there was 1 snow melt pool.

Parameter	^{3}H-α-T treated	Controls	Snow melt pool
oxygen (% saturation)	32.09 ± 3.26	42.51 ± 6.49	102.87 ± 10.60
alkalinity (meq acid/L water)	0.76 ± 0.05	0.64 ± 0.06	0.55 ± 0.03
conductivity (μmhos)	22.29 ± 1.00	23.75 ± 1.93	19.75 ± 2.94
pH	6.08 ± 0.04	6.08 ± 0.05	6.09 ± 0.05
water temperature (°C)	11.81 ± 0.42	11.61 ± 0.47	11.50 ± 0.49
depth (cm)	14.00 ± 0.26	14.61 ± 0.29	26.1 ± 1.9*

* mean value of 5 readings taken on May 19, 1993 (1 day after α-T application)

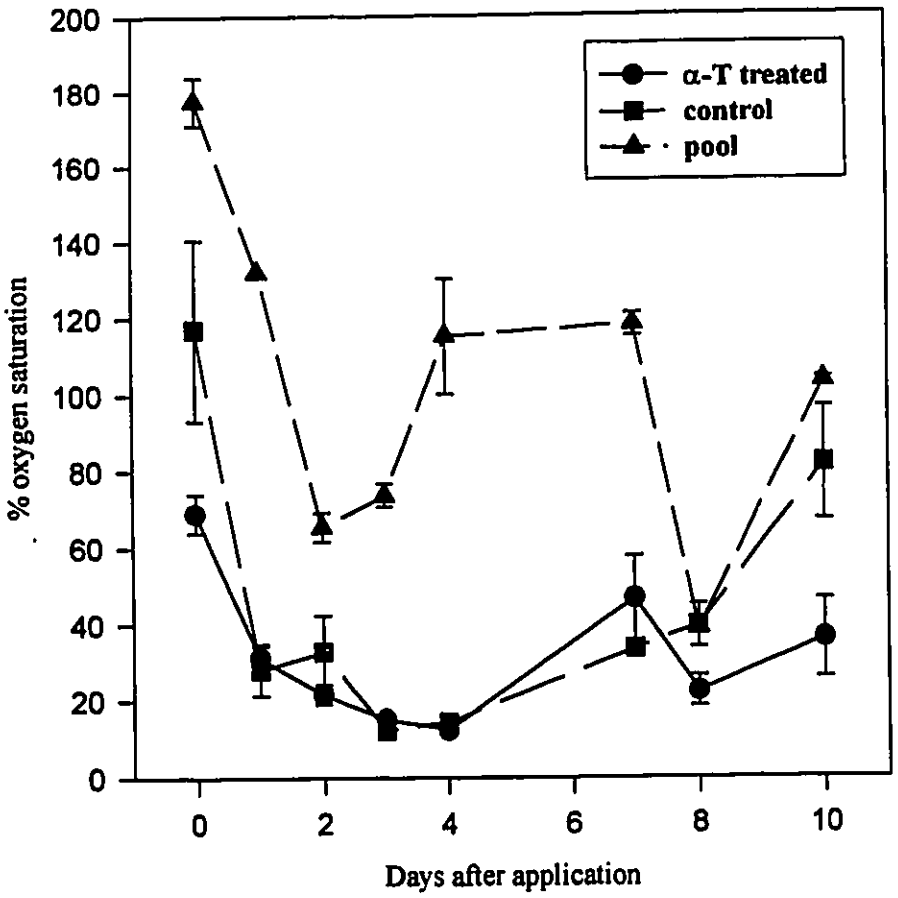


Figure 4.4. Dissolved oxygen content of water contained in *in situ* microcosms treated with ^{3}H-α-T and a natural snow melt pool as determined by the unmodified Winkler method. Error bars represent standard errors.

Table 4.3. A one-way repeated measures ANOVA comparing the oxygen content of the treated microcosms with the untreated control microcosms and a snow melt pool.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
enclosure[d]	5	68069.3	13613.8	172.17	0.0001
error	6	474.4	79.1	-	-
time	7	54469.39	7781.3	120.11	0.0001
time*enclosure	35	28048.5	801.39	12.37	0.0001
error (time)	42	2721.08	64.79	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

[d] enclosures includes treated microcosms, control microcosms, and the snow melt pool.

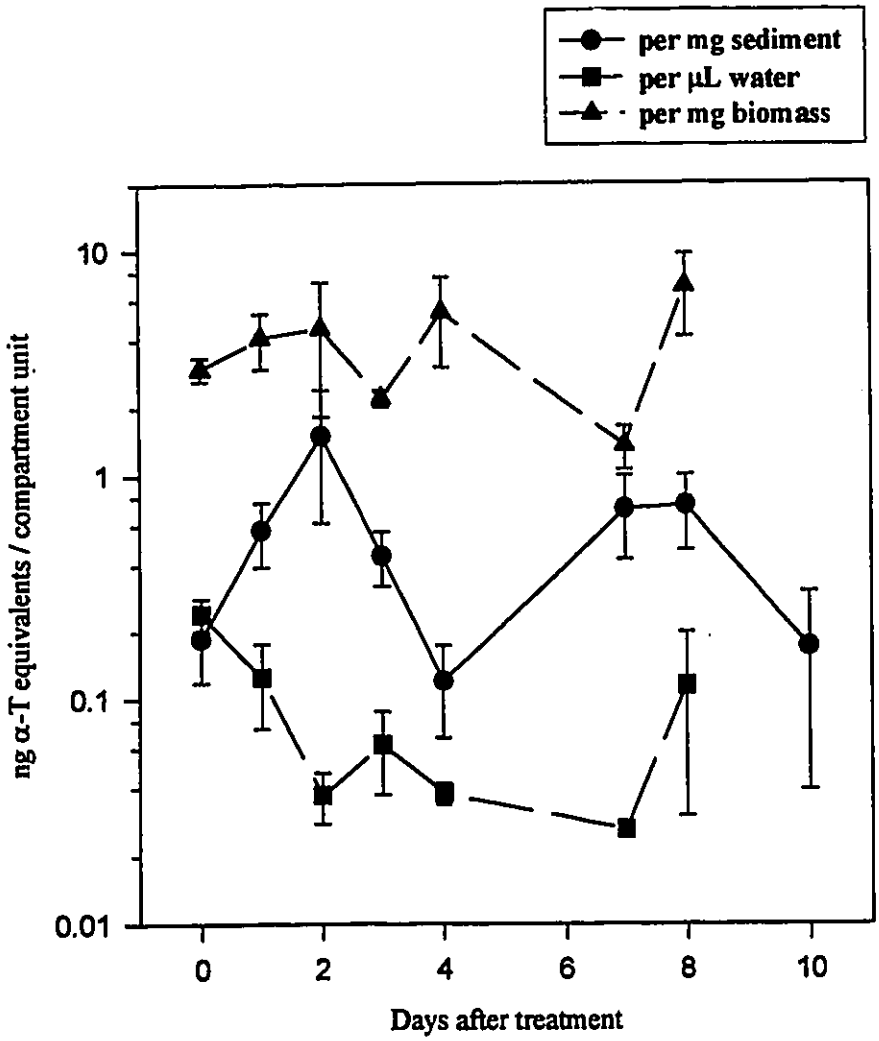


Figure 4.5. Fate of ^{3}H-α-T in the *in situ* microcosms: concentration of ^{3}H in the different compartments after applying 10 mg of ^{3}H-α-T in EC formulation on the water surface (s.a. = 0.18 m^{2}). Error bars represent standard error.

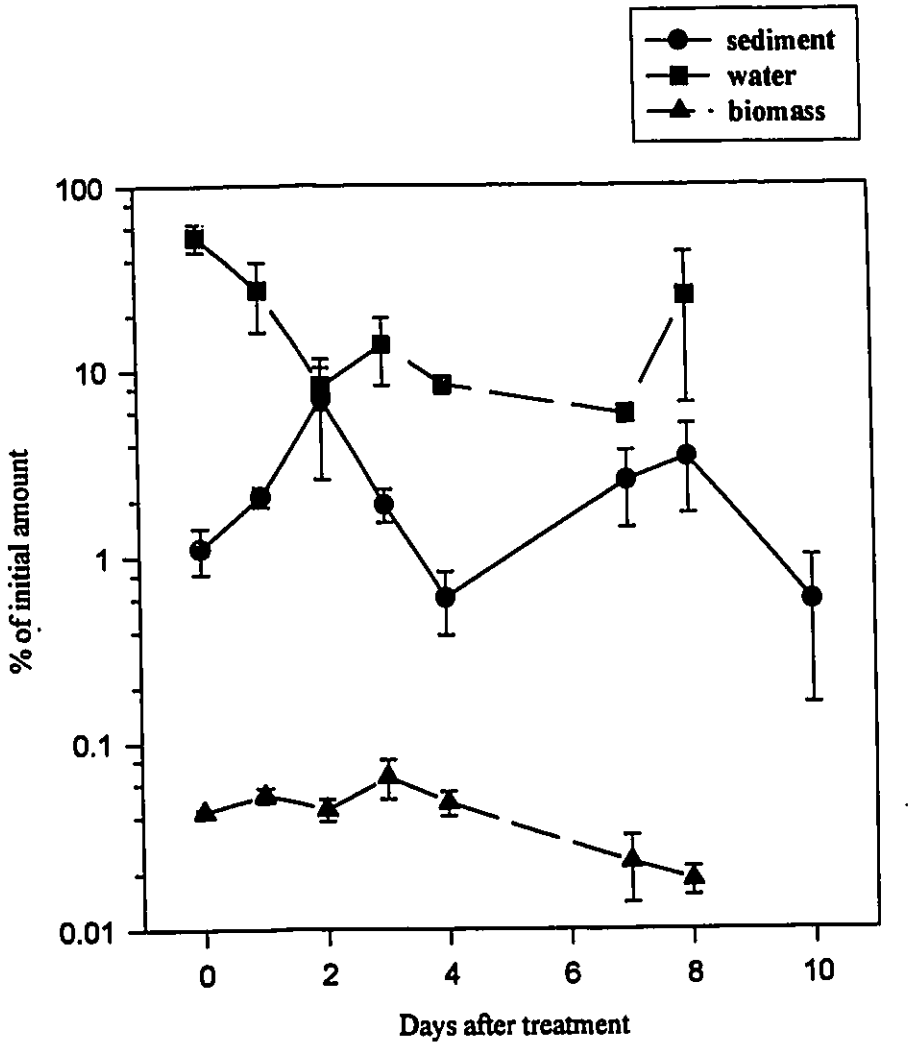


Figure 4.6. Fate of ^{3}H in the *in situ* microcosms: distribution in the different compartments after applying 10 mg of ^{3}H-α-T in EC formulation on the water surface (s.a. = 0.18 m^2). Error bars represent standard error.

Table 4.4. One-way repeated measures ANOVA* on a 10 day fate study of ^{3}H-α-T using *in situ* microcosms with the compartments, sediment, water and biomass comprised of *Aedes intrudens*, *Limnephilus indivisus* and *Daphnia magna*. The test was done based on the percent of initial amount of ^{3}H-α-T the each compartment.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
compartment	2	1.844	0.922	29.30	0.0008
error	6	0.189	0.031	-	-
time	6	0.219	0.037	3.38	0.0095
time*compartment	12	0.559	0.047	4.32	0.0003
error (time)	36	0.389	0.011	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

* A two-way repeated measures ANOVA testing for any variance as a result of the different microcosm units was not possible since there was not enough degrees of freedom.

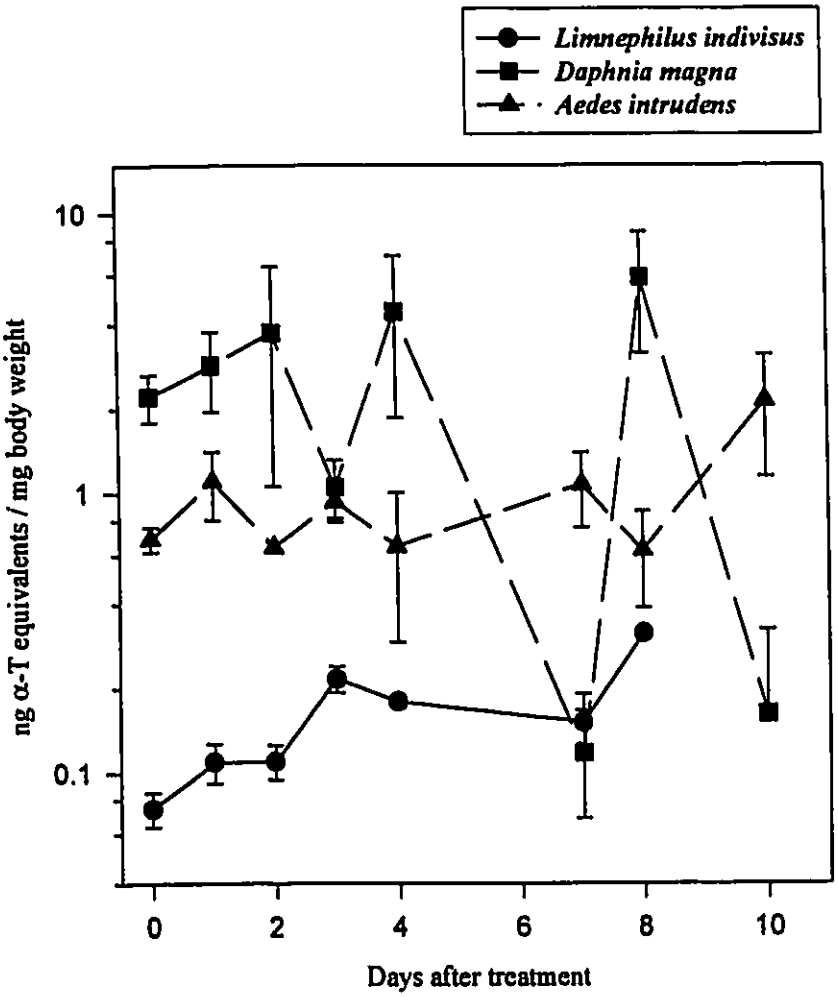


Figure 4.7. Amount of ^{3}H distributed in the three species that represent the biomass compartment in an *in situ* microcosm study after a single application of 10 mg ^{3}H-α-T on the water surface (s.a. = 0.18 m^2). Error bars represent standard error.

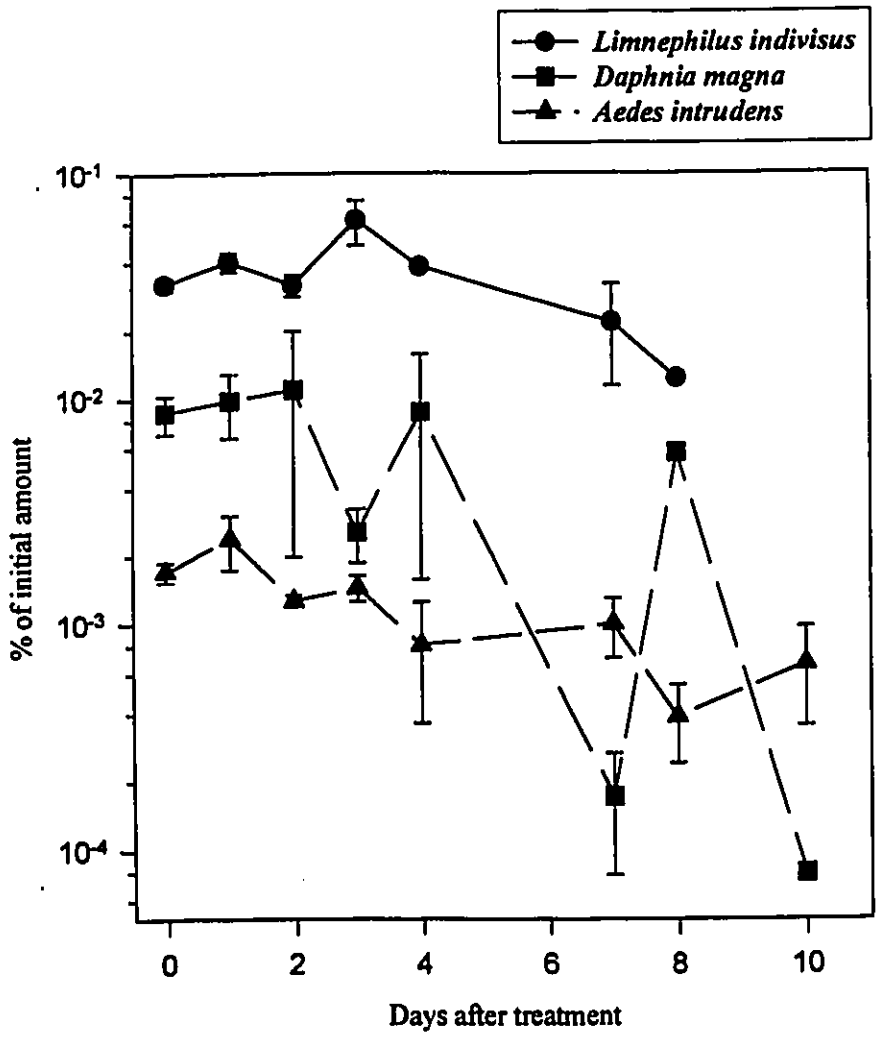


Figure 4.8. Distribution of ^{3}H in the three species that represent the biomass compartment in an *in situ* microcosm study after a single application of 10 mg ^{3}H-α-T on the water surface (s.a. = 0.18 m^2). Error bars represent standard error.

Table 4.5. A one-way repeated measures ANOVA* on a 10 day fate study of ^{3}H-α-T using *in situ* microcosms. with the species, *Aedes intrudens*, *Limnephilus indivisus* and *Daphnia magna*. The test was done based on the percent of initial amount of ^{3}H-α-T the each organism.

Source of variation	d.f.[a]	SS (Type III)[b]	MS[c]	F	p
species	2	2.41×10^{-3}	1.25×10^{-3}	61.73	0.0001
error	6	1.24×10^{-4}	2.49×10^{-5}	-	-
time	6	2.46×10^{-4}	4.09×10^{-5}	6.89	0.0001
time*species	12	3.03×10^{-4}	2.61×10^{-5}	3.63	0.0013
error (time)	36	2.58×10^{-4}	6.25×10^{-5}	-	-

[a] d.f. = degrees of freedom.

[b] SS = sum of squares.

[c] MS = mean squares.

* A two-way repeated measures ANOVA testing for any variance as a result of the different microcosm units was not possible since there was not enough degrees of freedom.

4.4 Discussion

The chemical characteristics of the water in the microcosms (and the natural pools) as summarized in Table 4.2 show that the water in the microcosms was acidic, poorly buffered, soft and anoxic. This characterization, along with a relatively cool temperature also described the natural temporary vernal pool at the field site. The only exception, besides a difference in depth, was the percent saturation of oxygen in the water.

Oxygen is the most fundamental parameter of fresh waters. It is an indication of primary production. Oxygen is produced by photosynthesizing phytoplankton (algae). The major consumption of oxygen in these bodies of water is by bacterial respiration at all depths, especially at the sediment-water interface. Chemical oxidation of dissolved organic matter (DOM) also depletes oxygen as does photochemical oxidation induced by UV light. Thus, as a result when DOM content is increased, bacterial levels are high and the water temperature is increased, the amount of dissolved oxygen in the water decreases [Wetzel 1983]. Oxygen is also a fundamental requirement for phototoxicity of α-T [Arnason *et al.* 1981*a*]. The highly anoxic conditions observed in the treated microcosms may have been due to the natural processes described above since the control microcosms were also anoxic. In addition, the small size of the enclosures could also lead to oxygen depletion by preventing water turnover. The low oxygen content of the microcosms was most likely the result of a large number of bacteria. The disturbance of sediment in the sampling processes could have led to circulating nutrients which leads to bacterial growth.

Organisms tend to concentrate lipophilic toxins in their bodies by bioaccumulation [Farrington 1989]. This trend was observed in the biomass from the 10 day fate study of ^{3}H-α-T. Although the proportion of ^{3}H relative to the initial amount added was the lowest in the biomass, the concentration when weight was taken into account was significantly higher than in either the water or the sediment.

The impact of α-T in application to the three test species in the microcosms was non-lethal. This was very different from the predicted toxicity by the laboratory bioassays or previous field trials. The difference may be due to factors such as low oxygen concentration, dilution, adsorption to suspended particles, volatilization from the water

surface, and microbial degradation. All these factors could have significantly reduced the initial concentration of α-T in the water, accounting for the very low percent recovery. The application procedure may have also affected the concentration of α-T. The compound was in an EC formulation, however, the mixture was sprayed onto the surface, rather than injected below the water surface. Pesticide solutions that are sprayed onto the surface tend to concentrate there in microlayers [Muir *et al.* 1985]. This increases the likelihood of volatilization, dilution, adsorption to particles, photolysis, and hydrolysis [Ernst *et al.* 1991]. Volatilization can be a significant route of disappearance for lipophilic pesticides when sprayed on the water surface. The organophosphorus insecticide, fenitrothion, can disappear from the surface in under 2.5 hours by volatilizing, leading to a total loss of 70% of the pesticide from the water [Maguire 1991]. This proportion is interesting to note since the percent recovery of ^{3}H on the day of application was only 54.4% and one day later had dropped to 29.6% indicating that 24 hours after surface application of the formulation, 70.4% of the ^{3}H was unaccounted. Since the compound was radiolabelled, degradation products would be detected in the sediment, water or biomass. In addition to the low percent recovery, the efficiency of extraction from the sediment and water was also low. The reduction of efficiency may also be attributed to the dissolved organic matter (DOM) in the water. A recent study has shown that the efficiency of hexane extraction is reduced as the concentration of DOM increases and this reduction is amplified by more lipophilic compounds [Maguire *et al.* 1995].

The DOMs in the water column may have reduced the phototoxicity of α-T to the exposed species. DOMs such as humic and fulvic acids are known to form complexes with polyaromatic hydrocarbons (PAH) resulting in the decrease of bioavailability to organisms. The reduction of phototoxicity by complexation has been observed in the PAH, anthracene, to species such as fathead minnows (*Pimephales promelas*), freshwater duckweed (*Lemna gibba*), and *Daphnia magna* [Oris *et al.* 1990, Gensemer *et al.* 1994]. This could account for the lack of lethality exhibited in the presence of α-T in the microcosms. It is also possible that the DOMs that provide protection against lethal toxicity may be a source of sub-lethal effects. A recent study has shown that DOMs complexed with lipophilic pesticides can aggregate and adhere to the spine and antennae

of *Daphnia magna*, impairing their ability to swim and feed [Hodge *et al.* 1993]. Due to the nature of the fate study, sub-lethal effects were not observed. DOM does not appear to affect the bioavailability of toxicants, such as α-T, that have adsorbed onto the sediment [Lores *et al.* 1993, Chin *et al.* 1990].

The different feeding behaviors of the species monitored may also affect the distribution of α-T in the biomass. *Daphnia* are filter feeders of algae, bacteria, fungi, protozoa and organic debris in the water column [Hebert 1978]. Daphnids are one of the most important primary consumers in fresh water bodies [Edmondson 1987, Martinez-Jeronimo 1994]. *Aedes intrudens* is a planktonic swimmer and diver that gathers and collects microorganisms and detritus from surfaces and sediments at the bottom of the pool, as well as feeding in the water column and at the air-water interface [Merritt *et al.* 1992]. *Limnephilus indivisus* is a detritivorous shredder of decomposing vascular plant tissue and the associated microflora at the bottom of the pool. These larvae also make a case from the vegetable material [Wiggins 1973, Cummins 1973, Wiggins 1977]. These three species are all important parts of the temporary vernal pool community [Wiggins 1973]. The caddisfly larvae shred the larger pieces of detritus and produce feces that increase the amount of fine organic particles in the water. These particles become the substrate for microbial colonization that is in turn food for the microcrustacea, such as *Daphnia magna*, and mosquito larvae [Barlocher *et al.* 1978]. Sublethal effects of α-T toxicity may result in alteration of feeding behaviour leading to effects at the population and community levels [Jones *et al.* 1991]. This may lead to more long-term effects that were not detected in the 10 day study.

Although there was no observed mortality in the three species, *Daphnia magna* accumulated the highest concentrations for its body weight. This was expected since cladocerans are typically very sensitive to toxins and bioassays with α-T indicated extreme sensitivity to its phototoxic effects. Other fate studies in aquatic environments have also found that cladoceran populations such as *Daphnia magna* were frequently adversely affected, even when other populations showed no signs of impact [Sundaram *et al.* 1991, Ernst *et al.* 1991, Schaefer and Miura 1990]. At α-T concentrations that would be lethal to the target mosquito larvae, *Daphnia magna* would be wiped out. Caddisfly larvae

would be somewhat more protected because although they also accumulate α-T, the reduced sunlight at the bottom of the pool and their protective cases would protect them from phototoxic effects. To a lesser extent, the caddisfly larvae may reduce contact with contaminated water by partially sealing themselves off. The target mosquito larvae would be the most susceptible since they must contact the surface of the water to breath air [Clements 1963]. This would increase the intensity of UV exposure from sunlight.

In summary, although acute toxicity bioassays in the laboratory indicated α-T poses a very high hazard to some aquatic organisms, such as *Daphnia magna*, this prediction did not translate to severe impacts in the fate study under natural conditions. This lack of toxicity differs from other field trials involving α-T. In field studies with naturals pools, simulated ponds, and streams, mortality of targets (mosquito and blackfly larvae) and non-target species occurred [Philogene *et al.* 1985, 1986, Dosdall *et al.* 1991, Arnason *et al.* 1988]. Reduction of exposure to available α-T in a natural system through physical and chemical processes is likely to lower the toxicological effects expected from laboratory studies, however, the anoxic conditions of the microcosms was most probably the reason no mortality occurred in this study. The previous field trials focussed on lethality towards the organisms, not the fate of the compound. The simulated ponds also did not always contain sediment. In addition, no oxygen measurements were taken in any of these studies. It could be concluded, however, that these bodies of water were likely not anoxic since α-T requires oxygen for toxicity [Arnason *et al.* 1981*a*].

Chapter 5. General Discussion

The rationale for this study was based on the knowledge that while the compound α-terthienyl is a hazardous substance due to its phototoxic mode of action, the risk to natural populations could be relatively low because of the reduced exposure. Exposure is controlled by rapid photodegradation, the requirement of sunlight to activate photosensitization and a potentially rapid clearance and metabolism by organisms.

The uptake of α-T from water was adequately described by compartmental kinetic functions in *Aedes atropalpus, Limnephilus indivisus*, and *Daphnia magna*. The whole body clearance was also explained by compartmental kinetics for the non-targets, *L. indivisus and D. magna* and these values were comparable to a previous study investigating the elimination from mosquito larvae [Hasspieler *et al.* 1988]. However, the conclusions from the elimination study in this project were in contrast to the previous studies [Hasspieler *et al.* 1988, Iyengar *et al.* 1987] which concluded that sensitivity towards α-T is inversely related to the rate at which an organism is able to metabolize and eliminate the toxin. The results from this study may be explained by the highly different nature of the species used. These organisms were chosen because they represented the variety of life found in the natural habitats. While metabolism has an important role in affecting a species' sensitivity, other factors are equally or more important. These factors include light penetration and body size (surface to volume ratio) and were most likely the source of the differing results from previous studies.

This study confirmed that metabolism of α-T is occurring in insects by identifying the presence of a metabolite in *Aedes atropalpus* larvae. Previous studies have shown that the detoxification enzyme systems are involved and clearance is enhanced when these enzymes are induced [Hasspieler *et al.* 1991, 1990, Iyengar *et al.* 1990, Feng *et al.* 1993]. The identification of a more hydrophilic metabolite has important implications about bioaccumulation. Although α-T is lipophilic (K_{ow} = 5.70) [Marles *et al.* 1991*b*] it is a plant allelochemical of a type that invertebrates potentially encounter in their environment.

This study has shown that three different invertebrates are capable of rapidly eliminating this compound from their bodies. The metabolism of α-T and the identification of a more hydrophilic compound (BCA) that exhibits reduced phototoxicity implies that the bioaccumulation potential of α-T is greatly reduced. The study confirms that this plant allelochemical is in fact, highly biodegradable and rapidly eliminated by several organisms.

The assessment of the fate of α-T in a more natural environment provided valuable information about the exposure component of the risk equation. The results showed that most of the accountable radioactivity from a radiolabeled (^{3}H) α-T remained in the water, thus exposure to organisms residing in or on the sediment was reduced. The amount of radiolabel in the biomass also appeared to decline through the duration of the fate study, most likely the result of metabolism by the organisms. The exposure to organisms in the water column such as *Daphnia magna* was also less than the predicted laboratory studies most likely due to the presence of dissolved organic matter that was capable of binding and reducing the phototoxicity of lipophilic α-T. The effects of DOM should be further investigated to make more definitive conclusions. One of the most interesting results of the fate study was the anoxic conditions that resulted in the microcosms. This was unexpected and has not been considered in previous field studies, however, this was the first time an artificial ecosystem has been used. The inclusion of the sediment and the suspected resulting bacterial growth was most likely the major factor leading to the depletion of oxygen. This result showed that the presence of oxygen was probably the most important factor for phototoxicity.

These studies demonstrated that, although the mode of action of α-T at the tissue and cellular level is not selective and poses a hazard to non-target species, the toxicity of α-T is modified by natural environmental factors that reduce the exposure and thus the sensitivity of non-target species. The two factors that are most important for phototoxic activity are UV light and the presence of oxygen. Organisms that occur in the sediment or at the water-sediment interface are at lower risk from α-T phototoxicity because of reduced light conditions and lower oxygen levels. Organisms that inhabit the water column are at greater risk, however, the highest risk remains for species that reside at the

surface of the water. The surface receives the most amount of UV light and surface water is normally saturated or supersaturated with oxygen [Wetzel 1983]. In addition, lipophilic compounds such as α-T can concentrate on the surface, even when using EC formulations. Since mosquito larvae must breathe air from the water surface α-T is selective towards them. However, mosquito larvae are not the only organisms that contact the water surface. There are many invertebrates, as well as amphibians that occur at the water surface and these organisms could be at increased risk to the phototoxic effects of α-T.

Future Studies

1. This study investigated the role of temperature in acute phototoxicity and found that temperature did not have an effect. A more involved study of the effects of temperature on the kinetics of accumulation and elimination would provide information about metabolic rate in the exposed organisms since invertebrate metabolism is temperature dependent [Mitchell *et al.* 1988] and temperature in snow melt pools can vary widely.

2. Simple yeast bioassays have shown that of the metabolites and degradation products, only the bis-thiophenes retained any phototoxic activity [Arnason *et al. In Press*]. Acute toxicity bioassays of the known metabolites and degradation products using the target and selected non-target species as well as toxicokinetics on active compounds could provide further evidence about the long term environmental effects of α-T.

3. Thus far, the focus of non-target studies have been invertebrates. Mosquito habitats do not generally include fish, however, amphibians breed in small forest ponds [Conant and Collins 1991]. α-T is not toxic to fish [Kagan *et al.* 1987], however the sensitivity of amphibians towards α-T is not well known and several species of forest frog tadpoles are known to be extremely sensitive to forest pesticides [Berrill *et al.* 1994]. The

impact on amphibian communities should be investigated for both lethal and sublethal effects.

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Appendix

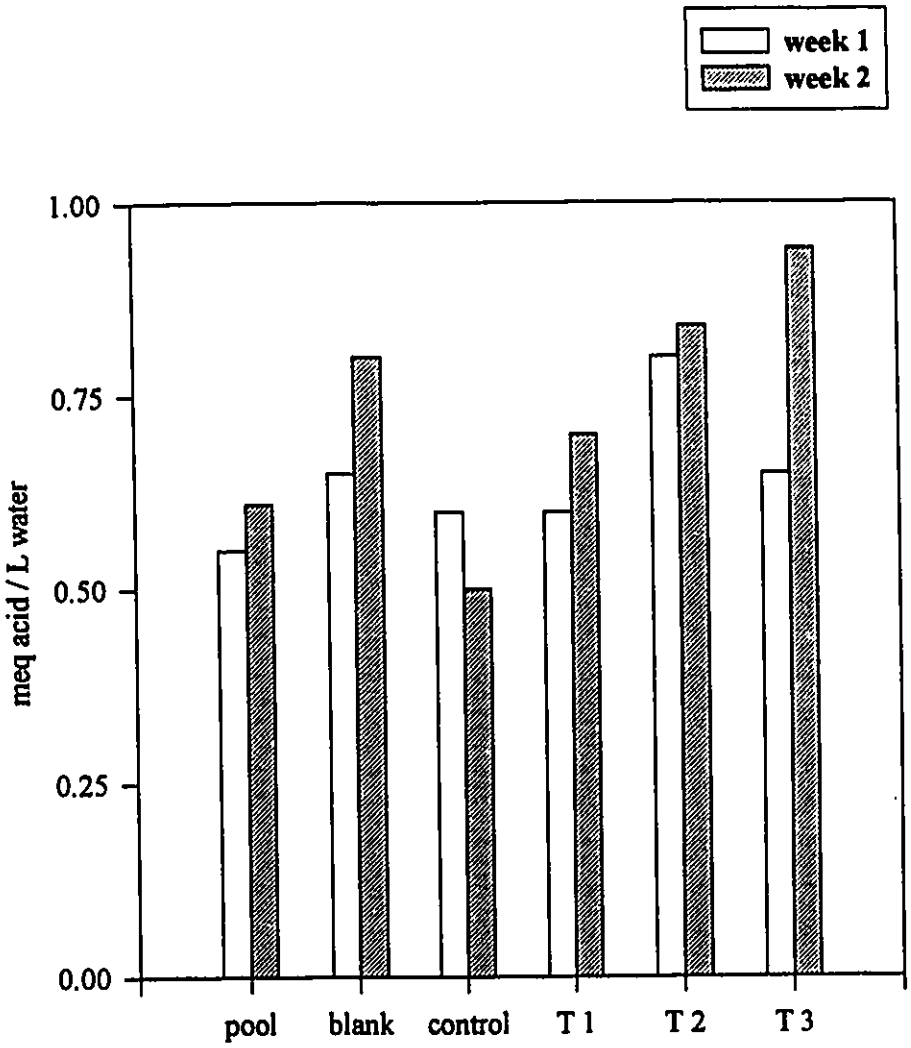

Figure A1. Total alkalinity of water contained in *in situ* microcosms treated with ^{3}H-α-T and a natural snow melt pool.

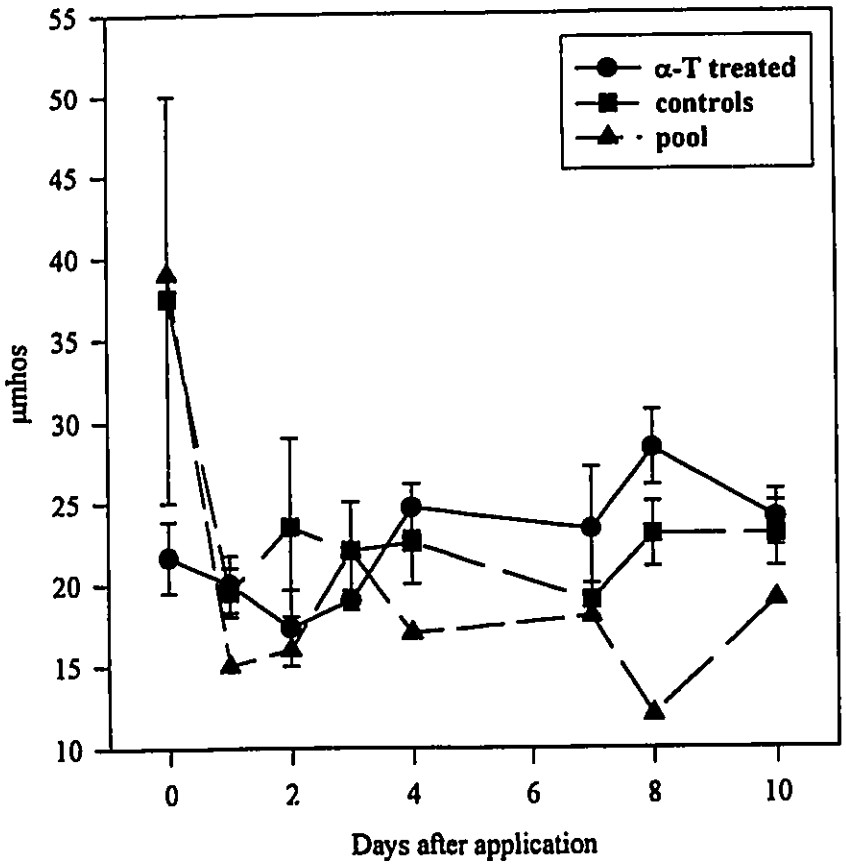

Figure A2. Specific conductance of water contained in *in situ* microcosms treated with ^{3}H-α-T and a natural snow melt pool. Error bars represent standard errors.

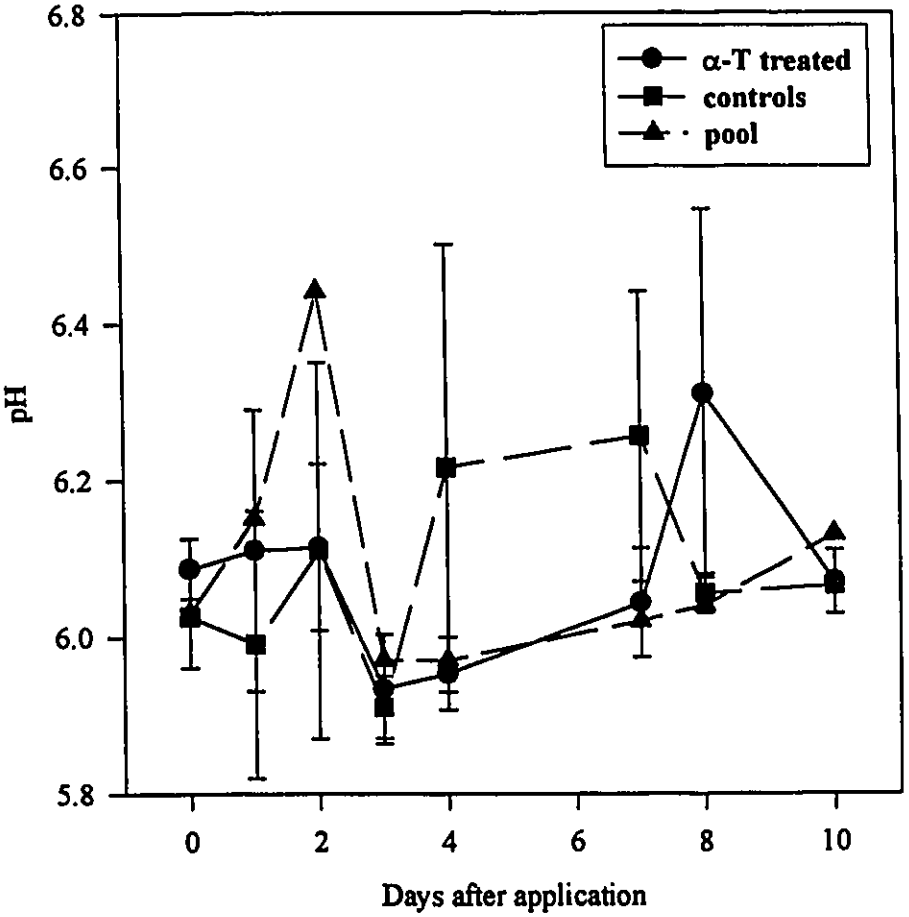

Figure A3. pH of water contained in *in situ* microcosms treated with ^{3}H-α-T and a natural snow melt pool. Error bars represent standard error.

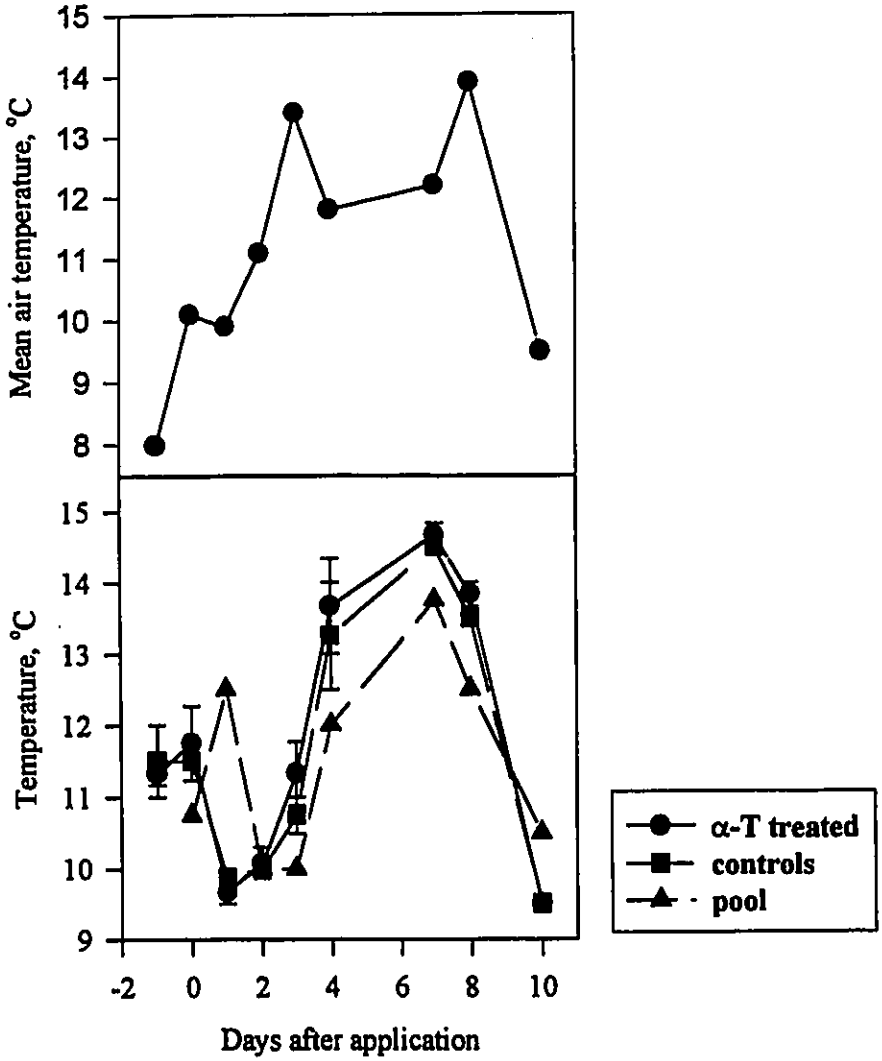

Figure A4. Water temperature in in situ microcosms treated with ^{3}H-α-T and a natural snow melt pool for the duration of the study. Mean air temperature is shown for comparison. Error bars represent standard error.

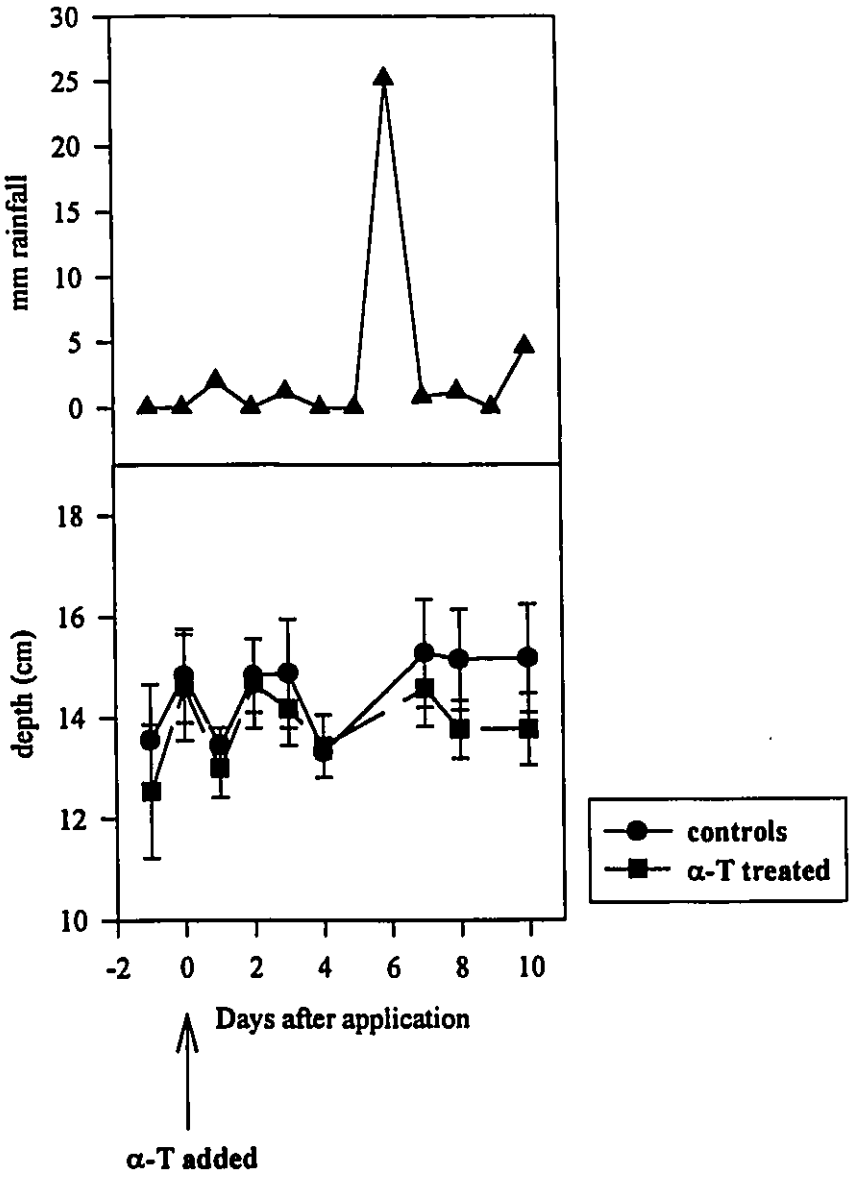

Figure A5. Depth of water contained in *in situ* microcosms treated with ^{3}H-α-T and the amount of rainfall during the study dates. Error bars represent standard error.