Understanding Groundwater-Surface Water Interactions (GSI) for Assessing Ecological Risk and Establishing Stressor Causality Linkages

by

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A Practicum submitted in partial fulfillment of the requirements for the degree of Masters of Ecosystem Science and Management (School for Environment and Sustainability) University of Michigan 2022

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Acknowledgments

Foremost, I would like to express gratitude to my advisor Professor G. Allen Burton for his continual support of my M.S. study and research. I am very grateful for his patience, content manner, guidance, and immense knowledge. Without the help of my advisor, lab mates, and fellow lab neighbors, this practicum would not have been made possible. I received tremendous help in purchasing the materials needed, data collecting, and sampling from Alison Rentschler and Elizabeth Nichols. Evelyn Faust and Anna McGlashen were very helpful in culturing species needed for the experiments. I received assistance in sampling from many people, namely from Alison Rentschler, Evelyn Faust, Sean Clark, and Elizabeth Nichols. I also want to acknowledge Rima Upchurch and Professor R. Donald Zak for their kindness in allowing access to utilize the Terrestrial Ecosystem Ecology Lab. I sincerely appreciate all those who assisted me in completing my practicum, especially under the circumstances and obstacles of the ongoing Covid-19 pandemic.
Abstract

Over half of the United States freshwater streams are impaired, many with a plethora of chemical contaminants. Since contaminants vary broadly in the toxicity and ecological risk, it is important to understand which site contaminants pose the biggest threat to the ecosystem. The in situ Toxicity Identification Evaluation (iTIE) technology was developed several years ago and continues to be refined and improved. Its purpose is to discern which chemical classes of common contaminants are causing the most toxicity at the site. This then allows site managers to target their restoration and remediation activities to remove the problem chemicals, such as ammonia, metals, non-polar organics or pesticides. This study further advanced the iTIE technology by: 1) characterizing the propensity of surface waters to infiltrate down into sediments during porewater iTIE testing; 2) determine whether sediment-water interface impervious discs reducing the downward penetration of surface waters, 3) improve the filtering of solids from porewaters during in situ sampling; and 4) determine if the acetyl-cholinesterase (AChE) assay can be used in the iTIE test on Hyalella azteca and Daphnia magna only exposed for 24 hrs – as an indicator of the presence of organophosphate insecticides. Results showed the surface waters will migrate downward and mix with the porewater during sampling. Sediments with less porosity slow the infiltration of the surface water. Rubber discs mounted to the iTIE chamber will also reduce the downwater flow. Small mesh (~250 micron) wrapped around the porewater sampler also reduces the transport of solids into the iTIE chamber. The AChE assay was successfully conducted on both test organisms, demonstrating it can be used in iTIE testing to separate out exposures and toxicity from organophosphate insecticides. The above findings advance the development of the iTIE and therefore our ability to more effectively restore and remediate chemically contaminated freshwater and marine sites.
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Acronyms

**AChE**: Acetylcholinesterase  
**BSA**: Bicinchoninic Acid Assay  
**CoC**: Contaminants of Concern  
**CSM**: Conceptual Site Model  
**DO**: Dissolved Oxygen  
**ERA**: Ecological risk assessment  
**GSI**: Groundwater Surface Water Interaction  
**HZ**: Hyporheic Zone  
**iTIE**: in situ Toxicity Identification Evaluation  
**PAH**: Polycyclic aromatic hydrocarbons  
**PCB**: Polychlorinated biphenyls  
**PFAS**: Per- and Polyfluoroalkyl Substances  
**TIE**: Toxicity Identification Evaluation  
**USEPA**: United States Environmental Protection Agency
Background

*Role of groundwater and surface water interaction (GSI)*

The hyporheic zone (HZ) is the volume of subsurface sediments where groundwaters and surface waters are actively exchanged (Schlesinger et al. 2020). This exchange is a critical part of the hydrologic cycle in water systems. Surface water supplies recharge to the underlying aquifer, where the groundwater can remain in storage for elongated periods of time (days, months, years, centuries, etc.), before dispersing back into the stream (Winter et al. 1998, Schlesinger et al. 2020). Nearly all surface-water features (i.e., streams, lakes, rivers, reservoirs, wetlands, and estuaries) interact with groundwater (Winter et al. 1998, U.S. EPA 2008). These interactions are referred to as upwelling and downwelling. Surface-water bodies gain water and solutes from groundwater systems (upwelling) and in other interactions the surface-water body is a source of groundwater recharge (downwelling).

The hyporheic zone is an important ecological component that enables community resilience to hydrological disturbance events (i.e., flooding, low flow, and drought) (Wood et al. 2010, Stubbington 2012). Various aquatic organisms at different trophic levels find habitat and refuge in the hyporheic zone including microbes, macroinvertebrates, and fishes. Organisms such as amphipods and isopods permanently reside within the shallow sediments and feed on the periphyton that grow within the HZ. Other benthic macroinvertebrates, such as *Trichoptera* (Caddisflies), use the hyporheic zone only for a portion of their life (Boulton et al. 2010). Fish rely on the stable temperature and dissolved oxygen conditions in the hyporheic zone for embryo development (Malcolm et al. 2005) and small fish like minnows find safety in the hyporheic zone from larger fish. These smaller fish also feed on the periphyton that grow there.
Groundwater surface water interactions in the hyporheic zone can influence the
distribution of organisms in the waterways (Boulton et al. 1998) and can alter hydrological
processes, water quality, and the health of ecosystems (Hendricks et al. 1993). For example,
nutrient cycling and dissolved oxygen (DO) in the hyporheic zone can modify habitat suitability
for organisms (Hendricks et al. 1993). Upwelling zones downstream can supply nutrients to the
water body, while downwelling zones upstream provide DO and organic matter to periphyton
and macroinvertebrates (Boulton et al. 1998). Upwelling usually results in elevated growth of
phytoplankton, which can lead to dissolved oxygen levels and other complications within the
water body. Downwelling causes changes in ground-water quality, and it typically occurs in
regions that have low biological productivity (Miracosta Oceanography 101 2021). Downwelling
zones upstream are important for periphyton because their growth can be stimulated by
deposition of organic matter in fine sediments (Navel et al. 2011). Periphyton growth and
availability directly affects the organisms that consume it.

GSI role in contaminant fate and effects

Upwelling and downwelling within the hyporheic zone can produce many routes of
contaminant exposures from multiple sources (Winter et al. 1998) and ultimately influence how
stressors from surface waters and sediments affect aquatic biota (Brunke and Gonser 1997). The
physical & chemical characteristics of contaminants, the geometry & temporal variations in the
HZ, and reactions that occur in the exchange of groundwater surface water interactions influence
the transport, fate, and effect of contaminated groundwater as it travels though the subsurface
prior to discharging to a surface-water body (U.S. EPA 2008). Temperature is one stressor that
can affect certain aspects of water quality. For instance, higher temperatures can reduce levels of
dissolved oxygen in a water body, which can negatively affect the growth and productivity of
aquatic life. Warmer temperatures in streams can accelerate chemical reactions and release excess nutrients into the stream as well (Duan et al. 2013). A stream’s water temperature can also influence the circulation of water flows.

Streams with groundwater inputs are more resilient to elevated water temperatures caused by warmer air, likewise streams having an influx of groundwater baseflows can tolerate drought periods (Hare et al. 2021). Groundwater discharge attributes to the generation of streamflow. Groundwater surface water interactions can provide stable hydrologic and temperature conditions which facilitate in the survival of aquatic organisms (Hare et al. 2021). However, upwelling in areas with contaminated soils can release highly toxic pollutants into the water column increasing bioavailability and exposure of pollutants to organisms that utilize and live within the hyporheic zone. Downwelling zones have greater dissolved oxygen than upwelling zones (Miracosta Oceanography 101 2021) which can counteract hyporheic exposures to stressors. Yet, biota in downwelling zones may be more at risk for impacts of metal toxicity, sedimentation, and surface water contaminants (Vadher et al. 2015), as oxidized environments can increase bioavailable forms of metals in sediments (Calmano et. al 1993).

Hyporheic flows influence sediment chemistry and the exposure of metals to aquatic biota. Many contaminants, chiefly heavy metal ions, have increased in aquatic systems due to natural and anthropogenic activities. Heavy metals are stored in three reservoirs upon entry in water systems: sediment, water, and biota, with sediment having the highest storage capacity (Hossain et al. 2021). Over 99% of the heavy metal load in aquatic systems are stored in the sediments (Hossain et al. 2021).

Many natural and anthropogenic heavy metals such as arsenic, copper, mercury, lead, cadmium, and zinc accumulate in sediments (Burton 2010). Metal bioavailability towards
aquatic biota is facilitated through hyporheic flows as upwelling and downwelling occur within
the HZ. Aquatic organisms gain exposure to heavy metals and other contaminants through direct
contact (dermal), ingestion and absorption of contaminated water and sediments, and
consumption of contaminated foods. In a short timespan (24 hours of exposure) at a high
concentration in a single exposure, organisms can exhibit adverse effects within 14 hours
(IUPAC 2006). This is known as acute toxicity. Long term exposures to heavy metals can lead to
chronic toxicity within aquatic organisms. Chronic toxicity can manifest as direct lethality/death
and more commonly as sublethal endpoints: decreased growth, reduced reproduction, and/or
behavioral changes (Randy 1995). Results of aquatic chronic toxicity tests can be used to
determine water quality guidelines and regulations for protection of aquatic organisms (Newman
2010). Acute toxicity testing can be used to evaluate potential hazards of an aquatic ecosystem
(ICCVAM 2018).

*Ecological risk assessments*

Ecological risk assessments (ERAs) are composed of an 8-step framework (see Text Box 1) and are conducted under the guidelines of the United States Environmental Protection Agency
(USEPA). An ecological risk assessment is the process for evaluating the impacts an
environment may have due to the exposure of one or more environmental stressor (U.S. EPA
2008). There are three phases of an ERA that are used to predict future effects and evaluate the
likelihood of past and current contaminant exposures within an ecosystem (U.S. EPA 2008):
problem formation, analysis, and risk characterization. These phases occur after planning.
During planning a risk management team consisting of risk manager(s), ecological risk assessors,
and other stakeholders is designed. Options, tasks, and goals are identified, and the natural
resources of concern are determined.
Problem formulation establishes the goals and focus of the risk assessment based on the planning results (i.e., contaminants of concern, endpoints to be evaluated, type of data to be used to assess risks to those endpoints). Conceptual site modeling occurs in problem formation, wherein, the toxicological relationships and exposure pathways between the contaminants and the assessment endpoints (i.e., contaminant migration pathways, chemical alterations, and organism life histories) are characterized. Problem formulation concludes with an analysis plan.

The analysis phase is divided into (1) exposure assessments and (2) effects assessments. During exposure assessments, species of concern, likelihood of stressor(s) exposure, and the degree of exposure is determined. Relationships between exposure level and possible adverse effects on biota is reviewed during effects assessments. Risk characterization includes two major components: risk estimation and risk description. Risk estimation compares the measured exposure level for each stressor and plant or animal population, community, or ecosystem of concern and the data on expected effects for that group for the exposure level. Risk description provides vital information for interpreting results.

*Limitations to ERAs when GSI is ignored*

Groundwater and surface water has been viewed as separate compartments of an aquatic ecosystem, causing an overlook of the ecosystem within the transition zone, the roles the zone has on the local ecosystem (i.e., Gibert et al. 1994), and the highly variable flow conditions in groundwater and surface interactions (U.S. EPA 2008, Schlesinger et al. 2020). Currently and in the past, ERAs have excluded the HZ in the design and conduct of the assessments (U.S. EPA 2008). Instead, emphasis is often on the associated biota of the sediment-water interface and surface water column regarding adverse ecological impacts (U.S. EPA 2008). The biota and ecological processes associated with the HZ are usually not accounted for during problem
formulation and CSM development. The ecological importance of the transition zone, the relationships and interactions among ground-water flow, surface-water hydrology, sediment dynamics, and the transition zone biota are usually omitted (U.S. EPA 2008). As a result, potential impacts of an environment can lead to unresolved problems and even more complications within an environment.

**GSI use in ecological risk assessments**

The existing and potential ecological effects of contaminated groundwater in the transition zone can be important considerations in site characterization and ecological risk assessment. In the design and conduct of an ERA that includes transition zones (HZ) and areas of groundwater discharge, it is especially important that the risk assessment team is interdisciplinary and inclusive of hydrogeologist/hydrologists (U.S. EPA 1997, U.S. EPA 2008) to effectively characterize the physical, hydrologic, and geohydrologic aspects of the site. This interdisciplinary focus is most effective when initiated during problem formulation (U.S. EPA 2008), being that the spatial and temporal variability in ecological systems can be different from the hydrogeological system. Hydrologists/hydrogeologists need to understand the local ecosystem, habitats, exposure pathways/linkages, and the ecological endpoints needed to be protected from contaminants of concern (U.S. EPA 2008). Likewise, ecological risk assessors need to understand the spatial and temporal variability in the transition zone locations and the potential mechanisms for transport of contaminants by GSI (U.S. EPA 2008).

During problem formation and the design of a conceptual site model the team should address the hydrologic regime of the site and its context in the watershed, occurrences of ecological exposures, organisms of concerns and ecosystem functions in the HZ, processes that are affected by contaminants during transport (e.g., abiotic transformations, biodegradation,
dispersion, diffusion, adsorption, dissolution, volatilization), the scope for the project, and additional data that may be needed to support the risk assessment (U.S. EPA 2008).

The EPA designed a 5-step framework to incorporate GSI and the transition zone into problem formation. The activities of the 5-step framework is as follows: (1) review available site-related chemistry data to identify known or potential contamination; (2) identify the hydrogeological regime and potential fate and transport mechanisms for ground-water contaminants; (3) identify ecological resources at areas of ground-water discharge, including associated transition zones; (4) identify ecological endpoints and surrogate receptors; and (5) develop a CSM and associated risk hypotheses (U.S. EPA 2008). In step 1, team members determine if there is a potential for groundwater contamination, and, if so, is surface water contamination related to groundwater contamination. Specifically, the team will focus on the
question: Is there known or potential groundwater contamination and/or sediment or surface-water contamination related to groundwater, and by what contaminants?

In step 2, areas of contaminated groundwater discharge (and associated transition zones) and the spatial and temporal variability in the magnitude and location of groundwater discharge are identified. Potential exposure points for ecological receptors are identified by chemical and hydrological data, direct investigations, and physical features. In step 3, risk assessors evaluate the conditions of the site and in the overlying surface water to identify the types of ecological resources that occur. In step 4, one or more measurement endpoints will be selected to evaluate each assessment endpoint. In step 5, information collected from previous steps will be used to create a CSM that identifies the relationships among the contaminant source, the environmental fate and transport of the contaminants in the groundwater, and the assessment endpoints that may be exposed to the contaminants (U.S. EPA 2008). The CSM should also identify the potential effects that the assessment endpoints may incur from the exposure.

Challenges of establish stressor to receptor causality

Establishing causal relationships between environmental stressors and observed effects in natural systems is difficult due to the many environmental factors. During the analysis phase, data is evaluated and characterized to determine how exposure to stressors is likely to occur. The potential and type of ecological effect of each stressor is characterize. Exposure levels are measured and effects for each stressor/receptor combination are quantified. According to U.S. EPA’s Guidelines for Ecological Risk Assessment (1998), exposure is analyzed by examining the (1) sources of stressors, (2) the distribution of stressors in the environment, (3) the extent of co-occurrence or contact with the stressors, and (4) stressor-response relationships.
Chapter 1: Approach Overview

Background

The in-situ Toxicity Identification Evaluation System (iTIE) identifies chemical classes that cause toxicity in surface water, pore water, and outfalls by separating chemical classes of contaminants of concern (CoCs) (Burton et al. 2004). Prototype 3 is the most recent prototype of the iTIE technology. The iTIES prototype 3 is a deployable system that allows for consistent and sensitive adjustments to pumping rates of waters through resin treatments that can separate potential toxicants such as: ammonia, heavy metals (i.e., Ag, Cd, Cu, Ni, Pb, Zn), and organic compounds including PCBs, PAHs, and PFAS (Burton et al. 2004). The iTIES deployment process has 5 steps: (1) identify the possible contaminants and pathways of concern in select appropriate absorbents, organisms, and endpoints; (2) preload iTIE in the laboratory or field; (3) deploy at site for 24 - 48 hours; (4) retrieve and assess endpoint responses; and (5) process water and/or resins for chemical analyses. Through the development process of the iTIES, 5 groups of chemicals have been successfully separated and compounds responsible for adverse biological impacts were confirmed (Burton et al. 2004).

The iTIE Prototype 3 improves certainty in the decision-making process regarding risk components (i.e., causality, bioavailability, source identification, and fate across ecosystem compartments) (Burton et al. 2004). The iTIE is also less expensive than the traditional Toxicity Identification Evaluation (TIE), which is a lab approach used by the USEPA. This technology can be a key component in evidence-based decision-making regarding sources of toxicity and impairment in receiving waters, but challenges remain before it can be a practical tool of use.

The iTIE exposure chamber is powered by a portable air pump that suctions pore water, via a Venturi system. The ambient water (surface or porewater) or effluent passes through the
selective sorption material into the exposure chamber containing the test organism(s). Selective absorption material and test organisms are absent in this laboratory study, as the focus was on other aspects of the iTIE.

Objective

The overall objective of my research was to advance the *in-situ* Toxicity Identification Evaluation technology and make for a more accurate field system by using a technical approach. My focus was to refine the porewater sampling option (e.g., verify sediment porewater sampling zone vs. surface water infiltration and gentle aeration of toxicity chamber porewater), through two primary goals: (1) prevent surface water infiltration from entering the iTIE and (2) develop methods that reduce oxygen bubbles yet provide a suitable level of DO to prevent stress in organisms.

Materials and Methods

The iTIE was tested to evaluate three iTIE design elements on porewater sampling: sediment grain-size influence, sediment surface barrier discs, and porewater filter sizes. Adjustments were made during testing to optimize the design. In each test, sediments were placed in beakers and homogenized. The laboratory studies were performed using water and sediments spiked with green tracer dye. Dye, at different concentrations, was used as an indicator to track water flow. Samples were measured at a wavelength of 406 nm on a spectrometer to analyze absorbances of dye. The figures (1-6) show the laboratory tests performed to identify the effects of sediment grain size, sediment disc barriers, and mesh filtration to prevent surface water and sediment from entering the iTIE chamber.
The green tracer dye was tested at 5 concentrations: (1) 2% (5 mLs of dye in 245 mLs miliQ water), (2) 0.2% (0.5 mLs of dye in 249.5 mLs miliQ water), (3) 0.02% (0.05 mLs of dye in 249.95 mLs miliQ water), (4) 0.002% (0.005 mLs of dye in 249.995 mLs miliQ water), and (5) 0.0002% (0.0005 mLs of dye in 249.9995 mLs miliQ water). Dye concentrations were developed in separate beakers before being added to the overline water of respected beakers. Sediments were saturated and homogenized in a plastic bucket and then added to beakers. Each beaker was filled with 900 mLs of saturated sediment, so that the iTIE opening was 2 inches away from the overline water. iTIEs were filled with milliQ water and inserted into the sediments. Afterwards, air pockets were filled by compacting the sediments and excess water was purged from each beaker. 150 mLs of clean milliQ water was added to the surface of the sediments as overline water then 5 mLs of green tracer dye at respected concentration were pipetted to overline water after 1 hour test run of the iTIE. iTIE was run at a pumping speed of 25 ml/hr for 6 hours.

Sediment discs were affixed to resin chambers using thread tape and covered with 1-2mm of sediment for stability to aid in the reduction of overline water and sediments from iTIE chamber. Sediment discs sizes were tested at 1.5 mm and 2.0 mm. Fine and coarse mesh types were tested for filtration and turbidity reduction. Samples were done in duplicate and collected every 30 minutes for absorbance measurement.
Figure 1. Green tracer dye at concentrations of 2% (5 mls of dye in 245 mls miliQ water, 0.2% (0.5 mls of dye in 249.5 mls miliQ water), and 0.02% (0.05 mls of dye in 249.95 mls miliQ water) being tested with duplicate in loamy soil type.

Figure 2. Porewater absorbances being tested in duplicate with iTIE 900 mLs (2 inches) from overline water in sand soil type.
Figure 3. Green tracer dye concentrations at 0.002\% (0.005 mls of dye in 249.995 mls miliQ water), and 0.0002\% (0.0005 mls of dye in 249.9995 mls miliQ water) tested with duplicate in sand soil type.
Figure 4. Porewater absorbance testing at 900 mls (2 inches) in loamy soil type.

Figure 5. Sediment discs made from rubber in two sizes at 1.5 mm (left) and 2 mm (right).
Figure 6. Display of iTIE with sediment discs, A (1.5 mm width) & B (2 mm width), being tested against control (no rubber disc) in loamy soil type.

Figure 7. Display of mesh types inside iTIE porewater adapter: fine mesh (left), coarse mesh (right).
Results

Three design variables were evaluated for optimizing porewater sampling, including the role of sediment type, surface sediment barriers, and mesh filters on the porewater sampler. Sediment type affected porewater absorbance and sediment-surface water infiltration into the iTIE. Sediment barriers create a blockade betwixt the sediment and porewater to aid in porewater absorbance. Mesh filters were used to filter surface water and sediment from entering into the iTIE. These results are highlighted as follows:

Sediment type: The rate of surface water entering the iTIE chamber was reduced in sandy soil with fine mesh. This observation was made in comparing iTIE absorbance and turbidity between loamy and sandy sediments.

Sediment surface barrier: The thicker rubber disc (B) was most effective at reducing surface water infiltration than the thinner (A) (Figure 10).

Mesh size: Fine mesh was most compatible with each soil type and most effective in reducing sediment and dye infiltration into the iTIE chamber. Turbidity was common when using course mesh. This result was determined by visual observations. (Figure 7)
Figure 8. Rate of absorbance of tracer dye into the iTIE chamber at a measurement of 900 ml in sand from the dyed overline water with dye concentration of 2% dye (5 mls of dye in 245 mls milliQ water). This test was to observe how well porewater absorbed through the iTIE 2 inches from the overline water.
Figure 9. Absorbance of tracer dye at the following concentrations:

- Control = 2% dye: 5 mls of dye in 245 mls mQ water
- 10-fold = 0.2% dye: 0.5 mls of dye in 249.5 mls mQ water
- 100-fold = 0.02% dye: 0.05 mls of dye in 249.95 mls mQ water

This test was run to observe the osmosis of the upper sediment transport of tracer dye at 10-fold and 100-fold compared to the control. As the concentration of tracer dye increased, the measure of absorbance increased.
Figure 10. Absorbanes of tracer dye at the following concentrations:

- Treatment = 2% dye: 5 mls of dye in 245 mls mQ water
- 1000-fold = 0.002% dye: 0.005 mls of dye in 249.995 mls mQ water
- 10000-fold = 0.0002% dye: 0.0005 mls of dye in 249.9995 mls mQ water

This test was run to observe the osmosis of the upper sediment transport of tracer dye at 1000-fold and 10000-fold compared to the control. As the concentration of tracer dye increased, the measure of absorbance increased. 1000-fold and 10000-fold treatments had relatively the same absorbance measurements. The treatment group absorbances was significantly higher than 1000-fold and 10000-fold groups.
Figure 11. Average absorbance of tracer dye with 2 types of rubber serving as blockade between the overline water and the sand below to prevent surface water from entering iTIE opening. The control group had no rubber. Both rubber discs: rubber A (1.5 mm) rubber B (2 mm rubber) reduced the flow of overline water from the sediment. Compared to the control, the flow of the overline water was reduced significantly. Rubber B was the most effective.

**Discussion**

This study provided results to better define whether overlying waters may be sampled when porewater sampling is the objective. If overlying waters are sampled, then the porewater concentrations will be diluted and, therefore, misleading in characterization of sediment contamination, toxicity, and causal CoCs. Improving this design aspect of the iTIES addresses exposure and effect uncertainties and prediction of site risk. Surface water infiltration into the porewater collection was affected by sediment grain size. Smaller grain sized sediments (e.g., silts and clays) reduce sediment porosity and potential for surface water infiltration. Surface water infiltration is also reduced by have an impermeable disc barrier that rests on the sediment surface, above the porewater sampler. Finally, using smaller mesh sizes on the iTIE porewater
intake attachment, reduces sediment particle infiltration into the iTIE unit. It is evident that additional design improvements are warranted to improve the usefulness of the iTIE technology. The current study made progress at improving the iTIE design.

Chapter 2: Acetylcholinesterase Analysis

Introduction

Acetylcholinesterase (AChE) is a key enzyme in the nervous system of animals. Its primary function is to catalyze the breakdown of acetylcholine and other choline esters that function as neurotransmitters. AChE terminates impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine. Organophosphate (OP) and carbamate esters can inhibit acetylcholinesterase (AChE) activity by binding to a serine residue in the enzyme active site (Bartlett et al. 2016). Hyalella azteca and Daphnia magna are commonly used organisms for sediment and surface water toxicity testing and will be used in iTIE site assessments. Bartlett et al. (2016) demonstrated the usefulness of using AChE testing of H. azteca to identify organophosphate pesticides in a watershed. The objective of this study was to evaluate whether or not this assay can be a useful chronic toxicity assay with H. azteca and D. magna as part of the iTIE technology. The following results only include the baseline for acetylcholinesterase analysis and protein determination.
Materials and Methods

Chemicals

Sulfuric acid, 2,3-dichlorophenoxyacetic acid, d14-trifluralin, dichloromethane, and pentafluorobenzyl bromide were purchased from Fisher Scientific. All chemicals for AChE and protein analyses were obtained from Sigma-Aldrich. Tris buffer (0.05 M) was prepared and adjusted to pH 8. Homogenizing buffer was prepared by adding 1% v/v Triton X-100 to Tris buffer, and 5,50-dithiobis-[2-nitrobenzoic acid] chromogen-buffer reagent (0.25 mM) was prepared in Tris buffer and adjusted to pH 7.4. Acetylthiocholine iodide substrate (0.156 M) was prepared daily. A quality control enzyme standard of electric eel cholinesterase was prepared daily at a concentration of 0.2 units/mL in homogenizing buffer. The bicinechonic acid working reagent was prepared daily by mixing bicinechonic acid solution with 4% (w/v) copper (II) sulfate pentahydrate in a 50:1 volumetric ratio. Protein standards were prepared by making serial dilutions of bovine serum albumin (BSA; 1.0 mg/mL) in homogenizing buffer (0mg/mL, 200mg/mL, 400mg/mL, 600mg/mL, 800mg/mL, and 1000mg/mL).

Test organisms

Hyalella azteca and Daphnia magna were obtained from United States Geological Survey (USGS) Midwest Laboratory in Columbia, Missouri and cultured at the University of Michigan in Ann Arbor, MI. Culturing conditions were stable at 22°-23°C with 16:8-h light:dark photo-period. Hyallella azteca were fed 1 gram Tetra fish feed 3 times a week. Daphnia magna were fed according to size. Neonates were given 15 ml of Selenastrum algae and adults were given 25 ml of algae.
Acetylcholinesterase analysis

Acetylcholinesterase activity was determined using 5 amphipods per replicate with 3 replicates. *Hyalitella azteca* and *Daphnia magna* were received from the control room, weighed for initial weight, transferred to a microcentrifuge tube and frozen at -80°C. Organisms were thawed and weighed again, before analysis. 500 μL of ice-cold homogenizing buffer was added to microcentrifuge tube along with the respected sample then homogenized using pellet pestle for 30 seconds. Samples were microcentrifuge for 10 minutes at 4°C at 10,000 g. Supernatant was removed and transferred from each sample into clean 1.5 mL tubes. Acetylcholinesterase determination was performed based on methods published by Ellman et al. and adapted for use in a microplate. Reagents were added to microplate via pipette in triplicate as follows 32 μL assay blank (homogenizing buffer), enzyme standard (electric eel acetylcholinesterase), or homogenate supernatant, 200 μL Ellman’s reagent (5,5’-dithiobis{2-nitrobenzoic acid}), and 8 μL acetylthiocholine iodide. Immediately after the addition of acetylthiocholine iodide substrate, absorbance was read at 406 nm in 2-minute intervals for 30 minutes using a microplate spectrophotometer. Acetylcholinesterase and protein determination was performed based on methods published by Ellman et al. 1961 and Bartlett et al. 2016.

Protein concentration in the homogenate supernatant was measured using the bicinchoninic acid protein assay by adding 25mL of assay blank (i.e., homogenizing buffer), enzyme standard, BSA protein standard, or homogenate supernatant, in triplicate to a microplate followed by the addition of 200mL of the bicinchoninic acid working reagent. The microplate was incubated at 25°C for 2 hours, and then the absorbance was measured at 562 nm on a spectrometer. Protein levels of the homogenate supernatant were calculated using the equation of
the line from the BSA standard curve. Specific activity for AChE was calculated based on the following equation:

\[
\text{Specific activity} = \frac{(A \times \text{Vol}_R \times 1000)}{(E \times \text{PL} \times \text{Vol}_H \times \text{PR})}
\]

Specific activity is micromol/min/g protein, A is the change in absorbance per minute, \( \text{Vol}_R \) is the reaction volume (300mL), 1000 is a unit conversion factor (g to mg), E is the extinction coefficient for 5,5-dithiobis[2-nitrobenzoic acid] chromogen-buffer reagent (1.36 x 10^4 M^-1 cm^-1), PL is the pathlength(0.875 cm), \( \text{Vol}_H \) is the homogenate volume (500mL), and PR is protein in the homogenate (mg/mL).

**Results**

The results demonstrate that we can perform AChE assays with *H. azteca* and *D. magna*, allowing for measures of their exposure and chronic toxicity effects from organophosphate insecticides. Baseline levels for BSA protein and AChE were measured, along with specific activity, in *Daphnia magna* and *Hyalella azteca*. AChE is the slope of the line divided by 2. The slope is the change for every two minutes (we only needed 1 minute) and BSA is the absorbance value of y. Statistical analysis is needed to further clarify baseline results. Baseline results were of a lesser value than that of Bartlett et al. 2016. The results are highlighted as follows:

- **Sample 1 for Daphnia**: BSA equaled to 0.417949 µg/mL, ACHE equals 0.00525 units/L, and Specific Activity equals 0.000506676 Bq/kg (Figure 1)

- **Sample 2 Daphnia**: BSA equals, 0.43 µg/mL, AChE 0.00405 units/L, and specific activity equals 0.00037991 Bq/kg (Figure 2)
- **Sample 1 H. azteca**: BSA equals 1.041282 µg/mL, AChE equals 0.1249 units/L and specific activity equals 0.004838251 Bq/kg (Figure 3)

- **Sample 2 H. azteca**: BSA equals 1.040333 µg/mL, AChE equals 0.1307 units/L and specific activity equals 0.005067544 Bq/kg (Figure 4).

*Figures 1: Baseline for BSA protein, AChE, and the specific activity for Daphnia sample.*
Figure 2: Baseline for BSA protein, AChE, and specific activity for Daphnia sample.

Figure 3: Baseline for BSA protein, AChE, and specific activity in H. azteca sample.
Discussion

Determination of acetylcholinesterase (AChE) activity is the appropriate tool for the diagnosis of organophosphate exposure. This short study provides a baseline threshold for in-situ toxicity tests of organophosphates in two popular toxicity test organisms and will allow for measures of chronic toxicity with only the 24 hr iTIE exposure. Enhancements are still needed to provide for a precise determination of AChE activity in macroinvertebrates.

Pesticides and other contaminants can be detrimental to surface waters. In some area concentrations exceed water quality. There is a need for more information on the impacts of pesticides on freshwater invertebrates. In situ exposures with Hyalella and Daphnia can be an effective way of adding a biological effects component to an ongoing water quality monitoring program to examine the impacts of pesticides and other contaminants to aquatic ecosystems. Evaluation of the in-situ toxicity of pesticides to Hyalella and Daphnia is needed during
preferably in pre-, peak-, and post-pesticide application to establish linkages between cause and effect for organophosphate pesticides. The next step in the research is to test for *Hyallela* and *Daphnia* exposure to different organophosphates in the field using iTIE exposures. The baselines help to reference for comparison purposes, but more calculation and statistical analysis is needed.


