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EVALUATING CHEMICAL TOXICITY: A NOVEL BEHAVIORAL ASSAY USING *DAPHNIA*

by

MAYA ZEIN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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DOCTOR OF PHILOSOPHY

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Approved by:

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Advisor Date

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DEDICATION

I dedicate this work to my dearest husband Karim Beydoun for his unconditional love and support throughout the years. A special feeling of gratitude to my parents, Hala Hammoud and Rauf Zein, for expanding my world by providing limitless opportunities for me to succeed. I also dedicate this dissertation to my best friend and brother, Mohammed Zein, for never leaving my side, my two younger siblings Jad and Rita and their beautiful mother Bertha Zein for believing in me. I also extend the dedication to my parents in law Ghada Saad and A. Aziz Beydoun and my brothers and sisters in law Miriam, Amin, Yara and Matt. I am especially thankful to my Grandparents, family and friends for their overwhelming love, and their constant words of encouragement.

In loving memory of my grandma...

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Chapter 1: Introduction

Insecticides, prescription and non-prescription drugs, personal care products, industrial chemicals, detergent metabolites and many other chemicals collectively known as *emerging contaminants*, have been detected in US streams (Kolpin et al. 2002) and their toxicity to the aquatic environment is not well understood. Not only does the term emerging contaminants refer to chemicals that have recently been introduced to the environment, but also those that have long been existing and their presence and impact has just been revealed (Daughton 2004).

Sources of these contaminants include wastewater treatment plants that are not capable of removing or inactivating contaminants that are biologically active at low concentrations and have low molecular weights. In some instances wastewater treatment methods may cause a parent compound to be transformed into more toxic metabolites. Other sources of emerging contaminants include surface runoff (urban and agricultural), groundwater and industrial discharges.

This dissertation work focuses on the development of a high-throughput method to detect and characterize sub-lethal toxicity caused by emerging contaminants on an aquatic keystone species, *Daphnia*. *Daphnia* are small crustaceans found in freshwater ecosystems world-wide including the Great Lakes watershed. In addition to the development of a high-throughput optical assay that utilizes *Daphnia*, the experiments utilize this assay to evaluate possible synergistic or additive effects caused by combinations of complex mixtures of chemical contaminants.

Throughout this dissertation, experiments focus on sub-lethal effects that may impact the fitness and survival of target organisms. Because of the status of *Daphnia* as a keystone species,

the results of this work provide insight into the impact emerging contaminants have on the ecosystem.

With thousands of potential emerging contaminants produced annually, the timely evaluation of potential toxicity is challenging because many of the existing basic ecotoxicology methodologies can be time consuming and expensive (Shaw 1998) . When referring to virtually endless possibilities for production of new chemicals, the individual chemical-by chemical approach adopted by regulatory agencies to monitor pollutants in the environment is essentially unachievable (Daughton and Ternes 1999) with the technologies now available.

Traditional toxicity studies have primarily focused on determining lethal concentration (e.g., LC_{50}). However, significant ecologically relevant effects can occur in organisms at concentrations well below LC_{50} levels. For example, altered motor function, behavioral alterations, and effects on development and reproduction are some of the challenges organisms face when exposed to sub-lethal concentrations of contaminants (Dodson and Hanazato 1995).

In order to assess the potential impact of emerging contaminants, the initial focus of this dissertation was to develop a high-throughput screening assay utilizing multiple freely swimming *Daphnia pulex* capable of assessing sub-lethal behavioral toxicity of aqueous compounds. The optical bioassay developed allows for a quick assessment of a wide-range of chemicals and their concentrations that can serve as a guide to subsequent toxicological studies and provide means to evaluate ecologically relevant behavior.

Exposure to contaminants in water typically involves complex chemical mixtures, which may contain compounds with synergistic or additive effects; thus, increasing the likelihood of toxicity and adverse impacts on ecosystems. This high-throughput optical assay can be used to directly identify additive or synergistic effects of chemicals. Differentiating *sub-lethal* from *lethal*

toxic effects, and determining how concentration and duration of exposure influences these outcomes, is critical to understanding toxicity of emerging contaminants and complex mixtures.

Bioassays for detecting toxicity in *Daphnia* have been developed to detect changes in life cycle thereby providing understanding of the exposure impact at the population level (Kashian and Dodson 2004) , others focused on monitoring physiological changes in motor and cardio-respiratory function of individual animal responses to chemicals (Pitts 2013). While useful, these approaches do not necessarily have a high-throughput capability for evaluating chemical toxicity. In addition to this potential for rapid screening, the optical bioassay can be used to assess toxicity of the complex mixtures that come from the effluent of wastewater treatment plants.

The primary goals of this research were to: 1) develop an assay that can detect and characterize sub-lethal behavioral responses to contaminants; 2) identify synergistic and additive effects within a class of chemicals that has similar mode of action, and between classes of chemicals that have different or unknown modes of action; and 3) detect sub-lethal behavioral effects of contaminants in environmental matrices (e.g. wastewater and community studies).

Chapter 2 presents a summary of the extensive literature in this area. Chapter 3 describes the scalable method developed for quantifying sub-lethal behavior using freely swimming *Daphnia*. During method development two hypotheses were assessed: 1) concentration-dependent behavioral responses in *Daphnia* can be quantified by measuring changes in their movement, and 2) compounds with similar modes of action elicit similar behavioral responses.

Chapter 4 discusses the new behavioral assay used to evaluate additive and synergistic effects for selected emerging contaminants. The following hypotheses were tested: 1. Compounds with similar modes of action cause additive, synergistic or antagonistic behavioral effects. 2. Compounds that are in different classes, based on mode of action or structure, can interact in an

additive, synergistic or antagonistic manner. 3. The biological effects of these interactions between chemicals are observed in environmental systems at relevant concentrations.

Chapter 5 explores how results obtained using the behavioral assay can provide insight into ecosystem function. To meet this objective, a community study was conducted to examine the susceptibility of *Daphnia* to predation following exposure to contaminants. The following hypothesis was tested: Acute sub-lethal exposure of *Daphnia* to diazinon causes an increase in susceptibility to predation by *hydra*.

Ecotoxicology is a very challenging field because of the complex relationships between organisms. With the new high-throughput assay for evaluating the toxicity of contaminants, this dissertation provides a novel tool for advancing understanding of ecotoxicology. By assessing some select contaminants using both the high-throughput optical assay and a community-level assay, this work provides an initial assessment of the relevance of toxicological findings to ecosystem function.

Chapter 2: Background

Emerging contaminants

In a landmark study by Kolpin et al. (2002) many pharmaceuticals and personal care products (PPCPs) were detected in rivers and streams throughout United States. Recent enhancements in analytical techniques allowed for lower limits of detection of such chemicals (Daughton 2001). Commonly described as *emerging contaminants*, these chemicals are newly detected in the environment or have long been present but their influence is just being recognized (Daughton 2004; EPA 2013). Major categories of emerging contaminants include prescription and non-prescription drugs, antibiotics, X-ray contrast media, reproductive hormones, detergent metabolites, disinfectants, plasticizers, fire retardants, insecticides, and insect repellants (Kolpin et al. 2002; Lishman et al. 2006; Snyder et al. 2003). In addition to uncertain impact on the environment, these substances are of increased concern because the number of these compounds detected is expanding (Murphy et al. 2012). Table 1 displays the most frequently detected compounds in the survey conducted by Kolpin et al (2002).

The most frequently detected compounds are from industrial, agriculture and residential uses. The higher concentrations detected included detergent metabolite 4-nonylphenol (Kolpin et al. 2002). Steroids, non-prescription drugs, insect repellents, and detergent metabolites are among the chemicals that are most frequently detected. Mixtures of these compounds occur in the environment, and it is still not clear what sort of interactive effects they might have on the ecosystem.

Table 1: Most frequently detected compounds in 139 US streams (Kolpin et al. 2002)

Compounds	*Use*	*Frequency of detection (%)*	*LogKow,measured (calculated)*
Coprostanol	Estrogen	~80%	(8.82)
Cholesterol	Plant/animal steroid	~80%	(8.74)
N-N-diethyltoluamide	Mosquito repellant	~80%	2.18 (2.26)
Caffeine	Stimulant	~75%	−0.07 (0.16)
Tris(2-chloroethyl)phosphate	Fire retardant	~75%	1.44 (1.63)
Triclosan	Antibiotic	~60%	NA
4-Nonylphenol	Surfactant	~60%	(5.92)
4-Nonylphenol monoethoxylate	Surfactant	~50%	NA
Ethanol, 2-butoxy-phosphate	Plasticizer	~45%	NA
4-Octylphenol monoethoxylate	Surfactant	~45%	NA
Bisphenol A	Plasticizer	~45%	3.32 (3.64)
Cotinine	Nicotine metabolite	~35%	0.07 (0.34)
4-Nonylphenol diethoxylate	Surfactant	~35%	NA
5-Methyl-1H-benzotriazole	Antioxidant	~30%	NA
Fluoranthene	PAH	~30%	5.16 (4.93)
1,7,-Dimethylxanthine	Caffeine metabolite	~30%	−0.22 (−0.39)
Pyrene	PAH	~25%	4.88 (4.93)
Trimethoprim	Antibiotic	~25%	NA
1,4-Dichlorobenzene	Deodorizer	~25%	3.44 (3.28)
Acetaminophen	Analgesic	~25%	0.46 (0.27)
Tetrachloroethylene	Solvent	~20%	NA
4-Octylphenol diethoxylate	Surfactant	~20%	NA
Erythromycin-H_2O	Antibiotic	~20%	NA
Estriol	Estrogen	~20%	2.45 (2.81)
Lincomycin	Antibiotic	~15%	0.59 (0.29)
Sulfamethoxazole	Antibiotic	~15%	0.89 (0.48)
Phthalic anhydride	Plasticizer	~15%	1.60 (2.07)
Carbaryl	Insecticide	~15%	NA

Sources

Chemicals of concern regarding human health and ecological impacts enter the environment via agricultural, industrial, pharmaceutical and household discharges (Kolpin et al. 2002).Among the many ways pesticides are used in agriculture is there use for pest management; although extremely important to preserve crops they are often misused and applied in quantities larger than needed. Pesticides end up in surface waters through run off or can leach into groundwater. Industrial chemical are released into the environment through discharges to water and air (Kolpin et al. 2002). Pharmaceuticals are released into the environment through agriculture (veterinary medicine) or aquaculture (fish farm activities) (Bueno et al. 2009), septic water systems

(Kolpin et al. 2002) and wastewater treatment plants that are not designed to treat such low molecular weights compounds(Kummerer 2009).

Administered drugs can be excreted by humans or animals as parent compounds, metabolites, or as transformational products and therefore introduced indirectly to our waters and the environment (Loffler et al. 2005) (Figure 1). Such chemicals are typically detected in concentrations of parts-per-billion levels, and their mode of action, i.e., the mechanism in which the drug alters the synthesis and transport of intracellular mediators such as neurotransmitters (Blumenthal 2011), in the environment is not well understood (Cleuvers 2003). Antibiotics and hormones used in agriculture, aquaculture, and veterinary medicine are also a source of these chemicals. Antibiotics and hormones used in agriculture, aquaculture, and veterinary medicine are also a source of these chemicals. Municipal water treatment processes do not adequately remove pharmaceuticals (Daughton and Ternes 1999), and in some cases may cause a parent compound to undergo further transformation, which leads to their persistence and bioaccumulation in the environment (Kummerer 2009). Many pharmaceuticals are resistant to photo-degradation and therefore remain biochemically active and persistent in the environment after they undergo treatment by wastewater plants (Brodin et al. 2013). For example, Brodin et al. (2013) detected benzodiazepines, a class of commonly used psychotherapeutic drugs , in rivers and streams at concentrations up to 0.4 $\mu g\, l^{-1}$ and in wastewater effluent up to 0.001 $\mu g\, l^{-1}$. At the concentrations observed, benzodiazepines had significant effects on the behavior and feeding rate of wild European perch.

Hospital effluents containing pharmaceuticals, disinfectants, and surfactants are also a large source for these emerging contaminants surface waters. High concentrations of

pharmaceuticals such as antibiotics (amoxicillin, ciprofloxacin), were detected in the effluent water of University Hospital of Santa Maria in Brazil (Henriques et al. 2012).

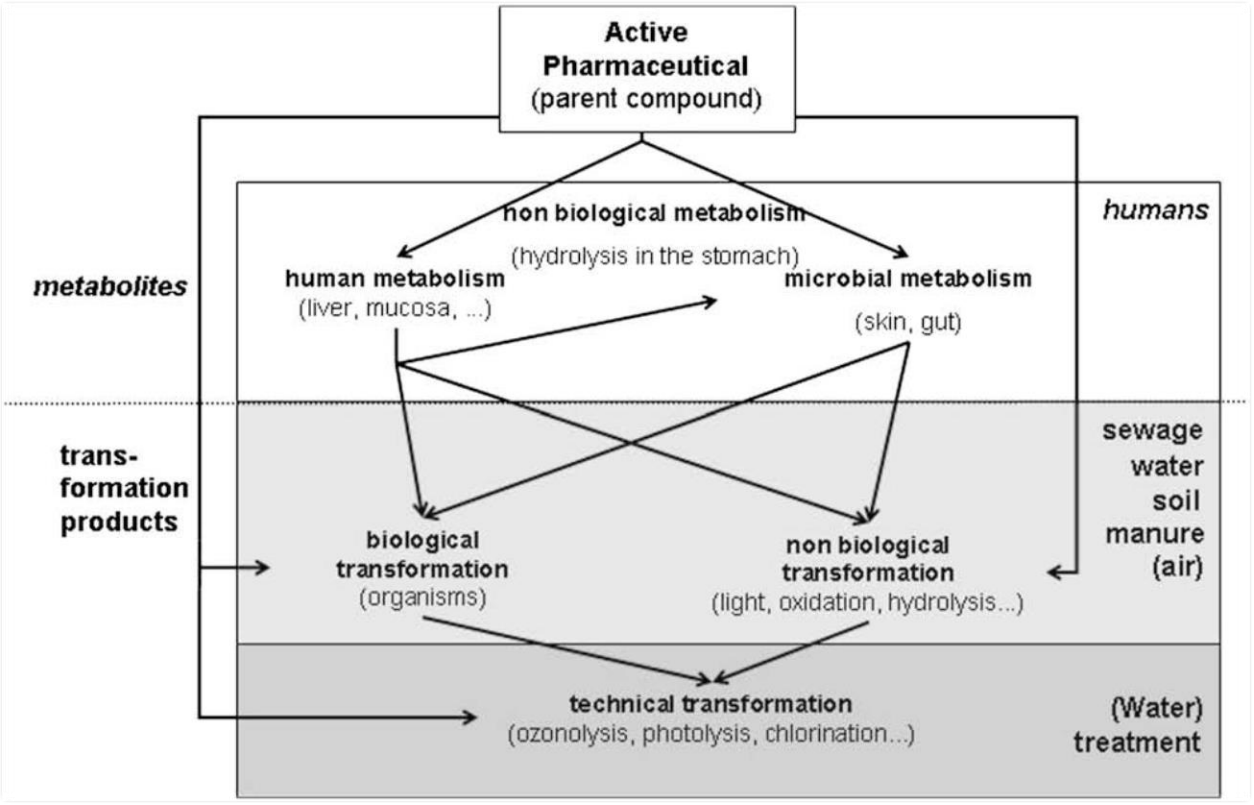


Figure 1: Parent compounds forming metabolites by biological and non biological processes (Kummerer 2009).

Environmental impact

Although contaminants in the environment have been heavily studied since the 50's there are still so many unknowns about the impact of chemicals in the environment. In studies examining pollution of water by emerging contaminants, reproductive and behavioral changes in fish, reptiles, mammals, and invertebrates were observed (Shultz et al. 2004). The veterinary use of the anti-inflammatory drug, diclofenac almost wiped out several species of vultures in India and Pakistan, (Oaks et al. 2004) (Shultz et al. 2004). Studies showing low concentrations of emerging

contaminants in the water without known ecological effects have caused an increase in public perception and awareness.

High throughput Bioassays

Toxicity bioassays have well been accepted and used in measuring toxicity of contaminants in the water to various aquatic organisms (Kimball and Levin 1985). They have provided insight on the effects of chemicals on an organism, its target receptors and tissues (Kimball and Levin 1985). Toxicity tests rely on standardized measures and endpoints that focus on the lethal concentration in which 50% of the test animals are killed (LC_{50}). However, significant impacts occur on organisms at concentrations well below LC_{50} levels, such as altered motor function in organisms, and effects on development and reproduction. Such sub-lethal effects can impact the fitness and survival of target organisms and affect ecosystem function (Dodson and Hanazato 1995). There is increasing interest in the development of high-throughput screening assays for evaluating the toxicity of the large number of chemical contaminants and mixtures (e.g., National Toxicology Program (http://ntp.niehs.nih.gov), Computational Toxicology Research (http://www.epa.gov/comptox/). One approach that has been employed is the use of optical assays. For example, zooplankton have been optically tracked in larger volume assay systems (>150 ml) with a primary focus on the study of swimming behavior relevant to function in aquatic ecosystems (Dodson et al. 1995; Lard et al. 2010). Such assay systems are very important to our understanding of zooplankton behavior, but limited in their utility for high-throughput toxicity screening. A more recent study by Richendrfer et al (2012) incorporated the use of high-throughput imaging system to demonstrate anxiety related behavior caused by sub-chromic concentrations of chlorpyrifos on zebrafish larvae. Assays such as this and the one presented in this research address the growing need for high-throughput screening tools to evaluate emerging contaminants.

Model organisms

A common organism used for aquatic toxicity testing is the *Daphnia pulex*, the freshwater crustacean. *Daphnia* is considered a model system for ecology, evolution and the environmental sciences. They are primary consumers of plankton (e.g., single cell algae, bacteria, protists), a primary food source for larger invertebrate and vertebrate species, and therefore are considered the base of the food chain in freshwater lakes. The importance of *Daphnia* as keystone species in freshwater ecosystems is well known, and the genus has become recognized as a model organism for studying aquatic ecosystems over the past several decades. *Daphnia* are very sensitive to biotic and abiotic changes in their environment and have developed specific adaptation strategies to cope with changes in temperature, water chemistry (e.g., dissolved oxygen), food supply, and predation. *Daphnia pulex* are ideally suited for studying toxicological and ecological effects, and are used as a screening tool for environmental contamination (Kashian and Dodson 2004) because of their large brood sizes, asexual reproduction, the ease of laboratory and field manipulation, and most importantly for having the highest genome homology to humans (Colbourne et al. 2011). The *Daphnia* genome has been termed "ecoresponsive" because of the very large number of genes, including many duplicated genes, and because of its phenotypic plasticity and adaptive responses to changing environmental conditions (Colbourne, Pfrender et al. 2011). *Daphnia* are frequently used to establish human and environmental health standards. These bioassays include acute toxicity tests that determine the lethal concentration in which 50% of the animals die (LC_{50}) and bioassays that examine population metrics (e.g., survival, sex ratio, growth, fecundity, and ability to molt). *Daphnia* have recently been identified as model organisms by the National Institute of Health (NIH) due to their ubiquitous distribution in surface waters, key ecological role in aquatic food chains, and sequenced genome (cite). Although *Daphnia* are routinely used in pesticide

testing, standard *Daphnia* toxicity tests were developed before emerging contaminants became an important issue, and rapid, high-throughput assays for detecting *sub-lethal* effects may serve as an effective and efficient measure of detecting sub-lethal effects.

Optical assay

Subsequent to acute toxicity testing, short term screening studies can be beneficial in providing much needed sub-lethal behavioral endpoints. The need for a rapid screening tool is vital for examining a wide range of chemicals and concentrations to guide subsequent studies and evaluate potential toxic effects of these contaminants in the environment. The optical bioassay proposed in this dissertation can serve as a more rapid high-throughput method to assess the toxicity of contaminants using freely swimming *Daphnia*. In addition to its potential to rapidly screen an array of contaminants, this assay can be used to evaluate ecological impacts and toxicity chemical mixtures such as wastewater effluents and influents**.**

Arthropods as utility compounds

Contrast to more traditional endpoints including survival and reproduction, behavioral responses of various species have been used as good indicators of toxic responses to various contaminants (Cailleaud et al. 2011). Swimming behavior in zooplankton including copepods and cladocerans such as *Daphnia* have been investigated in a number of studies (Cailleaud et al. 2011; Dodson and Hanazato 1995). Cailleaud et al (2011) investigated sub-lethal toxic effects of 4-nonyphenol on copepod's swimming behavior using digital monitoring (Cailleaud et al. 2011). Similarly Dodson and Hanazato (1995) used a video system to record zooplankton swimming behavior which was affected but sub-lethal concentrations of toxic xenobiotic. Other behavioral responses affecting the nervous system of *Daphnia* magna have been investigated (Duquesne and

Kuster 2010). In this study *Daphnia* proves to be a good indicator of sub-lethal toxicity caused by chemicals that affect the cholinergic system.

Combined Stressors: Evaluation of complex mixtures

Exposure to contaminants in the environment typically involves a complex of mixtures with varying toxicities, in addition to other environmental stressors such as low pH, low oxygen levels and elevated temperatures (Dodson and Hanazato 1995). These factors can interact synergistically and cause *Daphnia* to be even more susceptible to lower concentrations of contaminants in the environment. Mixture involves numerous chemicals with varying toxicities. The presence of some chemicals can have an additive, synergistic or antagonistic effect on the toxicity of other chemicals (NRC 1988), resulting in amplified or reduced interaction effect. Studies involving mixtures have shown reproductive and developmental impairment in a variety of aquatic species (Cailleaud et al. 2011).

The experiments outlined in this research examine behavioral effects of both individual and chemical mixtures on *Daphnia*.

Insecticides

Pesticides, which include insecticides, herbicides, rodenticides and fungicides, are among the many emerging contaminants detected in our waterways. This research focuses mostly on insecticides with different toxicological characteristics including organophosphates, carbamates, neonicotinoids and molt inhibitors. Each of these class of insecticides have different molecular targets and can have serious toxic effects on insects as well as humans (Klaassen 2008). Acetylcholinesterase and nicotine acetylcholine receptors are among the molecular targets affected by the organophosphates and neonicotinoids insecticides respectively. While there are structural differences between human and insect acetylcholinesterase enzymes (Pezzementi and Chatonnet

2010), insecticides that inhibit acetylcholinesterase can readily inhibit human acetylcholinesterase and cause toxicity.

Those insecticides possess properties such as chemical stability, lipophilicity, and slow rate of biotransformation causing them to bioconcentrate, and bioaccumulate, therefore become extremely persistent in the environment.

It is especially difficult to assess the ecological impact of various pesticides because of their diverse active ingredients and their unique characteristics such as their persistence in the environment.

Other prevalent chemicals

In addition to pesticides, there is an enormous amount of chemicals discharged from wastewater treatment plants. Prescription drugs, industrial chemicals, personal care products could all be part of the mixture. Triclosan, an antibacterial agent found in disinfectants and antiseptics, is used in a wide range of personal care products and has gained much attention over the years because of the considerable levels that have detected in humans, aquatic environment and wastewater samples (Kumar et al. 2010). They are of special concern because of their ability to bioaccumulate in fatty tissues (high K_{OW}), and their ability to undergo degradation to form dioxins, chemicals that are highly persistent in the environment, linked to cause cancer and major reproductive and development disorders (Kumar et al. 2010; Roh et al. 2009; Stasinakis et al. 2008; WHO 2010).

Chemicals such as surfactants are found in most personal care and household products, are fairly ubiquitous in the environment, and have been found in waste water effluent discharges (Li 2008). Alcohol ethoxylates, and alkylphenol ethoxylates are major classes of nonionic surfactants found in hospital effluent that can be further broken down to hydrophobic, high accumulative

compounds such as alkylphenols (Henriques et al. 2012). One of particular interest is the surfactant 4-nonylphenol, a breakdown of many detergents. Recent studies show 4-nonylphenol effects on swimming behavior of different species including guppies and in planarians, caused by cholinesterase enzyme inhibition (Cailleaud et al. 2011; Li 2008; Li 2012).

Acetyl cholinesterase is an enzyme that prevents the accumulation of the neurotransmitter acetylcholine that is responsible for continuous stimulation of neurons in the central nervous system. Acetyl cholinesterase inhibitors blocks the enzyme acetyl cholinesterase and thereby allowing continuous firing of neurons. Nonylphenol has been shown to interact with AChE-I, therefore has the potential to cause additive effect when combined with other chemicals with similar modes of action. Their concern in the environment is heightened due to studies showing endocrine disrupting effects (Li 2008, Cailleaud, Michalec et al. 2011, Li 2012, and they have been substituted in Europe with other detergent precursors because of their known toxicity to the aquatic ecosystem. Nonylphenol exist in our water system along with a mixture of contaminants, therefore their interaction with other contaminants specifically AChE-I is of major concern.

Wastewater Treatment Plants

One source of these contaminants found in the environment is from the discharge of wastewater treatment plants. Conventional plants are not capable of removing or inactivating contaminants that are biologically active at low concentrations and have low molecular weights. In some instances wastewater treatment methods may cause a parent compound to be transformed into more toxic metabolites (Lishman et al. 2006). In a study conducted by the EPA, five municipal wastewater treatment plants were screened for the presence of pharmaceuticals and personal care products (PPCPS). Primary and secondary treatments were evaluated for efficiency of removal. Secondary treatment method removal efficiency was compared to advanced or tertiary treatment

technologies that included nutrient removal such as phosphorus and nitrogen and chemical addition with filtration. Figure 2 illustrates a wastewater treatment plant with primary, secondary and tertiary treatment methods. It was found that while secondary treatment methods was efficient in removing steroids and various hormones it was not efficient in removing certain pharmaceuticals that were detected such as carbamazepine, an anticonvulsant drug and fluoxetine, an antidepressant also known as Prozac (Lubliner 2010). The advanced treatment methods primarily had longer biological contact time and tertiary filtration that allowed more efficient removal of PPCPS. It is important to note that chlorination can cause compounds to react with other chemicals, ozonation and ultraviolet light can break molecular structures causing transformation reactions. Most wastewater treatment plants are not equipped with tertiary treatment techniques.

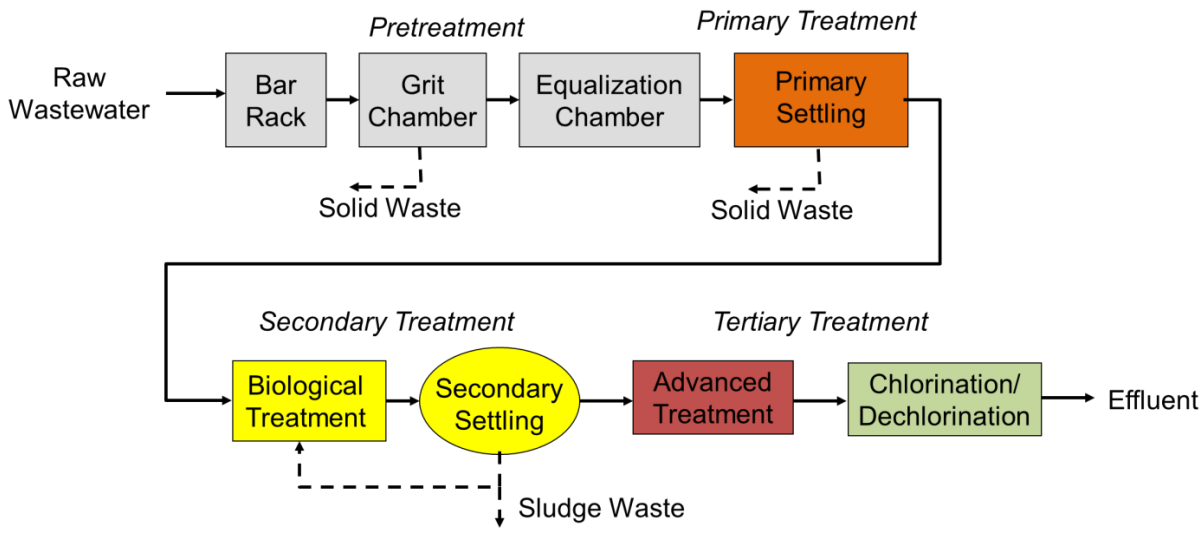


Figure 2: An idealized process plan for a wastewater treatment plant with tertiary treatment.

Contaminants in the environment tend to partition between different compartments such as water, solids, and biota. Octanol, a fatty alcohol with high molecular weight that is immiscible in water has been used to determine partitioning coefficients *(K_{ow}= $C_{octanol}/C_{water}$).* Octanol-water partition coefficients (K_{OW}), a measure of hydrophobicity, may in some cases be useful in predicting the fate of a drug (Hermens et al. 2013). K_{OW} has long been used in environmental chemistry and toxicology to establish exposure hazard and risk assessment based on quantitative structure activity relationship (QSAR)(Hermens et al. 2013). Parameters such as sorption and accumulation are a result of hydrophobicity; therefore chemicals with high K_{OW} are expected to sorb and partition to hydrophobic compartments. In the case of a pharmaceuticals compounds with high k_{ow} are distributed to more hydrophobic compartments such as lipids bilayers (fatty tissues), while hydrophilic compounds (low K_{ow}) tend to be in more "water-loving" environment such as blood. The partition between water and fatty tissue provides information for predicting partitioning of various other organic phases such as sediments and biota.

The compounds selected in this research represent those pesticides and other breakdown chemicals that are not removed from wastewater plants and are found prevalently in the environment.

Selected Compounds

Several chemicals that are known to be common water contaminants were examined. Different classes of insecticides and other chemicals were chosen to validate the optical assay. Three groups of insecticides with different modes of action were selected, these include: Cholinesterase inhibitors, Neonicotinoids, and molt inhibitors (*see appendix A for toxicity information of selected chemicals*).

The first group of insecticides, the cholinesterase inhibitors, included diazinon, chlorpyrifos (organophosphate insecticide), and physostigmine. Although physostigmine is not necessarily an insecticide, its mode of action as an acetyl cholinesterase inhibitor is well characterized, and can therefore serve as a model compound for the acetyl cholinesterase Inhibitors (AChE-I).

Chlorpyrifos

One of the widely used organophosphate insecticide in the US, chlorpyrifos, first introduced in 1965 by Dow Chemical is used abundantly in agricultural setting on a variety food crops, non-structural wood treatment and golf courses (Christensen 2009; EPA 2002). Chlorpyrifos inhibits the breakdown of the neurotransmitter acetylcholine causing it to accumulate in the synaptic cleft of insects (Christensen 2009). The accumulation of acetylcholine causes overstimulation of neurons that leads to neurotoxicity and death (EPA 2002). The reported Chlorpyrifos 48 hour LC_{50} in *Daphnia* is 1.7 µg/l (Tomlin 2011). According to the US Geological Survey, the breakdown of three mostly used organophosphate pesticides including chlorpyrifos and diazinon are much more toxic than the parent compounds on amphibians (USGS 2007).

Diazinon

Another widely used organophosphate insecticide, diazinon, widely used in agriculture to control insects on field crops, fruits and vegetables(Harper 2009). Prior to December 2004, diazinon was used as an active ingredient in household and gardening products(EPA 2012). In an effort to protect children and the environment, EPA began to phase out all residential use of diazinon, and in 2004 it was banned in non-agricultural products (EPA 2012). Diazinon agricultural products are still available as dusts, liquids, and concentrate (Harper 2009), it is persistent in the environment (*half –life 12-100 days depending on PH*) and moderately mobile.

Exposure to diazinon can be achieved through contaminated runoff or groundwater (ATSDR 2008). In fact, prior to the phase-out in 2004, diazinon was one of the most widely detected insecticides in surface waters(Harper 2009).

The second group of insecticides studied is the neonicotinoids. The use of neonicotinoids insecticides has been on the rise due to their selectivity towards insect receptors versus mammalian (Klaassen 2008). Imidacloprid and nicotine were the two insecticides chosen in this group to undergo testing. Nicotine was used as the model compound for neonicotinoids because its pharmacological properties are well known and have been well characterized and studies in the literature.

Nicotine

In the 1960s, nicotine was regarded as the first plant based insecticide in the form of tobacco extracts (Tomizawa 2013). In an effort to produce more potent and optimized nicotinoids insecticides, a new class was discovered, and termed neonicotinoids (Tomizawa 2013; Yamamoto et al. 1998). Nicotinoids and neonicotinoids both act as agonists to the nicotinic acetyl cholinesterase receptor (Yamamoto et al. 1998). The difference between the two is the higher selectivity of neonicotinoids to target insects rather than vertebrates. Neonicotinoids have high specificity for insects versus mammalian acetylcholine receptors (David et al. 2007).

Imidacloprid

Imidacloprid is a neonicotinoid registered for use by the US EPA in 1994 (Gervais 2010). The reported 48 hour LC_{50} for imidacloprid in *Daphnia* is 85mg/l (Gervais 2010). A study has shown sub-lethal exposures of *Daphnia* to imidacloprid resulted in decreased feeding rates, and lower responses to predator cues causing reduction in population growth rate (Gervais 2010).

Ecological relevance of selected compounds

Examining population dynamics due to contaminant exposure is central to ecotoxicology and the focus of regulatory agencies such as the EPA (Klaassen 2008). The impact of contaminants on animal behavior such as predator-prey interactions can cause major disturbances in population dynamics (Klaassen 2008).Studies examining behavioral effects of contaminants such as AChE insecticides have been linked to alter alarm response and homing in Chinook salmon (Scholz et al. 2006) and causing changes in swimming and feeding behavior in coho salmon (Klaassen 2008). A community is integrated in a complex way with many vital parts connected; slight changes that affect keystone species can causes have negative consequences across multiple trophic levels. For example, increased predation can result in declines in the prey population resulting in a cascade effect for the entire community. Exposure of prey to a chemical can result in behavioral changes such as increased swimming speed and therefore cause an increase in predator encounter frequency (Gerritsen and Strickler 1977). Measuring the extent in which invertebrates are susceptible to predation is important in examining the dynamics of arthropods communities (Spitze 1985).

In addition to the uptake of chemicals in the water via through their integument or gills, aquatic organisms are exposed to chemicals through contact with contaminated sediment or ingestion of contaminated food or water (Savino and Stein 1989). Considering the rate and amount at which pharmaceuticals, pesticides and other chemicals are used, evaluating and identifying their effects on behavior of *Daphnia* at environmentally relevant concentrations is a crucial first step in determining if water quality standards are needed for these compounds.

Therefore, the need to develop high throughput screening tools to detect those sub-lethal behavioral effects is important to establish some sort of understanding of the impact of those contaminants in the water.

Chapter 3: Optical Assay Development

Introduction

With advancement in analytical techniques, the number of new chemicals being detected in surface waters is rapidly increasing. In the first of its kind study (Kolpin et al. 2002), 139 streams throughout the United States were evaluated between 1999 and 2000, and 82 out of 95 target organic waste contaminants were detected in 80% of the waterways investigated. Chemicals detected included prescription and non-prescription drugs, antibiotics, reproductive hormones, detergent metabolites, disinfectants, plasticizers, fire retardants, insecticides, and insect repellant. Collectively, these substances are now commonly referred to as *emerging contaminants* (ECs) (Daughton 2004). The number of substances detected that are classified as ECs continues to expand (USGS 2013). Further complicating assessments of toxicity, these chemicals are part of a complex mixture of compounds(Cleuvers 2003). Evaluating toxicity of ECs is challenged by 1) limited means of assessment, 2) testing procedures that are time consuming and expensive and 3) understanding what biological endpoints are appropriate to evaluate human or ecosystem health. To obtain a more complete understanding of toxicity of aquatic pollutants a rapid and inexpensive method for quantifying sub-lethal effects is required. This chapter focuses on addressing this need through the development of a high-throughput optical screening assay capable of quantifying sub-lethal behavior in *Daphnia pulex*.

Even when compounds appear to be “safe” based on conventional testing, there is a growing body of literature documenting a broad range of sub-lethal effects such as reproductive and behavioral changes in fish, reptiles, mammals, and invertebrates (Holeton et al. 2011). A dramatic decline in wildlife populations in the Indian sub-continent due to emerging contaminants has been reported in the literature (Oaks et al. 2004; Shultz et al. 2004). These observations are

increasing concern over potential human exposure and resulting impacts on public health (Daughton and Ternes 1999; Murphy et al. 2012). Epidemiological studies suggest significant impacts on human development can already be detected (Bjorling-Poulsen et al. 2008; Crain et al. 2008). While data collected thus far are inconclusive, the risk of chronic low-level exposure to humans through drinking water, food or recreation is an area of active research.

Traditional methods for evaluating toxicity have primarily focused on determining lethal concentrations (LC_{50}). However, significant impacts occur on organisms at concentrations well below LC_{50} levels, such as behavioral responses, including altered motor function in organisms, and effects on development and reproduction. Such sub-lethal effects can impact the fitness and survival of target organisms and affect ecosystem function (Dodson and Hanazato 1995). Differentiating *sub-lethal* from *lethal* toxic effects, and determining how concentration and duration of exposure influences these outcomes, is critical to understanding toxicity of emerging contaminants and complex mixtures. As a result, there is increasing interest in the development of high-throughput screening assays for evaluating the toxicity of the large number of chemical contaminants and mixtures (e.g., National Toxicology Program (http://ntp.niehs.nih.gov), Computational Toxicology Research (http://www.epa.gov/comptox/))c. One approach that has been employed is the use of optical assays. For example, zooplankton have been optically tracked in larger volume assay systems (>150 ml) with a primary focus on the study of swimming behavior relevant to function in aquatic ecosystems (Dodson et al. 1995; Lard et al. 2010). Such assay systems are very important to our understanding of zooplankton behavior, but limited in their utility for high-throughput toxicity screening. A more recent study by Richendrfer et al. (Richendrfer et al. 2012) incorporated the use of a high-throughput imaging system to demonstrate the effect of sub-chronic concentrations of chlorpyrifos on zebrafish larvae in a 6-well plate.

In the present study, physostigmine and nicotine were chosen as prototypical compounds to validate the optical assay. In addition to these two model compounds, two other commonly used pesticides, chlorpyrifos and imidacloprid, were also evaluated. Physostigmine has been extensively used as a tool for studying physiological mechanisms, and its pharmacological properties as an acetylcholinesterase inhibitor (AChE-I) are well characterized (Taylor 2010). As an AChE-I physostigmine causes an increase in acetylcholine (ACh) in organisms that can over stimulate the nicotinic and muscarinic receptors (Figure 3) Chlorpyrifos is an organophosphate insecticide, which is an AChE-I, and therefore has a mode of action similar to physostigmine. Nicotine, formerly used as an insecticide (Ujvary 1997), acts directly on the nicotinic receptor (Figure 3*;* e.g. (Hibbs and Zambon 2010)) . Imidacloprid is a neonicotinoid insecticide that is an agonist with greater selectivity for the insect nicotinic receptor (Tomizawa 2004). Neonicotinoid insecticides are currently under increased scrutiny due to their possible association with bee colony collapse (Rebecca 2013).

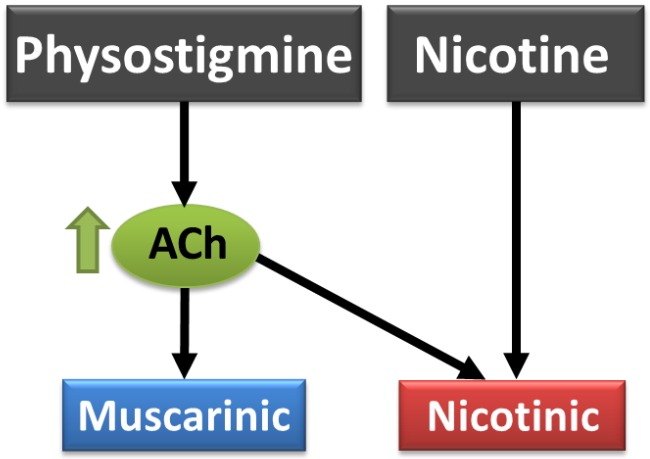


Figure 3: Mode of action of physostigmine and nicotine on muscarinic and nicotinic receptors. The acetylcholinesterase inhibitors (physostigmine, diazinon, and chlorpyrifos) inhibit the acetylcholinesterase enzyme (not shown), causing an increase in ACh levels (no longer broken down). Acetylcholinesterase inhibition results in stimulation of both muscarinic and nicotinic receptors. Nicotine only stimulates nicotinic receptors, not muscarinic receptors.

A common organism used for aquatic toxicity testing is the freshwater crustacean *Daphnia* (Kashian and Dodson 2004). *Daphnia* are primary consumers of plankton (e.g., single cell algae, bacteria, protists), are a primary food source for larger invertebrate and vertebrate species, and therefore are considered the base of the food chain in freshwater lakes (Kashian and Dodson 2002). The importance of *Daphnia* as keystone species in freshwater ecosystems is well known, and the genus has become recognized as a model organism for studying aquatic ecosystems over the past several decades. *Daphnia* are very sensitive to biotic and abiotic changes in their environment, and have developed specific adaptation strategies to cope with changes in temperature, water chemistry (e.g., dissolved oxygen), food supply, and predation (Caceres et al. 2007). The motor function of crustaceans, like *Daphnia*, is complex. Rhythmic behavior in *Daphnia* can be seen as the output of nervous system motor programs that are modulated by hormones of the neuroendocrine system (Christie 2011). Additionally, the *Daphnia* genome has been termed "ecoresponsive" because of the very large number of genes, including many duplicated genes, and because of its phenotypic plasticity and adaptive responses (Colbourne et al. 2011). *Daphnia* are ideally suited for studying ecotoxicological effects and are used as a screening tool for potential environmental contamination (Kashian and Dodson 2004).

To enhance our ability to assess the toxicity of emerging contaminants a scalable method for quantifying sub-lethal behavior using freely swimming *Daphnia* was developed. With this aim,

two hypotheses were evaluated: 1) concentration-dependent behavioral responses in *Daphnia* can be quantified by measuring changes in their movement, and 2) compounds with similar modes of action elicit similar behavioral responses.

Materials & Method

A single *Daphnia pulex* collected from Lake Michigan in 2008 was reared into a clone, and subsequently cultured in the laboratory until these experiments were conducted (2013). The *Daphnia* were housed in a 4 L jar in an incubator at 20^{o}C and exposed to equal light-dark cycles lasting 12 hours. A 50/50 algae mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* were used as food. The *Daphnia* were fed three times per week and their water was changed weekly. Artificial lake water, COMBO, was used as the culture medium as it has been shown to support the growth of both algae and zooplankton (Kilham et al. 1998).

Immediately prior to the experiments, *Daphnia* were removed from the culture with an eyedropper and passed through a screen mesh to ensure a *Daphnia* of uniform size (>1.4 mm in length) and approximately the same age were used during experiments. Select *Daphnia* were then randomly placed in isolated wells in a translucent 24-well plate. Each well has 256mm^2 in surface area to the air above and contained 3ml of aqueous solution when full. The 24 well plates allowed for limited natural vertical and horizontal swimming behavior by the *Daphnia*. For all experiments, a single animal was randomly placed into 1 of 6 wells in the middle of the 24-well plate containing different concentrations of the desired chemical (randomly assigned). On average, setup required approximately 5 min for the 6 *Daphnia* to be transferred before the experiment could begin. The isolation of animals in these 24 well plates is especially important to avoid animal interaction and enable efficient tracking (Figure 4).

Once the animals were added to the 24-well plate, the plate was placed on a raised platform where a standardized light source was projected from the bottom through a plastic paper diffuser. Fiber optic lighting was used to avoid overheating of the plates and *Daphnia*. Above the stage containing the 24-well plate, an Infinity2-1M monochrome camera with an AF Nikkor 28 mm lens

was used to capture live video recordings of the *Daphnia*'s movement. The camera was held at a fixed distance of ~ 56 cm from the plate surface providing 1280 X 1024 resolution. Live images were captured and recorded on the computer using Infinity Capture software (Lumenera, Ottawa, ON) and were saved in AVI format. Video analysis was performed using Image Pro Plus 7 software (Media Cybernetics, Rockville, MD) using the two-dimensional (2D) tracking module calibrated to measure animal movement. Prior to conducting experiments, spatial filtration was applied to flatten out the image and reduce background intensity variations and the spatial scale. The image was then sharpened to enhance fine details. Using this experimental setup, the processing techniques employed resulted in images that were void of background noise. Prior to quantification, images were calibrated to provide 2D distance measurements in millimeters.

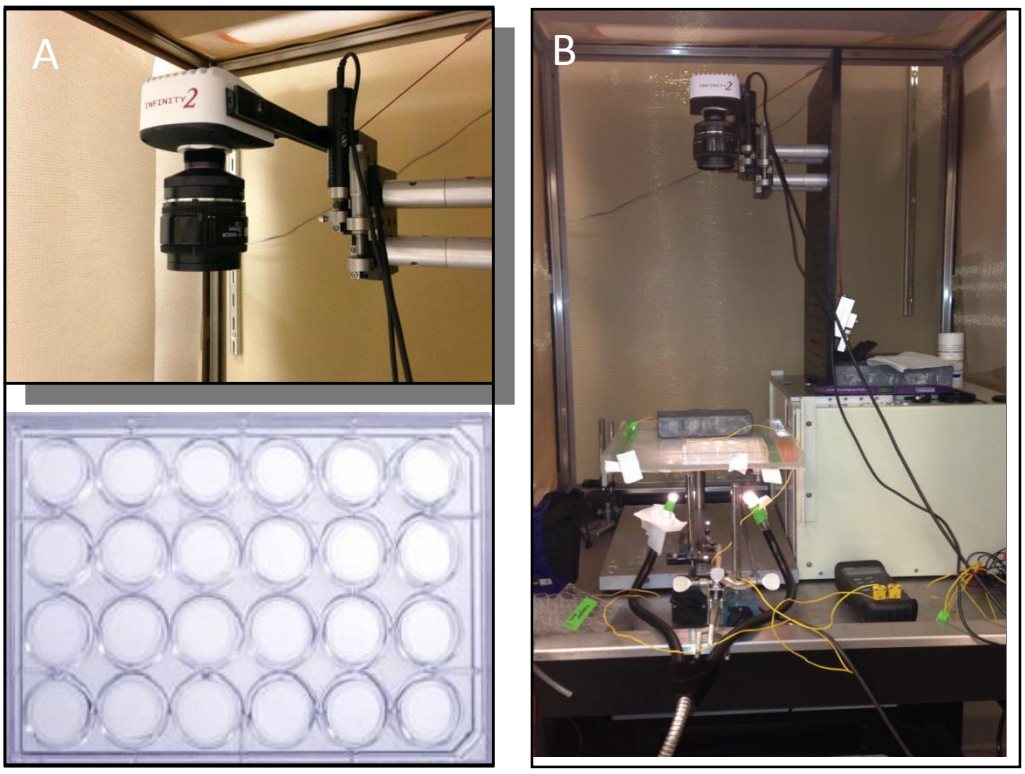


Figure 4: A. Infinity2-1M monochrome camera with an AF Nikkor 28 mm lens pointed at a 24 well plate. B. Optical tracking setup, camera held at a fixed distance from the 24-well plate on the raised platform with fiber optics lighting

The data analysis of videos is described in Figure 5. *Daphnia* were given a 10 min acclimation period after all animals were placed into individual wells to reduce the effects of the new environment on their behavior. After the initial 10 min exposure, 5 sec videos were recorded every 10 min for 90 min (Figure 5A). With an initial 10 min acclimation period and 90 min of optical tracking, *Daphnia* were exposed to each chemical for approximately 100 min by the end of each experiment. Every 5 sec recording resulted in a total of 145 images (i.e. frames; see Figure 5B), which were then used to track and quantify movement (Figure 5C). The video analysis software was then used to track, measure, and quantify (frame by frame) the movement of *Daphnia* (Figure 5C).

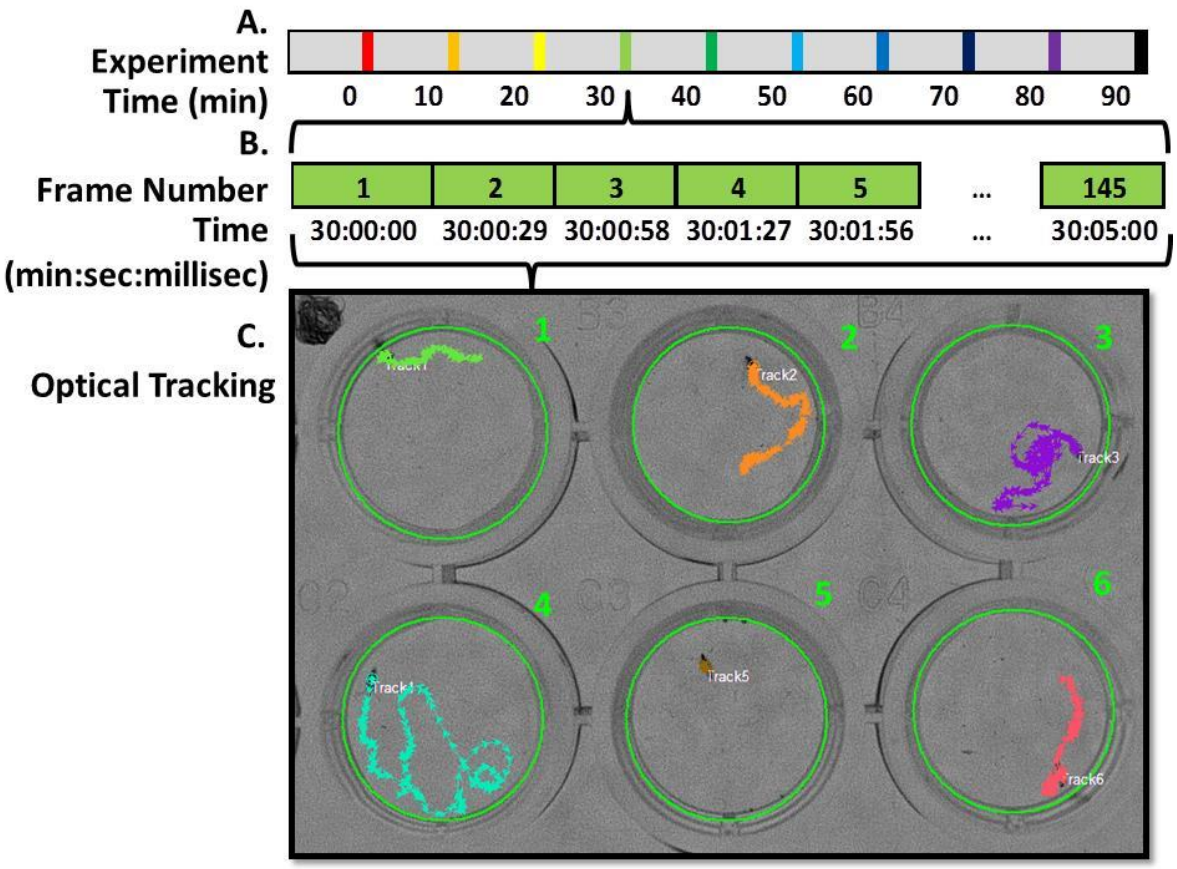
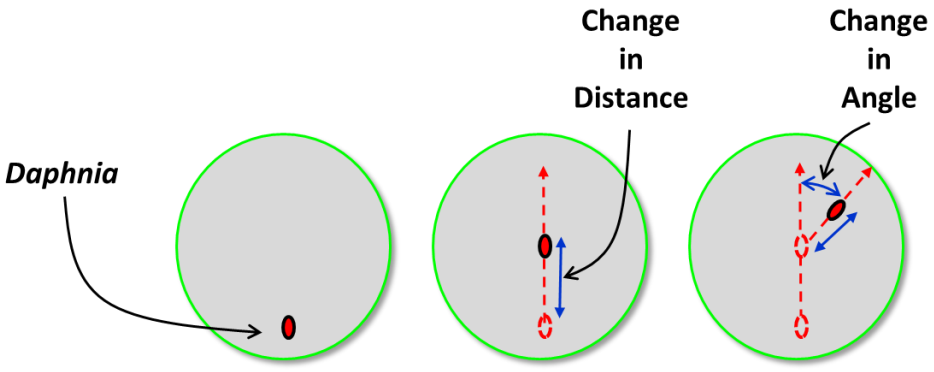


Figure 5: Sub-lethal effects measured during 100 min experiments: a) Exposure is initiated 10 min prior to the first image recording at t = 0 min and every 10 min thereafter until the end of the experiment (t = 90 min), b) during each 5 sec recording a total of 145 images (i.e. frames) are collected which c) are used to track and quantify movement

The cumulative distance *Daphnia* traveled and their angular change in direction were used to quantify movement (Figure 6). Cumulative distance was measured by summing the incremental distance moved between frames (n=145) over the course of a 5 sec video. The change in angle was measured by comparing the change in the direction of vectors from one frame to the next. For example, an initial vector can be defined by the change in position of the animal between frames 1 and 2 and a second vector can be defined by the change in position of the animal between frames 2 and 3 (Figure 6). The angle between these two vectors is the change in angle. For this analysis,

the change in angle reported is the average of the measure collected during each 5 sec measurement period (145 frames).



	Frame 1	Frame 2	Frame 3
Change in Distance (mm):	NA	10	8
Cumulative Distance (mm):	NA	10	18
Change in Angle (deg):	NA	NA	25

Figure 6: Example quantification of cumulative distance and change in angle.

Stock solutions of 1mM (physostigmine, nicotine) and 10mM (chlorpyrifos, imidacloprid), as well as subsequent serial dilutions, were made on the same day experiments were performed. The chlorpyrifos stock solution was made by dissolving the insecticide in acetone. All other chemicals used in this study were dissolved directly in COMBO. The highest concentration of chlorpyrifos studied contained 0.0025% acetone. The control solution used for experiments with chlorpyrifos contained 0.0025% acetone in COMBO water. To establish behaviorally relevant concentration ranges for the optical assay, *Daphnia* were exposed to 10-12 different concentrations of each chemical in 24-well plates and observed visually. Behavioral movements were observed continuously for 2 hours and then again for a few minutes at the 24 and 48-hour mark (Appendix

B). The six concentrations selected for the optical analysis were based on visual observations and bracketed LC_{50} reported (TOXNET 2013).

All statistical analyses were performed using Statistica (Version 10, Tulsa, OK, USA). The dependent variables were cumulative distance and change in angle. These measures were obtained at 10 min intervals during 90 min of optical tracking. Independent variables included time (0-90 min), concentration, well number, treatment (chemical), and temperature. Repeated measures analysis (time) was used to identify significant changes in the dependent variable (average cumulative distance or average angle) resulting from exposure to a certain chemical on *Daphnia* over the 90-min experiment. Analysis of covariance (ANCOVA) was conducted to control for between animal variations in basal motor activity. The covariate in this case was the level of activity at time zero, which varied between animals. By utilizing measures at t = 0 min as a covariate, the reduction in error variance increased the statistical power of the analysis. A least significant difference (LSD) post-hoc test was used to evaluate differences among means when there was a significant main or interaction effect in ANCOVA(Pitts et al. 1990). In the analysis of the data, each 24-well plate was considered a trial and each plate held 6 animals. In a typical experiment, there were 5 to 7 plates (30 to 42 animals).

Results

As can be seen from the example in **Figure 7**A, *Daphnia* were found to show a concentration-dependent effect of physostigmine exposure on swimming distance. The concentration of physostigmine increased from 0.25 μM in well number one to 4 μM in well number five. The control (concentration = 0) was in well number 6. Please note that during actual experiments, the placement of *Daphnia* and the concentration of each analyte were randomly assigned. As discussed below, physostigmine was found to induce a significant stimulatory effect

on swimming response as concentration increased until a threshold was reached at higher concentrations, and immobility was induced (well number five). Analysis results for physostigmine and chlorpyrifos are presented in Table 2(dependent variable is cumulative distance) and **Error! Reference source not found.** (dependent variable is angle). ANCOVA results for nicotine and imidacloprid are included as Supplemental Data (Appendix B).

The effect of the acetylcholinesterase inhibitors (AChE-I), physostigmine and chlorpyrifos, on behavior was evaluated in 5 trials (5 twenty-four well plates) for each individual chemical (n=30 animals per chemical). A significant concentration-dependent and chemical-dependent effect on the average cumulative distance was found due to altered swimming behavior (concentration x chemical interaction, $P < 0.05$, Table 2).

Table 2: Repeated measures analysis of covariance of cumulative distance for physostigmine versus chlorpyrifos.

Effect	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	**F value**	**P value**
Intercept	27944	1	27944	66.769	0.0000
Covariate (Time 0)	4101	1	4101	9.800	0.0030
Concentration Level	14143	5	2829	6.768	0.0001
Chemical	18805	1	18805	44.932	0.0000
Concentration x Chemical	5649	5	1130	2.700	0.0317
Error	19671	47	419		
Time	1978	8	247	1.632	0.1141
Time x Covariate	1533	8	192	1.265	0.2606
Time x Concentration	6018	40	150	0.993	0.4864
Time x Chemical	1118	8	140	0.922	0.4978
Time x Conc. x Chem.	7699	40	192	1.271	0.1329
Error	56963	376	152		

A post-hoc analysis of the model compound physostigmine showed that cumulative distances at concentration levels 2, 3, and 4 (0.5, 1 and 2 μM) were significantly greater than control (Figure 8A). The mean value at the 4 μM concentration was significantly lower than that at 2 μM, and the 4 μM concentration was not significantly different from control (time 0). However, optical tracking of the highest concentration of physostigmine concentration (4 μM) at 90 minutes demonstrated that three of the animals were immobile (moving less than 5 mm in 5 sec), and two of them were hardly moving (Figure 8). Motor function has been optically observed through the Daphnid exoskeleton after exposure of single animals to 4μM physostigmine at a magnification of 40x, and the swimming antennae and appendages no longer show spontaneous movement, but the heart is still beating (Pitts, D.K, Wayne State university, Detroit, MI, unpublished).

The cumulative distance response to chlorpyrifos resembled that of physostigmine (Figure 7A), with the highest concentration causing immobilization. However, in contrast to physostigmine, there was not a significant concentration-dependent increase in cumulative distance caused by mid-range concentrations of chlorpyrifos (concentration x chemical interaction, $P<0.05$, Table 2: Repeated measures analysis of covariance of cumulative distance for physostigmine versus chlorpyrifos.; LSD test, Figure 7A). Low concentrations of chlorpyrifos have also been shown to significantly affect the swimming behavior of zebra fish in a developmental study by Richendrfer et al (Richendrfer et al. 2012) that involves longer exposure periods and slightly lower concentrations. These results suggest that the motor behavior of zebrafish and *Daphnia pulex* can be affected at similarly low concentrations of chlorpyrifos, and that assays which compare these species maybe very useful in assessing aquatic toxicity in an invertebrate and vertebrate model.

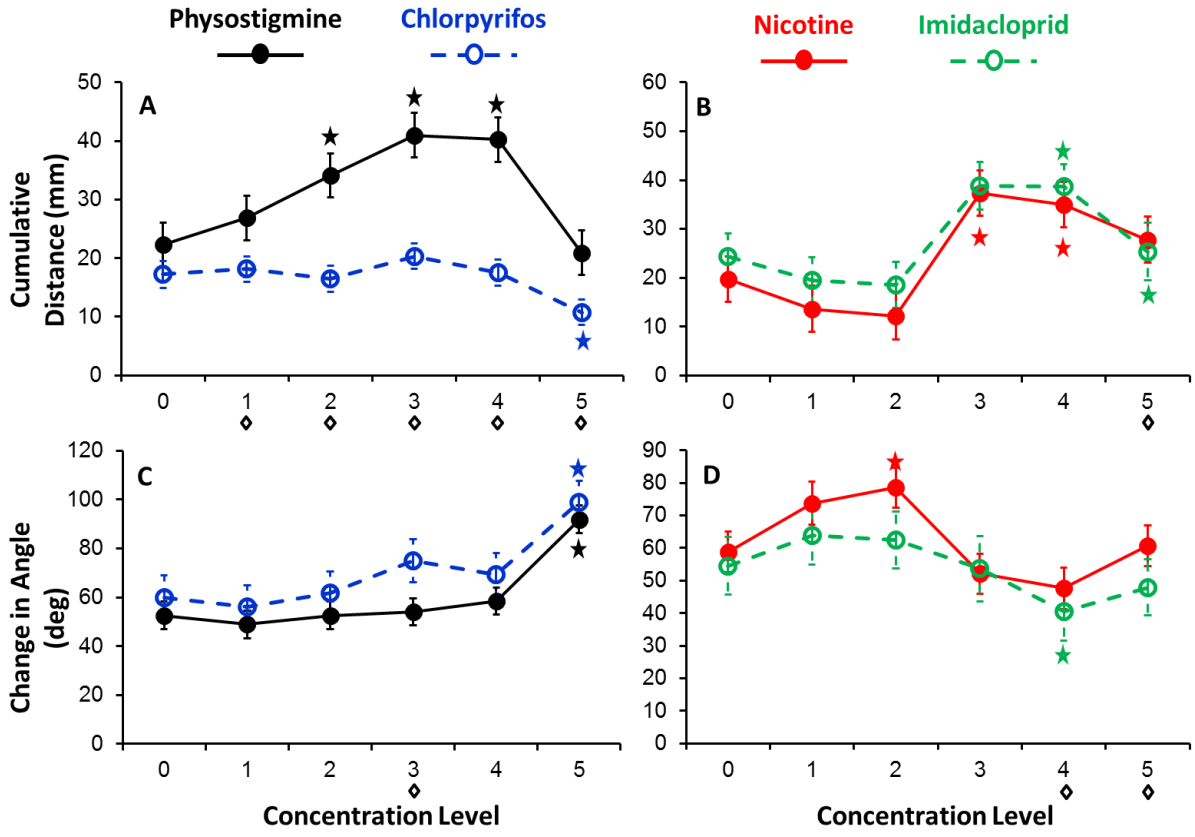


Figure 7: Behavioral responses of *Daphnia pulex* to AChE-I and neonicotinoids. Concentration levels 0-5 were: 0, 0.25, 0.5, 1, 2, and 4 µM for physostigmine (n=5); 0, 0.016, 0.3, 0.06, 0.12 and 0.25 µM for chlorpirfos (n=5); 0, 1, 4, 16, 64, and 256 µM for nicotine (n=6); and 0, 4, 16, 64, 256, 1024 µM for imidacloprid (n=5). Error bars are the standard error. Stars indicate significant ($p < 0.05$, LSD test) difference for each chemical relative to the control. Diamonds indicate significant ($p < 0.05$, LSD test) difference in the response observed between compounds with the same mode of action.

When the change in angle was evaluated for physostigmine and chlorpyrifos, a significant concentration-dependent increase was found (**Error! Reference source not found.**, concentration main effect, $P < 0.001$) that did not differ significantly across chemicals (**Error! Reference source not found.**) concentration x chemical interaction

P>0.20). A post-hoc analysis indicated that at the highest concentration of physostigmine (level 5, 4 µM), where immobility was observed, there was a significant increase in average angle (Figure 7, post-hoc analysis), and a virtually identical situation occurred for chlorpyrifos at the highest concentration level (0.25µM; Figure 7, post-hoc analysis).

Table 3: Repeated measures analysis of covariance of change in angle for physostigmine versus chlorpyrifos

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
Intercept	341139	1	341139	142.513	0.0000
Covariate (Time 0)	13865	1	13865	5.792	0.0201
Concentration Level	105041	5	21008	8.776	0.0000
Chemical	11425	1	11425	4.773	0.0339
Concentration x Chemical	3292	5	659	0.275	0.9245
Error	112506	47	2394		
Time	27924	8	3491	8.413	0.0000
Time x Covariate	7668	8	959	2.310	0.0199
Time x Concentration	37684	40	942	2.271	0.0000
Time x Chemical	4500	8	563	1.356	0.2145
Time x Conc. x Chem.	17398	40	435	1.048	0.3955
Error	1555993	376	415		

For cumulative distance, interactions with time were not significant (Table 1, P > 0.10). In Figure 7A, the effect of physostigmine on the cumulative distance *Daphnia* travel over time is broken down to show an example of the effect over time. The average of all means over time for a given concentration in Figure 8A is mathematically equal to the single mean for a concentration in Figure 7A. Contrast analysis (all means) indicated that the response observed during exposure to 1 and 2 µM of physostigmine was significantly different from control (P < 0.005), while the response observed during exposure to 4µM of physostigmine was not significantly different from control (P > 0.20) Figure 7 A.

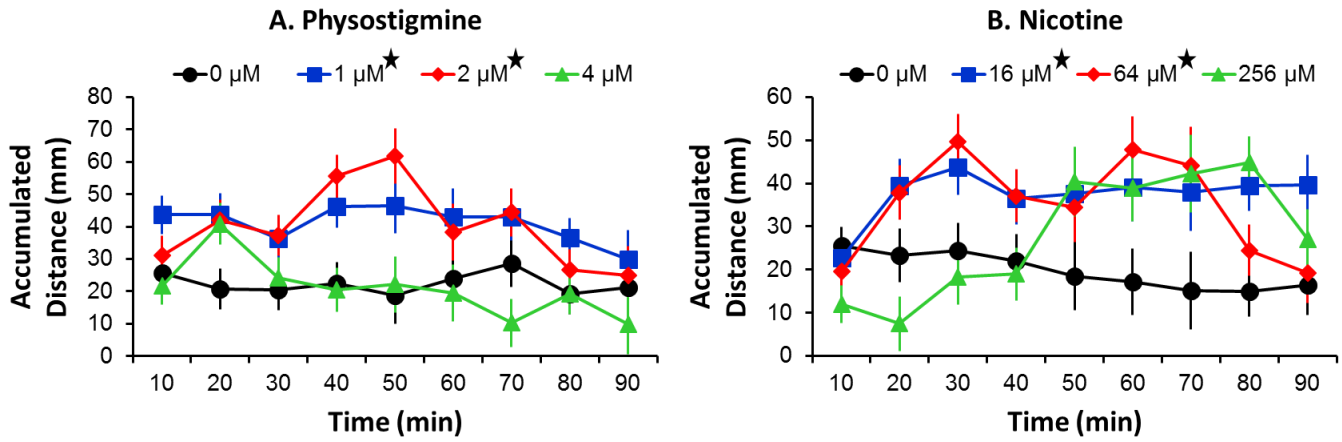


Figure 8: Mean response, with standard error, during optical tracking for the three highest concentrations. A) physostigmine, with control (n=6) and B) nicotine, with control (n=6). Stars indicate statistically significant ($p < 0.05$, LSD test) difference from control

The effects of the prototypical compound, nicotine, and the neonicotinoid, imidacloprid, each were examined in 5 trials (5 twenty-four well plates) for each individual chemical (n=30 animals per chemical). A significant concentration-dependent effect of these chemicals on cumulative distance was found (concentration main effect, $P<0.001$) that did not differ across chemicals (concentration x chemical, $P>0.20$) (see supplemental data Appendix C). A post-hoc analysis of the model compound, nicotine, showed that cumulative distances at concentration levels 3 and 4 (64 and 256 µM) were significantly greater than control (Figure 8 B). None of the animals were immobilized by the higher concentrations of nicotine over the 100 minute period of exposure. The general shape of the cumulative distance response curve to nicotine was strikingly similar to that of the neonicotinoid, imidacloprid (Figure 7B; chemical main effect, $P>0.10$; concentration x chemical effect, $P>0.20$). No sustained immobilization occurred at the highest concentrations of imidacloprid (1024 µM).

To illustrate the complexity of the effects of nicotine on the average cumulative distance, the response over time is depicted in Figure 8 B. The interaction between time and concentration

was significant (time x concentration interaction, $P < 0.001$). As discussed previously, the average of all means over time for a given concentration in Figure 8 B is mathematically equal to the single mean for a concentration in Figure 7B. For the contrast analysis (all means) depicted in Figure 8 B the response of *Daphnia* to 16 and 64 μM of nicotine were significantly different than from the control, while the overall difference in cumulative distance traveled was not significant for exposure to 256 μM versus the control ($P > 0.20$). However, by examining pairs of means from the 256 μM exposure data set using contrast analysis, the lower level response observed at t = 10 min and t = 20 min was found to be significantly different from the controls ($P < 0.05$), and the higher level response at t = 70 min and t = 80 min was also found to be significantly different from the controls ($P < 0.01$). This analysis suggests that at the highest nicotine concentration (256 μM) the swimming activity of the *Daphnia* was initially suppressed, but the animals were able to, at least partially, overcome this effect by 70 to 80 minutes into the exposure period.

When the change in angle was evaluated for nicotine and imidacloprid a significant concentration dependent change in angle was found (concentration main effect, $P<0.01$) that did not differ across chemicals (concentration x chemical interaction, $P> 0.20$). The response curves for the change in angle for nicotine and imidacloprid were strikingly similar (Figure 7D).

Discussion

As described previously, AChE-I and neonicotinoids were selected in this study because of their prevalent use and suspected ability to induce sub-lethal effects (Ashauer et al. 2011; Beketov and Liess 2008; Blacquiere et al. 2012; Groner and Relyea 2011). Acetylcholine and ACh receptors are found in both vertebrates and invertebrates (Pezzementi and Chatonnet 2010; Thany and Tricoire-Leignel 2011; Venter et al. 1988). Many of the insecticides found in surface

waters target cholinergic mechanisms, by either inhibiting acetylcholinesterase or directly stimulating ACh receptors (e.g., neonicotinoids).

Acetylcholinesterase normally terminates the bioactive effects of ACh by breaking ACh down into acetate and choline (Taylor 2010). As insecticides, AChE-I increase ACh to toxic levels by inhibiting this enzyme responsible for ACh degradation. When the enzyme is inhibited, overstimulation of all ACh receptor subtypes (e.g., muscarinic and nicotinic; Figure 3) would be expected to occur, and at sufficient concentrations this is lethal. While there are structural differences between human and insect acteylcholinesterase enzymes (Pezzementi and Chatonnet 2010), insecticides that inhibit acetylcholinesterase can readily inhibit human acetylcholinesterase and cause toxicity .

The neonicotinoids are another class of insecticides that target cholinergic mechanisms through a different mode of action. The neonicotinoids are direct ACh receptor agonists that bind directly to the receptor and show selectively for the insect nicotinic subtype (Figure 1) of the ACh receptor (Tomizawa 2013). Lethality results from over-stimulation of the insect nicotinic ACh receptor subtype. The insecticides referred to as *neonicotinoids* have been shown to be less toxic to vertebrates relative to the *nicotinoids*, such as nicotine and epibatidine (Tomizawa 2013).

The typical acute mode of action for these compounds that affect cholinergic function is to cause over-stimulation of ACh receptors. Insecticides that affect cholinergic function are known to be toxic to both vertebrates and invertebrates (Jett 2011; Tomizawa 2004). The relative potency and probability of toxicity depends on differences in toxicodynamic and toxicokinetic properties (Abdollahi and Karami-Mohajeri 2012; Jett 2011; Lloyd and Williams 2000; Rubach et al. 2010; Tomizawa 2013).

Two different dependent variables, cumulative distance and change in angle, were examined to evaluate the sub-lethal behavioral response of *Daphnia* to insecticides that affect cholinergic function via two modes of action. When the behavioral response patterns were compared, the response profile was found to be similar for compounds with the same mode of action but dissimilar for compounds with different modes of action (**Figure 7**). After 100 min of exposure to higher concentrations of AChE-I, physostigmine and chlorpyrifos, tended to result in immobility and the change in angle in the direction of movement was found to increase significantly. This increase in angular change corresponded to a decrease in cumulative distance (**Figure 7**). It is worth noting that the concentration of chlorpyrifos used was more than an order of magnitude lower than that of physostigmine because higher concentrations of chlorpyrifos were found to result in very rapid immobility (data not shown).

In contrast, the neonicotinoids, nicotine and imidacloprid, did not elicit long-lasting immobility during the study period, even though relatively high concentrations were utilized (maximum concentration of 256 μM and 1024 μM for nicotine and imidacloprid, respectively). For nicotine and imidacloprid, changes in the cumulative distance and the change in angle appeared to be mirror images of each other, with the maximum cumulative distance occurring at concentrations where the minimum change in angle occurred (**Figure 7**B,D). When the time course for the cumulative distance response to nicotine was examined (Figure 8 B), the highest concentration (256 μM) was found to cause an initial suppression of swimming behavior during the first 20 min of optical tracking, followed by a partial recovery and a significant increase in swimming distance at 70 to 80 minutes relative to the control. The observation that the animals could overcome the initial suppressive effects on swimming behavior during the highest nicotine concentration used (256 μM) suggests that *Daphnia* are able to partially overcome some of the

motor effects of nicotine and imidacloprid, at least on a short-term basis. This is supported by the significant increase in cumulative distance (Figure 7B, levels 3 and 4) and a decrease in the change in angle (Figure 7B, level 4). This effect on motor function in *Daphnia* was not observed for the two AChE-I, physostigmine and chlorpyrifos. It seems likely, based on the reported actions of AChE-I on invertebrates and vertebrates (Carvalho et al. 2003; Colovic et al. 2013; Rubach et al. 2010), that intense stimulation of all ACh receptors subtypes (Figure 3) by the ACE-I may be responsible for long-lasting immobility and death

One striking difference between physostigmine (a carbamate) and chlorpyrifos (an organophosphate) was the significant stimulatory effect of physostigmine on swimming behavior that was seen as an increase in cumulative distance at the mid-range concentrations, and was absent for chlorpyrifos (Figure 7A). Preliminary results suggest that another acetylcholinesterase inhibitor and insecticide, diazinon (an organophosphate), has a behavioral response profile similar to chlorpyrifos, one without the physostigmine-like stimulatory phase at low concentrations, but with immobilization at higher concentrations (data not shown). It is possible that the stimulatory phase seen with physostigmine, but not chlorpyrifos, could be related to toxicokinetic differences. Kretschmann et al. (2011) developed a toxicokinetic model for diazinon in *Daphnia magna* using the immobility LC_{50} as the behavioral endpoint, and found that there is a high degree of biotransformation of diazinon in *Daphnia magna* by cytochrome P450. Studies of vertebrates have shown that the carbamate, physostigmine, binds to the acetylcholinesterase enzyme and forms a covalent bond, which can be hydrolyzed, the compound released, and the effect reversed (Colovic et al. 2013). The actions of organophosphate AChE-Is are generally more long lasting than that of the carbamates.

Preliminary results from single animal studies in our laboratory suggests that the effects of physostigmine on Daphnid motor behavior can be at least partially reversed by several hours of perfusion with normal COMBO medium.

Limitations & Future Work

The expectation is that this assay method could easily be scaled up to screen a large number of compounds, and that the information obtained will complement other assays that focus on different endpoints, such as reproduction, mortality and growth rate. It is important to note even though well plates are not representative of *Daphnia*'s natural environment, standard toxicity tests using *Daphnia* as model organisms also employee artificial environments. It is expected that the behavioral effects will provide valuable insight into physiological processes in *daphnia.* These behavioral effects may occur in the natural environment and translate to other organisms, ultimately resulting in reductions in fitness. Effects on behavioral response can also result in population level impacts. For example, adverse population level impacts have occurred in many animal species as a result of behavioral changes associated with chemical exposure, those include failure to secure a mate and failure to escape predation (Hart 1993).

Conclusion

The optical assay developed was capable of detecting acute sublethal behavioral effects within the 90 min time period used in the present study. Significant deviations in both the cumulative distance and the change in angle support the first hypothesis posed, that concentration-dependent behavioral responses can be quantified by measuring changes in their movement. Similar responses were observed between prototypical compounds and insecticides with the same mode of action. This evidence directly supports the second hypothesis evaluated, that compounds with similar mode of action can produce similar behavioral responses. The method can easily be

scaled up to serve as a high-throughput screening tool to detect sub-lethal toxic effects of a variety of chemicals, chemical concentrations, specific chemical interactions and the effects of complex mixtures. Because this method can quantify sub-lethal effects relatively rapidly and inexpensively it has the potential to enhance our understanding of the toxic effects of ECs.

Chapter 4: Combined Stressors

Introduction

The toxicity of contaminants in the environment is the result of exposure to complex mixtures of chemicals, natural and anthropogenic, and conditions such as pH, oxygen levels and temperatures (Dodson and Hanazato 1995). The toxicity of specific chemicals can be amplified or reduced depending on the presence of other chemicals in solution (NRC 1988). Understanding these interactions is increasingly becoming important as more emerging contaminants continue to be detected in water (Daughton and Ternes 1999; Flaherty and Dodson 2005). Kolpin (et al. 2002) detected complex mixtures of pharmaceutical and personal care products, not removed by traditional wastewater treatment technologies, in 100% of 139 US streams monitored in a United States Geological Survey study. The detection of these chemicals is a cause for concern due to possible ecological impacts which include reproductive and developmental impairment in a variety of aquatic species (Cailleaud et al. 2011). These emerging contaminants are also mixed with industrial and agricultural contaminants which have been an environmental concern for decades.

It is estimated that 1.1 billion pounds of pesticides were used in the United States as of 2007, and most of the use (80%) was agricultural (EPA 2011). An pesticide intensive agricultural system poses a concern that by targeting pest species we may not only be endangering non native pest but also other biota including, such as through the use of insecticide and impacts to arthropods in the aquatic environment.

Many of the insecticides found in surface waters target cholinergic mechanisms, by either inhibiting acetylcholinesterase (e.g., diazinon and chlorpyrifos) or directly stimulating ACh receptors (e.g., neonicotinoids) like imidacloprid. Acetylcholinesterase normally terminates the

bioactive effects of ACh by breaking ACh down into acetate and choline (Taylor 2010). As insecticides, AChE-I increase ACh to toxic levels by inhibiting this enzyme responsible for ACh degradation. When the enzyme is inhibited, overstimulation of all ACh receptor subtypes (e.g., muscarinic and nicotinic; would be expected to occur, and at sufficient concentrations this is lethal.

Diazinon is an organophosphate insecticide, widely used in agriculture to control insects on field crops, fruits and vegetables (Harper 2009). Due to its known toxicity to aquatic organisms, it was banned for residential use in the United States. on December 31, 2004 (EPA 2002 ; Lee and Jones-Lee 2000). Following this ban, the concentrations of this compound and its occurrence in surface waters have decreased significantly (Banks et al. 2005). However, because it is readily transported, persistent, and continues to be legally used in agricultural, it is still detected in many surface waters (Hintzen et al. 2009). Similar to diazinon, nearly all home use of chlorpyrifos, another organophosphate insecticide, was banned in the US in June 2000. However, nearly 10 million pounds of chlorpyrifos are applied annually to agricultural watershed, with approximately half of the total mass being applied to corn crops (EPA 2002). . This ban has been effective in greatly reduced the concentration of chlorpyrifos in some areas (Banks et al. 2005) while some agriculturally areas where it is used continue tohave detectable levels in surface waters, particularly during summer and fall (Starner and Goh 2013). Neonicotinoids have high specificity for insects versus mammalian acetylcholine receptors (David et al. 2007). Imidacloprid is the most widely used neonicotinoid insecticide in agriculture (David et al. 2007; Sheets 2010). While few studies have directly assessed its prevalence in surface waters (Kreuger 2010; Lamers et al. 2011), it was detected in 89% of the samples collected from surface waters in three agricultural regions of California (Starner and Goh 2013).

Chemicals such as surfactants are found in most personal care and household products, are fairly ubiquitous in the environment, and have been found in waste water effluent discharges (Li 2008). 4-nonylphenol, a breakdown of detergents, has been increasingly found in surface waters (Cailleaud et al. 2011; Kolpin et al. 2002). 4-nonylphenol is not classified as an insecticide but has been shown to interact with AChE-I, therefore has the potential to cause interactive effects when combined with other chemicals with similar modes of action (e.g. known insecticides). Recent studies show 4-nonylphenol effects on swimming behavior of different species including guppies and in planarians, caused by cholinesterase enzyme inhibition (Cailleaud et al. 2011; Li 2008; Li 2012)**.** Nonylphenol exist in our water system along with a mixture of contaminants, therefore their interaction with other contaminants specifically AChE-I is of major concern. There is the distinct possibility that low levels of aquatic contaminants can interact in complex and unknown ways to cause more toxic effects than the lethality (e.g. LC_{50s}) of the individual constituents alone.

Evaluating the full ecological impacts of emerging contaminants will require assessments of toxicity that go beyond simple lethality tests and include an evaluation of sublethal effects. Effects that are not overtly lethal to individual organisms can nevertheless impact ecosystems. A behavioral alteration induced by chemical exposure can effect survival (e.g. predation) and in turn ecosystem functions. Other investigators have found that endpoints other than lethality are important in evaluating toxicity, and sub-lethal concentrations of contaminants can induce significant behavioral changes in aquatic organisms (Cailleaud et al. 2011; Flaherty and Dodson 2005; Ren et al. 2007). These behavioral changes may be maladaptive and have serious ecological consequences . Changes in behavior have proven effective in identifying toxic effects (Anderson et al. 2004) and these changes are widely used as biomarkers (Cailleaud et al. 2011). This study incorporates the use of the optical bioassay described in the previous chapter to measure the

sublethal behavioral response of *Daphnia pulex* exposed to individual chemical agents (e.g., diazinon, 4-nonylphenol), combination of chemicals: (1) chlorpyrifos, a chemical with in the same mode of action and of the class of pesticides, (2) imidacloprid, a chemical with a similar mode of action but form a different class of pesticides, (3) 4-nonylphenol, a chemical suspected as interacting with the AChE system and assumed to be unrelated, and finally chemical interactions within complex mixtures: treated wastewater containing an infinite of unknown compounds.

The following hypotheses were evaluated: 1. Compounds with similar modes of action cause additive, synergistic or antagonistic behavioral effects. 2. Compounds that are in different classes, based on mode of action or structure, can interact in an additive, synergistic or antagonistic manner. 3. The ecological effects of these interactions between chemicals are observed in environmental systems at relevant concentrations.

Materials & Methods

Because *Daphnia pulex* have long been recognized as an ideal organisms for studying ecotoxicological effects (Kashian and Dodson 2004) it was selected as the model organism for studying mixtures using the optical bioassay. *Daphnia pulex* collected from Lake Michigan were selected as model organisms for the synergistic studies. The *Daphnia* were housed in a 4 L jar of artificial lake water, COMBO (Kilham et al. 1998), in an incubator at 20^{o}C and exposed to 16 hours light followed by 8 hours of darkness (representing the longer days of the summer). Prior to experiments, *Daphnia* were poured out of the jar through a screen mesh to ensure similar size animals used in the experiments. *Daphnia* were then transferred to individual treatment beakers that had concentrations of drugs made up with a glass eyedropper. They were then transferred to isolated wells in a translucent 24-well plate. Each well has 256mm^2 in surface area to the air above and contained 3ml of aqueous solution when full. The 24 well plates allowed for limited natural

vertical and horizontal swimming behavior by the *Daphnia*. For all experiments, a single animal was randomly placed into 1 of 6 wells in the middle of the 24-well plate containing different concentrations of the desired chemical (randomly assigned). The time in which the animal is placed in individual beakers with made up concentration to the time of analysis is about 5 minutes.

Effluent wastewater was collected from the Detroit Water and Sewage Department (DWSD) located on Jefferson Avenue in Detroit, Michigan. The effluent from the DWSD has undergone the following treatments: Primary treatment (equalization tanks), secondary treatment (activated sludge), and advanced treatment (addition of FeCl3 to remove phosphorus) (Figure 9).

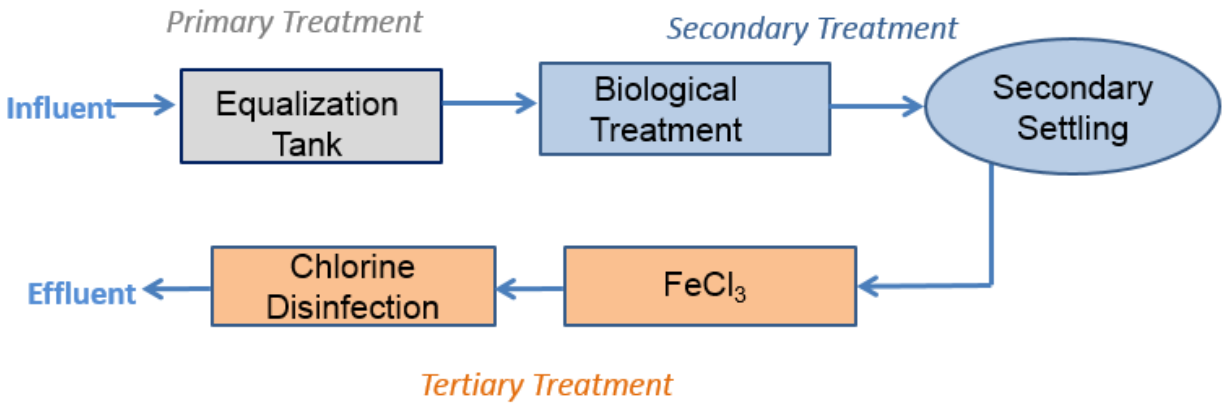


Figure 9: Schematic diagram of DWSD treatment plant

The sample was kept in a glass container and transferred in a dark box into the lab where they were immediately cooled in the fridge. The *Daphnia* was kept in the wastewater for 24 hours before the experiment took place. Control *daphnia* were maintained in COMBO water.

The 24-well plate was then placed on a raised platform where a standardized light source was projected from the bottom through a plastic paper diffuser. Fiber optics lighting was used to

avoid overheating of the plates and *Daphnia.* An Infinity2-1M monochrome camera with an AF Nikkor 28 mm lens was held at a fixed distance of ~ 56 cm above the plate surface providing 1280 X 1024 resolution. The camera was used to capture live video recordings of the *Daphnia*'s movement in the individual well plates. Live images were captured and recorded on the computer using Infinity Capture software (Lumenera, Ottawa, ON) and were saved in AVI format. Video analysis was performed using Image Pro Plus 7 software (Media Cybernetics, Rockville, MD) using the two-dimensional (2D) tracking module calibrated to measure animal movement. Prior to conducting experiments, spatial filtration was applied to flatten out the image and reduce background intensity variations and the spatial scale. The image was then sharpened to enhance fine details. Using this experimental setup, the processing techniques employed resulted in images that were void of background noise. Prior to quantification, images were calibrated to provide 2D distance measurements in millimeters.

Prior to recording, the *Daphnia* are kept for a period of 10 minutes in the 24-well plate to allow for acclimation. After the initial 10 min exposure, 5 sec videos were recorded every 10 min for 90 min. *Daphnia* were therefore exposed to each chemical for approximately 100 min by the end of each experiment. The video was then transferred for optical tracking analysis using image-pro plus software. The software allows tracking of the 145 frames generated by the 5 second videos.

Stock solutions of the following chemicals were made up the same day of the experiments. 10mM diazinon stock was made by dissolving the chemical in acetone. Serial dilutions were carried out to get the following concentrations of diazinon (0, 0.0625, 0.125, 0.25, and 0.5µM). Chlorpyrifos stock (10mM) was also dissolved in acetone and the following serial dilutions were made (0, 0.0156, 0.0312, 0.0625, 0.125, and 0.25µM). 10mM imidacloprid stock solution was

made by dissolving the chemical in COMBO water, the following concentrations of imidacloprid were generated (0, 4, 16, 64, 256, 1024µM). 4-nonyl-phenol stock was dissolved in acetone and the following concentrations were made (0, 0.25, 0.5, 1, 2, 4µM). The six concentrations selected for the optical analysis were based on visual observations and bracketed LC_{50} reported (TOXNET 2013). Combined effects of the following chemicals were investigated at specific concentrations. Four concentrations of diazinon concentrations (0.0625, 0.125, 0.25, 0.5µM) were added to one concentration of chlorpyrifos (0.125µM). The same concentrations for diazinon were added to imidacloprid (64µM), and 4-nonylphenol (0.5µM).

Data generated from Image pro plus is then transferred to STATISTICA for statistical analysis. The dependent variables were cumulative distance and change in angle. These measures were obtained at 10 min intervals during 90 min of optical tracking. Independent variables included time (0-90 min), concentration, well number, treatment (chemical), and temperature. Repeated measures analysis (time) was used to identify significant changes in the dependent variable resulting from exposure to a certain chemical or a combination of tow on *Daphnia* over the 90-min experiment. Analysis of covariance (ANCOVA) was conducted to control for between animal variations in basal motor activity. The covariate in this case was the level of activity at time zero, which varied between animals.

Results

Diazinon

Diazinon caused a significant concentration-dependent change in cumulative swimming distance (Figure 10A, concentration effect, $P< 0.05$). The cumulative swimming distance increased with a peak at the lowest concentration (0.125µM) and then it declined from this peak at higher concentrations, where the mean values were below the control level at 1 and 2 µM. This

concentration-dependent effect of diazinon on cumulative swimming distance was time-dependent (concentration x time effect, $P<0.05$). Figure 10B shows the time-dependent changes in cumulative swimming distance for the lowest and highest diazinon concentrations (0.125 and 2 µM). The animals were observed to be hardly moving or immobile at the higher concentrations (1µM – 2µM), with 6 out of 6 animals immobilized after about 90 minutes of exposure to the 2uM concentration. Note the stimulatory effect of diazinon could be observed throughout most of the time-course for the lowest concentration of diazinon (0.125 µM), while the stimulation of swimming behavior was only observed in the first 10 minutes after exposure to 2 µM diazinon. The concentration (0.125µM) is significant than control (LSD $P<0.05$).

Diazinon exposure also resulted in a significant concentration-dependent change in angle (Figure 10C, concentration effect, $P<0.001$), with the mean for angle at its lowest value at 0.125 µM, where cumulative swimming distance was greatest, and highest at 2 µM, where the mean cumulative swimming distance was lowest. The concentration-dependent effect on angle was also time-dependent (concentration x time, $P<0.001$). Figure 10D illustrates the time-dependent effect of diazinon at 0.125 µM and at 2uM. The mean angle for the 2uM concentration reaches a plateau at around 60 minutes (6 out 6 animals immobilized).

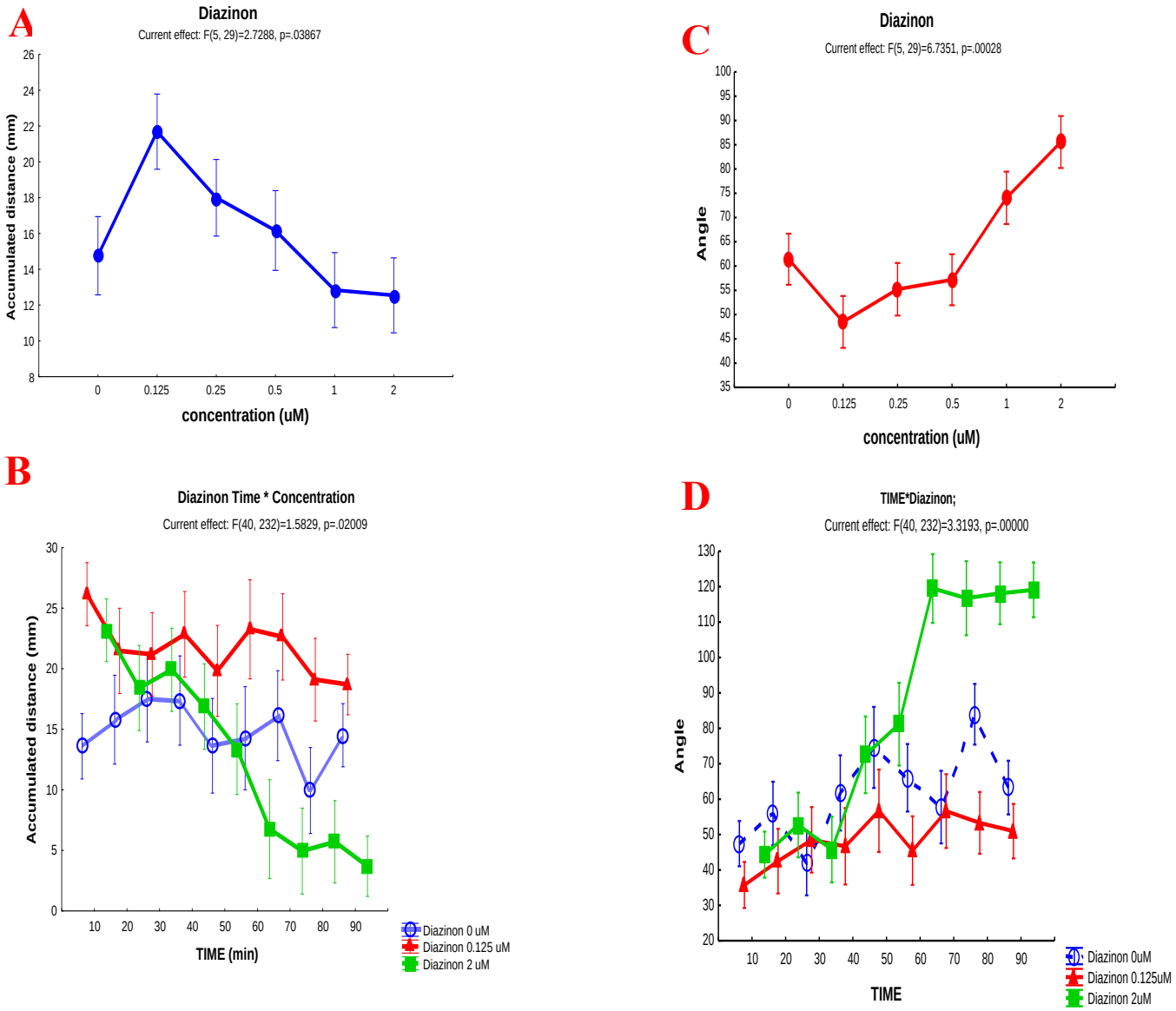


Figure 10: A) Concentration dependent effect of diazinon on Accumulated distance**. B)** Shows the time-dependent changes on cumulative swimming distance. **C**) Concentration dependent effect of diazinon on angle **D**) time-dependent effect of diazinon on Angle

4-nonylphenol

4-nonylphenol produced a significant concentration dependent change in cumulative distance (Figure 11A, $P<0.05$). The cumulative distance for the three highest concentrations of 1, 2, and 4µM, were significantly different from control (LSD test, $P<0.005$). There was a non-significant trend for a time-dependent effect of concentration ($P\sim 0.126$). As can be seen in Figure 11B, the highest concentration of 4-nonylphenol caused a reduction in the cumulative swimming distance, which plateaued after about 40 minutes of exposure.

4-nonylphenol changed angle in a significant concentration-dependent manner (Figure 11 C, $P<0.05$). The highest mean value for angle occurred at 4 µM, where 6 out of 6 animals were found to be immobilized. In Figure 11 D a significant time-dependent effect of concentration can be observed (concentration x time effect, $P<0.005$), and the effect of the highest concentration, (4 µM) is seen to plateau at round 50 minutes during the exposure.

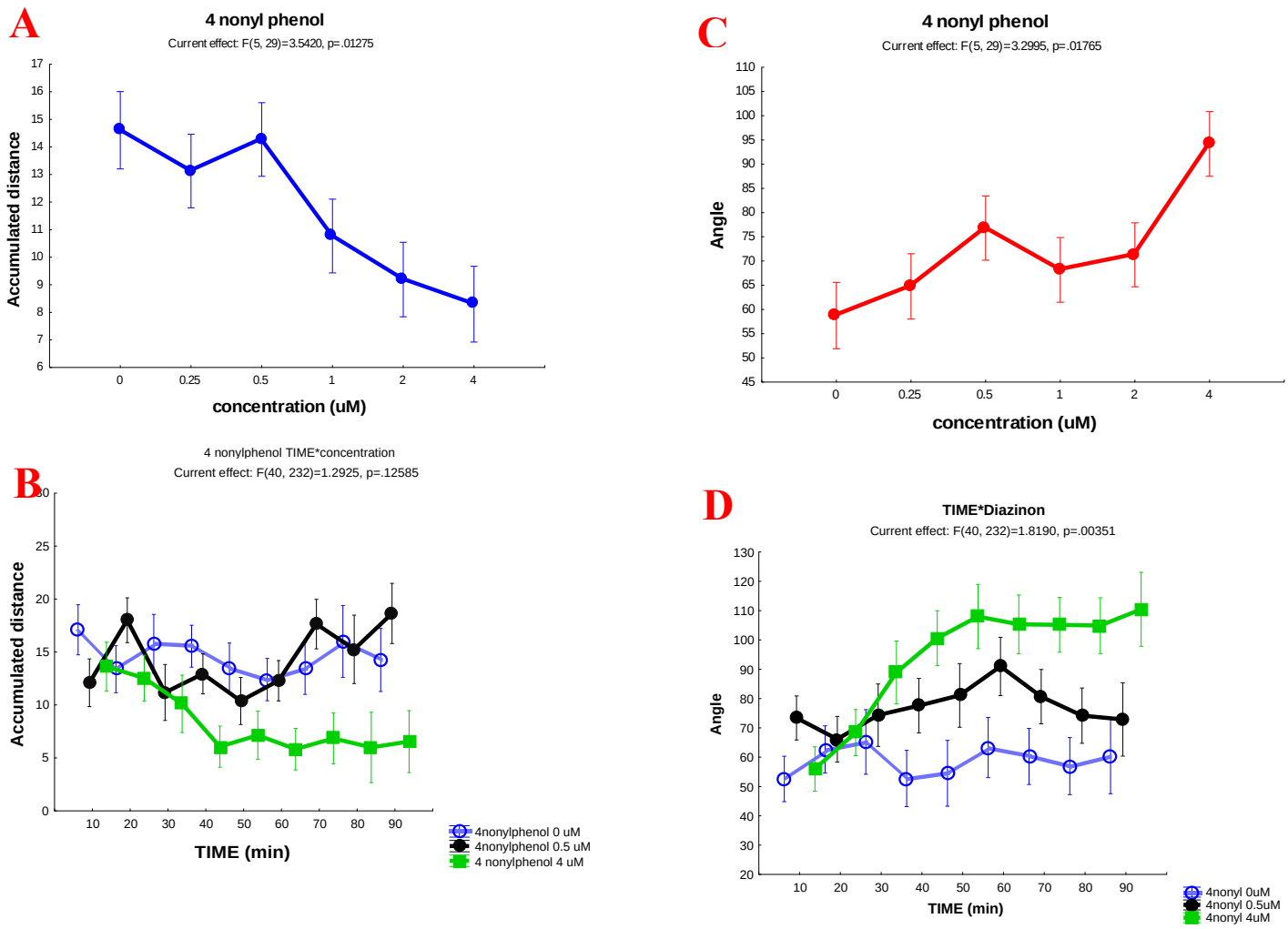
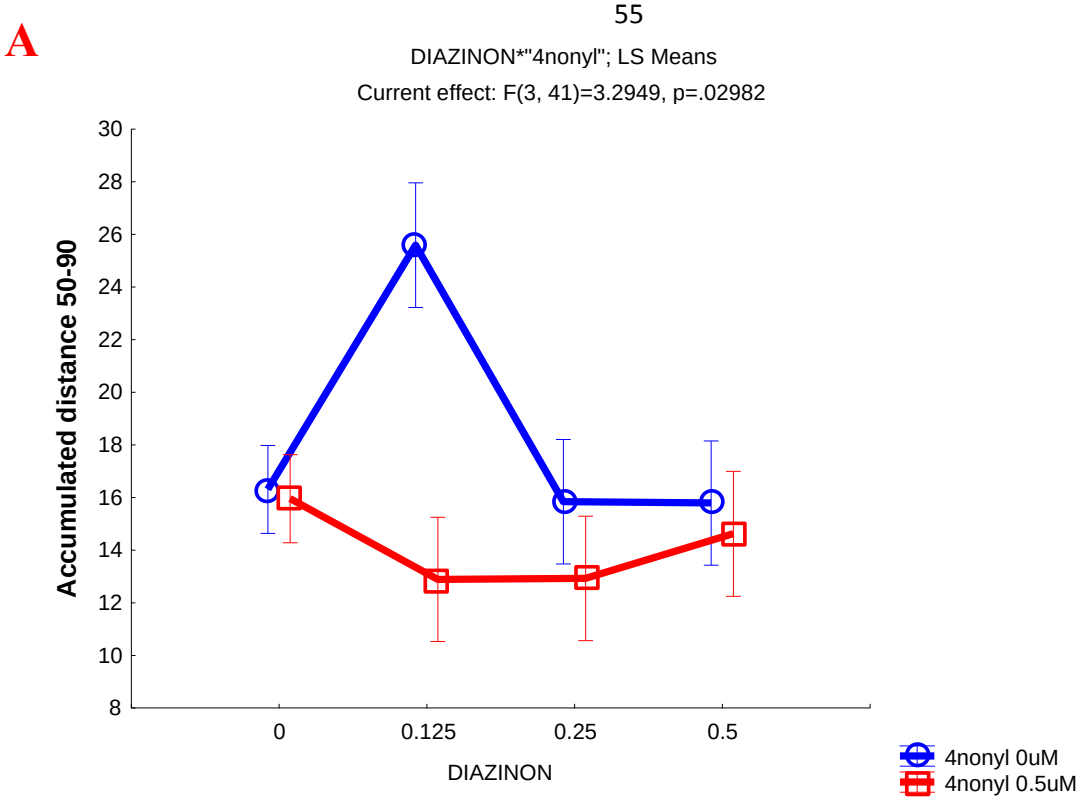


Figure 11: **A**) Concentration dependent effect of 4-nonylphenol on Accumulated Distance. **B**) Time-dependent effect of 4-nonylphenol on accumulated distance. **C**) Concentration dependent effect of 4-nonylphenol on angle **D**) time-dependent effect of 4nonylpheol on Angle.

Diazinon & 4-nonylphenol

The concentration-response relationship of diazinon (0, 0.125, 0.25, and 0.5 µM) was examined in the presence or absence of 4-nonylphenol (0, 0.5 µM) during the 50 to 90 minute exposure period. There was a significant 4-nonylphenol effect (Figure 12A, $P<0.01$) on cumulative swimming behavior. There was also a significant diazinon-concentration by 4-nonylphenol interaction ($P<0.05$), indicating a significant interaction between the two chemicals. A LSD post-hoc test indicated a significant difference between groups at a diazinon concentration of 0.125 µM. When angle was examined, there was a trend towards a diazinon-concentration by 4-nonylphenol interaction ($P\sim 0.084$).



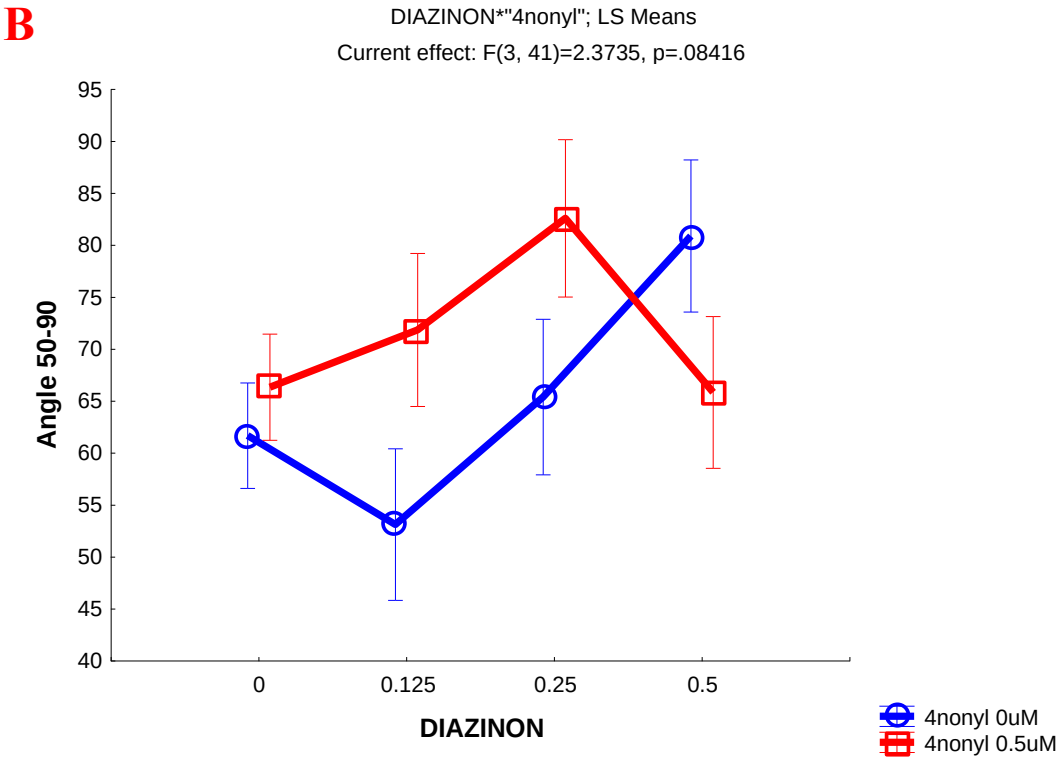
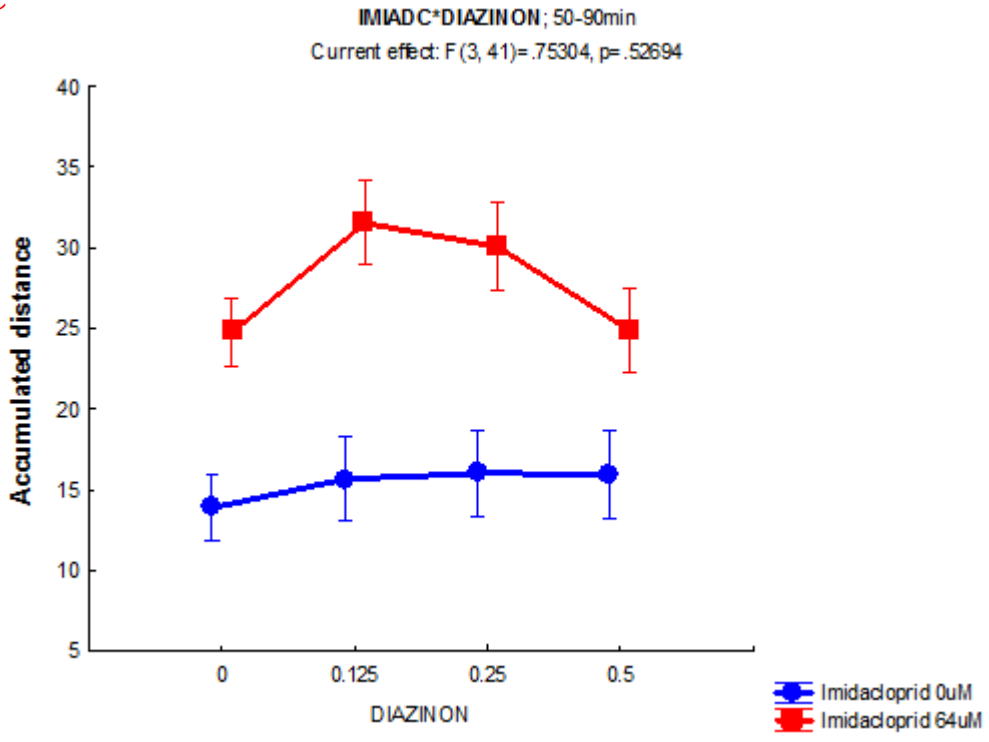


Figure 12: Interaction between 4-nonylphenol and diazinon. **A)** Accumulated distance **B)** Angle **C)** Interaction between Imidacloprid and diazinon effects on accumulated distance

Diazinon & Imidacloprid

The concentration-response relationship of diazinon (0, 0.125, 0.25, and 0.5 µM) was examined in the presence or absence of imidacloprid during the 50 to 90 minute exposure period. Based on previously published results (Zein et al. 2013), that showed an imidacloprid concentration of 64 µM was used to test for an interaction between these chemicals. There was a significant imidacloprid effect (Figure 12 C, $P<0.001$) on cumulative swimming behavior. The diazinon-concentration by imidacloprid interaction was not significant ($P>0.50$), indicating an essentially parallel upward shift in the diazinon concentration-response curve in the presence of imidacloprid. (Descriptive statistics outlined in appendix C)

C



Diazinon & Chlorpyrifos

Diazinon and chlorpyrifos are both organophosphorus compounds that inhibit acetylcholinesterase via a similar mechanism of action (AChE-I). Using a more rapid sampling period (recording videos every 5 minutes), the interaction between a single concentration of diazinon (2.0 µM) and a single concentration of chlorpyrifos (0.25 µM) was examined over time, and the rate of development of chemical effects on swimming behavior was determined. The chlorpyrifos concentration was selected based its ability to cause immobility within approximately 90 minutes (Zein et al. 2013). The effect of diazinon and chlorpyrifos on swimming behavior is depicted in Figure 13. When the time-course of the effects of chemical exposure on cumulative distance was examined, a trend towards a time x chlorpyrifos x diazinon interaction was observed ($P\sim 0.082$, Figure 13 Panel A). In the presence of either AChE-I agent alone, or in combination, there was a reduction in mean cumulative distance traveled after 70 minutes. Diazinon alone or in combination with chlorpyrifos tended to cause a more rapid decrease in cumulative swimming distance than chlorpyrifos alone. When the time-course for the effects of chemical exposure on angle was examined (Figure 13 Panel B), there was a significant chlorpyrifos effect ($P<0.01$), diazinon effect ($P<0.01$), and time x chlorpyrifos x diazinon interaction ($P<0.001$). At the point of intersection of the response curves with an angle value of 90, a perpendicular to the time axis provides approximate time values of 80 minutes for each of the two chemicals alone, and a value of approximately 60 minutes for the combination of diazinon plus chlorpyrifos. Contrast analysis, comparing line segments across treatments at 50, 60, and 70 minutes, also strongly suggest a more rapidly developing increase in angle for the combined chemicals relative to diazinon alone ($P<0.01$) and chlorpyrifos alone ($P\sim 0.052$).

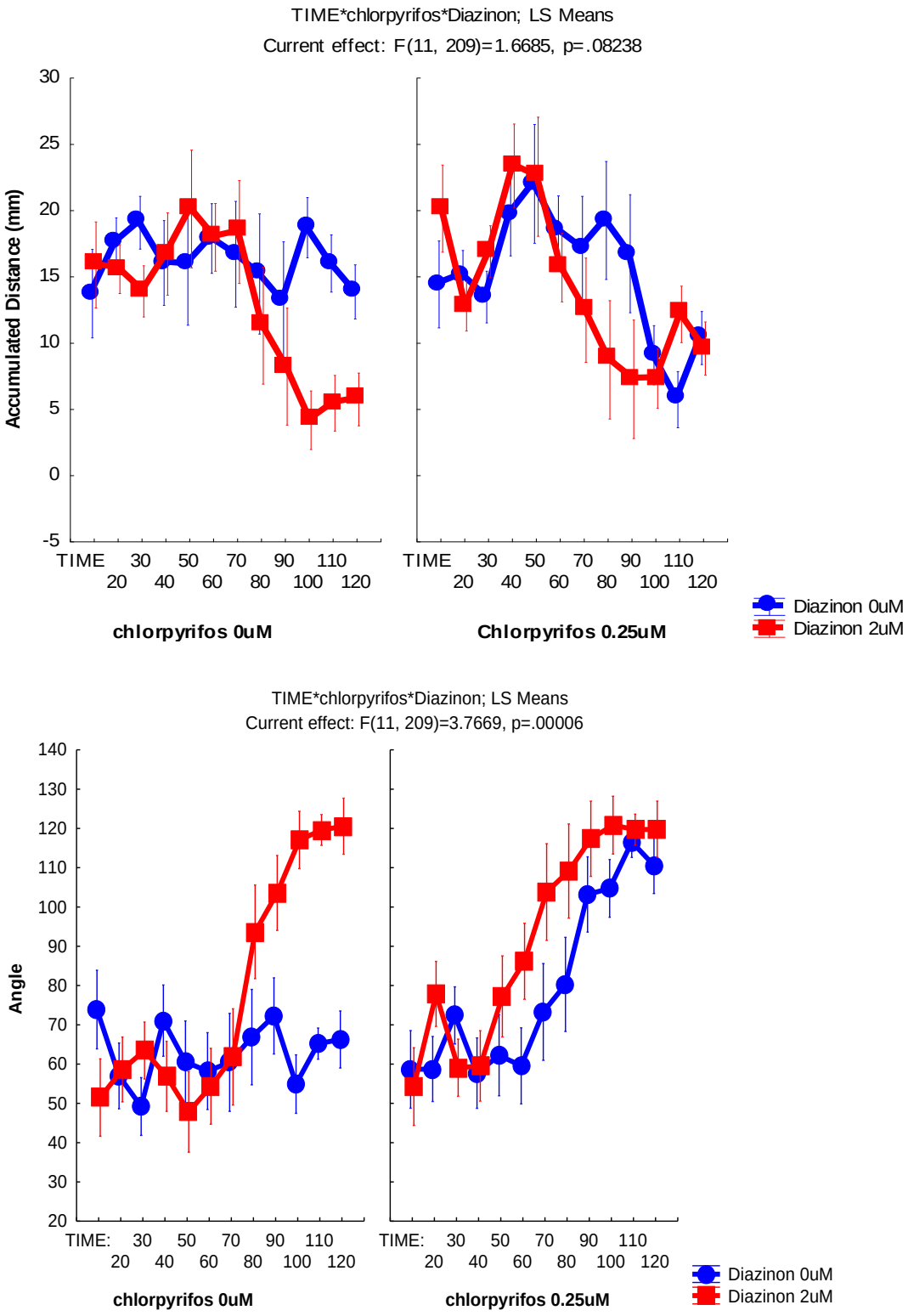


Figure 13: Interaction between chlorpyrifos and diazinon over 120 minutes and their combined effect on *daphnia*.

Diazinon & Wastewater Effluent

The concentration-response relationship of diazinon was examined in two different media: wastewater effluent or COMBO water (control). When the 50 to 90 minute period of exposure was examined, Diazinon was found to cause a significant increase in cumulative swimming distance at 0.125 µM (Figure 14A, LSD test, $P<0.01$). A significant media effect on cumulative swimming distance was also found ($P<0.05$), with the cumulative swimming distance for wastewater being less than that for COMBO water. The media x diazinon-concentration interaction was not significant (Figure 14 B, $P\sim0.16$), suggesting a similar depression of cumulative swimming distance across all three diazinon concentrations. The LSD post-hoc test identified a significant difference between groups at diazinon concentrations of 0.125 ($P<0.05$) and 0.5uM ($P<0.05$). At the diazinon concentration of 0.5uM all of the animals in COMBO media were still moving at 90 minutes, while all of the animals in wastewater were completely immobilized.

The wastewater effect on angle was significant ($P<0.001$), and found to be dependent on diazinon concentration (media x concentration effect, $P<0.05$). The mean values for angle were found to be significantly different between groups at each concentration studied (LSD test, $P<0.005$ for all three). In contrast to what was observed for COMBO media (see **Figure 11**C, D), the effect of 0.5uM diazinon on angle in the wastewater treatment group was significantly different from the wastewater control (LSD test, $P< 0.05$) and comparable to the large increase in angle observed at the 2.0 uM diazinon (**Figure 10**C, D).

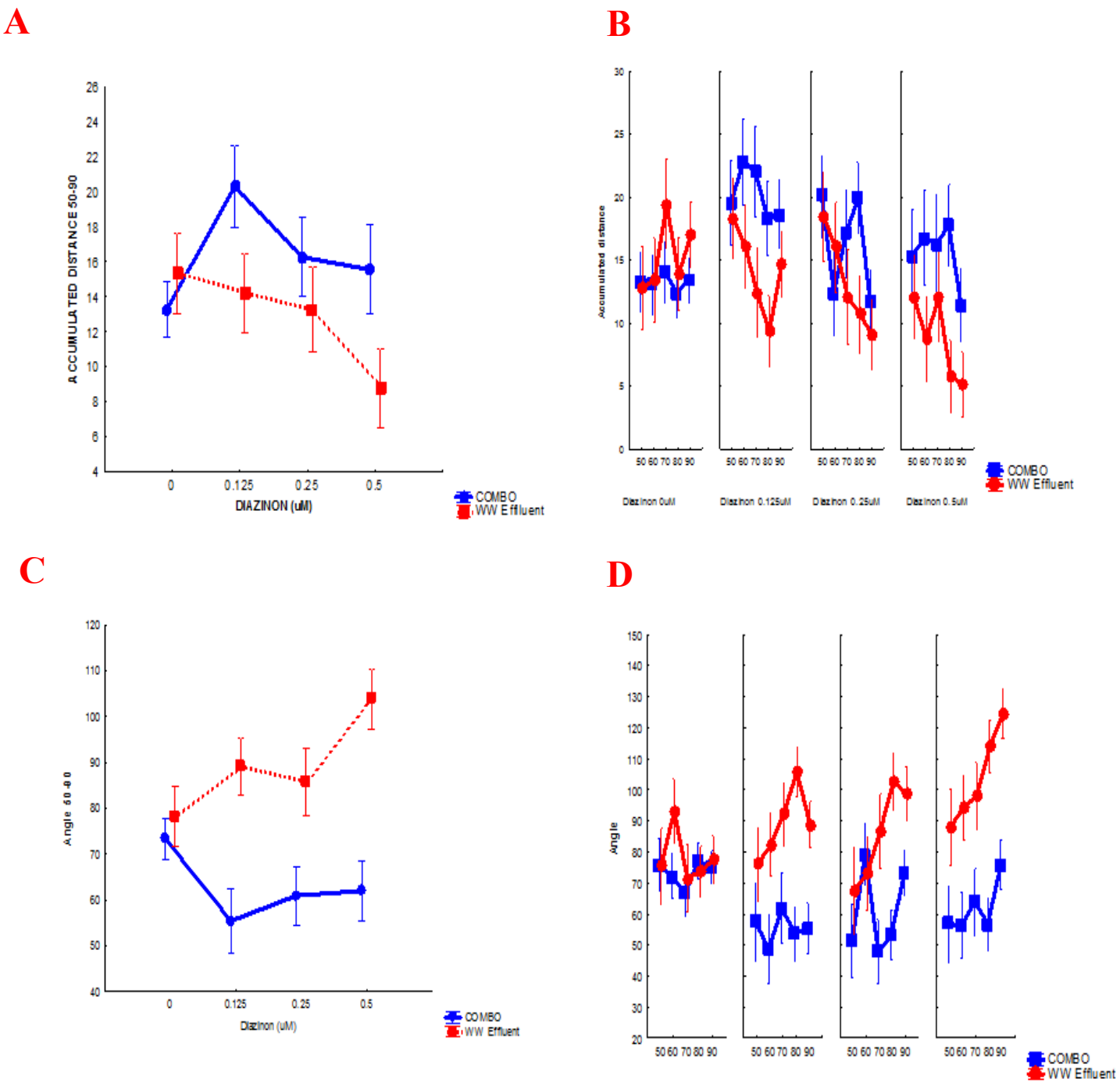


Figure 14: Interaction between diazinon and wastewater effluent.

Discussion

AChE-I, neonicotinoids and the metabolite 4-nonylphenol were selected in this study because of their prevalent use and suspected ability to induce sub-lethal effects (Ashauer et al. 2011; Beketov and Liess 2008; Blacquiere et al. 2012; Groner and Relyea 2011). Two different dependent variables, cumulative distance and change in angle, were examined to evaluate the sub-lethal behavioral response of *Daphnia* to mixtures. Cumulative distance was measured by summing the incremental distance moved between frames (n=145) over the course of a 5 sec video. The change in angle was measured by comparing the change in the direction of vectors from one frame to the next. In chapter 3, when the behavioral response patterns were compared, the response profile was found to be similar for compounds with the same mode of action but dissimilar for compounds with different modes of action (Zein et al. 2013). After 100 min of exposure to higher concentrations of acetylcholinesterase inhibitors, physostigmine, chlorpyrifos (Zein et al. 2013) and diazinon (Figure 10B) resulted in immobility and the average angular change in the direction of movement increased significantly. This increase in angular change corresponded to the decrease in accumulated distance (Figure 10C).

The AChE-I, diazinon, causes a stimulatory effect similar to that of physostigmine (Zein et al. 2013), in which a concentration-dependent increase in swimming distance corresponds to a decrease in mean angular change. The stimulatory peak depicted as a significant increase in accumulated distance is at a lower concentration than that of physostigmine 0.125µM (Figure 10A), this initial increase in swimming distance is followed by immobilization at the high concentration of 2µM (Figure 10A,B). Chlorpyrifos the other AChE-I also shows physostigmine like stimulation response in terms of accumulated distance and angle. The concentration-dependent effects of diazinon changed over time, with the largest changes occurring at about 50 minutes (Figure 10 B,

D). The interpretation of combined effects of chemicals on swimming behavior focused on the time period from 50-90 minutes (time point with the highest statistical power).

The concentration response curve for 4-nonylphenol shows a stimulatory peak followed by immobility at higher concentrations. The highest concentration of 4-nonylphenol caused a reduction in the cumulative swimming distance, which plateaued after about 40 minutes of exposure. The highest mean value for angle occurred at 4 µM, where 6 out of 6 animals were found to be immobilized. 4-nonylphenol shows a similar response curve to AChE-I diazinon (Figure 11 A), stimulatory phase depicted as increase in accumulated distance followed by immobility at higher concentrations.

As previously mentioned the time course showing behavioral effects starts at minute 50. For the reasons states above, the focus on time point 50-90 minutes was to examine potential chemical interactions (e.g., additive, synergetic) between diazinon and 4-nonylphenol. The three lower concentrations of diazinon were chosen to study this interaction with a single 4-nonylphenol concentration (0.5µM). The 3x 2 design corresponds to the tracking limitation. Figure 12 A shows a significant 4-nonylphenol effect on diazinon. 4-nonyphenol may have suppressed the stimulation effect of diazinon and thereby enhancing diazinon's potential to cause immobility. 4-nonylphenol's significant effect on diazinon is also depicted in Figure 12 B in which the angle is much higher when both chemicals are combined suggesting increased effect on immobility.

One striking difference between physostigmine (a carbamate) and chlorpyrifos (an organophosphate) was the significant stimulatory effect of physostigmine on swimming behavior that was seen as an increase in cumulative distance at the mid-range concentrations, and was absent for chlorpyrifos (Zein et al. 2013). It is possible that the stimulatory phase seen with physostigmine, but not chlorpyrifos, could be related to toxicokinetic differences. (Kretschmann

et al. 2011) developed a toxicokinetic model for diazinon in *Daphnia magna* using the immobility LC50 as the behavioral endpoint, and found that there is a high degree of biotransformation of diazinon in *Daphnia magna* by cytochrome P450. Studies of vertebrates have shown that the carbamate, physostigmine, binds to the acetylcholinesterase enzyme and forms a covalent bond, which can be hydrolyzed, the compound released, and the effect reversed (Colovic et al. 2013). The actions of organophosphate AChE inhibitors are generally more long lasting than that of the carbamates.

A different approach was taken when investigating the interaction between chlorpyrifos and diazinon. Both compounds are organophosphate cholinesterase inhibitors with very similar mode of action; they are potent inhibitors that can readily cause immobility at relatively low concentrations. It is therefore easier to detect additive effect of those drugs by looking at the rate of development of behavioral alterations. Evaluating a specific endpoint such as immobility (e.g., floor effect) may be difficult in assessing the additive effect of one drug has on the other, by measuring the rate in which they eventually become immobile. This is a non-equilibrium state over time. The concentration examined for diazinon and chlorpyrifos were: 2µM and 0.25µM respectively.

When the time-course for the effects of chemical exposure on angle was examined at the point of intersection of the response curves with an angle value of 90, a perpendicular line to the time axis provides approximate time values of 80 minutes for each of the two chemicals alone, and a value of approximately 60 minutes for the combination of diazinon plus chlorpyrifos. The difference in the time it takes to reach an angle value of 90, suggests that there is a more rapid increase in angle for the combination of both chemicals relative to each chemical alone.

The concentrations of diazinon examined in wastewater media were 0, 0.125, 0.25, and 0.5 µM. At the highest concentration of diazinon (0.5µM) there were no immobility when the media was combo water; however the same concentration in wastewater resulted in immobility for all the animals. As with the previous interaction studies the focus was on time 50-90 (min). Accumulated distance results: wastewater media had an effect somewhat similar to our study of 4-nonylphenol in that it tended to suppress stimulatory effect of diazinon at low concentrations and enhance the ability of diazinon to reduce swimming distance and eventually cause immobility suggesting that diazinon is more toxic with the 24 hr. wastewater media treatment. Lower concentration with diazinon in wastewater study looked similar to higher concentrations of diazinon alone which suggests that diazinon got more toxic after exposing to wastewater.

Limitations & Future Work

There are constituents in the wastewater that may be enhancing the toxicity of individual chemical contaminants, however this wastewater sample has not been characterized, even when we see similarity of wastewater to 4-nonylphenol the concentrations of 4-nonylphenol were not examined (4-nonylphenol was not measured in wastewater) and this is something that needs to be further examined .

Future work should implement extraction method specific for 4-nonylphenol, which will allow to further characterize the toxic component that is adding to the effect of diazinon. Future studies should measure actual concentrations in the wells rather than nominal concentrations as used in this study.

Conclusion

Chapter 3 demonstrated the utility of the optical assay for examining toxic effects of acetylcholinesterase inhibitors and nicotine agonists (mechanisms associated with insecticides).

The present study validates the capability of the assay to look at interactions between different chemical agents. It also shows the utility of the assay in comparing different chemical classes (detergents metabolite, insecticides) and examining wastewater effluent (e.g., interaction between chemical classes including complex mixtures). The results of *daphnias*' behavioral responses to combined stressors, shows the usefulness of the bioassay in detecting additive effects as well as the complexity and unpredictability of toxic effects resulting from combining various stressors.

Complex mixtures, as found in real environmental situations, may make the assessment of the toxicity of individual chemical contaminants more challenging, and this also complicates the task of regulatory bodies responsible for protecting the public and providing a safe living environment.

Chapter 5: Environmental relevance

Introduction

Aquatic invertebrates are often used as model organisms for studying the effects of water contaminants. Although such experiments provide important insight into the effect of one chemical or a chemical mixture might have on an individual species, it does not reflect outcomes on species interaction within a community (Preston et al. 1999). Predator- prey interactions are an important aspect of aquatic ecosystem function, and changes in predator prey interactions can influence population dynamics (Preston et al. 1999). Examining changes in population dynamics due to contaminant exposure is central to ecotoxicology. Regulatory agencies, such as the Environmental Protection Agency (EPA), are also concerned with changes in ecosystem function, and one way to assess this is by investigating changes in population dynamics (Klaassen 2008).

An alteration in predator-prey relationship can have cascading effects in an entire aquatic community via disruptions in the food web. For example, increased predation can result in declines in the prey population thereby distressing the entire community. There are many potential causes for an increase in predation. A change in predation can be associated with alterations in either predator and/or prey. A specific alteration in either predator or prey may not always be clearly demonstrated, but rather quantified as a change in survival without knowing which one or both is affected.

Measuring the extent to which invertebrates are susceptible to predation is important in examining the dynamics of arthropod communities (Spitze 1985). Predator encounter frequency may be influenced by body size, and swimming speed (Gerritsen and Strickler 1977).

Susceptibility of prey to predation can be affected by changes in swimming behavior.

Hyperactivity or increased irregular swimming caused by exposure to sub-lethal concentration of contaminants may increase encounter rates with predators (Brooks et al. 2009; Havens and Hanazato 1993). Studies with *Daphnia* demonstrated the rate of attack by the predator, *Chaoborus americanus,* declined as the swimming speed of *Daphnia* decreased (Spitze 1985). To the best of our knowledge this paper is the first to show diazinon effect on invertebrate predator-prey interactions. Other studies involving insecticides affecting the cholinergic system (diazinon, chlorpyrifos) focused on vertebrate predator-prey interactions (Sandahl et al. 2005; Scholz et al. 2000).

Hydra littoralis are sessile predatory invertebrates that are ubiquitous in freshwater systems. *Hydra* feed on *Daphnia* and other small invertebrates. They have a single tube-like body consisting of a head at one end and a basal disc at the other (Martinez 1998). They are especially unique in their ability to renew their epithelial cells, which are in a constant mitotic cycle (Martinez 1998). Such characteristics have led many researchers to suggest that *hydra* is immortal. *Hydra* have tentacles that are used for capturing prey. They can extend their reach by stretching the tube-like body and also by stretching the tentacles. These tentacles have stinging cells called nematocysts that eject neurotoxins and paralyze daphnia (Rachamim and Sher 2012*)*. Studies have shown *hydra* to be useful indicators of pollution because of their high sensitivity to contaminants and other environmental stressors (Beach and Pascoe 1998; Pollino and Holdway 1999). In addition, to using mortality as an indicator of pollution, sublethal endpoints using Hydra have also been useful, for example, the rate of asexual reproduction has been used as an index for estimating biological effects caused by sublethal concentrations (Stebbing and Pomroy 1978).

The effect that sublethal concentrations have on swimming behavior has been evaluated to determine how contaminants affect the risk of predation (Preston et al. 1999). Preston et al. (1999)

studied behavioral of rotifers after exposure to sublethal concentrations of the contaminant, pentachlorophenol. They measured the number of encounters, ingestions, and swimming speed using a computer tracking system. They demonstrated that predator-prey relationships are sensitive to contaminant exposure, that the nature of the contaminant effect depends on the species examined, and that the effects of contaminants on predator-prey relationships can provide an understanding of the potential impact on ecosystems.

To assess the possible impact of diazinon on predator-prey relationships, this study utilized *Hydra littoralis* and *Daphnia pulex* as predator and prey. Chapter 4 demonstrated that diazinon exposure resulted in both stimulatory and inhibitory influences on *Daphnia* (e.g., increase swimming distance over time and immobility, respectively). Alterations in swimming behavior may affect the survival of *Daphnia*, which in turn may affect the entire food web due to the importance of *Daphnia* both as a grazer of phytoplankton and a food source for fish.. In the present study, the following hypothesis was evaluated: Acute sub-lethal exposure of *Daphnia* to diazinon causes an increase in susceptibility to predation by *hydra*.

Materials & Methods

Hydra littoralis were obtained from Carolina Biological supply company (Burlington, NC). were kept in 4 L of spring water from Carolina Biological Supply Company. Hydra were fed *Daphnia pulex* everyday to maintain a healthy population and maintained under dim lighting at 21^{o}C.

A single *Daphnia pulex* collected from Lake Michigan in 2008 was reared into a clone, and subsequently cultured in the laboratory. The *Daphnia* were housed in a 4 L jar in an incubator at 20^{o}C and exposed to equal light-dark cycles lasting 16 hours. A 50/50 algae mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* were used as food. The *Daphnia* were

fed three times per week and their medium was changed weekly. Artificial lake water, COMBO, was used as the culture medium for *Daphnia* as it has been shown to support the growth of both algae and zooplankton (Kilham et al. 1998).

A 10mM diazinon stock solution was made by dissolving it in acetone. Serial dilutions were carried out to achieve a concentration of 0.25µM. *Daphnia* were exposed to a single concentration of diazinon (0.25µM) for twenty minutes (figure 1), a concentration shown in previous experiments (chapter 4) to cause an increase in swimming activity. 160 *Daphnia* were screened through a fine mesh to obtain similar body sizes. 20 hydras were collected with a glass pipette and inserted into individual petri dishes (60 X 15mm). The time-line for the experiments is outlined in more detail below (Figure 15).

Experiment 1) Hy: One *Hydra* was added to individual petri dishes containing spring water. Five *Daphnia* were placed in each of these five petri dishes using a glass pipette. These *Daphnia* were not exposed to diazinon. **(Petri dishes, N =5; Daphnia, n =25)**

Experiment 2) HD + Hy: As described above, *Hydra* was added to individual petri dishes containing spring water. Five *Daphnia* were placed in each of these five petri dishes using a glass pipette. In this experiment *Daphnia* were pre-exposed to a high concentration of diazinon of (0.25µM) for 10 minutes. **(N=5, n=25)**

Experiment 3) LD + Hy: Since the methods used in experiment 2 resulted in the transfer of a small amount of diazinon to the petri dish containing *hydra*, the amount of diazinon transferred by the pipette was estimated. Equivalently sized drops from the glass pipette were weighed and the concentration of diazinon in the petri dish containing hydra was estimated to be diluted 1:10. This experiment served as a control for the diluted diazinon concentration present in experiment 2. **(N=10, n=50)**

Experiment 4) HD: Another control for the effects of diazinon on survival was examined, in which *Daphnia* were pre-exposed to a high concentration of diazinon (0.25µM) then transferred to a petri dish with spring water without *Hydra*. **(N=1, n=10)**. Note: This experiment was limited by the number of available *Daphnia*.

A two-way analysis of variance (ANOVA) with repeated measures (time) was used to evaluate main effects. A least significant difference post-hoc test was used to compare the means after ANOVA. .

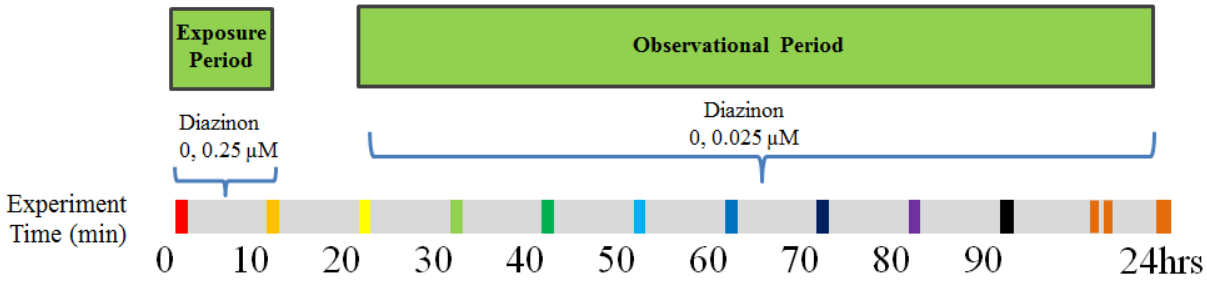


Figure 15: Exposure duration and types of treatment. Daphnia were either pre-exposed to 0.25µM of diazinon or just combo water (0 µM) containing no diazinon for a period of 10 minutes. The observational period starts at minute 20 up to 90 minutes, and again at the 24 hour time point. The observational period may not contain any concentration of diazinon (0 µM) just spring water, or it may contain (0.025µM) of diazinon.

Experiment 1	Experiment 2	Experiment 3	Experiment 4
Hy	HD + Hy	LD + Hy	HD
Hy: ***Hydra***	HD: **0.25 diazinon**	LD: **0.025 diazinon**	HD: **0.25 diazinon**
Hydra & Daphnia	Pre-exposed *Daphnia* to a 0.25 µM diazinon & *Hydra*	*Daphnia* & *Hydra* exposed to a 0.025 µM diazinon	Pre-exposed *Daphnia* to a 0.25 µM diazinon

Figure 16: Code and Exposure Protocol. Experiment 1 contains Hydra and Daphnia without any exposure to diazinon. Experiment 2 contains a pre-exposed Daphnia to a 0.25 µM of diazinon. Experiment 3 both Daphnia and Hydra are exposed to 0.025 µM diazinon. Experiment 4, Daphnia is pre-exposed to diazinon and observed in the absence of Hydra.

Results

A single *Hydra* is depicted in Figure 17A. This black and white digital photograph illustrates the body and its tentacles. Although *Hydra* is relatively sessile, the tentacles dramatically elongate to capture prey. Upon contact with prey *(D. pulex*) the tentacles wrap around the body of the *Daphnia* (Figure 17B). Figure 4 shows *Hydra* to capturing more than one *Daphnia* at a time.

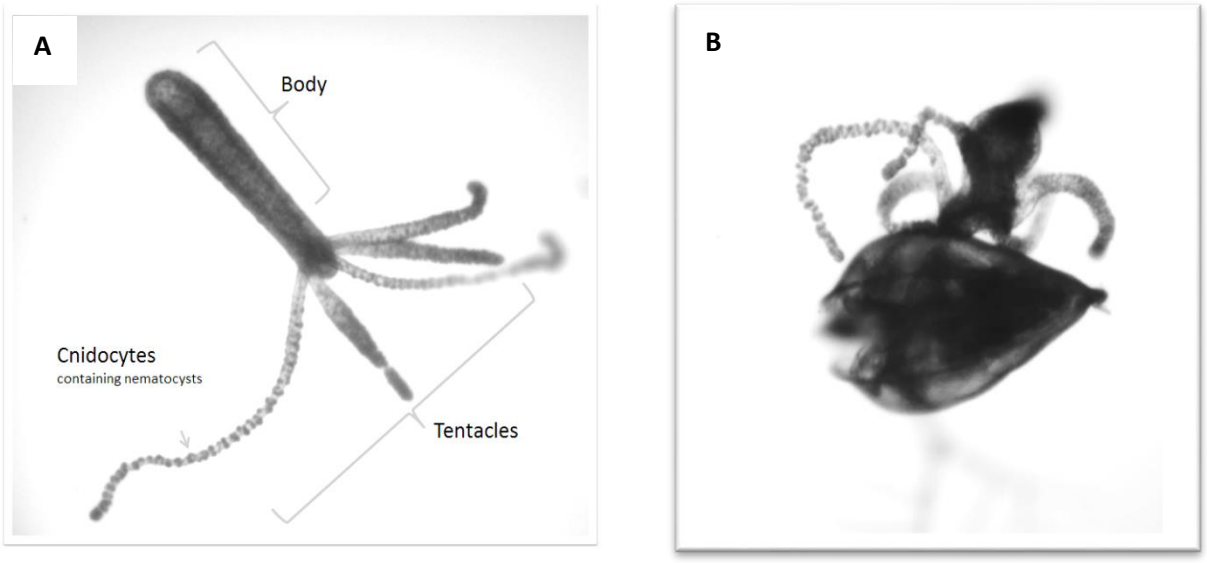


Figure 17: A. Black and white Photo of a single Hydra under 40X magnification. B. Photo of a single Hydra wrapping its tentacles around one Daphnia pulex.

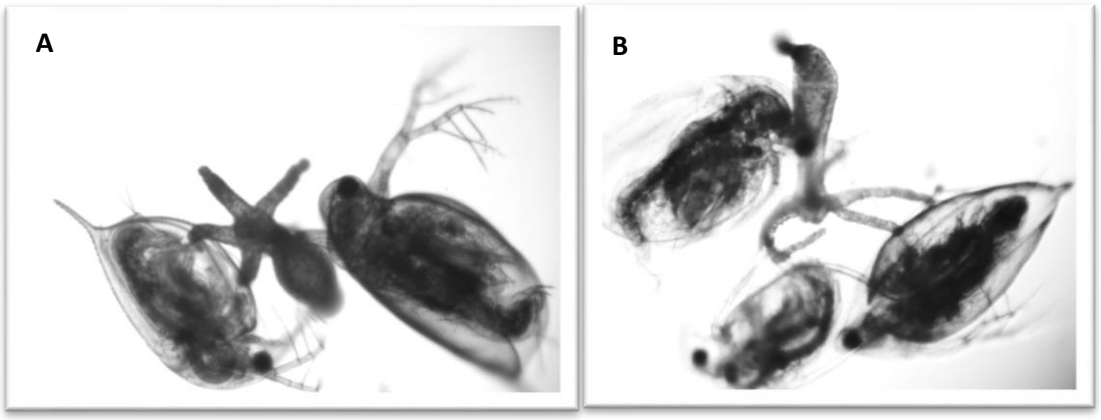


Figure 18: A. Photo of a single Hydra under 40X magnification capturing 2 Daphnia pulex. B. Photo of a single Hydra trying to capture a third Daphnia.

When Hy, HD + Hy, and LD + Hy were compared, there was a significant treatment ($P<0.01$) and time effect ($P<0.001$). The interaction between treatment and time was not significant ($P>0.5$). This indicates that the treatment effect does not dependent on time. The time course for the *three* treatments between 20 minutes and 24 hours are similar, and this is consistent with a non significant time X treatment interaction mentioned above (***Figure 19***). The fourth

treatment HD was not included in the 2-way ANOVA comparing treatments due to sample size differences.

When treatments were compared ignoring time, the HD + Hy treatment was significantly different from the other two treatments, Hy and LD + Hy ($P<0.01$) in both cases.

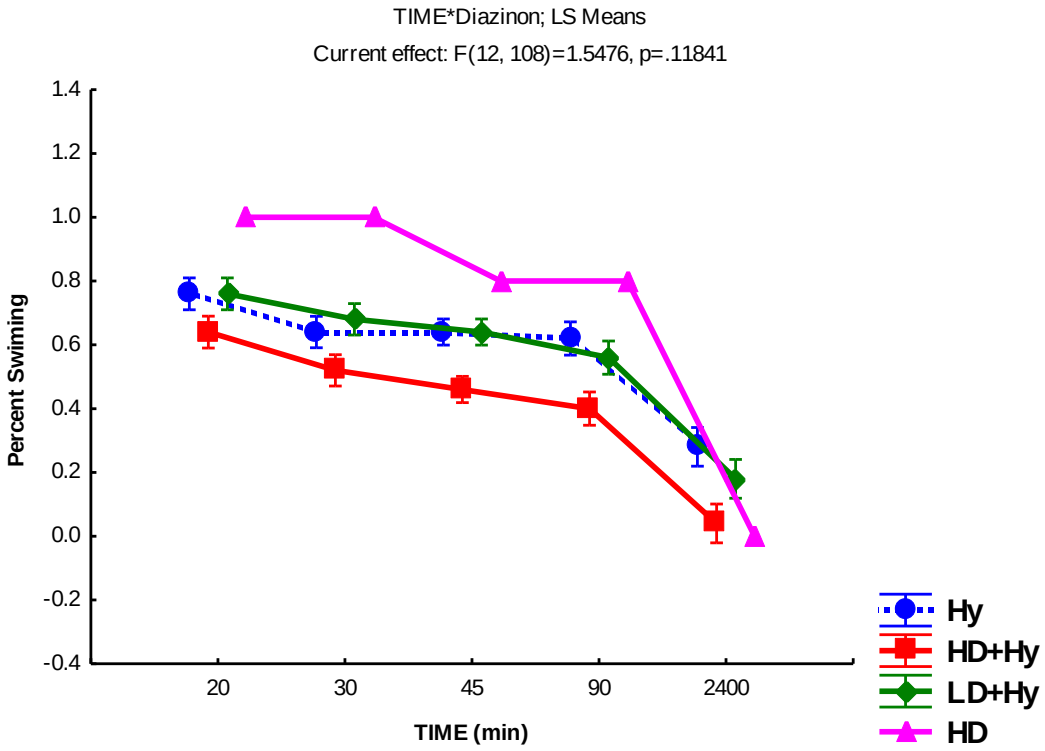


Figure 19: The percent of freely swimming hydra over 24 hours.

The mean proportional values at the last 24 hour time point using (LSD) , HD + Hy was significantly different from Hy ($P<0.005$), however HD + Hy was not significant from LD + Hy ($P\sim0.056$). LD + Hy was also not significantly different from Hy ($P> 0.10$) in both cases.

Since there is a significant treatment effect and not a significant interaction effect, the rate of loss of freely swimming *Daphnia* appears to be similar across treatments. This suggests that

there is a diazinon effect (HD+Hy vs. Hy or LD+Hy) that occurs at 20 minutes following exposure and this rate of loss does not increase over time.

The mean proportional value at the 20 minute time point after initial diazinon exposure to (0.25µm), showed that all the HD treated *Daphnia* were freely swimming (**Figure 19**). The mean value for proportion of freely seeing animals is reduced in all treatments that included *Hydra* (Hy) (**Figure 20**). The treatment that included high diazinon exposure plus *Hydra* (HD+Hy) had the lowest mean proportion of freely swimming *Daphnia*.

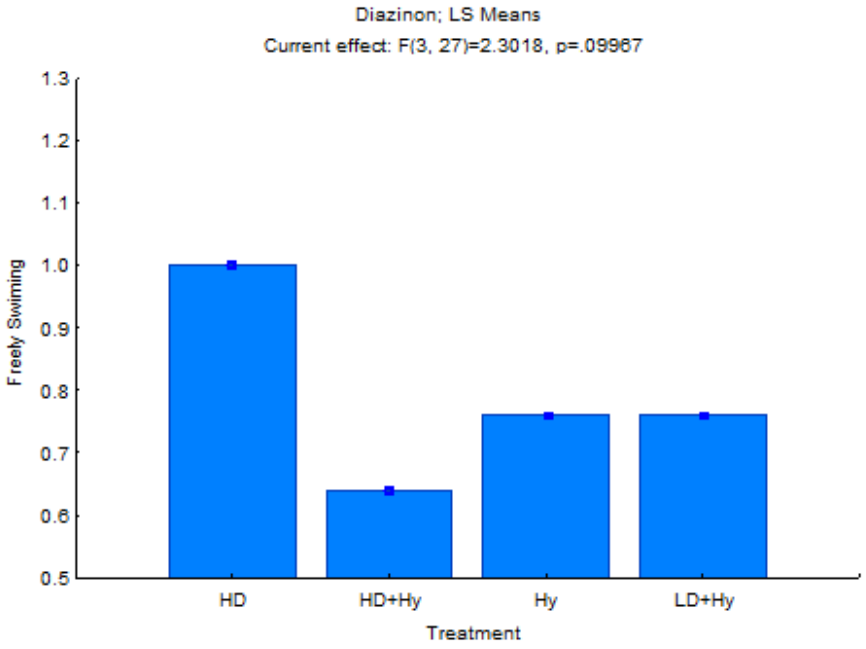


Figure 20: Analysis of freely swimming Daphnia at 20 minutes. Daphnia exposed to diazinon but not Hydra were all still alive (HD, proportion of freely swimming 1.0). The other treatment groups all contained Hydra and were significantly lower than HD.

Discussion

Previous acute toxicity studies (Chapters 3, 4) have shown that diazinon and other acetylcholinesterase inhibitors significantly alter swimming behavior at concentrations that are sub-lethal over a 90 minute observational period. Behavioral stimulation as measured by increasing cumulative swimming distance can be seen both at lower exposure concentrations (e.g., 0.125 µM), and early in the time-course for higher exposure concentrations (e.g. 0.25 µM). The diazinon-induced stimulatory effects on swimming behavior are most likely seen at lower concentrations and more transiently at higher concentrations because they are dependent on concentration gradient and the rate and extent of acetylcholinesterase inhibition (Kretschmann et al. 2011). As concentration is increased, and the percent inhibition of acetylcholinesterase crosses a threshold (Kretschmann et al. 2011), an inhibitory effect on behavior dominates, with immobility appearing within the 90 minute observational period as the final end-point of the response (e.g., to 2 µM).

In the present study, a short 20 minute exposure of *Daphnia* to 0.25 µM diazinon was used to perturb the swimming behavior of *Daphnia,* and to determine if diazinon exposure could alter the rate of predation by *Hydra*. The previous acute toxicity findings focused on a single species and the ability of diazinon, an insecticide commonly found in aquatic ecosystems, to alter behavior. A key question remains – does exposure to diazinon show the potential to alter species interactions? The focus of the present study on predator-prey interactions begins to address potential impacts on species interactions within a community. This is a first, small step, towards addressing the larger question about potential impacts of chemical contaminants like diazinon on

aquatic ecosystems. This community study is the first one to examine diazinon's effects on invertebrate predator-prey relationships

For the treatment groups that included *hydra* there was a significant treatment effect that was similar over time. The finding that the treatment x time interaction was not significant supports the idea that the rate of loss of freely swimming daphnia over time was similar for the three hydra treatment groups. A post hoc analysis of the three hydra treatment groups showed that the high diazinon exposure was significantly different from the other two *Hydra* treatment groups (Hy+LD, Hy). Since 0.25 µM diazinon effects on swimming behavior were previously demonstrated in chapter 4, one possible explanation for these results is that the relatively brief exposure of *Daphnia* to diazinon impaired *Daphnid* swimming, and this caused an increase in predation at the earliest time point measured during the observational period (20 min). Diazinon exposure appeared to cause an increase in the proportion of daphnia captured by *Hydra* at the 20 minute time point. After the 20 minute observational time point, the rate of loss of freely swimming *Daphnia* looks similar for all 3 treatments. One possible explanation for this finding is that the diazinon effects on behavior responsible for increased predation occur early on in the observational period. It is possible that a stimulatory effect on Daphnia was occurring during the first 20 minute observational period, and that this increased the rate of predator-prey interactions. Since the swimming behavior of *Daphnia* was not tracked within these community experiments, this possibility cannot be addressed in the present study.

When *Daphnia* were exposed to diazinon alone a large effect on *Daphnid* survival was not observed over the first 90 min of exposure (80% survival at 90 minutes). For all three treatment groups containing *Hydra,* the mean percent of freely swimming *Daphnia* was lower than the diazinon alone control group at 20 minutes after exposure conditions. All four treatment groups

appear to have a similar rate of loss of freely swimming *Daphnia* over time from 20 minutes to 90 minutes. However, after 24 hours of exposure to 0.25 µm diazinon all of the 10 animals were immobilized. This is consistent with the findings reported in chapter 4. It should be noted, however, a limitation of this study was the inability to include this control treatment group (HD) in the statistical analysis along with the three other treatment groups (Hy, Hy+HD, Hy+LD) due to sample size issues (see results).

Limitations & Future Work

This experiment demonstrates how changes in swimming behavior caused by sublethal exposure to chemicals can affect predator-prey interactions. Community studies are complex and involve many biotic and abiotic factors. Given the complexity of both predator-prey interactions and the toxicokinetic and toxicodynamic aspects of exposure to a contaminant like diazinon, future studies will need to focus on multiple concentrations, a lower range of concentrations, behavioral measurements made more frequently over time, and include environmentally relevant exposures that result in both predator and prey being exposed to the same contaminant concentration. In addition, systematic examination of environmental space as a variable (petri dish size), numbers of animals and ratios of prey to predators would give experimental control over the rate of predator-prey interactions. This may be particularly valuable when studying a predator like *Hydra*, which is sessile. These considerations would lead to a greater understanding of predator-prey dynamics and could lead to the development of models applicable to larger systems.

Conclusion

This study is the first to address the potential effects of the insecticide, diazinon, on invertebrate predator-prey relationships, and it suggests that diazinon may adversely affect the prey, *Daphnia pulex,* in a manner that makes it more susceptible to predation by an invertebrate

predator like *Hydra*. This is an important next step in trying to evaluate potential impact of contaminants at ecosystem level. Such ecotoxicological studies are essential for regulatory bodies, such as the EPA, to make realistic extrapolations about the consequences of emerging contaminants on interconnected community structure and the ecosystems upon which we depend (Daughton 2004; EPA 2003).

Chapter 6: Significance & Conclusions

Advancement in analytical techniques enable for limits of detection for many emerging contaminants, pharmaceuticals and personal care products (PPCPS)(Daughton 2001). Compounds that tend to be biologically active at low concentrations have caused heightened concerns, especially when they occur in water samples as part of a complex mixture. Ecological impacts of these chemicals are challenging to assess and concern about potentially affecting public health is increasing. PPCPs and other emerging contaminants have been reported in sediments, soils, surface water and groundwater(Lubliner 2010). These chemicals are introduced into the environment as parent compounds or metabolites and can be chemically modified into transformation products that may impair physiological processes in exposed organisms, alter reproductive, endocrine or immune system function, and ultimately affect fitness and survival (Daughton 2004).

With thousands of different chemicals produced annually, proper monitoring and evaluation of toxicity has not kept pace and this has profound ecological implications. Traditional toxicology testing methods focus on examining toxic effects of conventional pollutants, those that are regulated, and high volume industrial chemicals that compromise only a small portion of pollutants worldwide (Daughton 2001).

This study addresses the need to identify low level effects (sub-lethal), and the interactions between multiple chemicals(e.g., additive, synergistic and antagonistic interactions) (Daughton 2004; EPA 2003). A need for high-throughput screening assays that evaluate the toxicity of contaminants and complex mixtures has also been identified (e.g., National Toxicology Program (http://ntp.niehs.nih.gov).

The dissertation is unique in that it incorporates multiple disciplines including toxicology, pharmacology, environmental engineering and ecology. Such an approach is essential for addressing very complex systems such as ecosystems and the impact of emerging contaminants on ecosystem health (Daughton 2004; EPA 2003).

A novel optical method for toxicity testing was developed that has the potential of becoming a high throughput assay system by the detection of sub-lethal behavioral changes in aquatic organisms. *Daphnia*, a keystone species, with high sensitivity to environmental changes, proved to be ideally suited for this kind of assay. Their short life cycle, large brood size, asexual reproduction, and rapid reproduction rate, makes them especially easy and relatively inexpensive to culture and maintain in a laboratory environment.

In the first set of experiments (Chapter 3) the ability of the optical assay developed to detect acute sub-lethal behavioral effects within the 90 min observational period was demonstrated. Significant concentration-dependent alterations in swimming behavior were detected. Changes in both the cumulative swimming distance and the change in swimming angle support the first hypothesis posed, that concentration-dependent behavioral responses can be quantified by optically tracking changes in swimming behavior. Similar responses were observed between prototypical compounds and insecticides that shared the same mode of action. This evidence directly supports the second hypothesis evaluated, that compounds with similar mode of action can produce similar behavioral responses. Furthermore, these results support the basic underpinnings of the "Read-Across Hypothesis" where prior knowledge associated with the drug development phase could be used to predict potential environmental impacts of drugs based on their mode of action, concentration in target and non- target organisms, and conserved biology (Rand-Weaver et al. 2013). However, this hypothesis does not fully address the more complex

problem of the interactions between chemicals, with known or unknown biological actions, and their effects on biota.

There is increasing concern about the environmental impact of emerging contaminants exposure to complex of mixtures of chemicals. Chapter 4 addresses the concern that low levels of aquatic contaminants can interact in complex and unknown ways to elicit more toxic effects that may be greater than the reported toxicity of individual constituents. The utility of the assay demonstrated in chapter 3 was again used in chapter 4 to determine if there were interactions between chemicals to which daphnia were exposed (e.g., additive, synergistic or antagonistic effects).In particular, the interactions between chemicals classified as similar modes of action and chemicals not classified as having similar modes of action were examined. Chapter 4 validated the capability of the assay to look at interactions between different chemical classes. The utility of the assay in evaluating the interaction between compounds with similar modes of action was clearly demonstrated, supporting the first hypothesis that compounds with similar modes of action may cause additive, synergistic or antagonistic behavioral effects. The interaction between compounds with different or unknown modes of action was examined using the same assay. Insecticides with different modes of action were found to significantly interact in an additive or synergistic manner. Similarly, a detergent metabolite was also found to have additive or synergistic effects on behavior with an insecticide. These findings support the second hypothesis, that compounds that are in different classes, based on mode of action or structure, can interact in an additive, synergistic or antagonistic manner. When one insecticide was combined with the complex mixtures of substances that normally occur in wastewater (DWSD sample), the insecticide became more toxic, suggesting an additive or synergistic effect was occurring with some chemical(s) in the uncharacterized wastewater. This supports the final hypothesis in chapter 4, that the biological effects of these

interactions between chemicals can be observed in environmental systems at relevant concentrations. The behavioral responses of *daphnia* to combined stressors, showed the usefulness of the bioassay in detecting additive effects as well as the complexity and unpredictability of toxic effects resulting from combining various stressors.

Chapter 5 explores how results obtained using the behavioral assay can provide insight into ecosystem function. Chapter 4 demonstrated the behavioral effects of diazinon on swimming behavior in *daphnia*, and both stimulatory (e.g., increase swimming distance over time) and inhibitory influences (immobility) on this behavior were found. Similar contaminant exposure levels were examined in the community study to determine the susceptibility of *daphnia* to predation following exposure to diazinon. Results suggesting an increase in the proportion of *daphnia* captured after exposure to diazinon support the hypothesis that, acute sub-lethal exposure of *daphnia* to diazinon may cause an increase in susceptibility to predation. Although this study is suggestive of a possible effect of diazinon on predator-prey relationship, additional community oriented studies will need to be conducted in order to completely understand the influence of an insecticide, like diazinon, on predator-prey relationships.

Risk Assessment

This work can be expanded and scaled up to include chronic studies that look at very low concentrations over longer periods of time. Also, in addition to screening parent compounds special attention should be given to bioactive metabolites and transformation products. It is important to note that while one chemical might not appear to be inherently toxic at levels found in the environment, the many potential interactions with both biotic and abiotic stressors strongly suggest that we need to re-evaluate our current regulatory standards. For regulatory agencies to provide adequate safety and protection of ecosystem health we may need to consider doing more thorough environmental impact studies before introducing new chemical substances. The

assessment of the toxicity of chemicals, one substance at a time, is insufficient when many aquatic systems have multiple contaminants present. In general there is a need for policies that begin to reverse a common assumption that all chemicals are safe unless proven otherwise (ECOS 2010) .

There has been increased attention given to toxicokinetic-toxicodynamic models when dealing with ecotoxicological research and risk assessment (Kretschmann et al. 2011). Toxicokinetic models, which focus on the rate of biouptake and biotransformation within an organism, may be used to better assess and understand sublethal effects (Ashauer et al. 2011). When such toxicokinetic models are combined with toxicodynamic models, this may help establish patterns associated with certain chemical classes, and specific responses organisms, and therefore serve as valuable predictors (Ashauer et al. 2011). These kinds of models can aid in decision making and risk management (Rand-Weaver et al. 2013). It is important to realize that the data necessary to evaluate all chemicals in use cannot be obtained and ecological risk assessment on all species within an ecosystem is impractical. This points to the need for more recent attempts at predictive models such as the "Read-Across Hypothesis" that are going to be especially important for evaluating environmental impact (Rand-Weaver et al. 2013).

Predicting the fate of contaminants in the environment has long been dependent on the octanol-water partition coefficients (K_{OW}). $K_{OW(S)}$ have proven useful in acute toxicity studies and quantitate structure activity relationship (QSAR) models (Hermens et al. 2013). However, other methods need to be explored to deal with the complex chemical mixtures seen in aquatic ecosystems and complex interactions with biota. The optical tracking method described in these studies can be scaled up to be a true high through-put bioassay capable of quantifying sub-lethal effects rapidly and inexpensively. In addition, the assay has the potential to enhance our understanding of the toxic effects of chemical contaminants, as individual chemicals, combinations

of chemicals, and as complex mixtures. In particular, a more thorough analysis of how complex chemical mixtures interact with biota as a stressor will be essential for regulatory bodies, such as the EPA, to make realistic extrapolations about the consequences of emerging contaminants on interconnected community structure and the ecosystems upon which we depend (Daughton 2004; EPA 2003).

APPENDIX A: CHEMICAL INFORMATION

Chemical Name: **Imidacloprid**

Reference Material (RM)

1. General Information

Formula: C9H10C1N502
CAS-No.: [138261-41-3]
Usage: Insecticide
Molar Mass: 255.66 g/Mole
Recomm. Storage temp.: room temp

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	Complying
Assay (HPLC)	99.9 %
Melting Range	144.0-144.5 °C
Water (Karl Fischer)	0.15 %
Date of Analysis	08.May.2009

MW = 255.66 g/mol
0.2256 g/L or mg/ml 1MM

M = C x V
M = 0.2556 $\frac{mg}{ml}$ x 5 ml (Deionized water)
= 1.278 mg = 0.001278 g
10MM Stock = 0.01278g to weigh in 5 ml deionized

If we use 3 ml water
0.2556 x 3 ml = 0.76698 mg
0.00076698 g
10 MM stock = 0.0076g

Chemical Name: **Chlorpyrifos**

Reference Material (RM)

1. General Information

Formula: C9H11C13NO3PS
CAS-No.: [2921-88-2]
Usage: Insecticide
Molar Mass: 350.59 g/Mole
Recomm. Storage temp.: 2-8 °C

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	Complying
Assay (HPLC)	99.9 %
Melting Range	40.9-41.9 °C
Water (Karl Fischer)	0.06 %
Date of Analysis	04.Jun.2009

1MM:

MW $\frac{350.59 \text{ g/mol}}{1000 \text{ ml}}$ = 0.35059 mg/ml or g/L

Acetone: 5ml = V_1

M = C x V

= 0.35059 $\frac{\text{mg}}{\text{ml}}$ x 5 ml = $\frac{1.753 \text{ mg}}{1000}$ = 0.001753 g

10 MM stock = 0.0001753 x 10 = 0.01753 g

48 hour LC50= 1.7ug/l
Concentrations studied: 0.016, 0.032, 0.0625,0.125, 0.25µM

Chemical Name: **Diazinon**

Reference Material (RM)

1. General Information

Formula: C12H21N2O3PS
CAS-No.: [333-41-5]
Usage: Insecticide
Molar Mass: 304.35 g/Mole
Recomm. Storage temp.: 2-8 °C

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	Complying
Assay (GC)	98.5 area %
Refractive Index	1.4972
Date of Analysis	21.Mar.2012

MW = 304.35 g/mol
Density = 1.117 g/cm^3

1MM stock = 0.3044 g/L
= 0.3044 mg/ml

Acetone: 5 ml
Need = 0.3044 $\frac{\text{mg x 5 ml}}{\text{Ml}}$ = 1.52 mg
= 0.00152 g

Density = $\frac{\text{Mass}}{\text{Volume}}$

V= $\frac{0.00152\ \text{g}}{1.117\ \text{g}}$ x 1 ml = 0.00136 ml (vol of drug needed in 5 ml)

To create 10 mM solution = 0.00136 ml x 10
= 0.0136 ml
= 13.6 µl/ml
48 hour LC_{50} 0.522 ppb, 0.8ug/l

Concentrations studied: 0,0.125,0.25,0.5,1,2µM

Chemical Name: **4 - Nonylphenol**

Reference Material (RM)

1. General Information

Formula:
CAS-No.: 104-40-5
Usage:
Molar Mass: 220.35
Recomm. Storage temp.:

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)
Assay (GC)
Refractive Index
Date of Analysis

1 MM:

$$MW = \frac{220.35\ g/mol}{1000\ ml} = 0.22035\ mg/ml \text{ or } g/L$$

Acetone: 5 ml

$$M = C \times V$$
$$= 0.22035\ \frac{mg}{ml} \times 5\ ml = \frac{1.10175\ mg}{1000} = 0.0011\ g \times 10$$

10 mM Stock: = 0.011 g

LC_{50} pulex 0.14 mg/L (48 hrs)
LC_{50} magna 0.18 mg/L (24 hrs)

EC_{50} 104-190 µg/L

Concentrations studies: 0, 0.25, 0.5, 1, 2, 4µM

Chemical Name: **Nicotine Hydrogen tartrate salt**

Reference Material (RM)

1. General Information

Formula:
CAS-No.: 65-31-6
Usage:
Molar Mass: 462.41
Recomm. Storage temp.:

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

   Identity (NMR)
   Assay (GC)
   Refractive Index
   Date of Analysis

MW = 462.4 g/mol
0.4624 mg/ml or g/L

1mM Stock:

M = C x V
= 0.4624 mg/ml x 20
= 9.248 mg = 0.009 g

Concentrations for optical assay 0 µm, 1 µm, 4 µm, 16 µm, 64 µm, 256 µm

Chemical Name: **Physostigmine**

Reference Material (RM)

1. General Information

Formula:
CAS-No.: 64-47-1
Usage:
Molar Mass: 324.39
Recomm. Storage temp.: 2-8^{oc}

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (confidence level = 95%, n=6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

   Identity (NMR)
   Assay (GC)
   Refractive Index
   Date of Analysis

1 mM Stock = 0.324 g/L
MW = 324.4 g/mol

$$C = \frac{M}{V}$$

$$M = C \times V$$
$$= 0.324 \frac{mg}{ml} \times 20 \text{ ml combo}$$
$$= 6.48 \text{ mg} = 0.00648 \text{ g}$$

Concentrations for optical assay
0, 0.25, 0.5,1,2,4 µm

APPENDIX B: VISUAL OBSERVATIONS

	Variable	Normal Swimming (NS)	Fast Swimming (FS)	Spinning (SP)	Irregular swimming (IS)	Hardly Moving (HM)	Immobile
Code		1	2	3	4	5	6

	Observer	maya	Reema	Suleena	Candice	ramzi	selmir	Anu
Code		1	2	3	4	5	6	7

	Chemical	4-nonylphenol	Nicotine	Imidaclop	Diazinon	physostigmine	chlorpyrifos
code		6	1	2	3	4	5

NS: daphnia swimming horizontally around the entire well, constant movement
FS: Daphnia moving faster from point A to B , sometimes could be more of sporadic movement
SP: daphnia clearly making circles around itself
IS: Daphnia either bouncing or hitting the wall (on and off flickering movemet)
HM: Daphnia having a really hard time moving in the well , makes very tiny movements
IM: daphnia clearly immobile not swimming at all, although body parts could still be moving.

The behavioral effects were assessed using a scoring system 1-6 (specify types +/-). 1- Normal Swiming (daphnia appears to behave as the control, swiming horizontally across the entire well) sometimes includes swimming in a circle or arc in well, but not exhibiting spinning. 2-Fast swiming: faster swimming across the well seems more active continous movement (swimming at higher velocity than normal/control) . 3-Spinning: daphnia clearly making circles (rotating in a small area-circular diameter no more than 2 x the length of the animal) around itself minimum 3 rotations. 4-Irregular swiming: Daphnia either bouncing or hitting the wall of the well animal with forward progression 5-Hardly moving:
Daphnia appendages and heart may still be moving, do show some attempt at forward progression but intermittently immobile , makes very tiny movements with minimum forward progression. 6-Immobile: daphnia clearly immobile not swimming at all, although body parts could still be moving.

4-nonylphenol

Date	Observer	Chemical	Concentration(um)	Well #	Plate #	Time0	Time 10	Time20	Time30	Time40	Time50	Time60	Time70	Time80	Time90	Time 24hr	Time 48hr
5_8_13	4	6	0	A1	1	1	1	1	1	1	1	1	1	1	1	1	
5_8_13	4	6	0.0625	A2	1	1	1	1	1	1	1	1	1	1	1	5	
5_8_13	4	6	0.125	A3	1	1	1	1	1	1	1	1	1	1	1	5	
5_8_13	4	6	0.25	A4	1	1	3	1	1	3	1	2	1	1	1	6	
5_8_13	4	6	0.5	A5	1	1	1	1	1	1	1	1	1	1	1	6	
5_8_13	4	6	1	A6	1	1	1	2	1	1	5	5	1	5	5	5	
5_8_13	4	6	2	B1	1	1	1	5	5	5	5	5	5	5	5	6	
5_8_13	4	6	4	B2	1	4	5	5	5	5	6	6	6	6	6	6	
5_8_13	4	6	16	B3	1	5	5	5	5	6	6	6	6	6	6	6	
5_8_13	4	6	32	B4	1	5	5	6	5	6	6	6	6	6	6	6	
5_9_13	1	6	0	C1	1	1	1	1	1	1	1	1	1	1	1		
5_9_13	1	6	0.0625	C2	1	1	1	1	1	1	1	1	1	1	1		
5_9_13	1	6	0.125	C3	1	1	1	1	1	1	1	1	1	4	4		
5_9_13	1	6	0.25	C4	1	1	1	1	1	4	4	4	3	4	5		
5_9_13	1	6	0.5	C5	1	1	1	1	1	1	5	5	5	5	5		
5_9_13	1	6	1	C6	1	1	1	1	1	5	6	5	6	5	6		
5_9_13	1	6	2	D1	1	1	1	5	5	6	6	6	6	6	6		
5_9_13	1	6	4	D2	1	1	1	6	6	6	6	6	6	6	6		
5_9_13	1	6	16	D3	1	1	5	6	6	6	6	6	6	6	6		
5_9_13	1	6	32	D4	1	1	5	6	6	6	6	6	6	6	6		
5_13_13	3	6	0	A1	1	1	1	1	1	1	1	1	1	1	1		
5_13_13	3	6	32	A2	1	2	3	5	6	6	6	6	6	6	6		
5_13_13	3	6	16	A3	1	1	2	4	4	4	4	4	4	5	5		
5_13_13	3	6	4	A4	1	1	1	4	4	4	4	4	5	6	6		
5_13_13	3	6	2	A5	1	1	1	4	5	5	4	4	3	5	4		
5_13_13	3	6	1	A6	1	1	4	4	1	1	1	1	1	1	1		
5_13_13	3	6	0.5	B1	1	1	1	1	1	1	1	1	1	1	1		
5_13_13	3	6	0.25	B2	1	1	1	1	1	1	1	1	1	1	1		
5_13_13	3	6	0.125	B3	1	1	1	1	1	1	1	1	1	1	1		
5_13_13	3	6	0.0625	B4	1	1	1	1	1	1	1	1	5	5	5		
5_14_13	5	6	0	C1	1	1	1	1	1	1	1	1	1	1	1		
5_14_13	5	6	0.0625	C2	1	1	1	1	1	1	1	1	1	3	3		
5_14_13	5	6	0.125	C3	1	1	1	1	1	1	1	1	1	1	1		
5_14_13	5	6	0.25	C4	1	1	1	1	1	1	1	1	1	1	1		
5_14_13	5	6	0.5	C5	1	1	1	1	1	1	4	5	5	4	5		
5_14_13	5	6	1	C6	1	1	1	1	4	5	5	5	6	6	6		
5_14_13	5	6	2	D1	1	1	1	5	5	6	6	6	6	6	6		
5_14_13	5	6	4	D2	1	1	4	4	5	6	6	6	6	6	6		
5_14_13	5	6	16	D3	1	1	4	5	5	6	6	6	6	6	6		
5_14_13	5	6	32	D4	1	1	1	1	1	1	1	1	1	1	1		

Imidacloprid

Date	Observer	contaminant code	Concnetration	Well #	Plate #	Time0	Time 10	Time20	Time30	Time40	Time50	Time60	Time70	Time80	Time90	Time 24	Time 48h
2_4_13	7	2	0.5	A1	1	1	1	1	1	1	1	1	1	4	4	4	6
2_4_13	7	2	1	A2	1	1	1	1	1	1	1	1	1	3	1	3	5
2_4_13	7	2	2	A3	1	1	1	1	1	1	1	1	1	1	1	4	6
2_4_13	7	2	4	A4	1	1	1	1	1	1	1	1	1	1	4	3	5
2_4_13	7	2	8	A5	1	1	1	1	1	1	1	1	1	4	4	4	3
2_4_13	7	2	16	A6	1	1	1	1	1	1	1	4	4	4	3	4	5
2_4_13	7	2	32	B1	1	1	1	1	1	1	1	4	4	4	4	4	4
2_4_13	7	2	64	B2	1	1	1	1	1	1	1	4	4	4	4	4	3
2_4_13	7	2	128	B3	1	1	4	3	4	4	4	4	4	4	4	4	3
2_5_13	7	2		A1	1	1	1	1	1	1							2
2_5_13	7	2		A2	1	1	1	1	1	1							6
2_5_13	7	2		A3	1	1	1	1	3	3							4
2_5_13	7	2		A4	1	1	1	4	3	4							3
2_5_13	7	2		A5	1	1	1	4	4	4							6
2_5_13	7	2		A6	1	1	4	3	3	4							6

Chlorpyrifos

Date	Observer	contaminant code	Concnetration um	Well #	Plate #	Time0	Time 10	Time20	Time30	Time40	Time50	Time60	Time70	Time80	Time90	Time 24h
3_26_13	1	5	1	B3	1	1	1	1	1	1	1	1	1	1	1	1
3_26_13	1	5	4	B4	1	1	1	1	1	1	1	1	1	1	1	6
3_26_13	1	5	16	B5	1	1	1	1	1	1	1	1	1	1	1	6
3_26_13	1	5	64	C1	1	1	1	1	1	1	1	1	1	1	1	6
3_26_13	1	5	256	C2	1	1	1	1	4	1	4	4	4	1	3	6
3_26_13	1	5	1024	C3	1	1	1	3	6	6	6	6	6	6	6	6
3_26_13	1	5	4096	C4	1	1	3	6	6	6	6	6	6	6	6	6
3_26_13	1	5	0	C5	1	1	1	1	1	1	1	1	1	1	1	6
3_26_13	1	5	0	D6	1	1	1	1	1	1	1	1	1	1	1	6
3_27_13	1	5	0	A1	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	1	A2	1	1	1	1	1	1	1	1	1			6
3_27_13	1	5	4	A3	1	1	1	1	1	1	1	1	4			6
3_27_13	1	5	16	A4	1	1	1	1	1	1	1	1	4			6
3_27_13	1	5	64	A5	1	1	1	1	1	1	3	4	6			6
3_27_13	1	5	256	A6	1	1	1	3	6	6	6	6	6			6
3_27_13	1	5	1024	B1	1	1	6	6	6	6	6	6	6			6
3_27_13	1	5	4096	B2	1	1	6	6	6	6	6	6	6			6
3_27_13	1	5	0	B3	1	1	1	1	1	1	1	1	1			6
3_27_13	1	5	0	B4	1	1	1	1	1	1	1	1	1			5
3_27_13	1	5	0	B5	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	B6	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	C4	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	C5	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	C6	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	D4	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	D5	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	0	D6	1	1	1	1	1	1	1	1	1			1
3_27_13	1	5	1	A1	2	1	1	1	1	1	1					6
3_27_13	1	5	4	A2	2	1	1	1	1	1	1					6
3_27_13	1	5	16	A3	2	1	1	1	1	1	1					6
3_27_13	1	5	64	A4	2	3	3	3	6	6	6					6
3_27_13	1	5	256	A5	2	6	6	6	6	6	6					6
3_27_13	1	5	1024	A6	2	6	6	6	6	6	6					6
3_27_13	1	5	4096	B1	2	6	6	6	6	6	6					6
3_27_13	1	5	0	B2	2	1	1	1	1	1	1					6

Diazinon

Date	Observer	contaminant code	Concnetration	Well #	Plate #	Time0	Time 10	Time20	Time30	Time40	Time50	Time60	Time70	Time80	Time90	Time100	Time 24
2_25_13	1	3	0.0625	A1	1	1	1	1	1	1	1	1	1	4	4	4	5
2_25_13	1	3	0.125	A2	1	1	1	4	1	1	4	6	6	6	6	6	6
2_25_13	1	3	0.25	A3	1	1	4	4	4	4	4	4	3	4	4	4	6
2_25_13	1	3	0.5	A4	1	1	4	4	4	4	4	3	6	6	6	6	6
2_25_13	1	3	1	A5	1	1	4	4	6	6	6	6	6	6	6	6	6
2_25_13	1	3	0	A6	1	1	1	1	1	1	1	1	1	1	1	1	1
2_12_13	1	3	1	A1	1	1	1	1	1	1	1	1					1
2_12_13	1	3	4	A2	1	1	4	2	3	3	3	3					1
2_12_13	1	3	16	A3	1	1	1	4	1	1	4	4					6
2_12_13	1	3	64	A4	1	1	1	1	4	4	6	6					6
2_12_13	1	3	256	A5	1	1	5	5	4	6	6	6					6
2_12_13	1	3	1024	A6	1	1	1	1	6	6	6	6					6
2_12_13	1	3	4096	B1	1	1	1	6	6	6	6	6					6
2_12_13	1	3	0	B2	1	1	1	1	1	1	1	1					1

APPENDIX C: STATISTICAL OUTPUT

Nicotine

	Nicotine repeated Measures Analysis of variance				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	53142.63	1	53142.63	45.26878	0.000000
MaxAcc Dist-0	1210.22	1	1210.22	1.03091	0.318342
concnetration	29540.14	5	5908.03	5.03267	0.001933
Error	34044.13	29	1173.94		
TIME	4880.62	8	610.08	3.56214	0.000647
TIME*MaxAcc Dist-0	4890.70	8	611.34	3.56950	0.000634
TIME*concnetration	17693.73	40	442.34	2.58277	0.000005
Error	39733.97	232	171.27		

	Nicotine Repeated Measures Analysis of Variance				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	207028.1	1	207028.1	100.8542	0.000000
MeanAngle-0	8452.9	1	8452.9	4.1179	0.051700
concnetration	37296.0	5	7459.2	3.6338	0.011285
Error	59529.7	29	2052.7		
TIME	4811.9	8	601.5	1.1267	0.345965
TIME*MeanAngle-0	11243.0	8	1405.4	2.6326	0.008869
TIME*concnetration	33069.6	40	826.7	1.5487	0.025462
Error	123850.0	232	533.8		

Nicotine LSD test; Accumulated Distance Probabilities for Post Hoc Tests
Error: Between MSE = 1173.9, df = 29.000
Include condition: v5=1

Cell No.	concnetration	{1} 19.700	{2} 13.460	{3} 10.969	{4} 37.839	{5} 35.314	{6} 28.276
1	0		0.351828	0.195790	0.010135	0.024769	0.203620
2	1	0.351828		0.708261	0.000904	0.002474	0.032425
3	4	0.195790	0.708261		0.000326	0.000917	0.013690
4	16	0.010135	0.000904	0.000326		0.704627	0.157743
5	64	0.024769	0.002474	0.000917	0.704627		0.294632
6	256	0.203620	0.032425	0.013690	0.157743	0.294632	

Nictoine LSD test; variable DV_1 (combined data in combined 6_20)
Probabilities for Post Hoc Tests
Error: Between MSE = 2052.7, df = 29.000
Include condition: v5=1

Cell No.	concnetration	{1} 56.673	{2} 69.508	{3} 79.176	{4} 52.466	{5} 50.795	{6} 62.928
1	0		0.151779	0.015184	0.633096	0.505560	0.478898
2	1	0.151779		0.276649	0.060343	0.040357	0.456517
3	4	0.015184	0.276649		0.004693	0.002883	0.072555
4	16	0.633096	0.060343	0.004693		0.849333	0.239922
5	64	0.505560	0.040357	0.002883	0.849333		0.174660
6	256	0.478898	0.456517	0.072555	0.239922	0.174660	

Imidacloprid

Imidacloprid Repeated Measures Analysis of Variance

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	9615.73	1	9615.73	3.972556	0.055731
MaxAcc Dist-0	15178.10	1	15178.10	6.270547	0.018152
concnetration	19811.67	5	3962.33	1.636964	0.181732
Error	70195.63	29	2420.54		
TIME	717.35	8	89.67	1.163002	0.322501
TIME*MaxAcc Dist-0	1716.23	8	214.53	2.782436	0.005871
TIME*concnetration	2437.85	40	60.95	0.790471	0.811908
Error	17887.47	232	77.10		

Effect	SS	Degr. of Freedom	MS	F	p
Imidacloprid Repeated Measures Analysis of Variance					
Intercept	38231.7	1	38231.75	11.11994	0.002347
MeanAngle-0	16713.6	1	16713.62	4.86126	0.035552
concnetration	16485.5	5	3297.09	0.95898	0.458816
Error	99705.7	29	3438.13		
TIME	8522.0	8	1065.25	2.31354	0.020965
TIME*MeanAngle-0	9438.9	8	1179.86	2.56244	0.010740
TIME*concnetration	13485.3	40	337.13	0.73219	0.881092
Error	106822.6	232	460.44		

Imidacloprid LSD test; Accumulated Distance
Probabilities for Post Hoc Tests
Error: Between MSE = 2420.5, df = 29.000
Include condition: v5=2

Cell No.	concnetration	{1} 17.120	{2} 11.783	{3} 12.480	{4} 26.399	{5} 38.925	{6} 49.222
1	0		0.577363	0.627827	0.335170	0.028648	0.002030
2	4	0.577363		0.941832	0.133520	0.007650	0.000453
3	16	0.627827	0.941832		0.152319	0.009154	0.000553
4	64	0.335170	0.133520	0.152319		0.196200	0.022500
5	256	0.028648	0.007650	0.009154	0.196200		0.285764
6	1024	0.002030	0.000453	0.000553	0.022500	0.285764	

Imidacloprid LSD test; Angle
Probabilities for Post Hoc Tests
Error: Between MSE = 3438.1, df = 29.000
Include condition: v5=2

Cell No.	concnetration	{1} 55.254	{2} 62.496	{3} 62.162	{4} 55.147	{5} 46.396	{6} 52.865
1	0		0.526089	0.545232	0.992500	0.438822	0.833777
2	4	0.526089		0.976576	0.520038	0.164337	0.400384
3	16	0.545232	0.976576		0.539065	0.172980	0.416737
4	64	0.992500	0.520038	0.539065		0.444313	0.841113
5	256	0.438822	0.164337	0.172980	0.444313		0.570902
6	1024	0.833777	0.400384	0.416737	0.841113	0.570902	

APPENDIX D: COMMUNITY STUDY STATISTICS

Effect	Hydra + Daphnia community study results				
	SS	Degr. of Freedom	MS	F	p
Intercept	40.76827	1	40.76827	556.8308	0.000000
Diazinon	0.91093	2	0.45547	6.2210	0.005998
Error	1.97680	27	0.07321		
TIME	5.31573	4	1.32893	93.2464	0.000000
TIME*Diazinon	0.08907	8	0.01113	0.7812	0.620139
Error	1.53920	108	0.01425		

LSD tests for all points

Variable	Dh+H vs H Time 0-90				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	0.512000	1	0.512000	7.252886	0.012015
Error	1.906000	27	0.070593		

Variable	DH+H vs DL+H time 0-90				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	0.480500	1	0.480500	6.806663	0.014626
Error	1.906000	27	0.070593		

Variable	DH+H vs. DH time 0-90				
	Sum of Squares	Degr. of Freedom	Mean Square	F	p
M1	0.567364	1	0.567364	8.037155	0.008575
Error	1.906000	27	0.070593		

Cell No.	LSD test; Treatment	TIME	{1} .76000	{2} .64000	{3} .64000	{4} .62000	{5} .28000	{6} .64000	{7} .52000	{8} .46000	{9} .40000	{10} .04000
1	0	20		0.026630	0.026630	0.009996	0.000000	0.100597	0.001375	0.000086	0.000004	0.000000
2	0	30	0.026630		1.000000	0.708685	0.000000	1.000000	0.100597	0.014858	0.001375	0.000000
3	0	45	0.026630	1.000000		0.708685	0.000000	1.000000	0.100597	0.014858	0.001375	0.000000
4	0	90	0.009996	0.708685	0.708685		0.000000	0.782464	0.170032	0.029694	0.003189	0.000000
5	0	2400	0.000000	0.000000	0.000000	0.000000		0.000004	0.001375	0.014858	0.100597	0.001375
6	1	20	0.100597	1.000000	1.000000	0.782464	0.000004		0.026630	0.001038	0.000018	0.000000
7	1	30	0.001375	0.100597	0.100597	0.170032	0.001375	0.026630		0.263577	0.026630	0.000000
8	1	45	0.000086	0.014858	0.014858	0.029694	0.014858	0.001038	0.263577		0.263577	0.000000
9	1	90	0.000004	0.001375	0.001375	0.003189	0.100597	0.000018	0.026630	0.263577		0.000000
10	1	2400	0.000000	0.000000	0.000000	0.000000	0.001375	0.000000	0.000000	0.000000	0.000000	
11	2	20	1.000000	0.100597	0.100597	0.056207	0.000000	0.100597	0.001375	0.000086	0.000004	0.000000
12	2	30	0.271247	0.581091	0.581091	0.408450	0.000000	0.581091	0.029694	0.003189	0.000225	0.000000

Cell No.	LSD test;for the three treatments (excludinh HD) Var2	{1} .58800	{2} .41200	{3} .56400
1	Hy		0.003069	0.660946
2	HD+Hy	0.003069		0.009132
3	LD+Hy	0.660946	0.009132	

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ABSTRACT

EVALUATING CHEMICAL TOXICITY: A NOVEL BEHAVIORAL ASSAY USING *DAPHNIA*

by

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Degree: Doctor of Philosophy

Pharmaceuticals, and personal care products (PPCPs), and other emerging contaminants, such as pesticides, are increasingly being detected in the environment. Important sources of these contaminants are wastewater treatment plants and agriculture. Many of these contaminants are biologically active at low concentrations, and may impair physiological processes in exposed organisms, alter reproductive, endocrine or immune system function, and ultimately affect fitness and survival. These chemicals are often found in the environment as complex mixtures, and this complicates their evaluation of their toxicity. There is a need for high-throughput assays to rapidly assess the toxicity of these emerging contaminants. A behavioral assay utilizing freely swimming *Daphnia pulex* was developed to evaluate the sub-lethal chemical effects. *Daphnia*, a keystone species, are small planktonic invertebrate crustaceans (0.5-5.0mm) in freshwater ecosystems. They are commonly used for aquatic toxicity testing because of high sensitivity to changes in their environment. This novel optical bioassay was validated with a series of model compounds that have known modes of action. By measuring changes in their swimming activity, concentration-dependent behavioral responses in *Daphnia* were observed and quantified by the assay.

Compounds with similar modes of action were found to elicit similar behavioral responses. Mixtures of compounds were then evaluated using the optical assay to identify possible synergistic, additive or antagonist effects. Additive effects at environmentally relevant concentrations were observed between mixtures of contaminants with similar modes of action, from different classes, and in the presence of wastewater effluent. Finally, in order to address potential ecosystem impacts, alterations in predator-prey interactions caused by exposure to an insecticide were observed in a community study. A prototype of a high-throughput assay that has great utility for evaluating the biological effects of chemicals and chemical mixtures was developed. This assay has demonstrated that chemicals within wastewater may interact in complex ways to enhance toxicity, and may have important implications for regulatory agencies. The assay may also serve as a valuable ecotoxicological tool for studies aimed at assessing chemical contamination on ecosystem health.

AUTOBIOGRAPHICAL STATEMENT

As a native of Lebanon, I moved to Canada at the age of 16 to pursue higher education. After graduating from the University of Windsor with a bachelor's degree in Biology, I decided to follow my passion in Environmental Sciences and toxicology. I researched the best programs within the region and found that the program at Wayne State University perfectly suited my goals. The Occupational and Environmental Health Science program at Wayne State University was especially interesting as it not only covered environmental health and safety but also the field of toxicology. Upon joining the program, I became deeply involved in the student chapter and eventually applied for the graduate student assistant position. During my studies I interned at BASF, The Chemical Company, as an associate toxicologist and gained some valuable experience. The remarkable faculty I was working with, encouraged me to move on and apply for a doctorate program. I found a perfect home in Civil and Environmental Engineering, a field that has endless possibilities. I knew that I wanted to work on a project that encompassed much of my backgrounds in biology, toxicology, and environmental engineering. As a result my research turned out to be quite unique and multidisciplinary. My research focused on a multidisciplinary approach that would enhance our understanding of the toxic effects of chemical contaminants, as individual chemicals, and as complex mixtures. Such interdisciplinary approach is essential for addressing very complex systems such as ecosystems and the impact of emerging contaminants on ecosystem health. As I reflect on my educational accomplishments, I am excited to graduate and pursue my career in toxicology and environmental sciences. My experiences at Wayne State University have greatly altered my life, and for that, I am most grateful