SERDP

FINAL REPORT

In Situ Toxicity Identification Evaluation (iTIE) Technology for Assessing Contaminated Sediments, Remediation Success, Recontamination and Source Identification – Phase II

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ACRONYMS AND ABBREVIATIONS

AChE	Acetylcholinesterase
AFDW	Ash-free dry weight
ASTM	American Society for Testing and Materials
CoC	Chemical of concern
DO	Dissolved oxygen
DI	Deionized
DoD	Department of Defense
DoDI	Deoxygenated deionized
EGLE	Michigan Department of Environment, Great Lakes, and Energy
EPA	Environmental Protection Agency
FEP	Fluorinated ethylene propylene
FSW	Filtered seawater
HDPE	High-density polyethylene
GAC	Granular activated carbon
IEW	Ion-enriched water
iTIE	*in-situ* Toxicity Identification Evaluation
iTIES	*in-situ* Toxicity Identification Evaluation System
LDPE	Low-density polyethylene
MDL	Minimum detection limit
MHRW	Moderately hard reconstituted water
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PFAS	Per- and polyfluoroalkyl substances
PFOS	Perfluorooctanesulfonic acid
PTFE	Polytetrafluoroethylene
RL	Reporting limit
SEED	SERDP Exploratory Development
SON	Statement of Need
SWRCB	California State Water Resources Control Board
TIE	Toxicity Identification Evaluation
WoE	Weight-of-evidence

KEYWORDS

Chemical mixtures, Mixture toxicity, Causal linkages, Weight-of-evidence, Restoration success, Remediation success, Biological fractionation, Perfluorooctanesulfonic acid (PFOS), Per- and polyfluoroalkyl substances (PFAS)

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The *in-situ* Toxicity Identification Evaluation (iTIE) concept and first prototype iteration were originally developed by Burton and Nordstrom (2004). Subsequent prototype and protocol refinements were made in thesis studies completed by Steigmeyer (2015), Meyer (2016), and Nichols (2023), as well as a practicum completed by Calloway (2022). Phase I of this project was funded by the Strategic Environmental Research and Development Program (SERDP; Contract No. W912HQ18C0019, Project No. ER18-C4-1181), and was completed in 2019. The following report details Phase II of the project, also supported by SERDP (Contract No. Contract No. 21-C-0052, Project No. ER18-1181). This research would not be possible without the support of personnel from the Burton Lab, NIWC Pacific, Coastal Monitoring Associates, and other UM SEAS research labs and administrative departments. Dr. Karen Alofs of the University of Michigan provided facilities, equipment, and advice for fish care. Dr. Andrew Gronewold of the University of Michigan provided advice for data analysis. Sally Petrella of Friends of the Rouge provided advice for field deployments.

ABSTRACT

INTRODUCTION AND OBJECTIVES: The Department of Defense (DoD) has called for realistic, cost-effective *in-situ* technologies that can strengthen causal relationships between toxicants and adverse biological effects, especially at sites impacted by multiple stressors. Some previously developed methods used to establish stressor-causality linkages, such as the United States Environmental Protection Agency (USEPA) laboratory toxicity identification evaluation (TIE) method, can be confounded by excessive sample manipulations and temporal limitations. The *in-situ* Toxicity Identification Evaluation (iTIE) protocol was created to address this gap. The iTIE is a biological fractionation protocol in which water from an impacted site is differentially fractionated through an array of diagnostic resins and directly exposed to test organisms *in-situ*. It is intended to be a cost-effective substitute for Phase 1 TIE methods in tiered risk assessments.

TECHNICAL APPROACH: In Phase II of this SERDP Project ER18-1181, the iTIE prototype was expanded into the comprehensive iTIE system (iTIES). The current iTIES includes diverless porewater sampling, a multi-pump system allowing for precise control of iTIE exposure pump rates, and a passive oxygenation system allowing gentle aeration of porewater. The iTIES can separate a wide range of chemicals of concern (CoCs), including ammonia, heavy metals, organophosphate pesticides, per- and polyfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other organic toxicants of varying polarities. Numerous organisms and biological endpoints are compatible with iTIE testing, including fish teratogenicity and enzymatic bioassays.

RESULTS: Several iTIES prototype components were evaluated for efficacy in Phase II of this project. 1) The diverless porewater sampling system, also known as the Trident probe, can effectively sample porewater with minimal surface water infiltration with the proper setup specifications. 2) After several laboratory tests and prototype adjustments, it was found that an oxygenation coil with oxygen-pressurized silicone tubing is the most effective configuration for aerating marine porewater with high dissolved sulfide content. 3) Resin conditioning methods were optimized to ensure that resins do not induce stress to organisms during iTIES use. Following prototype testing and optimization, the iTIES was successfully used in a variety of field settings, both marine and freshwater, to identify and rank CoC classes predominantly causing toxicity at a site.

BENEFITS: The iTIES should be explored as an option for incorporation into DoD tiered risk assessments of impacted sites. Once chemical toxicity is detected at a site, the iTIES can be used to identify which CoC classes are present, establish causal linkages between those CoCs and toxicity responses, and rank CoCs causing the most stress. Compared to USEPA Phase 1 TIE methods, the iTIES testing protocol is more sensitive, cost-effective, and representative of realistic site conditions.

EXECUTIVE SUMMARY

INTRODUCTION

This report details the second phase of SERDP Exploratory Development (SEED) project ER18-1181. SERDP has previously issued Statements of Need (SONs), calling for "innovative approaches for both monitoring and implementing *in-situ* remediation of impacted aquatic sediments" that "ultimately reduce costs". At sites with multiple stressors present, there is a need for realistic, cost-effective *in-situ* tools that can quantitatively demonstrate causal linkages between chemical classes and adverse biological effects. At present, stressor-toxicity linkages are commonly determined through qualitative methodologies. Existing quantitative methods, like the United States Environmental Protection Agency (USEPA) toxicity identification evaluation (TIE) method, are often confounded by excessive sample manipulation and fail to capture realistic site conditions. These challenges hinder effective management and remediation decision-making, which risks the unaddressed impairment of critical ecosystems and the unnecessary loss of time and public resources.

The overall purpose of this project was to develop and refine a novel *in-situ* monitoring technology, which can be used to establish stressor-causality linkages via realistic, cost-effective exposures. The *in-situ* Toxicity Identification Evaluation system (iTIES) is a biological fractionation technology in which site water is continuously sampled, oxygenated, fractionated by an array of resins, and exposed to test organisms *in-situ*. Different resins are selected for each iTIES deployment depending on site knowledge (i.e., chemicals expected to be present). This iTIES provides direct quantitative linkages between the presence of chemicals and observed toxicity. After Tier 1 of an ecological risk assessment, in which chemical toxicity is detected as site stressor, the iTIES can be utilized in a Tier 2 or 3 assessment to determine which CoC classes are predominantly causing toxicity and their relative toxicity contributions.

The following tasks were completed in Phase I of this project: 1) the development of the Prototype 3 iTIE Unit, an acrylic two-chamber unit that is robust, easily deployable and broadly applicable; 2) proof-of-concept deployments of the iTIE technology in several lab and field-based applications; 3) resin optimization studies, where several candidate resins, ideal resin volumes, and optimal flow rates were identified and tested; and 4) a cost comparison investigation, where it was proven that iTIE methodologies require approximately half the time to complete compared to traditional U.S. EPA TIE methods.

OBJECTIVES

The overall objective of Phase II, the current project iteration, was the continued development and verification of the iTIES as a widely applicable technology and testing protocol for incorporation into weight-of-evidence site characterization studies. At the end of Phase I, the following sub-tasks were identified as key focus areas for Phase II: Task 1: refinement of the iTIE prototype to allow for porewater sampling and diverless deployment; Task 2: the testing of early life stage fish; Task 3: an expansion of available sublethal chronic endpoints in invertebrate test organisms; Task 4: a continuation of resin optimization efforts; Task 5: additional field verifications in marine and freshwater environments; and Task 6: development of a decision-making framework to guide iTIES implementation.

TECHNOLOGY APPROACH

Task 1: Refinement of the iTIE prototype to allow for porewater sampling and diverless deployment

This task builds upon the Prototype 3 iTIE Unit from Phase I of the SEED project. A full iTIE system was developed using the Prototype 3 iTIE Unit as its foundation (Section 2). The latest iTIES prototype was designed to be suitable in a wide variety of deployment settings and study goals. The ability to sample and test porewater was a key focus of Phase II.

Site porewater is continuously collected using a porewater sampler known as the Trident probe (Chadwick et al., 2003). The Trident probe can be installed and retrieved diverlessly in waters up to 10 meters deep. The Trident probe's ability to collect porewater while limiting surface water infiltration was a focus of study during this SEED project (Section 3.1). Porewater often contains CoCs at far greater concentrations than found in surface water. Additionally, chemical stressors in surficial sediments disproportionately impact benthic macrofaunal health and community biodiversity, compared to other ecological compartments (Brown et al., 2000; Reynoldson, 1987). The inadvertent drawdown of surface water can dilute CoC concentrations in sampled porewater, which may confound conclusions regarding the stress caused by sediments. To address this, the research team conducted an in-lab tracer dye study, where the Trident was assessed at a variety of sampling depths in sediments of varying textures.

Given the low dissolved oxygen content of porewater, another priority for Phase II was the construction of a system capable of providing gentle aeration to sampled water. The latest iTIES prototype includes an oxygenation system, through which water flows after it is sampled by the Trident probe. After porewater is aerated, it is exposed to resins and test organisms contained in Prototype 3 iTIE units, and collected in sample bottles for post-exposure chemistry analysis. Water movement through the iTIES is driven by a series of peristaltic pumps, which can be precisely programmed to ensure optimal oxygenation and resin exposure time. Several tubing materials were assessed for efficacy for the oxygenation system, specifically when aerating water containing dissolved sulfide (Section 3.2).

All iTIES components are housed within easily transportable Pelican coolers. The iTIES prototype underwent iterative assessment and optimization through a series of laboratory tests. Following optimization, the prototype was used in a series of field deployments at a variety of sites, both marine and freshwater (Sections 6 and 7).

Task 2: Testing of early life stage fish

Early life-stage fish are an important class of organisms for toxicity testing due to their sensitivity to chemicals and significance for ecosystem structure. The objective of Task 2 was to validate embryo-larval fish as viable test organisms for iTIE applications and to confirm teratogenicity as a detectable endpoint in brief exposure periods. This was primarily accomplished through field deployments utilizing embryo-stage fish as test organisms, with survival, teratogenicity, and ash-free dry weight (AFDW) as key endpoints (Sections 6 and 7). A series of lab-based toxicity tests were also conducted using embryo-stage fish prior to the use of fish in field deployments (Section 5.1).

Task 3: Expansion of available sublethal chronic endpoints in invertebrate test organisms

In Phase I of this SEED project, proportion of survival was used as the primary toxicity endpoint. Task 3 necessitated the addition of available sublethal chronic endpoints for usage in an iTIES deployment. Section 5.2 describes efforts to use acetylcholinesterase (AChE) activity in freshwater amphipods (*Hyalella azteca*) as an endpoint to detect toxicity due to exposure to organophosphate pesticides. *H. azteca* adults were exposed to water spiked with chlorpyrifos in a series of in-lab iTIE experiments using various resins targeting organics, including C18 and Oasis HLB. Resulting AChE specific activity was quantified in bioassays.

In addition, several chronic endpoints were assessed for application in various lab- and field-based iTIE deployments (Sections 5.3, 6, and 7). Quantifiable chronic endpoints that were evaluated include reproduction timing, reproduction rates, and growth as AFDW.

Task 4: Continuation of resin optimization efforts

The iTIES is intended to be applicable at a broad range of sites impacted by varying chemical stressor combinations. Resins must be able to selectively target classes of CoCs while themselves not inducing stress to organisms. Task 4 entailed the continued testing of diagnostic resins for iTIE usage. A standardized *Daphnia magna* sub-chronic toxicity test method was used to assess potential toxicity caused by each resin. Section 4.2 details efforts to explore new resins for adsorption of PFAS. Section 4.3 describes an investigation of conditioning methods for Chelex, a resin effective in binding heavy and transitional metals, but also noted for causing stress in freshwater organisms. Section 4.4 explores various conditioning methods for granular activated carbon (GAC), a general adsorbent for heavy metals, organics of mixed polarities, and natural stressors like sulfides, though GAC has also been found to negatively impact organisms if unproperly conditioned.

Task 5: Additional field verifications in marine and freshwater environments

Task 5 necessitated the completion of additional field deployments of the iTIES at a variety of field sites. Sites were chosen and deployments were designed based on different areas of focus for iTIES optimization and verification. In chronological order of deployment:

1) The iTIES was field-tested at several relatively clean freshwater sites to verify the performance of technological components, including Third Sister Lake and Fleming Creek in Ann Arbor, MI (Section 7).

2) Sexton and Kilfoil Drain in Taylor, MI, receives effluent from the Detroit Metropolitan Airport during high flows. CoCs associated with airports, including ammonia and per- and polyfluoroalkyl substances (PFAS), were expected to be present in sediment in the drainage. An iTIE exposure was completed at the drainage using *Pimephales promelas* embryos (Section 7.2).

3) Paleta Creek in National City, CA, is a marine/estuarine site with previous detections of heavy metals, pesticides, polycyclic aromatic hydrocarbons, and dissolved sulfide. Site characteristics including depth, hydrology, and chemical mixtures make Paleta Creek ideal for iTIES prototype testing. In the most recent iTIES deployment,

porewater was sampled, fractionated, and exposed to *Atherinops affinis* embryos and *Americamysis bahia* larvae for 8 hours (Section 6.1). Following the observation of significant toxicity at the site, fractionated water samples were transported back to the lab, vigorously aerated, and exposed to *A. affinis* embryos for 48 hours for additional insight. Sulfide concentrations were quantified in porewater before and after exposure to the iTIES oxygenation coil.

4) Clark's Marsh in Oscoda, MI, is a freshwater site with a legacy of PFAS impacts. The site was chosen to verify the ability of the iTIES to detect PFAS toxicity (Section 7.4). Due to logistical constraints, an *ex-situ* deployment of the iTIES was completed utilizing *P. promelas* embryos and *Chironomus dilutus* larvae as test organisms (Section 7.4).

5) The Rouge River in Detroit, MI, is a freshwater stream with an highly industrialized and urbanized watershed. Multiple chemical stressors including metals and organic toxicants have historically been detected in the sediment near the river's mouth. An iTIES deployment was completed near the river's mouth using *P. promelas* embryos and *C. dilutus* larvae (Section 7.5).

Task 6: Development of a decision-making framework to guide iTIES implementation

Specific conditions must be satisfied to successfully integrate the iTIES into a successful higher-tiered site assessment. Task 6 requires the development of a decision-making framework to help site managers determine if and how to incorporate the iTIES into an effective assessment.

RESULTS AND DISCUSSION

Task 1: Refinement of the iTIE prototype to allow for porewater sampling and diverless deployment

The current iTIES prototype is a robust, broadly usable field technology that allows for porewater sampling and testing, gentle aeration of sampled water, diverless deployments at depth, and applicability in a wide range of environments. Through a series of lab tests, field deployments, and iterative refinements, the current system prototype has been evaluated and successfully deployed in a variety of settings.

The Trident porewater sampler was found to effectively sample porewater without significant surface water infiltration in multiple sediments. A minimum sampling depth of three inches is needed to prevent infiltration and preserve porewater sample integrity. However, the Trident was unable to sample porewater from one sediment with a high proportion of finer particles. Further investigation is recommended to determine whether the dynamics observed with this sediment are likely to occur with other clayey sediments.

The iTIES oxygenation system is capable of oxygenating anoxic porewater, even when initially saturated with dissolved sulfide. Based on findings from in-lab experiments, the oxygenation system was built using gas-permeable silicone to efficiently deliver oxygen to porewater and oxidize dissolved sulfides into less toxic sulfur forms.

Additional enhancements were implemented in the iTIES through the duration of Phase II. The pumping sub-system was upgraded to include a booster pump, allowing for the effective transport of water to and through the iTIES. A drip chamber was also installed within the iTIES to remove gas bubbles from water, which may disrupt water flow and endanger organisms.

Task 2: Testing of early life stage fish

Early life-stage fish were successfully used in numerous lab-based iTIE tests and field iTIES deployments, in both marine and freshwater applications (Sections 5.1, 6 and 7). In addition to survival as an indicator of acute toxicity, several chronic toxicity endpoints were measurable in fish following 24- or 48-hour iTIE exposures, including teratogenicity and growth. Embryo-stage fish are ideal test organisms for iTIE experiments, due to their sensitivity, transportability, and low space requirements during exposure. Additional endpoints may be investigated for iTIE usage, including heart rate, olfaction, and genetic effects.

Task 3: Expansion of available sublethal chronic endpoints in invertebrate test organisms

Several sublethal chronic endpoints in invertebrate test organisms were verified for iTIE usage. Ash-free dry weight was verified as a simple endpoint that is responsive to many toxicants (Sections 5.3 and 7). AChE-specific activity in amphipods was also effectively used to causally link organophosphate pesticides to organism toxicity in iTIE exposures (Section 5.2). Results suggests the potential for other enzymatic bioassays to be used with the iTIES. Some reproductive endpoints including brood timing may be influenced by stress induced by the iTIE procedure itself and thus warrant further study (Section 5.3).

Task 4: Continuation of resin optimization efforts

Oasis WAX (Waters) was determined to be an optimal PFAS resin for use in iTIES experiments due to its strong survival and reproduction data compared to other candidate resins (Section 4.2). Oasis WAX was effectively used in an iTIE experiment at Clark's Marsh, allowing for the establishment of a stressor-toxicity linkage for PFAS.

Conditioning protocols were optimized for two crucial iTIE resins, Chelex and GAC (Sections 4.3 and 4.4, respectively). For use in freshwater deployments, Chelex must be converted from its sodium form (as manufactured) to its calcium form to preclude impacts to water hardness and pH that may induce stress to organisms. GAC must be conditioned with a period of aeration or other physical disturbance, which was found to mitigate water quality impacts to dissolved oxygen (DO), pH, conductivity, and suspended solids. When properly conditioned, the two resins are powerful diagnostic treatments for iTIES experiments.

Task 5: Additional field verifications in marine and freshwater environments

1) The iTIES prototype was successfully field-tested at Third Sister Lake and Fleming Creek. The prototype configuration developed in this project phase is recommended

for future iTIES designs. Biological results from the deployments indicate that both sites are relatively clean and may be designated as appropriate reference sites for environmental risk assessments in southeast Michigan.

2) The iTIES deployment at the Sexton and Kilfoil Drain provided some evidence of chemical toxicity, like due to non-PFAS organic CoCs. However, the results of the deployment were confounded by low control group survival. Control group mortality was attributed to issues with fish culturing protocols, which were rectified in later iTIES experiments.

3) At Paleta Creek, the iTIES silicone-based oxygenation system effectively aerated porewater while decreasing dissolved sulfide concentrations. A high degree of chemical toxicity was detected, with widespread mortality in *A. bahia* larvae in all treatments after only 8 hours of exposure. *A. affinis* embryos exposed for 8 hours to unfractionated water also had extremely low survival (10%). The Oasis HLB and Oasis WAX treatment groups both had full survival, indicating that an organic CoC, likely pyrethroids, were the dominant stressor. The Chelex treatment group had an improved but still lower proportion of survival (40%), indicating that metals are a secondary cause of toxicity at the site. Additionally, *A. affinis* embryos exposed for 48 hours to vigorously aerated water samples experienced complete mortality, eliminating sulfides as a dominant stressor while underscoring the site's high degree of toxicity.

4) PFAS was confirmed as the dominant stressor at Clark's Marsh. For PFAS-sensitive *C. dilutus* larvae, the highest survival was observed in the Oasis WAX treatment group, associated with the lowest concentrations of PFAS. The zeolite treatment groups for both species also saw moderate survival, indicating that a chemical class targeted by the resin, such as ammonia, is another dominant stressor at the site. Finally, relationships were able to be established between chronic toxicity at the site and PFOS, copper, nickel, and zinc, indicating that heavy metals are potentially a source of stress at the site.

5) The dominant stressors at the Rouge River were found to be heavy metals and PFAS. *C. dilutus* larvae had the highest survival rate in the Oasis WAX treatment. *P. promelas* larvae, which are less sensitive to PFAS but more sensitive to heavy metals, had the highest survival rate in the Chelex treatment. Some CoC classes including PAHs and PCBs were not detected at the site, contrary to expectations.

Task 6: Development of a decision-making framework to guide iTIES implementation

A decision-making framework was developed with five tiered steps to aid decision-making for remediation, recovery or site status projects. First, an appropriate reference condition must be established. It must be determined whether chemical or non-chemical stressors dominate at a site, including chronic toxicity and bioaccumulation potential. Next, a Go/No-Go decision can be made on whether to conduct iTIE exposures to establish stressor-causality linkages and CoC ranks. If successful weight-of-evidence evaluations have been conducted, the likely dominant stressor classes will have been verified.

IMPLICATIONS FOR FUTURE RESEARCH AND BENEFITS

The iTIES is an effective, durable, user-friendly, highly sensitive, and broadly applicable diagnostic tool that can be used to strengthen stressor-causality linkages and rank CoC classes at sites impacted by multiple stressors. The iTIES can be deployed diverlessly to sample sediment porewater with minimal surface water infiltration and gently aerate the porewater to prevent organism stress due to hypoxia. An array of available resins can be used to separate CoC classes including ammonia; heavy metals; nonpolar organics like organophosphates, PAHs, and pyrethroids; and polar organics like PFAS. Early life-stage fish are compatible with iTIE testing, with teratogenicity and growth as highly sensitive endpoints for chronic toxicity. Other sub-chronic endpoints like AChE-specific activity were verified with the iTIES, indicating the potential for additional enzymatic bioassays to be applied to the testing technology. Data can be produced linking the presence of CoCs with toxicity, thus reducing personnel hours requirements compared with traditional testing protocols.

The progress made in Phase II continue to suggest that the iTIES should become a standard diagnostic assessment technology for incorporation into weight-of-evidence studies. After Tier 1 assessments suggest that chemicals may be causing toxicity at a site, the iTIES should be integrated as a Tier 2 or 3 level methodology to determine which CoC classes should be targeted in further study or remediation efforts. The potential remains for additional diagnostic resins, test organisms, and toxicity endpoints to be verified and applied for iTIES use.

1.0 INTRODUCTION

This report details the second phase of SERDP Exploratory Development (SEED) project ER18-1181. This project is intended to address the continuing Department of Defense (DoD) need for realistic, cost-effective *in-situ* tools that can quantitatively demonstrate causal linkages between chemical classes and adverse biological effects, at sites where multiple stressors are present. Often, stressor-toxicity linkages are established through weight-of-evidence methodologies that are costly and ultimately subjective in conclusion. Some quantitative methodologies, such as the U.S. Environmental Protection Agency (EPA) laboratory toxicity identification evaluation (TIE) method, suffer from a lack of realism due to excessive sample manipulation. These manipulations fundamentally alter the basic characteristics of water and sediment samples, including pH, redox potential, and chemical bioavailability, leading to false conclusions drawn regarding sample toxicity. Additionally, ex-situ experiments often fail to capture temporal site variability and realistic site conditions.

The presence of such a gap hinders effective management and remediation decision-making, which risks the unaddressed impairment of critical ecosystems and the unnecessary loss of time and public resources. Thus, there is an urgent need for "innovative approaches for both monitoring and implementing *in-situ* remediation of impacted aquatic sediments" that "ultimately reduce costs" while providing an "improved understanding of the utilization of lower cost bioavailability measures as surrogates for higher trophic level sampling events".

The overall purpose of this project was to develop and refine a novel *in-situ* monitoring technology, which could be used to establish stressor-causality linkages via realistic, cost-effective exposures. The *in-situ* Toxicity Identification Evaluation system (iTIES) is a biological fractionation technology that systematically removes toxicity-causing chemical classes from sampled water and exposes the partially cleaned water to test organisms. The iTIES provides direct quantitative linkages between the presence of chemicals and observed toxicity, strengthening conclusions in site characterizations and ecological risk assessments regarding causality, bioavailability, toxicant source, and fate.

The first phase of this project was completed in 2019. The following tasks were completed in Phase I: 1) the development of the Prototype 3 iTIE Unit, an acrylic two-chamber unit that is robust, easily deployable and broadly applicable; 2) proof-of-concept deployments of the iTIE technology in several lab and field-based applications, including in both marine and freshwater settings; 3) resin optimization studies, where several candidate resins, ideal resin volumes, and optimal flow rates were identified and tested; and 4) a cost comparison investigation, where it was proven that iTIE methodologies require approximately half the time to complete compared to traditional U.S. EPA TIE methods.

The objectives of Phase II, the current project iteration, were as follows: Task 1: refinement of the iTIE prototype to allow for porewater sampling and diverless deployment; Task 2: the testing of early life stage fish; Task 3: an expansion of available sublethal chronic endpoints in invertebrate test organisms; Task 4: a continuation of resin optimization efforts; Task 5: additional field verifications in marine and freshwater environments; and Task 6: development of a decision-making framework to guide iTIES implementation.

2.0 ITIES PROTOTYPE OVERVIEW

This section addresses Task 1: Refinement of the iTIE prototype to allow for porewater sampling and diverless deployment. The Prototype 3 iTIE Unit, previously developed in SEED Phase I, is a thoroughly tested and proven field technology, and forms the basis for iTIE System. The current iTIES prototype is a robust, broadly usable field technology that allows for porewater sampling and testing, gentle aeration of sampled water, diverless deployments at depth, and applicability in a wide range of environments. Through a series of lab tests, field deployments, and iterative refinements, the current system prototype has been evaluated and successfully deployed in a variety of settings. A photograph of the full iTIES deployed at a field site is shown in Figure 2.1.1, while a schematic diagram of the iTIES is shown in Figure 2.1.2.

Figure 2.1.1: iTIES deployment at the Rouge River, Detroit, MI. In the foreground is the iTIE Cooler Sub-System, which contains iTIE resin treatments and test organism groups, as well as the oxygenation coil and sample collection bottles. Next to the iTIE Cooler are the two pump cases, which drive water movement through the entire system. The Trident can be seen above the pump cases, installed in the river channel near shore.

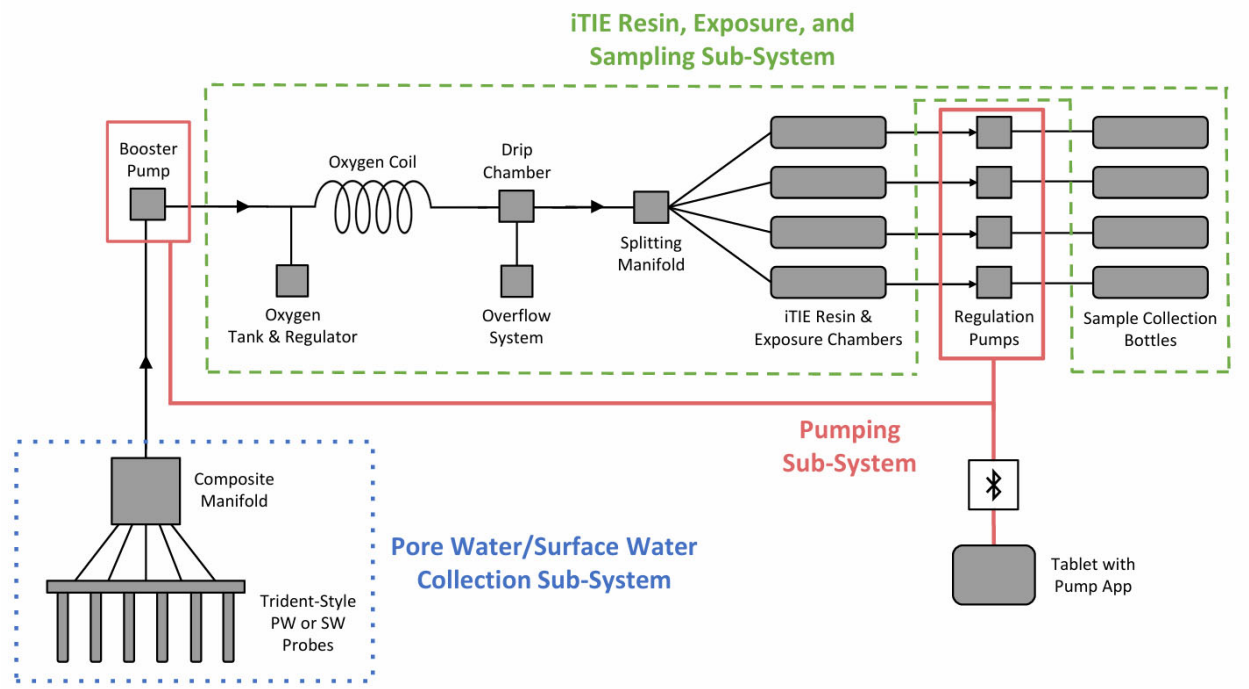

Figure 2.1.2: A schematic representation of the iTIES prototype. The prototype is divided into three sub-systems, outlined by different color outlines. Water is sampled in the bottom left of the diagram in the "Pore Water/Surface Water Collection Sub-System". Black lines with arrows show the direction of water flowing through the system.

2.1 WATER COLLECTION SUB-SYSTEM

Previous iTIES prototypes were capable of sampling and evaluating surface water using a simple weighted intake tube, shown in Figure 2.1.1. This intake weight is compatible with standard ¼" outer diameter tubing, which attaches directly to the rest of the iTIES. Task 1 of this project was to expand the prototype's capabilities to include the testing of porewater.

Figure 2.1.1: iTIES intake tube weights.

In the current prototype, porewater can be collected using a Trident porewater sampler (Figure 2.1.2). The Trident porewater sampler was originally developed by Chadwick et al. (2003) for DoD applications. It is a rugged, highly customizable technology comprised of a stainless-steel base witha series of mounted porewater probes. Each assembled porewater probe has an insertable length of 15 cm, an outer diameter of 2.5 cm, and an inner diameter of 2.1 cm. Each probe includes a filtration cover, including a tight-fitting inner screen (mesh size 500 um) and an outer sheath (slit length 3.8 cm; slit count n=24, mesh size 500 um). The interstitial space between the inner screen and outer sheath is filled with glass beads, which act as a filtration media for suspended solids. A probe tip (height 2.3 cm) on each probe secures the glass bead layer and allows for ease of insertion into the sediment. The Trident sampler has six probes oriented in a perfect hexagonal pattern, with adjacent probes 11.4 cm apart. A tube (1/8" outer diameter, 1/16" inner diameter) is attached to each probe to transmit water to a manifold mounted on the main Trident base. Site water is drawn through each probe up to the manifold via a vacuum created by a portable onshore peristaltic pump.

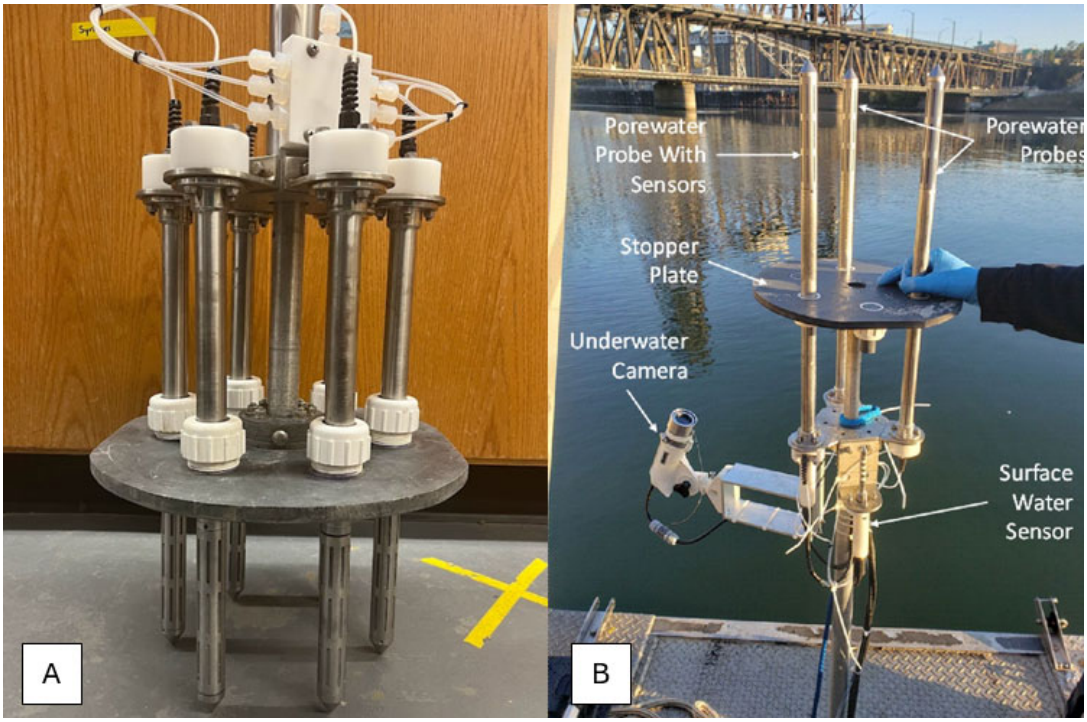

Figure 2.1.2: The Trident porewater sampling system (Chadwick et al., 2003). A) The version of the Trident used with the iTIES. This is a six-probe system with glass bead filtration layers, a stopper plate to control sampling depth and limit surface water infiltration, and push-pole diverless installation capabilities. B) Another Trident configuration. This is a three-probe system with a stopper plate and several auxiliary sensors attached.

An ellipsoidal stopper plate (radius 16.8-18.2 cm) is fixed to the probes. The position of the stopper plate is adjustable and controls the depth at which the Trident probes collect porewater. A stabilizing rod is attached to the stopper plate, anchoring its position on the Trident. Probes are inserted until the stopper plate is aligned and fully contacts the sediment surface. This

controls porewater sampling depth and forms a seal with the sediment, limiting the inadvertent drawdown of surface water when collecting porewater. Given that porewater typically contains higher concentrations of chemical stressors than overlying waters, it may be of concern to investigators that porewater is effectively isolated with minimal surface water infiltration. A study assessing this is described in Section 4.1.

The Trident is installed diverlessly via a direct-push system (Figure 2.1.3). A series of push poles are affixed to the Trident frame using locking pins. These push poles can be used to press the Trident probes into the sediment either using a hand-powered or hydraulic hammer. If push poles are left in place after Trident installation is complete, objects can collide with the push poles, risking equipment damage and the dislodgement of the Trident from the sediment. To prevent this, the push poles can be disengaged after installation using a release pin system.

Figure 2.1.3: Trident probe installation. The Trident probe is installed using a series of push poles. After installation, these push poles can be disengaged and removed, leaving the Trident in place in the sediment. Tubing remains connected to the Trident, routing porewater to the iTIES. A rope is used to remove the Trident at the end of deployment.

Auxiliary devices can be attached to the Trident body for concurrent data collection during iTIES deployment. Devices can include sensors measuring conductivity, temperature, and other water quality parameters; passive sampling devices for additional assessment of surface water quality; and underwater cameras.

2.2 PUMPING SUB-SYSTEM

All water movement through the iTIES, including site water collection, is driven by pressure differentials created by peristaltic pumps. The iTIES prototype was expanded to include a series of portable peristaltic pumps at strategic locations in the iTIES. These ensure that water effectively moves into and through the system at precise rates.

The pumping sub-system is divided into two parts, the first of which is the "booster pump" (Figure 2.2.1A). The booster pump is connected directly to the water collection sub-system and supplies water to the rest of the iTIES. The booster pump is comprised of a single peristaltic pump housed within a portable Pelican iM2275 case (waterproof rating IP67). The peristaltic pump used is a Versa pump (EcoTech Marine), which is a precision peristaltic pump capable of pump rates as low as 0.5 uL/min and as high as 300 mL/min. This pump is programmable via the Mobius app on iOS and Android. The pump is connected to tubing ports are mounted on the exterior of the pump case for ease of use during a field deployment. DC power is provided via a lithium-iron-phosphate battery (Bioenno; 24-volt; 10 ampere-hours). This pump case can maintain a charge for a minimum of four days. A direct AC plug-in option is also available on the exterior of the case. The case panel has a three-way switch, where the user can toggle between the two different power sources, as well as a 5-amp fuse that protects the case's electronics. The section of the case's interior at risk for water leakage is packed with absorbent material and partitioned from the section of the case's interior containing electronics.

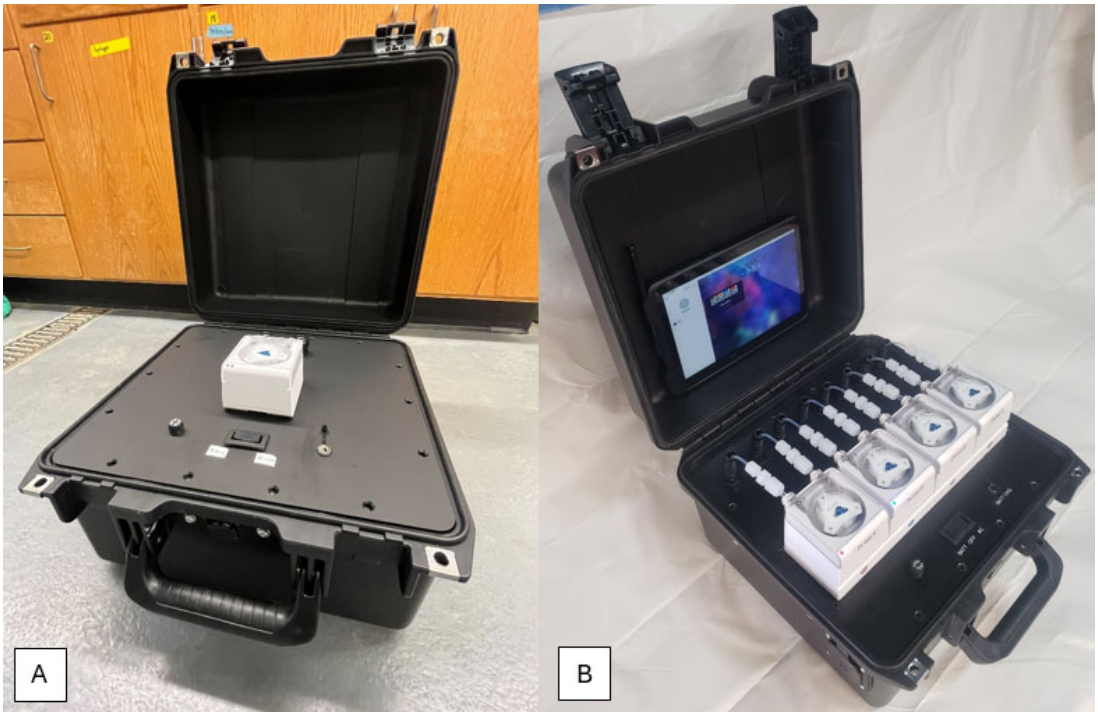

Figure 2.2.1: The iTIES Pumping Sub-System. A) The booster pump case, containing a single peristaltic pump. B) The quad pump case, containing four peristaltic pumps. A tablet is mounted inside the lid of the quad pump case, which can be used to program and operate both pump cases when connected to the internet.

The second part of the pumping sub-system is the "quad pump", named so because it contains four peristaltic pumps (Figure 2.2.1B). Each peristaltic pump is directly connected to the outflow of an iTIE organism chamber, allowing for the precise control of the slow flow rate required through each iTIE treatment (n=4). All pumps are individually programmable using the Mobius app. The quad pump is a Versa 4-Pump Base Station (EcoTech Marine) housed in a Pelican iM2275 case. The case is set up nearly identically as the booster pump case, including DC and AC power options, a three-way switch, and case interior partitioning. This case utilizes a 20-ampere-hour lithium-iron-phosphate battery, which can also hold a charge for four days. The two main differences between the case setups are the quad pump case has: 1) a 20-ampere-hour lithium-iron-phosphate battery, and 2) eight 2-meter tubes exiting the pump case, each directly connected to either an in-port or out-port of one of the four peristaltic pumps.

The present pumping sub-system arrangement, particularly the addition of a booster pump, was determined after a series of lab and field verification tests. Prior to the addition of a booster pump, water movement into and through the system was driven entirely by quad pumps. This was effective in deployments focused on surface water or even sandy sediments. However, when resistance was introduced, either by attempting to collect porewater from finer sediments or transport water up a vertical lift, it became impossible to maintain a sufficient pressure differential to effectively move water to the system. Furthermore, the maintenance of a vacuum (negative pressure) in the iTIES oxygenation coil (described below) facilitates the formation of gas bubbles, which impede water flow through the coil, disrupt resin beds, and reduce sampled water volumes. A booster pump ensures that a strong enough vacuum can be created to sample porewater from fine sediments and move water up any present vertical lift. It also separates that vacuum from the rest of the iTIES, precluding the excessive formation of gas bubbles.

While all pump flow rates can easily be calibrated, perfect calibration is never possible. A resulting imbalance between booster and quad pump rates can produce either positive or negative pressure in the iTIES interior, both of which can be highly disruptive. Thus, an overflow system was introduced to the iTIES interior, described in Section 2.3.

2.3 ITIES COOLER SUB-SYSTEM

After site water is collected and transported to shore, the rest of the iTIES is housed inside a durable, easily transportable Pelican 50QT Elite cooler. iTIES components in the interior of the cooler, including the oxygenation coil, drip chamber, iTIE unit holders, and sample bottle shelf, are built into a high-density polyethylene (HDPE) seaboard rack (Figure 2.3.1). This rack is removable from the Pelican cooler. The bottom of the Pelican cooler contains a port through which tubing can be threaded to the pumping sub-system. Blue Ice packs can be mounted to the Pelican cooler lid for temperature regulation.

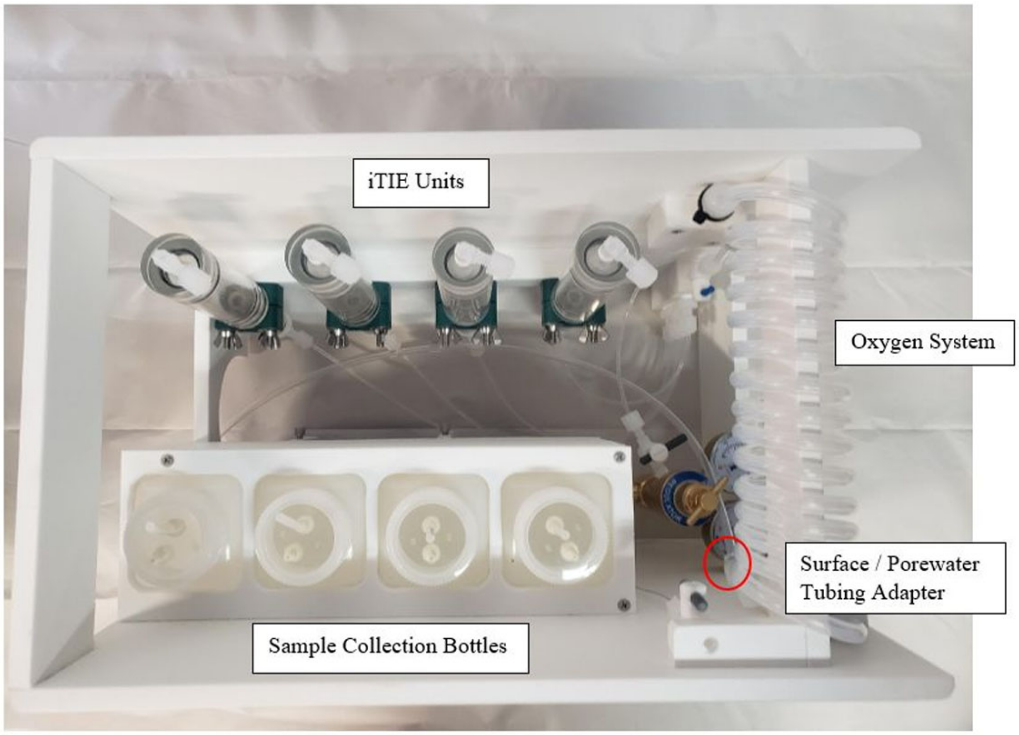

Figure 2.3.1: The iTIES Cooler Sub-System. All components comprising this sub-system are constructed into an HDPE seaboard rack, which fits inside a Pelican 50QT cooler. This sub-system includes an oxygenation system, mounts for four iTIE units, and four sample collection bottles.

2.3.1 iTIES Cooler Sub-System: Oxygenation Coil

Some site water, especially porewater, has naturally low dissolved oxygen content. Without proper oxygenation, anoxic conditions can cause mortality and morbidity in test organisms, upending the results of iTIES experiments. It is critical for the completion of Task 1 (Refinement of the iTIE prototype to allow for porewater sampling and diverless deployment) for the iTIES prototype to be capable of aerating anoxic porewater.

After sampled water exits the booster pump case, it is transmitted via 1/4-inch tubing to the oxygenation coil situated inside the iTIE Cooler. This tubing is directed through the port at the bottom of the iTIE Cooler, so the cooler can be closed during deployment. The oxygenation coil is comprised of a small-diameter (outer diameter 1/8-inch, inner diameter 1/16-inch gas-permeable tube containing pressurized oxygen, threaded through a larger tube through which sample water is conveyed (outer diameter 3/8-inch, inner diameter 1/4-inch) (Figure 2.3.2). Oxygen gas is supplied to the system using an off-the-shelf pressurized oxygen cylinder (Bernzomatic), which is stored underneath the sample bottle shelf. The gas cylinder is connected to a gas regulator, facing upward for ease of access during deployment. The regulator should be set to provide a pressure of 20-40 psi. The pressurized interior tubing is one continuous piece, threaded through each end of the exterior water conveyance tubing. Both ends of the exterior tubing are attached to a hollow HDPE “coupler,” which joins the two tubes together and allows water to be routed into and out from the coil. The end of the pressurized interior tubing is capped using a stopcock valve that can be opened to relieve gas pressure.

Figure 2.3.2: The iTIES oxygenation system. The oxygenation coil (top of picture) is built from nested tubing. The interior tubing of the coil is gas-permeable and pressurized with oxygen, which is supplied by an oxygen canister (hidden) that is controlled by a gas regulator (bottom left). Sampled water is exposed to the pressurized tubing as it flows through the exterior tubing.

The tubing is wrapped around an HDPE bar on the iTIE cooler rack, forming a coil to conserve space. As water slowly flows through the exterior tubing, it is gently oxygenated through contact with the gas-permeable interior tubing. The coil is approximately 343 cm long, with a water-fillable volume of approximately 81.4 mL. If the booster pump is programmed to operate at a rate of 100 mL/hour, then water will be exposed to the oxygenation coil for nearly 49 minutes.

Different gas-permeable tubing materials were evaluated for performance with anoxic water containing high dissolved sulfide content. This study is described further in Section 4.2. From this study, it was determined that platinum-cured silicone is the optimal tubing material for diffusing gas into site water and oxidizing dissolved sulfide into less toxic sulfur forms. Some field deployments were conducted using platinum-cured silicone as the interior oxygenation tubing; other field deployments used fluorinated ethylene propylene (FEP) interior tubing, which is slightly less gas-permeable than silicone. One important consideration is that silicone readily adsorbs chemicals, making it a difficult material to decontaminate. Thus, users should regularly exchange silicone tubing material to prevent contamination from occurring.

2.3.2 iTIES Cooler Sub-System: Drip Chamber and Overflow Bottle

After water passes through the oxygenation coil, it reaches an overflow system comprised of a drip chamber and an overflow bottle (Figure 2.3.3). This component is effective in sorting and removing gas bubbles, which often form within the oxygenation coil interior. Additionally, this component allows for excess water to exit the system. This aerated, unfractionated water can be collected for analysis.

Figure 2.3.3: The iTIES drip chamber. This small chamber is oriented vertically, with the perpendicular tubing located near the top of the chamber. As water flows into the chamber, gas bubbles and excess water are sorted and diverted through the perpendicular tubing to an overflow collection bottle.

The drip chamber is comprised of a piece of wide-diameter PVC tubing oriented vertically and connected to the system in-line (outer diameter 3/4-inch, inner diameter 5/8-inch, length 5.5 cm). It is crucial that the inner diameter is wide enough that there is no capillary action within the tubing, allowing water to collect at the bottom of the drip chamber and gas to collect at the top. Water exits the bottom of the drip chamber and is directed to a splitting manifold, where it is drawn up into iTIE resin fractionation chambers. A second tube (FEP; outer diameter 1/4-inch; inner diameter 3/16-inch) exits from near the top of the drip chamber. This tube can be routed into a bottle to collect overflow water for water quality monitoring and post-exposure chemistry analysis.

2.3.3 iTIES Cooler Sub-System: iTIE Prototype 3 Unit

The iTIE Prototype 3 Unit was introduced in Phase I of this project (Figure 2.3.4). Each unit was constructed from acrylic pieces that fit together with butyl rubber O-rings providing watertight seals. Water flows upward through each unit, entering through the bottom and exiting from the top. A peristaltic pump is connected to the top of each unit, driving water movement. The current iTIES prototype contains four iTIE units, which are mounted vertically inside the iTIE Cooler, as well as four peristaltic iTIE pumps, housed in the quad pump case.

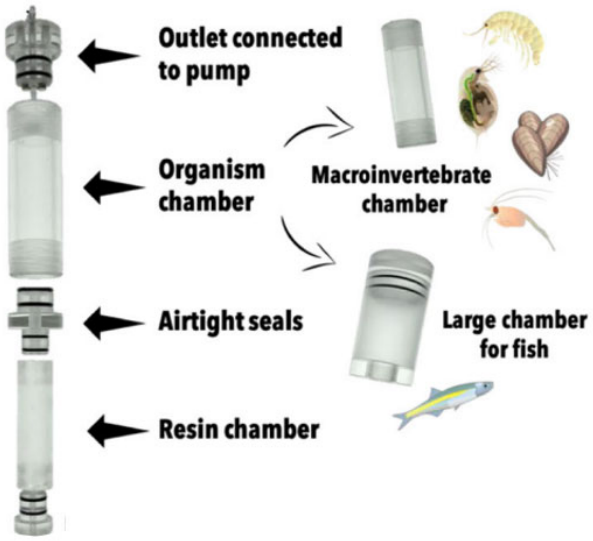

Figure 2.3.4: The iTIE Unit Prototype 3. Water flows upward through the bottom fitting, first passing through the resin chamber and then passing through the organism chamber. Two possible configurations are shown, a macroinvertebrate chamber and a large fish chamber. The macroinvertebrate chamber was used in every study described in this report.

After water leaves the splitting manifold and enters the iTIE units, it first flows through the resin chambers. Each resin chamber has a fillable volume of 16 mL, fillable depth of 5.5 cm, outer diameter of 2.6 cm, and inner diameter of 1.9 cm. Each chamber can be packed with a diagnostic resin, contained on either side with pads of inert glass wool. The water is differentially fractionated as it flows through the resin matrix, with a resin exposure time dependent on the programmed pump rate (typically between 40-80 minutes). The resin fractionation chamber and organism exposure chamber are connected by an acrylic middle fitting, which is covered with 20 um mesh to prevent the leakage of fine resin material into the organism chamber. Finer mesh can be substituted if needed.

Test organisms are then exposed to partially fractionated water in organism exposure chambers. Each organism chamber has a fillable volume of 40 mL, fillable depth of 7.6 cm, outer diameter of 3.8 cm, and inner diameter of 2.5 cm. These chambers are large enough to house some common test organisms, including embryonic fish and invertebrates. Substrates can be placed into organism chambers to improve organism health during deployment. Water exits the organism chambers through a top fitting, which is covered with nylon mesh.

2.3.4 iTIES Cooler Sub-System: Sample Bottles

Finally, processed water is collected in sample bottles housed in the iTIE Cooler. Since water movement is driven by the peristaltic pumps in the quad pump case, water is conveyed to the quad pump case and back into the iTIE Cooler through a pair of tubes (outer diameter 1/8-inch, inner diameter 1/16-inch). Sample bottles can be pre-loaded with preservation reagents for accurate post-deployment water chemistry analysis. The current bottle rack is built to hold 500 mL HDPE bottles, though bottle material and size is customizable.

3.0 PROTOTYPE VERIFICATION STUDIES

3.1 TRIDENT SURFACE WATER INFILTRATION

The sediment-water interface is a critical zone of interest for the iTIES because toxicants that are not broken down, bioaccumulated, or otherwise diluted often accumulate in sediment and its interstitial spaces (Burton, 1991). Toxicants that collect in porewater often include substances that are not typically found in surface water or that are in forms that are more likely to be absorbed into sediments, such as heavy metals and PFAS (Burton et al., 2011; Evich et al., 2022). Additionally, chemical stressors in surficial sediments disproportionately impact benthic macrofaunal health and community biodiversity, compared to other ecological compartments (Brown et al., 2000; Reynoldson, 1987).

By coupling the Trident with the iTIE system, test organisms are exposed to site porewater in a more time- and cost-effective manner, improving the realism of exposures. However, a remaining concern with the use of the Trident is the potential for surface water infiltration during porewater extraction. Porewater typically contains higher concentrations of toxicants than surface water, so surface water infiltration can dilute toxicant concentrations in samples and lead to an underestimation of site toxicity. As the Trident removes water from the sediment, a void space is created, referred to as the "zone of influence" (Figure 3.1.1). The zone of influence for each individual Trident probe is spherocylindrical, while the combined zone of influence for a multi-probe Trident is likely roughly cylindrical with rounded edges. Surface water infiltration will occur if the zone of influence meets the sediment surface. Increasing the sampling depth reduces the likelihood of surface water infiltration. Additionally, the stopper plate acts as a physical barrier between the sediment surface and the zone of influence, limiting the potential for surface water drawdown. This study aimed to address the following objectives: (1) verify that the Trident can effectively extract porewater with minimal surface water infiltration; (2) determine the optimal sampling depth at which no surface water drawdown occurs in a variety of sediments; and (3) characterize the tested sediments to assess how particle size composition affects Trident probe performance.

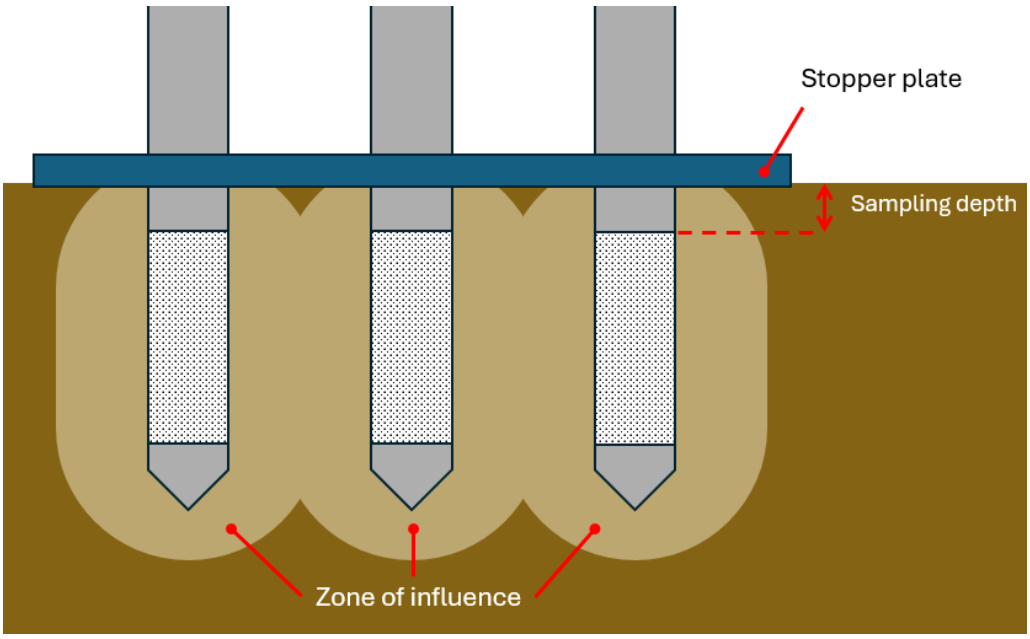

Figure 3.1.1: The Trident probe's "zone of influence". The intake port around each cylindrical Trident probe is 15-cm long (dotted region). Sampling depth is the distance between the tops of the intake ports and the sediment surface. The zone of influence is the space from which porewater is extracted by the Trident. The stopper plate acts as a barrier, separating the zone of influence from the sediment surface.

3.1.1 Methods

Sediment Collection and Processing

Three sediments were tested in this experiment, designated with the names "Sediment A", "Sediment B", and "Sediment C". Sediment A was a commercial all-purpose sand procured from a hardware store. Sediment B was a terrestrial sediment collected from a wooded area in Saginaw Forest, Ann Arbor, MI. Sediment B was qualitatively estimated to contain moderate fractions of clay and silt due to its small granular aggregation properties and relative permeability. Sediment C was a terrestrial sediment collected near a residential area located on the University of Michigan's North Campus in Ann Arbor, MI. Sediment Cwas estimated to have the highest clay content of the three sediments, due to its stickiness, impermeability, and formation into massive aggregates. Sediments B and C were each processed through a 5.6-mm sieve to remove coarse fragments and incubated via saturation with deionized (DI) water.

Surface Water Infiltration: Basin and Reservoir

A series of laboratory tracer dye tests were conducted. A system was designed to represent the hydrological dynamics of the sediment-water interfaces at which the Trident would be utilized (Figure 3.1.2). This system was composed of two major components: a sediment basin and a larger water reservoir. The sediment basin was comprised of a 17-gallon HDPE cylindrical Chem-Tainer with radius 22.5 cm and three rows of half-inch diameter holes drilled around its circumference to allow for water movement. The rows were placed 6, 8, and 10 inches above the bottom of the basin to approximately align with Trident probe intakes. Clean mesh was

secured over the holes to minimize loss of sediment from the basin. Tests with high-clay/silt sediments used 80-µm mesh, while tests with low-clay/silt sediments used 250-µm mesh.

The Chem-Tainer was then placed in the center of a larger reservoir and filled with approximately 20 gallons of a test sediment. If the sediment contained coarse fragments, the sediment was pre-processed through a 5.6 mm sieve prior to placement in the basin. The reservoir was then filled with tap water until the sediment was saturated with water, and approximately one inch of surface water was present above the sediment surface. Aluminum foil was used to cover the sediment surface to limit suspension of fine particles. Water levels inside and outside the basin were allowed to equilibrate for several hours.

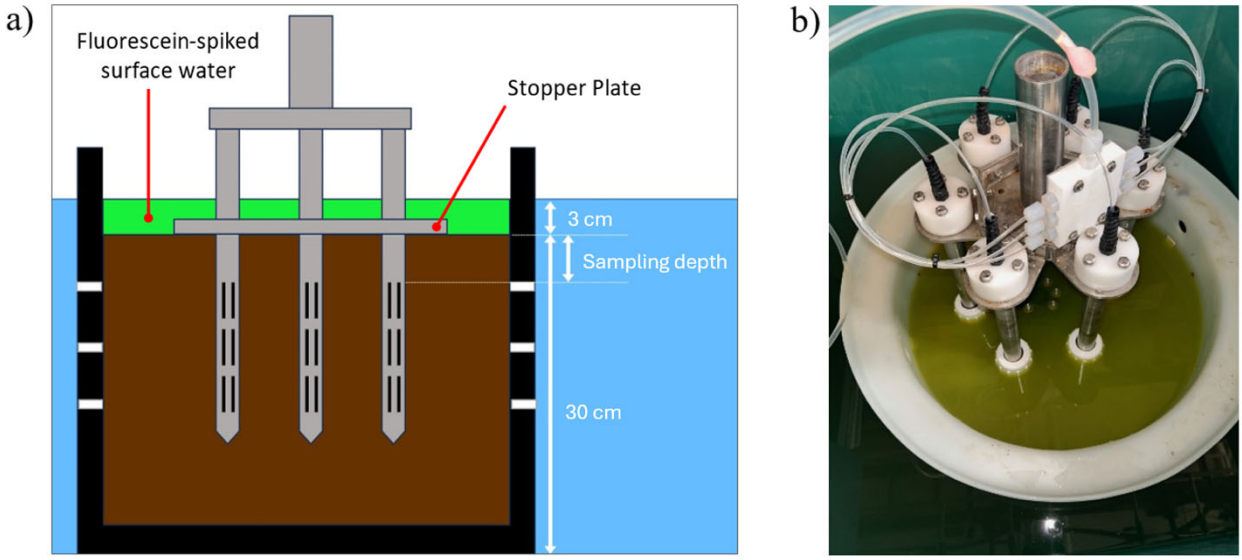

Figure 3.1.2: The Trident tracer dye experimental design. A) A diagram showing the tracer dye experimental setup. A 17-gallon Chem-Tainer basin (black) is situated inside a larger reservoir and filled with a test sediment (brown). The outer reservoir is filled with water (blue) until the sediment is fully saturated and approximately one inch of surface water is present above the sediment surface. Water levels inside and outside the basin are allowed to equilibrate. The sampling depth of the Trident is specified by adjusting the position of the stopper plate. The Trident is inserted into the sediment. After Trident insertion, the surface water is spiked with a fluorescein-based tracer dye (green). **B)** a photograph of the experimental setup.

Surface Water Infiltration: Trident Installation and Operation

The Trident used in these experiments is comprised of six water sampling probes mounted to a stainless-steel base. A stopper plate is attached to the probes, which is designed to limit the potential for surface water infiltration by forming a seal with the sediment surface during Trident insertion. The stopper plate on the Trident can be adjusted vertically, controlling sampling depth. A variety of sampling depths ranging from 1-3 inches were tested for each sediment. The prepared Trident was inserted into the sediment in the Chem-Tainer and hammered in place using a fence post driver, to ensure a proper seal between the stopper plate and sediment surface. A calibrated peristaltic pump (EcoTech Marine) was connected to the Trident with ¼-inch FEP tubing.

After Trident installation, the surface water atop the sediment basin was spiked with 1 mL of a fluorescein-based tracer dye. This tracer dye was selected due to its highly fluorescent yellow-green color, distinct absorbance curve with a peak at 490 nm, and non-toxic and biodegradable properties. The tracer dye was dispersed throughout the surface water.

A series of water samples were collected at the beginning of each experiment: (1) triplicate reservoir samples, to ensure the absence of fluorescein outside the sediment basin; (2) triplicate porewater samples to establish baseline absorbance patterns for each sediment with no dye present; and (3) triplicate surface water samples to establish absorbance patterns after the addition of tracer dye. Approximately 2 mL of each sample were collected and placed in a clean cuvette. The peristaltic pump was operated for 24 hours at a rate of 2 mL/min, the maximum pump sampling rate for a typical iTIE application. Beginning at hour 16 of each experiment, porewater samples were collected hourly from the peristaltic pump. Reservoir and surface water samples were sampled again in triplicate at the end of each experiment.

Sediment A was tested twice with a sampling depth of 2 inches and twice with a sampling depth of 3 inches. Sediment B was tested once with a sampling depth of 2 inches and twice with a sampling depth of 3 inches. Sediment C was tested three times with a sampling depth of 3 inches.

Surface Water Infiltration: Sample Analysis

Cuvettes containing samples sat undisturbed for 48 hours to allow the settling of suspended solids out of solution. Then, samples were analyzed using a spectrophotometer to measure absorbance as a function of wavelength. Absorbance (unitless) was recorded between the wavelengths 310 nm and 610 nm, at intervals of 30 nm. Fluorescein tracer dye causes detectable absorbance increases at wavelengths 400-520 nm, with a pronounced peak at 490 nm (Figure 3.1.3A). The dye's absorbance of other wavelengths between 310-610 nm is negligible. The absorbance patterns of sediments free of dye roughly follow a cubic polynomial curve between the relevant wavelengths (Figure 3.1.3B). Conversely, sediments containing dye do not follow a cubic absorbance curve due to the absorbance spike at 490 nm.

Cubic regression models with 95% confidence intervals were constructed for each sample using absorbances between wavelengths 300-370 nm and 550-610 as model inputs. These models estimate the absorbance pattern of the sediment in each sample. Then, for each sample, the measured absorbance value at 490 nm was compared to the 95% confidence interval of the sample's regression model. If the sample's absorbance at 490 nm fell above the modeled range, then the sample was determined to contain dye, a sign of surface water infiltration.

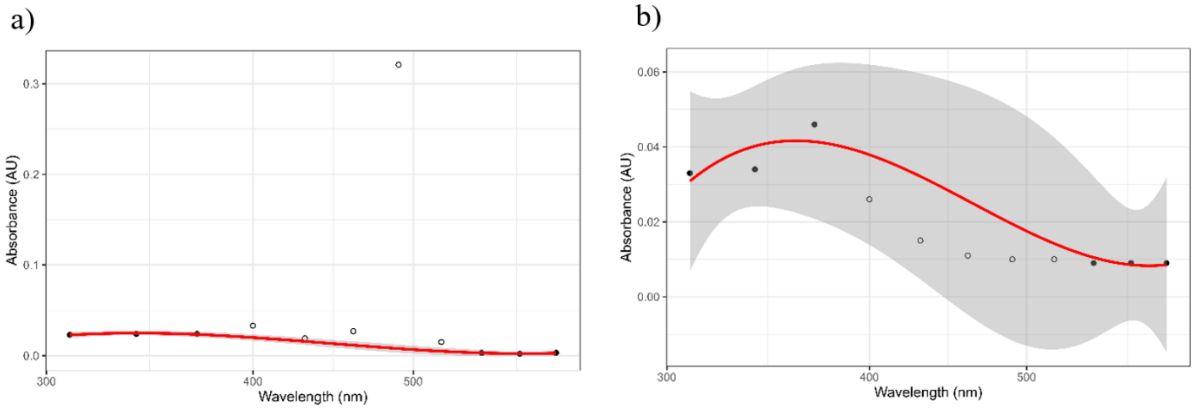

Figure 3.1.3: Expected absorbance patterns of Trident infiltration experiment components. A) Pure surface water spiked with fluorescein dye. **B)** Pure sediment porewater with no dye. Both graphs also include a cubic polynomial model (red). Each model was constructed using measured absorbance values at wavelengths 310-370 nm and 550-610 nm, to create an estimated absorbance pattern of the sediment in each sample. Fluorescein causes an absorbance spike at 490 nm. Samples containing fluorescein had absorbances at 490 nm falling outside of the cubic model's predictive range.

Particle Size Distribution: Sieve Analysis

The particle size distributions of the three test sediments were established using two standard methods: sieve analysis and hydrometer analysis. Sieve analysis procedures, used to determine distribution of particle sizes larger than 75 µm, were adapted from ASTM D6913-04 (ASTM, 2004). The method was modified to incorporate wet sieving and oven-drying each sediment specimen as part of the pre-processing procedure. This change was made to account for the significant amount of fine material in the sediments, which caused conglomeration and reduced the accuracy of the standard method.

A sub-sample of each test sediment was obtained, oven-dried at 105°C and weighed in a tared container. Sub-samples were then wet-sieved through a No. 200 sieve to separate the fine material (≤75 µm) from the coarser fractions. The coarse fraction retained on the No. 200 sieve was oven-dried and weighed. The difference between the initial sample weight and the dry mass of the coarse fraction was used to calculate the percentage of material finer 75 µm.

The oven-dried coarse fraction was placed in a stack of sieves arranged in descending order of mesh size (Table 3.1.1). The stack was placed in a mechanical sieve shaker (W. S. Tyler) for 15 minutes to ensure thorough separation of particle sizes. The contents of each sieve frame were emptied into tared weigh boats with brushes used to ensure that all particles were removed from the sieves. Each fraction was weighed. The resulting masses were used to calculate the cumulative percentage retained and the cumulative percentage passing for each sieve size. Two to four sieve replicates were produced for each sediment of interest.

Table 3.1.1: Sieve numbers and diameter sizes used in sieve analyses.

Sieve Number	Diameter (µm)
No. 10	2000
No. 20	850
No. 40	425
No. 60	250
No. 100	150
No. 140	106
Pan	

Particle Size Distribution: Hydrometer Analysis

Hydrometer analysis was used to determine distributions of particles smaller than 75 µm in diameter. Analyses were completed following ASTM D7928 (ASTM, 2021). A 152H hydrometer (Thermo Fisher Scientific) was used for these experiments. Sub-samples of moist sediments weighing 80-110 grams were collected and processed through a No. 10 sieve. A dispersing solution was prepared by dissolving 5 g of sodium hexametaphosphate in DI water. The sediment sub-sample was combined with the stock solution, soaked briefly, and emulsified in a blender for at least two minutes to create a slurry. This slurry was transferred to a clean beaker and allowed to soak overnight.

The following day, the slurry was rinsed into to a 1-L graduated cylinder (the "test cylinder"), and the cylinder was filled to 1 L using DI water. A second 1-L graduated cylinder (the "control cylinder") was filled with DI water and 5 g of sodium hexametaphosphate. The hydrometer was placed in the control cylinder and allowed to stabilize. The water level on the hydrometer stem was recorded as the "zero correction factor." The distance between the water level and the top of the meniscus was recorded as the "meniscus correction factor". The test cylinder was sealed with Parafilm and repeatedly inverted for two minutes to ensure uniform suspension of the sediment. The cylinder was then placed on a level surface and the time of placement was recorded. For finer or clayey sediments, up to five drops of isopropyl alcohol were added to the surface after inversion to reduce foam; if applied, the same amount of alcohol was added to the control cylinder.

Hydrometer readings (top of meniscus) and temperatures were recorded at specific intervals: 1 min, 2 min, 4 min, 8 min, 15 min, 30 min, 1 hour, 2 hours, 4 hours, and 24 hours. The hydrometer was returned to the control cylinder after each reading. Each insertion or removal of the hydrometer was performed slowly to avoid excessive disturbance of the sediment suspension. Temperatures were measured with a digital thermometer at specific intervals after hydrometer readings were measured: 1 min, 30 min, 1 hour, 2 hours, 4 hours, and 24 hours. After the final measurements were recorded, the contents of the test cylinder were rinsed into a pre-weighed Pyrex pan using a DI water squirt bottle. The pan was placed in an oven at 105°C to dry overnight. The dried sediment was weighed, subtracting the 5 g of sodium hexametaphosphate. Two to four hydrometer replicates were produced for each sediment of interest.

Hydrometer and temperature measurements were processed using modified calculations to obtain estimates of equivalent particle diameter and cumulative finer-than percentages (Hossain et al., 2022). Particle size distributions from sieve analyses and hydrometer analyses were combined and averaged for each test sediment. A low-degree polynomial regression was created for each sediment type to generate a continuous gradation curve.

3.1.2 Results

Surface Water Infiltration

Figure 3.1.4 shows the absorbance patterns of end-run porewater samples collected during surface water infiltration tests for Sediment A. The gray shaded region on each graph represents the 95% predictive interval around each cubic regression model. For Sediment A, when sampling depth is set to 2 inches, absorbance at 490 nm exceeded the bounds of the 95% predictive interval. This is evidence of surface water infiltration during porewater extraction. At a 3-inch sampling depth, the absorbance at 490 nm stayed within the 95% predictive area, indicating no surface water infiltration.

Similar patterns were observed for Sediment B (Figure 3.1.5). Sediment B experienced significant surface water infiltration at a sampling depth of 2 inches, evidenced by an apparent absorbance spike at 490 nm. Conversely, when sampling depth was set to 3 inches, absorbance remained within the bounds of the 95% predictive area, indicating no detectable infiltration.

All experiments using Sediment C were confounded and terminated early. Porewater movement was observed to stop within one hour of the initiation of each trial. This likely occurred due to the clogging of Trident probe intakes by fine sediment particles.

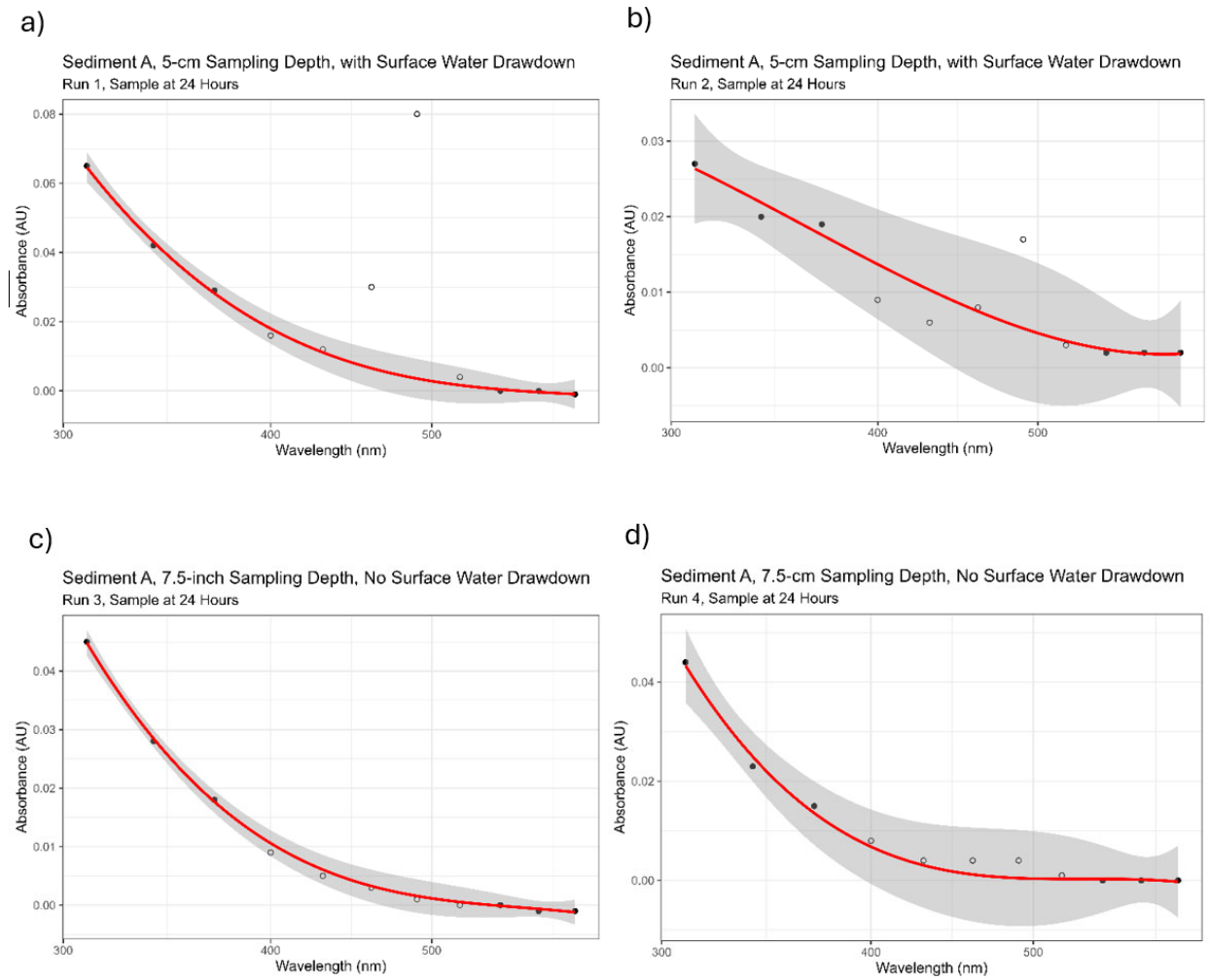

Figure 3.1.4: Absorbance patterns of porewater samples collected for Sediment A after 24 hours. Graphs **(a-b)** show absorbance for runs where sampling depth was set to 2 inches. Graphs **(c-d)** show absorbance for runs where sampling depth was set to 3 inches. All points (filled and unfilled) represent measured absorbance values. Filled points (310-370 nm; 550-610 nm) were used to create cubic regression models (red lines) and 95% predictive intervals (gray shaded regions).

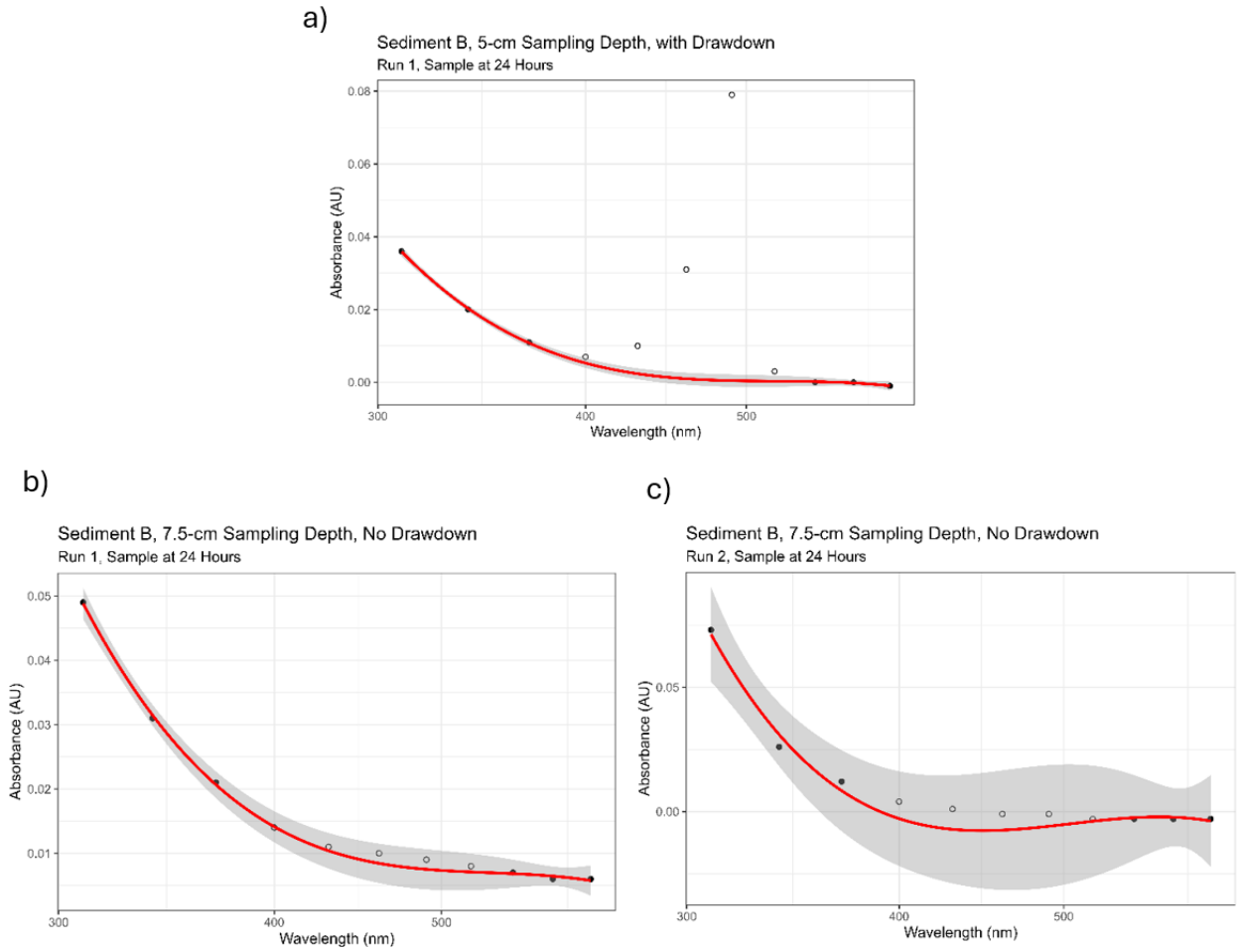

Figure 3.1.5: Absorbance patterns of porewater samples collected for Sediment B after 24 hours. Graph (a) shows absorbance for runs where sampling depth was set to 2 inches. Graphs (b-c) show absorbance for runs where sampling depth was set to 3 inches. All points (filled and unfilled) represent measured absorbance values. Filled points (310-370 nm; 550-610 nm) were used to create cubic regression models (red lines) and 95% predictive intervals (gray shaded regions).

Particle Size Distribution

Particle size distributions for the three test sediments are shown in Figure 3.1.6. Sediment A had a negligible proportion of silt and clay. This aligns with expectations for all-purpose sand. Sediments B and C had comparatively higher proportions of fine particles. Unexpectedly, the two sediments had similar particle size distributions, despite qualitative characteristics that suggest greater differences.

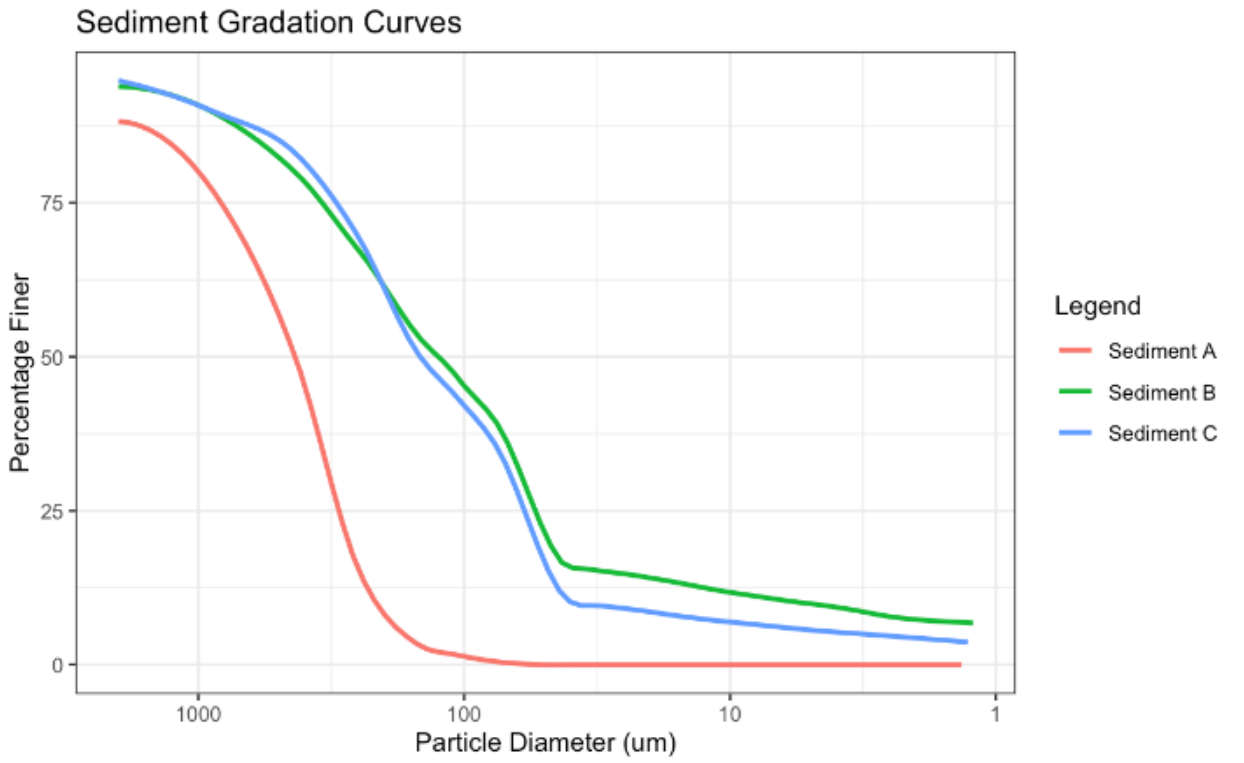

Figure 3.1.6: Average particle size distribution curves for Sediments A, B and C. Each curve including datapoints from two or three replicates for each sediment type. Curves were created using low-degree polynomial regression models. The horizontal axis uses a reverse logarithmic scale.

3.1.3 Discussion

The results of the tracer dye experiments indicate that at typical iTIES pump rates, an appropriate sampling depth of 3 inches is necessary to prevent surface water infiltration and preserve porewater sample integrity. This applies to a range of sediments, including sandy sediments like Sediment A and sediments with moderate silt-clay content like Sediment B. Deeper sampling depths may be required to limit surface water infiltration at faster pump rates. Alternative interventions such as increasing the size of the stopper plate may also be assessed.

Porewater was unable to be extracted from the third test sediment, Sediment C. Trident probe intakes quickly became clogged by this sediment, leading to the early termination of surface water infiltration trials. These results, as well as qualitative observations like a high degree of impermeability compared to the other two test sediments, led the research team to hypothesize that Sediment C is comprised of significantly higher proportions of finer particles. However, particle size distribution analyses refuted this expectation, revealing unanticipated similarities between Sediments B and C. It is unclear why these sediments had such similar particle gradations and yet were qualitatively so dissimilar. Given the prevalence of fine-particle sediments in impacted depositional areas, further investigation is recommended to determine whether the dynamics observed with Sediment C are likely to occur with other clayey sediments.

In conclusion, this study confirms that the Trident porewater sampling device performs effectively at the 3-inch sampling depth, mitigating surface water infiltration and ensuring

sample integrity in specific sediment conditions. While limitations remain regarding fine-grained sediments, the insights gained provide a foundation for refining the technology and optimizing its application in environmental research including iTIES applications. Continued development and testing will enhance the Trident's utility and versatility, contributing to more accurate assessments of sediment-porewater interactions in complex aquatic environments.

3.2 OXYGENATION COIL & SULFIDES

Task 1 necessitated that the iTIES be capable of aerating anoxic porewater samples prior to organism exposure. The iTIES contains an oxygenation coil for this purpose, described in greater detail in Section 3.3.1. An early iteration of the iTIES was constructed with an oxygenation coil made of semi-permeable FEP. The current version of the iTIES was utilized in an iTIE experiment at Paleta Creek, a marine/estuarine site in National City, CA, in August 2023. This exposure was intended to last for 24 hours. However, approximately five hours into the exposure, high mortality and morbidity were observed in test organisms, and the experiment was terminated. Low dissolved oxygen levels were measured in water after exposure to the oxygenation coil. Also, strong sulfide odors were apparent to all personnel, though they were not quantified at the time.

The research team hypothesized that high concentrations of hydrogen sulfide and low concentrations of dissolved oxygen in porewater were the primary drivers of mortality. Sulfide is often naturally present in the porewater of marine sediments (Sims & Moore, 1995). Theoretically, as oxygen diffuses into porewater, it should oxidize sulfide into less toxic sulfur forms like sulfate. However, if porewater is fully saturated with sulfide and kept fully contained within the iTIE system, oxygen may be prevented from diffusing into the water via the oxygenation coil. As a result of the 2023 Paleta Creek iTIE deployment, the research team concluded that further investigation of the oxygenation coil in the presence of sulfide saturation was necessary, with the potential for prototype refinement.

3.2.1 Apparatus Design

To investigate the effectiveness of the iTIE oxygenation coil in sulfide-saturated water, the research team designed a series of laboratory experiments testing: (1) different gas-permeable tubing materials, (2) varying oxygenation pressures, and (3) sulfide-free and sulfide-saturated water. Several apparatuses were constructed to match the specifications and recreate the dynamics of the oxygenation coil, each containing a different candidate tubing material (Figure 3.2.1). Each apparatus consisted of a 125-mL glass jar nested within a 500-mL glass jar. The intermediate space between the two jars was filled with test water, representing space within the oxygenation coil occupied by sampled water. Gas-permeable tubing was coiled around the smaller jar, allowing it to be exposed to test water. The tubing ends were threaded through holes in each apparatus lid and connected to a pressurized oxygen canister and a stopcock valve. Lids also contained a rubber hose barb safety vent to prevent pressure buildup in the apparatus. Each apparatus contained a closed-loop DO-measuring system. Water was drawn by a tube connected to a peristaltic pump, exposed to a YSI ProODO sensor through a PVC T-shape connector, and replaced to the opposite side of the apparatus. This allowed for continuous detection of DO levels within the apparatus over time.

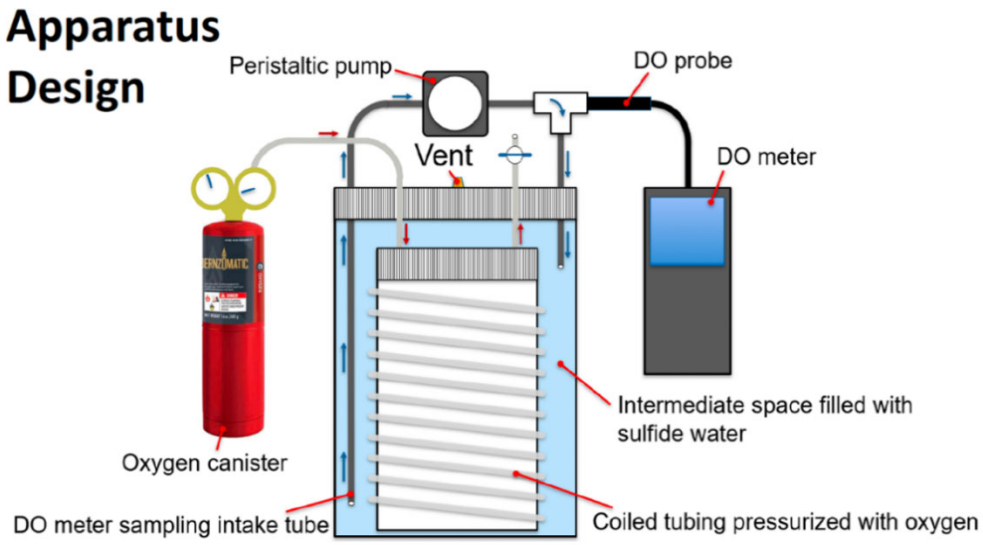

Figure 3.2.1: Oxygenation tubing apparatus schematic diagram. The small interior jar is coiled with tubing pressurized with oxygen. The intermediate space filled with sulfide-spiked test water. Test water is continuously sampled through the intake tube, pumped through the T-shaped connector, and replaced in the apparatus' intermediate space on the opposite side. The T-shape connector is fitted with a DO probe, which measures real-time DO concentrations in the test water. A peristaltic pump moves water through the DO measurement loop.

Four candidate tubing materials were assessed: FEP, low-density polyethylene (LDPE), polytetrafluoroethylene (PTFE), and silicone (Figure 3.2.2). The research team hypothesized that silicone tubing would be the best candidate, since silicone is known as a highly permeable elastomer. In addition, the research team hypothesized that increasing oxygen pressure would enhance the tubing oxygen diffusion rate.

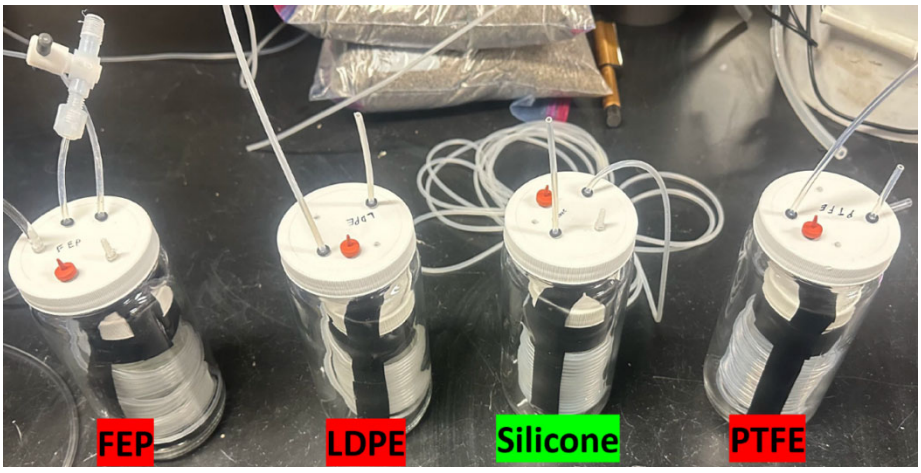

Figure 3.2.2: A photograph of the four oxygenation coil apparatuses. Candidate tubing materials included fluorinated ethylene propylene (FEP), low-density polyethylene (LDPE), silicone, and polytetrafluoroethylene (PTFE).

3.2.2 De-Oxygenated Deionized Water

All four apparatuses were first tested with de-oxygenated deionized (DoDI) water to assess the gas permeability of the different tubing materials in the absence of dissolved sulfide. Deionized water was de-oxygenated via bubbling with nitrogen gas until DO was measured to be below 2 mg/L. The water was then gently pumped into each apparatus, minimizing inadvertent aeration. At time t=0, the oxygen canister regulator was pressurized, representing the moment that site water enters the iTIE oxygenation coil. The FEP and LDPE apparatuses were assessed at the following oxygen canister pressures: 40, 50, 60, 70 and 75 psi. The PTFE apparatus was tested at 75 psi only. The silicone apparatus was tested at a maximum of 40 psi to avoid bursting the tubing. For each test, DO was measured every five minutes for one hour, approximately matching the minimum residence time of site water in the iTIE oxygenation coil during a typical iTIE deployment. Oxygenation rates for each test were calculated by the following formula:

$$Rate\ (mg * L^{-1} * min^{-1}) = \frac{(Final\ DO) - (Initial\ DO)}{Time}$$

Equation 3.2.1: Formula to calculate oxygenation rate (mg/L/min) for each apparatus test.

The results of the DoDI experiments are shown in Figures 3.2.3 through 3.2.6. In the FEP experiments, DO levels increased from 2.26-3.01 mg/L at the beginning of the tests to 4.8-6.9 mg/L after one hour of exposure. Experiments with LDPE and PTFE tubing showed similar oxygen increases. For FEP and LDPE, oxygenation rates did not noticeably increase with higher oxygen canister pressure. Also, DO never became supersaturated after exposure to any of the three rigid plastics. Meanwhile, for silicone tubing at an oxygenation pressure of 40 psi, DO increased from 2.4-3.4 mg/L to 19.2-19.6 mg/L after one hour of exposure (Figure 3.2.6).

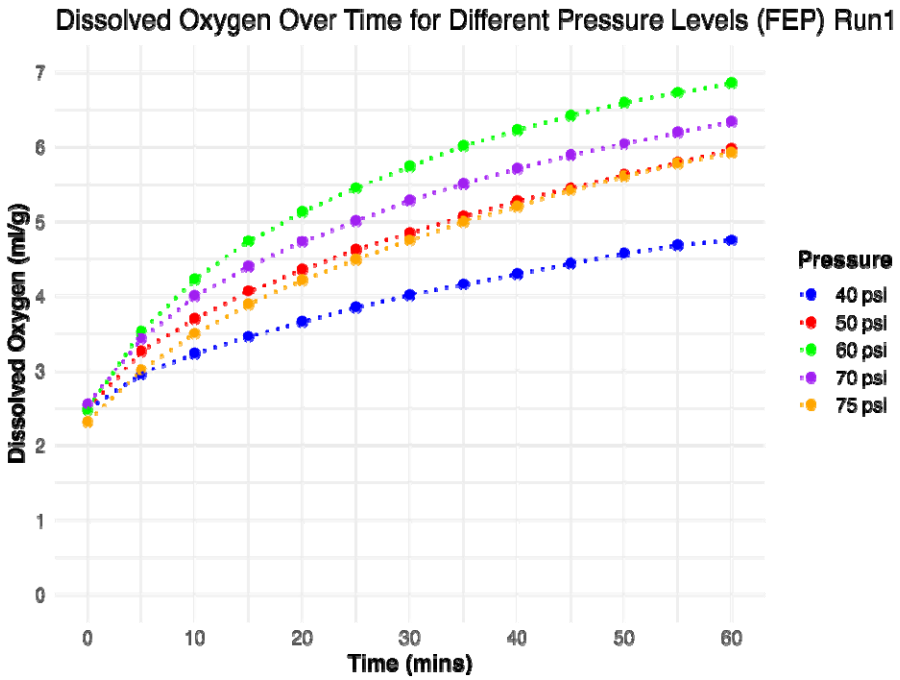

Figure 3.2.3: Dissolved oxygen concentrations over time during FEP apparatus tests with DoDI water. Oxygen canister pressures were set to 40 psi, 50 psi, 60 psi, 70 psi, and 75 psi. Dissolved oxygen concentrations were recorded every 5 minutes.

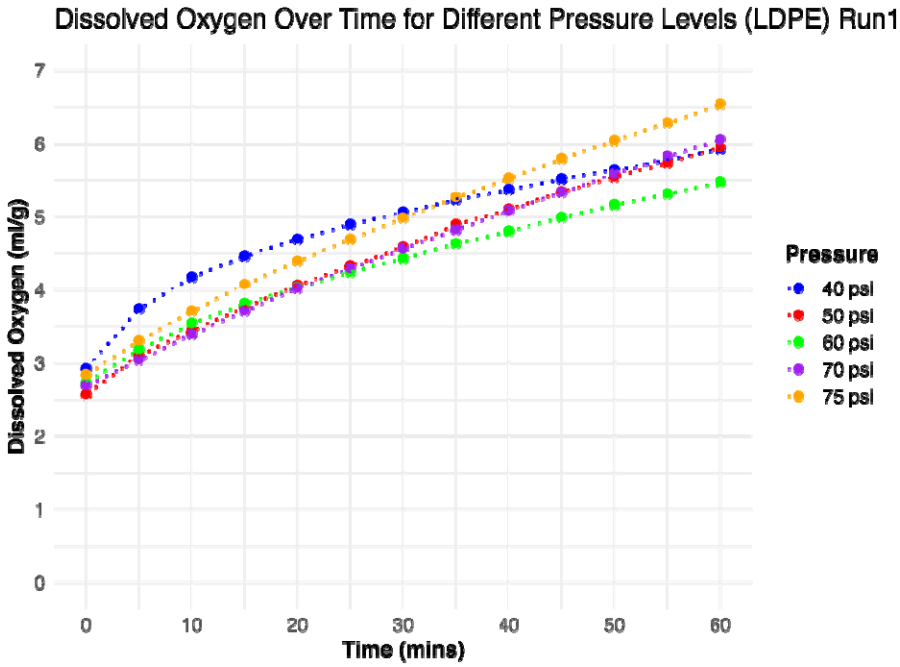

Figure 3.2.4: Dissolved oxygen concentrations over time during LDPE apparatus tests with DoDI water. Oxygen canister pressures were set to 40 psi, 50 psi, 60 psi, 70 psi, and 75 psi. Dissolved oxygen concentrations were recorded every 5 minutes.

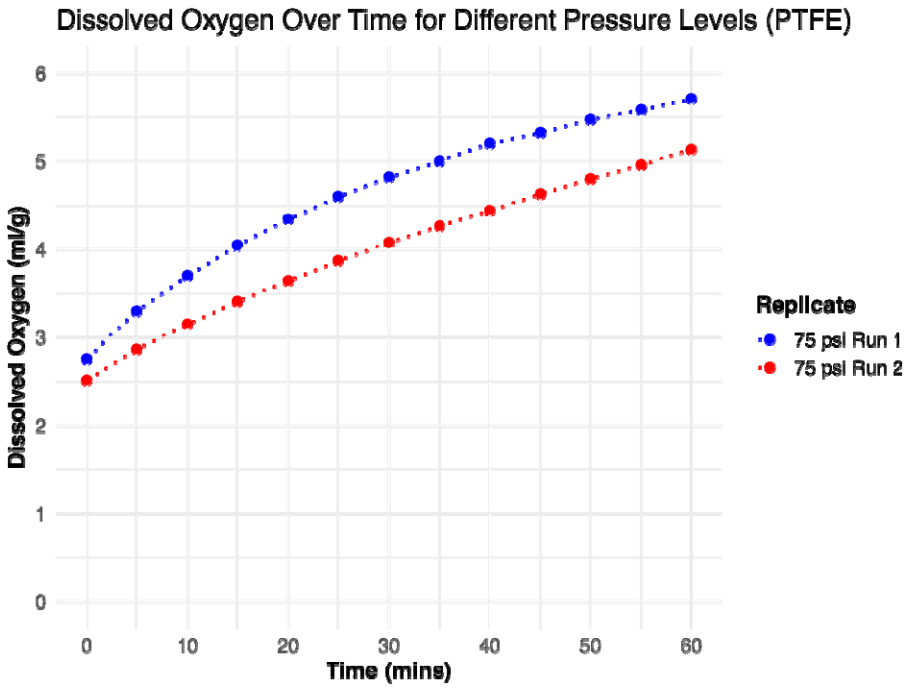

Figure 3.2.5: Dissolved oxygen concentrations over time during PTFE apparatus tests with DoDI water. Oxygen canister pressures were set to 75 psi only. Dissolved oxygen concentrations were recorded every 5 minutes.

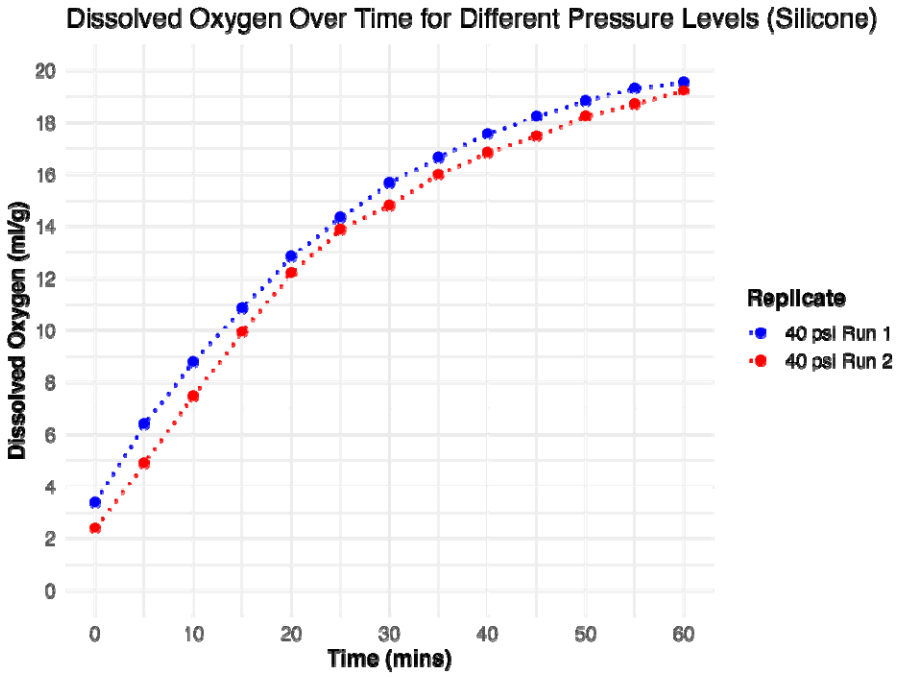

Figure 3.2.6: Dissolved oxygen concentrations over time during silicone apparatus tests with DoDI water. Oxygen canister pressures were set to 40 psi only. Dissolved oxygen concentrations were recorded every 5 minutes.

Oxygenation rates among the four aeration tubing materials were analyzed using ANOVA and Tukey's HSD tests (Table 3.2.1). A violin plot displaying the distributions of oxygenation rates for the four aeration tubing materials is shown in Figure 3.2.7. No significant differences were found between the performance of FEP, LDPE and PTFE tubing. Therefore, the latter two plastics were eliminated as candidates. On the other hand, silicone tubing significantly outperformed the three rigid plastic tubings, despite the silicone having a lower oxygen canister outlet pressure. After these tests, silicone was designated as the primary candidate tubing material for use in the iTIE oxygenation coil.

Table 3.2.1: Post-hoc Tukey's HSD test results comparing oxygenation rates between tubing materials with DoDI water.

Comparison	Mean Difference	95% CI (Lower - Upper)	Adjusted p-value	Statistical Significance
LDPE - FEP	-0.0096	(-0.0204, 0.0012)	0.0906	Not Significant
PTFE - FEP	-0.0114	(-0.0290, 0.0062)	0.2940	Not Significant
Silicone - FEP	0.2169	(0.1993, 0.2346)	<0.0001	Significant
PTFE - LDPE	-0.0018	(-0.0198, 0.0162)	0.9922	Not Significant
Silicone - LDPE	0.2266	(0.2086, 0.2446)	<0.0001	Significant
Silicone - PTFE	0.2283	(0.2056, 0.2511)	<0.0001	Significant

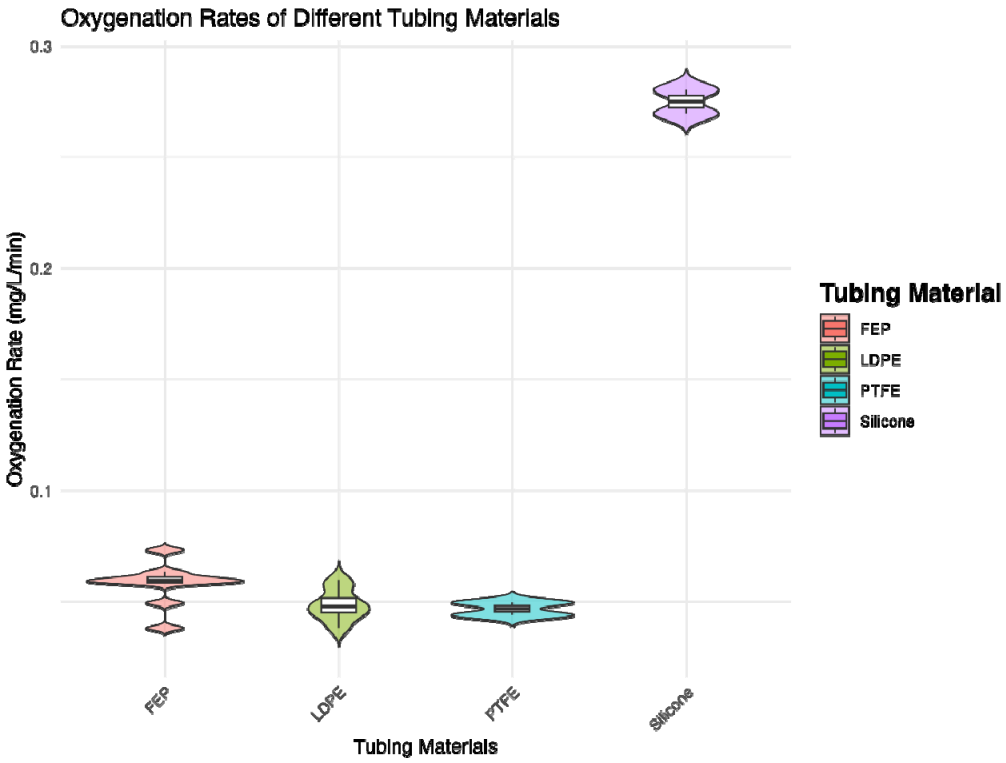

Figure 3.2.7: Violin plot of oxygenation rate in mg/L/min of different tubing materials with DoDI water. Silicone shows a more consistently higher oxygen diffusion rate than the other tubing materials.

3.2.3 Hydrogen Sulfide Water

The FEP and silicone apparatuses were assessed using DoDI water spiked with hydrogen sulfide. Sulfide-spiked test water was prepared by bubbling DI water with nitrogen gas until hypoxic and adding hydrogen sulfide-saturated water (Ricca Chemical) to varying sulfide concentrations, approximately between 10-100 mg/L to match a range of potential concentration expected at typical impacted marine sites. Actual sulfide concentrations were confirmed via iodometry before and after each run. The spiked water was then gently added to each apparatus to avoid volatilization of dissolved sulfides. The FEP tubing was tested at an oxygen canister pressure of 75 psi, while the silicone tubing was tested at 40 psi. DO levels were recorded every five minutes for one hour. Hydrogen sulfide concentrations were quantified before and after each experiment using Standard Method 4500-S2-F: Sulfide by Iodometry (Eaton & Franson, 2005).

The FEP apparatus test results with hydrogen sulfide water are shown in Figure 3.2.8. The apparatus was tested with water containing three initial concentrations of dissolved sulfide: 6.028, 35.058, and 75.004 mg S^{2-}/L. In all three experiments, DO levels increased moderately to 5.0-5.4 mg/L. These DO concentrations are safe for aquatic life, but close to causing hypoxia stress in some species. Thus, an FEP tubing oxygenation coil may underperform when sulfides are present.

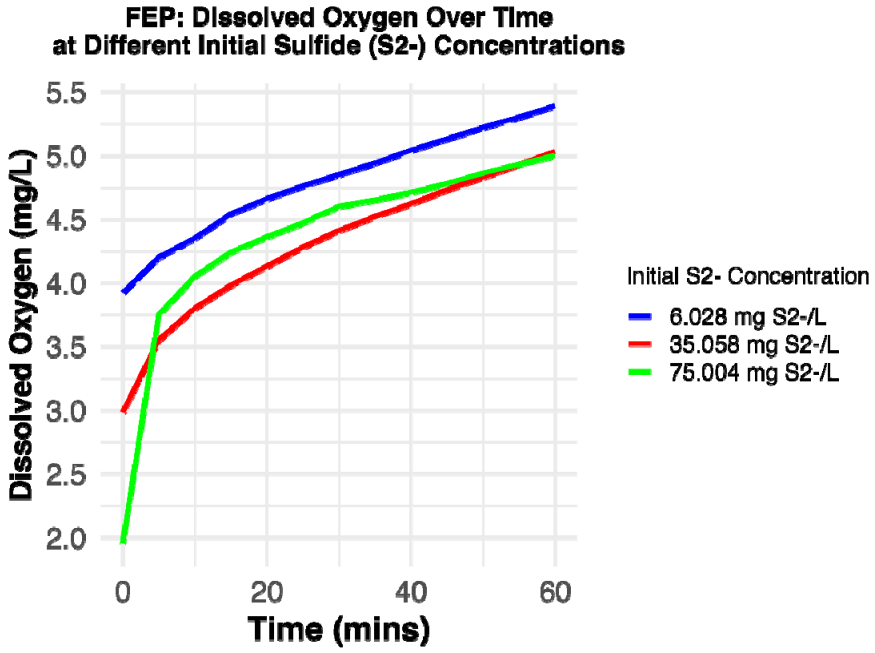

Figure 3.2.8: Dissolved oxygen concentration over time with the FEP apparatus with hydrogen sulfide water. The oxygen canister pressure was set to 75 psi. The initial hydrogen sulfide concentration was recorded at 6.028 mg S^{2-}/L, 35.058 mg S^{2-}/L, and 75.004 mg S^{2-}/L.

Changes in sulfide concentrations after exposure to the FEP apparatus are shown in Figure 3.2.9. At an oxygen pressure of 75 psi, the FEP apparatus decreased sulfide concentrations in test water by an average of 23.1% after one hour of exposure.

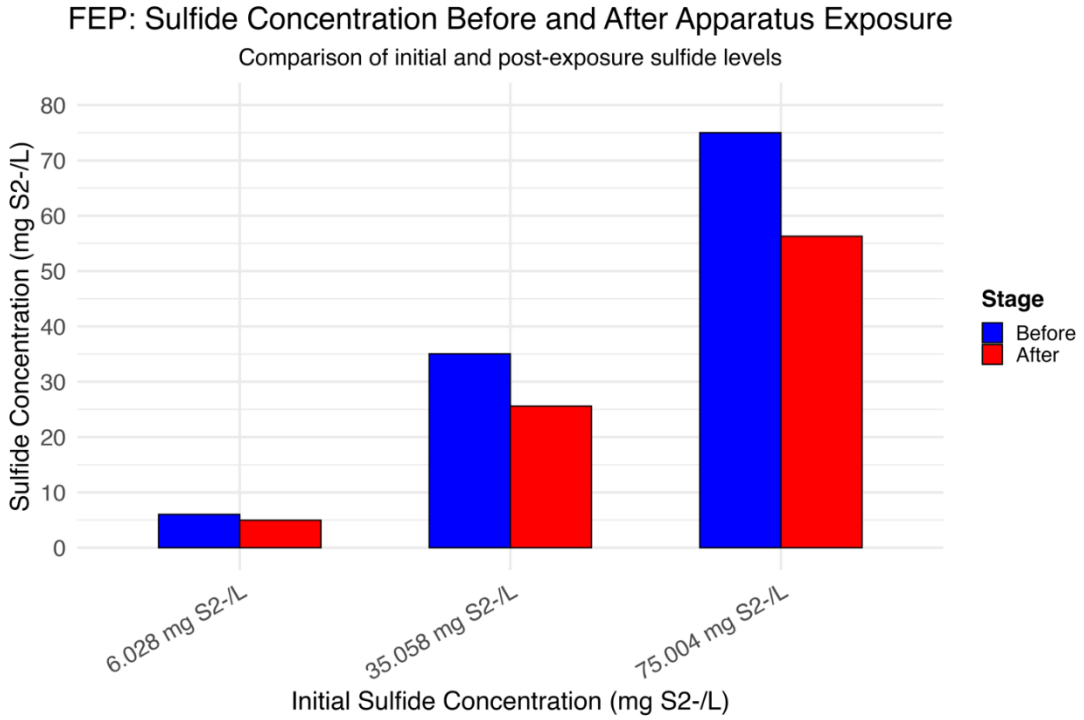

Figure 3.2.9: Sulfide concentrations before and after FEP apparatus exposure in test water with varying initial sulfide concentrations. The aeration tubing in the apparatus was pressurized with oxygen at 75 psi.

DO increased noticeably more quickly in the silicone apparatus when exposed to test water with varying initial dissolved sulfide concentrations (Figure 3.2.10). The apparatus was tested with three initial concentrations of dissolved sulfide: 10.230, 30.586, and 83.054 mg S^{2-}/L. At all three sulfide levels, DO increased from hypoxic levels (1.05-3.23 mg/L) to supersaturation (17.32-21.01 mg/L) after one hour of exposure. These results suggest that an oxygenation coil constructed with silicone may perform significantly better than one made from FEP, particularly in oxygenating porewater containing dissolved sulfides.

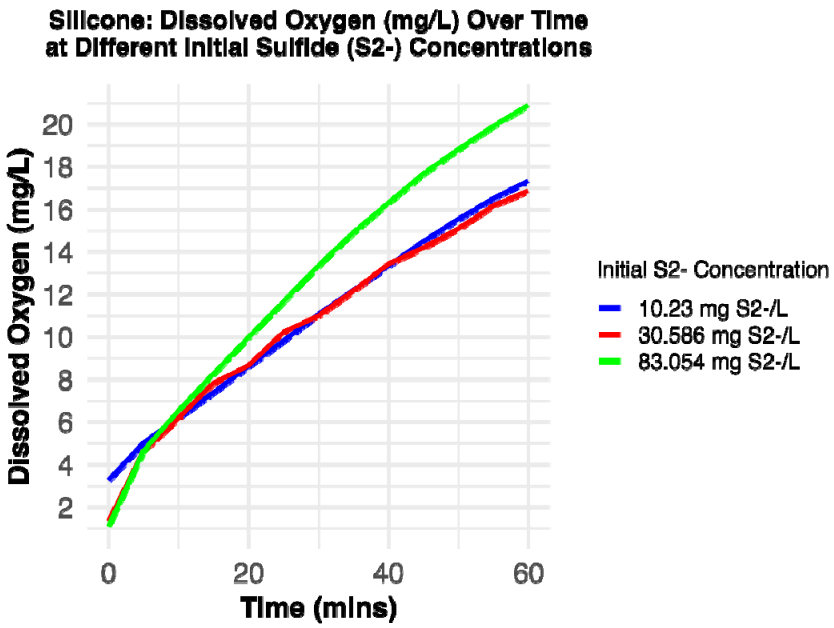

Figure 3.2.10: Dissolved oxygen concentrations during the silicone apparatus test with hydrogen sulfide water. The oxygen canister pressure was set at 40 psi. The initial hydrogen sulfide concentration varied at 10.23 mg S^{2-}/L, 30.586 mg S^{2-}/L, and 83.054 mg S^{2-}/L. The dissolved oxygen concentration is recorded every 5 minutes for 1 hour.

Changes in sulfide concentrations after exposure to the silicone apparatus at a pressure of 40 psi are shown in Figure 3.2.11. On average, the silicone apparatus decreased sulfide concentrations by 66.5%. These reductions in sulfide content are far more pronounced than the reductions observed with FEP tubing. From these results, silicone appears to be the ideal tubing candidate for use within an iTIES oxygenation coil.

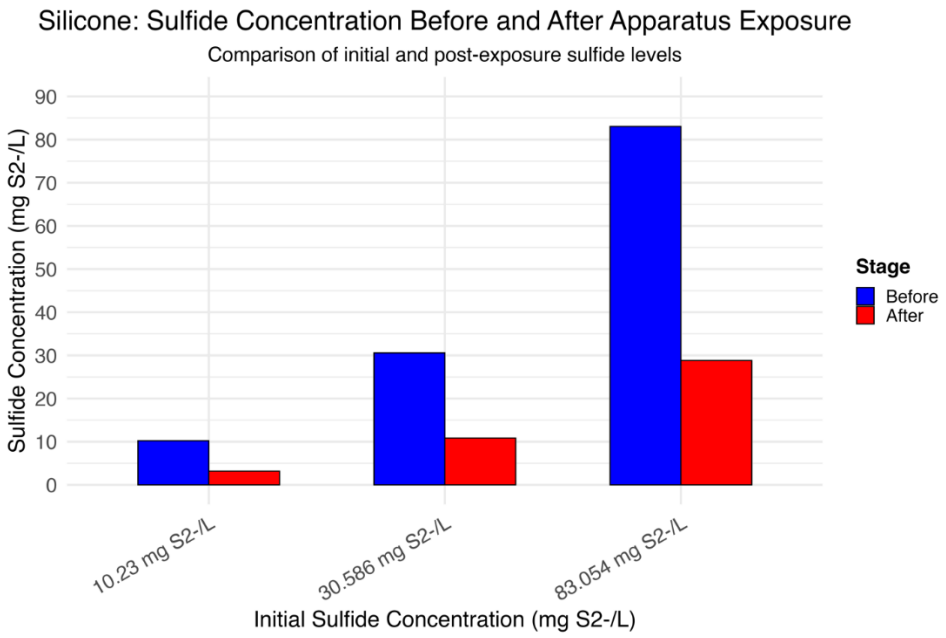

Figure 3.2.11: Sulfide concentrations before and after silicone apparatus exposure in test water with varying initial sulfide concentrations. The aeration tubing in the apparatus was pressurized with oxygen at 40 psi.

3.2.4 Prototype Amendments

Several prototype amendments were made based on the results of the apparatus tests. First, the oxygenation coil interior tubing was changed to silicone. Small pieces of FEP tubing were fitted on the ends of the silicone tube to allow for airtight connections with the oxygen canister and stopcock valve. The coil's exterior tubing was also substituted with reinforced PVC (Tygon) tubing. The length, cross-sectional radii, and other characteristics of the coil remained unchanged.

Additionally, a drip chamber was added to the iTIES directly below the oxygenation coil's exit. This was integrated to account for the excessive formation of oxygen gas bubbles in the coil, which was observed following the installation of silicone tubing. These bubbles disrupt the flow of water through the coil and pose a risk to resin beds and organisms. The drip chamber effectively sorts gas bubbles from the oxygenated water and routes them from the system. Excess sampled water is also routed from the system to an overflow collection bottle. This water can be monitored for water quality parameters like DO and analyzed for chemistry post-exposure.

The refined iTIES oxygenation coil was tested in a lab setting. After two hours of exposure to the oxygenation coil with a canister pressure of 20 psi, sulfide concentrations decreased by approximately 53.8% (from 91.67 to 42.36 mg S^{2-}/L), while DO increased from 1.79 mg/L to 14.73 mg/L. With a canister pressure of 40 psi, sulfide content decreased by 63.9% (from 104.74 to 37.84 mg S^{2-}/L), while DO increased from 1.47 mg/L to 19.17 mg/L.

After prototype adjustments were evaluated in-lab, a second iTIE deployment was completed at Paleta Creek in August 2024. This deployment is detailed in Section 7.1.

4.0 DIAGNOSTIC RESIN OPTIMIZATION

This section describes efforts to address Project Task 4: a continuation of resin optimization efforts. Resins are granular substances with physicochemical properties that allow them to selectively remove CoCs from water. During each iTIES deployment, diagnostic sorptive resins were chosen depending on site knowledge. Each resin typically only targets a narrow range of toxicant classes, allowing for precise control of which toxicants that organisms are exposed to in each exposure chamber. The following resins have been successfully used in iTIES exposures:

- Ambersorb 560, which can bind with general organics but is marketed to be effective against 1,4-dioxane
- C18, which targets nonpolar organic CoCs like organophosphates, PAHs, and pyrethroids
- Chelex, which binds with problematic heavy metals like nickel, copper, zinc, arsenic, cadmium, and lead
- GAC, which adsorbs a wide variety of CoCs including metals, organics, and sulfides
- Oasis HLB, which binds with general organic CoCs
- Oasis WAX, which targets general organic CoCs but is particularly effective with PFAS
- Zeolite, which primarily targets ammonia but can also bind with metals and PFAS.

Every resin requires proper conditioning prior to use to ensure that the resin itself does not cause toxicity to test organisms. For example, if a resin significantly alters the pH of influent site water during a deployment, then the toxicity observed in the associated organism group might be falsely attributed to toxicants in the site water. Because of this, several common diagnostic resins and conditioning procedures were assessed as sources of potential toxicity.

4.1 DAPHNIA MAGNA TOXICITY TEST METHOD

Each diagnostic resin was evaluated using a method based on ASTM Method E1706-19, a standard 7-day *Daphnia magna* toxicity test protocol. Resins were conditioned and placed into iTIE resin chambers between pads of glass wool, a lab-grade inert substance. *D. magna* neonates are collected from an in-house culture. The culture water used is known as Moderately Hard Reconstituted Water (MHRW), comprised of DI water fortified with $NaHCO_3$ (165 mg/L), NaBr (2 mg/L), $MgSO_4$ (101.1 mg/L), KCl (9.9 mg/L), and $CaSO_4$ (69.2 mg/L). Ten *D. magna* neonates (approximately 4-5 days post-hatch) are placed inside each iTIE organism chamber in MHRW. *D. magna* groups are fed 2 mL of Sel-Cero, which is comprised of *Raphidocelis subcapitata* algae (1×10^7 cells/L) and cerophyl. The iTIE units are connected to peristaltic pumps and receive MHRW at a rate of 10 mL/hour. Organisms are exposed for either 24 or 48 hours. Once per day, water quality measurements are collected, including DO (YSI ProODO), pH (Thermo Orion Star A121), conductivity (YSI ProDSS), hardness (test strip), alkalinity (test strip), and ammonia (test strip).

Additionally, two control groups were established concurrently with each resin test: a lab control group and glass wool control group. Lab control groups (n=10 organisms) were established in individual 100 mL beakers containing 80 mL of MHRW. Beakers were placed in a temperature-controlled room at 21±1°C and a 16L:8D photoperiod. Lab control organisms were fed 0.6 mL of Sel-Cero at the beginning of the iTIE exposure. Glass wool control groups (n=10 organisms) were established in an iTIE unit with a resin chamber only containing glass wool. The glass wool control group was treated like other iTIE resin groups. Both control groups must have at least 80% survival to meet minimum test acceptability criteria.

At the end of each iTIE exposure, organism survival was enumerated as a measure of acute toxicity. Surviving organisms were placed in 100 mL beakers containing 80 mL of MHRW in the temperature-controlled room. Culturing procedures include water changes and feedings (0.6 mL of Sel-Cero) once every two days. In some tests, organisms were cultured until all surviving organisms had brooded once (7-day procedure). In other tests, organisms were raised for 21 days, until all organisms had had three broods. Chronic toxicity endpoints include time to first brood, number of neonates per brood, and ash-free dry weight (AFDW).

4.2 PFAS RESIN INVESTIGATION

PFAS are an emerging class of CoCs at various target sites. Because of this, it is important for the iTIES to have a resin that can successfully target this broad chemical class. To select a resin for this role, the research team identified three candidate resins marketed for PFAS adsorption: Oasis WAX (Waters), Strata-X-AW (Phenomenex), and Dexsorb (Cyclopure). Toxicity tests were performed to determine which of these resins induces minimal stress to organisms and are therefore compatible with use in an iTIE exposure.

4.2.1 Methods

Each resin was conditioned in adherence with manufacturer recommendations. Oasis WAX and Strata-X-AW were conditioned by first rinsing with a small amount of methanol to remove artifacts from the surface of the resin particles, and to help water bind to the resin. Then, both resins were placed on 20 µm Nitex mesh and rinsed thoroughly with DI water. After rinsing, the resins were submerged in excess DI water and allowed to equilibrate. The above process was repeated daily for at least three days prior to test initialization. Dexsorb was conditioned with repeated rinses and submersions in excess DI water daily for at least three days.

The three resins were assessed using the 21-day toxicity test schedule. Organisms were exposed in iTIE units to the three resins for 24 hours. After exposure, surviving organisms were counted and cultured until reaching at least the third brood. Data collected at the end of the culturing portion of this test included survival at various points throughout the duration of the test and average number of neonates per brood. Because there was a decline in survival at the halfway point of the test, total survival is discussed more broadly at various points of the test.

4.2.2 Results and Discussion

Some trends are clearly visible in organism survival proportions from this test (Figure 4.2.1). Within a few days following the exposure portion of the test, the Strata-X-AW treatment exhibited complete mortality. Given that this mortality was observed well before any other

survival declines in other treatment groups, it can be concluded that the resin caused acute toxicity to test organisms. The survival trends for Oasis WAX and Dexsorb are less conclusive. Both resin treatment groups had adequate survival until the halfway point of the culturing period, but they declined below the culture groups soon after. Given that the control groups saw a decline in survival as well, it is difficult to determine the degree to which the decrease in survival was due to resin treatments or due to overall culture health.

Reproduction endpoints convey a more positive narrative for Oasis WAX and Dexsorb (Figure 4.2.2). For these resin treatments, no significant difference was found in average number of neonates per brood compared to the control groups. This endpoint suggests that these resin treatments did not cause reproductive stress. However, this datapoint may have been impacted by the decline in organism health during the latter half of the culturing period and should thus be regarded with caution.

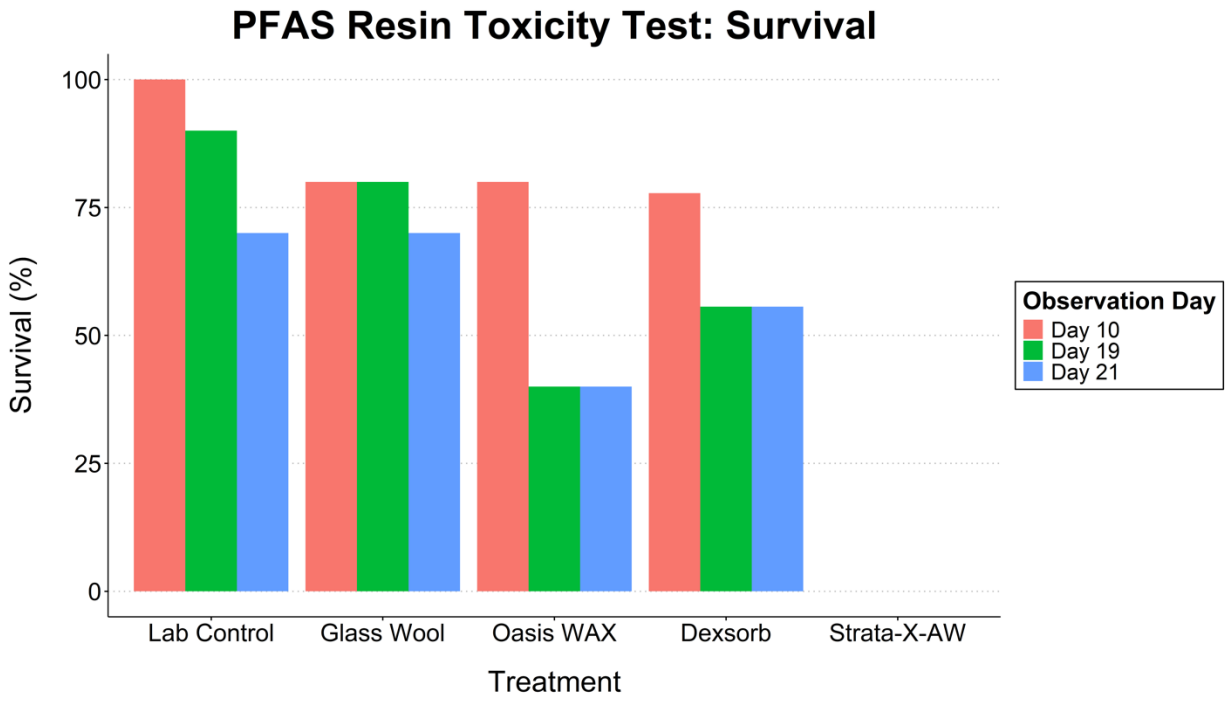

Figure 4.2.1: Total survival for the PFAS resin *D. magna* chronic toxicity test. Dexsorb survival is out of 9 organisms due to an experimental error. Note that survival here is recorded at multiple checkpoints. Day 10 represents a rough midpoint of the test, Day 19 is the penultimate culturing day, and Day 21 is the final day of the culturing portion.

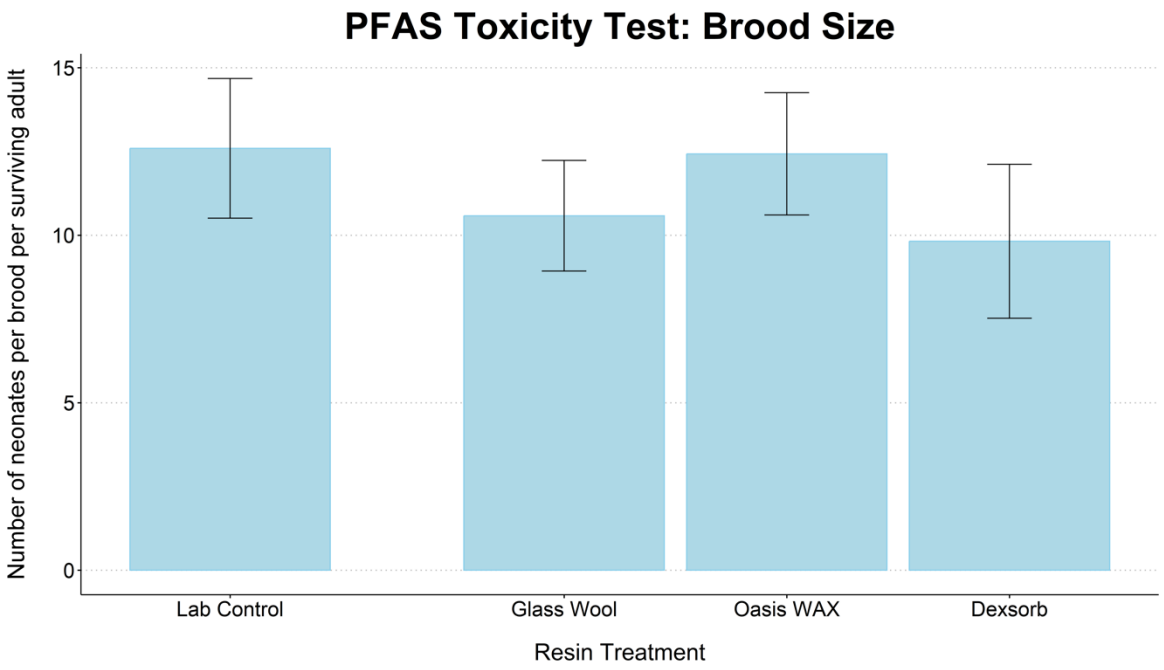

Figure 4.2.2: Number of neonates per brood for the PFAS resin *D. magna* chronic toxicity test. Neonates were counted during the culturing portion. Error bars are the standard deviation within each group. No significant difference was found between groups.

Oasis WAX was determined to be the optimal PFAS resin for use in iTIES experiments due to its strong survival and reproduction data compared to the other resins. There is evidence from reproduction endpoints that Oasis WAX does not cause chronic stress. However, the decline in survival during the culturing portion of the experiment compared to the glass wool control suggests that Oasis WAX may cause a degree of stress to test organisms. This resin has since been used successfully in multiple field deployments without observing adverse effects on organism health.

4.3 CHELEX CONDITIONING INVESTIGATION

Third-row transition metals and heavy metals are among the most common toxicant classes expected at industrial impacted sites. As such, it is necessary to identify a reliable and effective resin for adsorption of heavy metals in iTIES deployments. In the past, this role was fulfilled by Chelex 100 (Bio-Rad Laboratories), a resin with a high affinity for divalent and transition metal species (Bio-Rad Laboratories, n.d.; Sigma-Aldrich, 1996). The resin had been used successfully in previous exposures utilizing marine test organisms. However, in laboratory iTIE experiments utilizing freshwater test organisms, high mortality was observed in iTIE groups after fractionation using the standard sodium form of the resin. Despite thorough rinsing with DI water, the resin continued to alter water quality parameters outside of bounds supporting aquatic life, including raising pH as high as 11 and decreasing hardness levels to less than 25 mg $CaCO_3$/L. Due to this, the research team investigated different conditioning methods to reduce the ability of Chelex to change water quality parameters and induce stress to organisms.

4.3.1 Methods

Chelex 100 is manufactured in its sodium form. The recommended conditioning protocol includes rinsing with DI, submersion in DI, and equilibration over a period of several days. The resulting resin is referred as "sodium-form Chelex" or "Chelex-Na".

According to the manufacturer, Chelex has an extremely low affinity for sodium, a higher affinity for calcium and magnesium, and an extremely high affinity for transition and heavy metals (Bio-Rad Laboratories, n.d.). In solutions with moderate hardness levels, Chelex preferentially removes calcium and magnesium ions from solution, replacing them with sodium ions and thereby reducing water hardness (Bio-Rad Laboratories, n.d.). To avoid this effect, the research team converted a sample of Chelex 100 resin from sodium form to calcium form (Chelex-Ca) prior to iTIE use. This conversion involves replacing Na^+ ions with Ca^{2+} and Mg^{2+} ions at the iminodiacetate functional groups on the resin surface (Bio-Rad Laboratories, n.d.). To accomplish this, the resin was submerged in excess 2N $CaCl_2$ two times and allowed to sit overnight prior to the beginning of the rinsing and equilibration process.

Additionally, the research team sought to mitigate Chelex-Na's alkalizing properties while maintaining resin effectiveness. Sodium-form Chelex has an equilibrium pH of 11, a property confirmed through initial testing (Bio-Rad Laboratories, n.d.). The manufacturer recommends mitigating this effect by rinsing with 0.5M sodium acetate buffer and DI water. However, the research team found that repeated rinsing with and submersion in DI water was adequate to neutralize the surface chemistry of Chelex.

Toxicity tests were performed using Chelex-Na and Chelex-Ca. These tests followed the 7-day *D. magna* toxicity test procedure. The following treatments were used: Chelex-Na (1 rep), Chelex-Ca (2 reps), and lab and glass wool controls. Data collected at the end of the culturing period included total survival, number of neonates per brood, and time to first brood. Growth measurements via AFDW were not collected for this test.

Reproduction endpoints, including time to first brood and number of neonates per brood, were compared using ANOVA and post-hoc Tukey multiple comparisons of means tests with 95% family-wise confidence intervals. For this test, the time to first brood dataset did not satisfy the normalization assumption.

4.3.2 Results and Discussion

There was a notable difference in survival between Chelex-Na and Chelex-Ca treatments (Figure 4.3.1). At the conclusion of the culturing portion, the lab control, Chelex-Ca, and Glass Wool Control treatments all had at least 90% survival. In contrast, the Chelex-Na treatment had only one surviving organism by the end of the test. It should be noted that the 9 deaths in the Chelex-Na group occurred during the exposure portion of the test, demonstrating that the sodium form of the resin caused acute toxicity to test organisms. Water quality was measured for the effluent water of each treatment group at the 24-hour and 48-hour mark. At the 48-hour mark, Chelex-Na had significantly altered the pH, conductivity, and hardness of the source water (Table 4.3.1). In addition, Chelex-Ca had a mildly altered pH value, but all other water quality metrics appeared roughly the same as the controls. Acute toxicity in the Chelex-Na treatment was likely caused either by the alkaline pH, low hardness values, or both. It is possible that a

sudden change in these metrics induced shock to test organisms, leading to mortality. While Chelex-Ca also lowered the source water pH, it does not appear to have caused acute toxicity.

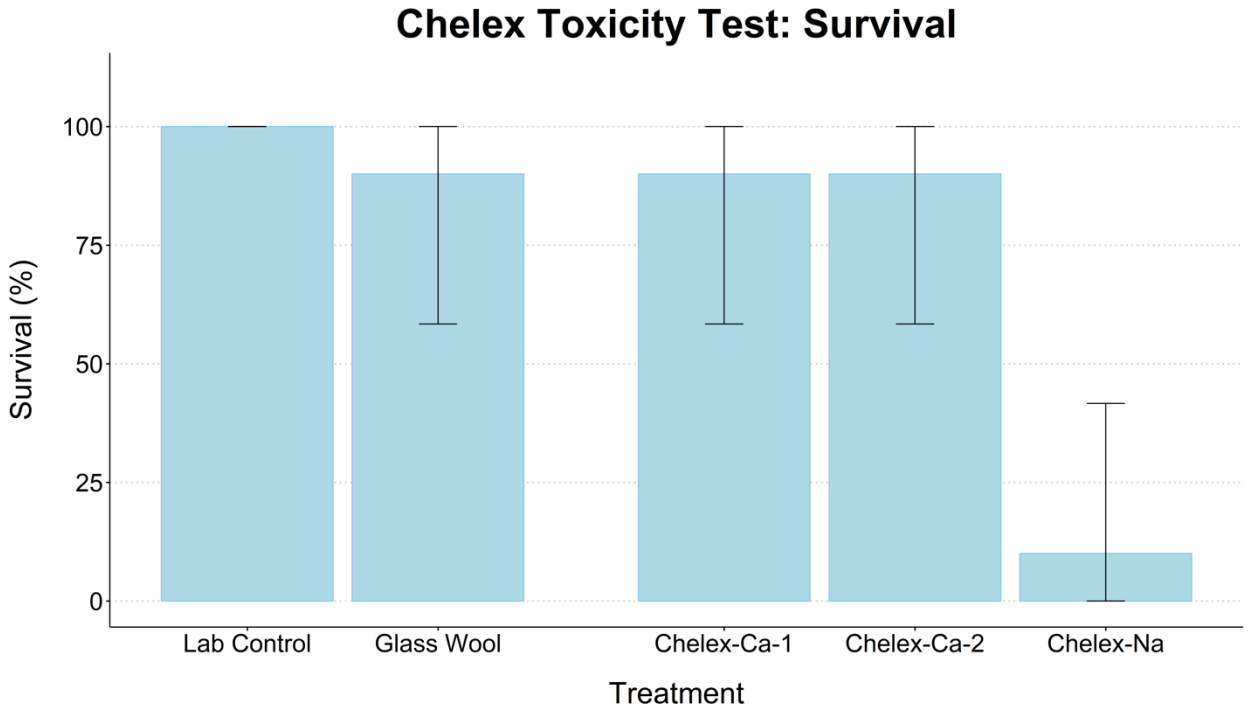

Figure 4.3.1: Total survival for the Chelex *D. magna* toxicity test. The two treatment groups utilizing a calcium-form Chelex are denoted as "Chelex-Ca-1" and "Chelex-Ca-2", while the treatment group utilizing sodium-form Chelex is denoted as "Chelex-Na". The error bars indicate standard deviations within each treatment (n=10 organisms), truncated between 0 and 100%.

Table 4.3.1: Water quality metrics measured at the 48-hour mark of the Chelex iTIE exposure. Underlined measurements denote the ones that are explicitly discussed. Asterisk (*) denotes a rough estimate from water quality measurements that were taken with test strips. All other metrics were measured with meters. NA = measurement that was not taken (human error).

Water Quality Metric	Source Water	Chelex Ca-form Rep. 1	Chelex Ca-form Rep. 2	Chelex Na-form	Glass Wool Control
Temperature (°C)	21.2	21.9	21.8	21.9	22.0
DO (mg/L)	7.23	6.49	6.70	6.55	6.94
pH	8.17	7.04	7.07	9.47	8.06
Conductivity (µS/cm)	508	493	491.1	603	520
Hardness (mg/L $CaCO_3$) *	NA	150	150	0-25	150
Alkalinity (mg/L) *	80	40-80	40-80	120	80
Ammonia (mg/L) *	0	0	0	0	0

The reproduction toxicity endpoints convey a trend that is mostly consistent with the survival data, with some important caveats. For the average number of neonates per brood, significant differences were found between the lab control group and every iTIE group (Figure

4.3.2). On average, the lab control group had roughly double the average brood size of the iTIE treatments. However, brood sizes were not significantly different among iTIE groups.

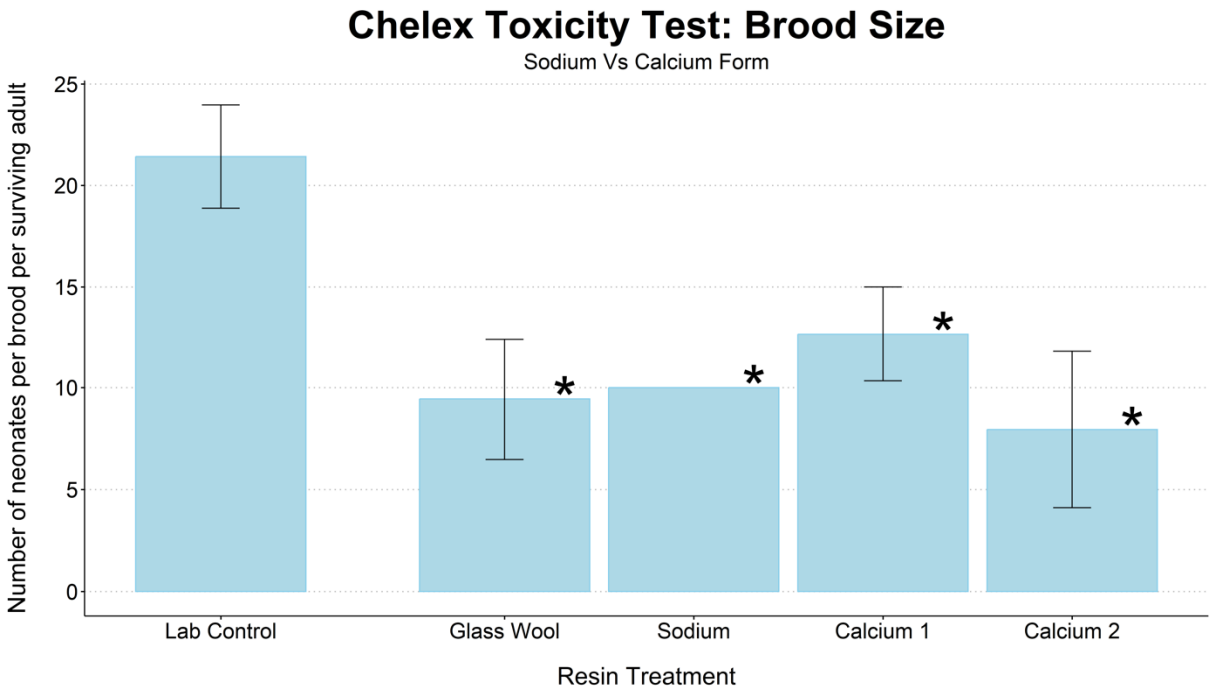

Figure 4.3.2: Average number of neonates per brood for the Chelex *D. magna* chronic toxicity test. The error bars indicate standard deviations within each treatment (n=10 organisms). An asterisk (*) denotes a statistically significant difference from the Lab Control group ($p < .05$).

The same trend can be seen in time to first brood (Figure 4.3.3). Significant differences were found between each iTIE group and the lab control group, but no significant differences were found within the iTIE groups themselves. On average, the lab control organisms had their first brood multiple days earlier than the organisms subjected to the iTIE protocol, regardless of resin treatment.

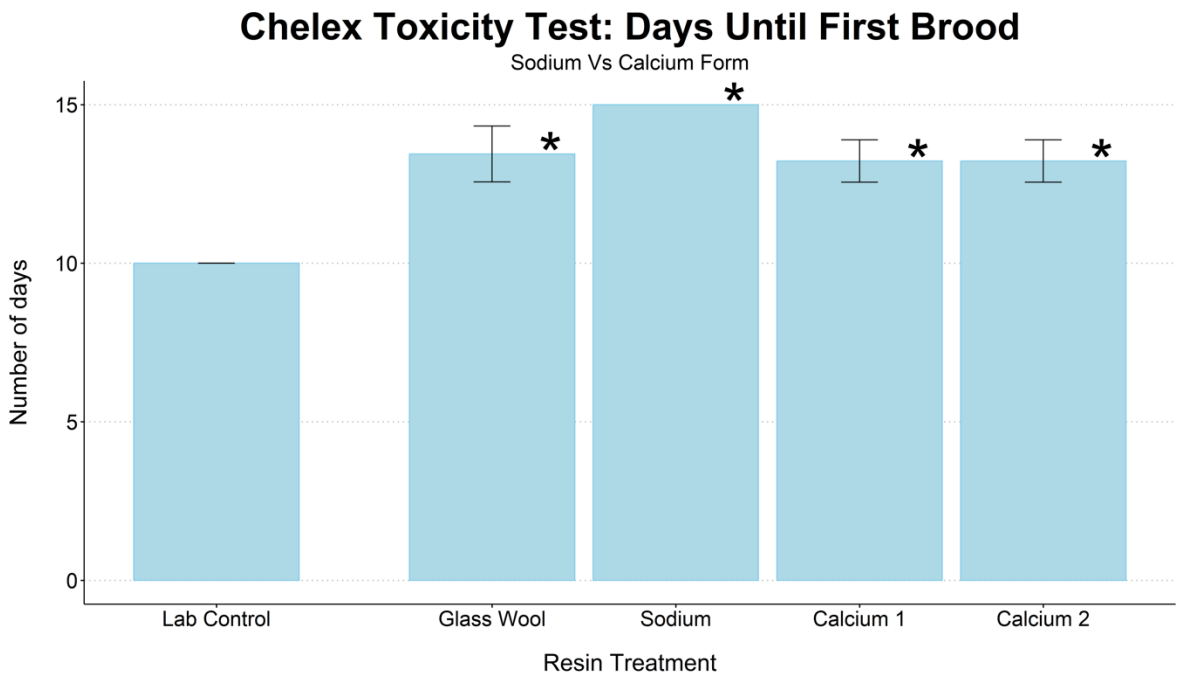

Figure 4.3.3: Average number of days until first brood for the Chelex *D. magna* toxicity test. Day 1 is the collection date of organisms (<24 hours old). The error bars indicate standard deviations within each treatment (n=10 organisms). An asterisk (*) denotes a statistically significant difference from the Lab Control group ($p < .01$).

The sodium form of Chelex causes high acute toxicity to *D. magna*. The single surviving organism did not display significant chronic toxicity in reproduction endpoints. However, this sample size was very small (n=1), and thus conclusions must be considered with this caveat.

Mechanisms for the Chelex resin's impact on influent water quality are only speculative, but available information about the surface chemistry of the resin agrees with observed phenomena. The functional group on Chelex has a higher affinity for Ca^{2+}/Mg^{2+} than Na^{+}, so Chelex-Na reduces hardness in influent source water by exchanging Ca^{2+}/Mg^{2+} ions with the Na^{+} ions bound to its surface (Bio-Rad Laboratories, n.d.). By equilibrating Chelex-Na in a calcium chloride solution, sodium ions occupying active sites on the resin are replaced with calcium ions, preventing that ion exchange from occurring in sampled water exposed to organisms. This form of the resin should still be effective for removing metal toxicants from the source water without directly causing toxicity to test organisms.

Additionally, the functional groups in their deprotonated form are inherently basic, reaching equilibrium at a pH of 11 (Bio-Rad Laboratories, n.d.). The resin utilizes paired iminodiacetate ions at its surface to chelate its metal targets, a mechanism that may abstract protons from the source water and cause an initial spike in pH (El-Bahy, 2018). Repeated conditioning via DI rinses and equilibrations may mitigate the alkalizing effect of Chelex by protonating some iminodiacetate functional groups.

When properly conditioned, Chelex-Ca is a robust candidate for the removal of transition and heavy metal toxicants from site water. For use in freshwater iTIE experiments, it is recommended that Chelex is thoroughly conditioned by: 1) equilibration in a calcium solution to convert binding sites from sodium form to calcium form, and 2) repeated rinsing and

equilibration in DI water to mitigate the resin's tendency to increase water pH. When properly conditioned, Chelex-Ca is a robust candidate for the removal of transition and heavy metal toxicants from site water.

Interestingly, the results of this toxicity test show that the iTIE protocol itself may have been a source of stress to test organisms. This trend was also noted in a similar investigation on granular activated carbon (Section 4.4), and it is a topic that warrants its own investigation (Section 4.5).

4.4 GRANULAR ACTIVATED CARBON (GAC) CONDITIONING INVESTIGATION

Granular activated carbon (GAC_ is a sorptive medium for a wide array of CoCs, including general organic chemicals and sulfides. GAC has the potential to serve as a "catch-all" medium in iTIE applications due to its varied surface chemistry, porosity, and high surface area. However, there are barriers to the usage of this medium if it is not prepared properly prior to use. For example, preliminary tests using unconditioned GAC have shown that GAC can greatly diminish DO concentrations and alter pH levels of influent water while releasing suspended solids from its surface. Because of this, the research team investigated various conditioning strategies to prevent GAC from causing organism stress in iTIE experiments.

4.4.1 Methods

All experiments utilized a bituminous petrogenic GAC sourced from Marineland® (Black Diamond® Media Premium Activated Carbon). Other types of GAC, including biogenic GAC, should be tested separately prior to iTIE use. The general GAC conditioning protocol included thorough rinsing with DI water on a 500-µm sieve, followed by submersion in excess DI water and overnight equilibration. In some instances, certain GAC treatments were continuously aerated using an air stone while equilibrating in DI water.

The first experiment evaluated an extended GAC conditioning method. GAC was rinsed and equilibrated daily for 12 days. For the first nine days of conditioning, the GAC medium was equilibrated in unaerated DI water. During the final three days, the GAC equilibration water was aerated continuously using an air stone. Two iTIE groups were set up using the conditioned GAC (*D. magna* neonates, 4-5 days post-hatch, n=10 per group). Additionally, two glass wool control groups (n=10) and one lab control group (n=10) were established. Exposures lasted for two days. Organisms were cultured post-exposure for five additional days. Data collected at the end of the test included total survival, number of neonates per brood, time to first brood, and growth in the form of AFDW. Water quality measurements were collected throughout the test duration. For the chronic toxicity test, it should be noted that neither the lab control nor the glass wool control organisms reached the 20 neonates per brood threshold recommended by ASTM Method E1706-19.

A second experiment evaluated a shortened GAC conditioning method. In this test, the conditioning period lasted for two days only. In one treatment, GAC was rinsed and equilibrated with DI water with no aeration (GAC-Unaerated). In a second treatment, GAC was rinsed and equilibrated in continuously aerated DI water (GAC-Aerated). A third treatment utilized unconditioned GAC, which was only wetted with a small amount of MHRW immediately prior to use (GAC-Unconditioned). iTIE groups were established for each of the three resin

treatments, as well as a glass wool control group and a lab control group. Exposures lasted for 24 hours. Total survival was recorded at the end of exposure, as well as water quality parameters. Organisms were not cultured post-exposure.

For time to first brood and number of neonates per brood, treatment groups were compared using a Tukey multiple comparisons of means test with 95% family-wise confidence intervals. The time to first brood data did not meet the normalization assumption. Similarly, a one-sided Student's T-test was used to compare AFDW measurements between groups.

4.4.2 Results and Discussion

In the first experiment evaluating an extended conditioning period, all treatment groups had high survival counts throughout the test duration (Figure 4.4.1). Relatedly, there were no noticeable differences in any of the seven standard water quality metrics during the exposure portion of the toxicity test (Table 4.4.1).

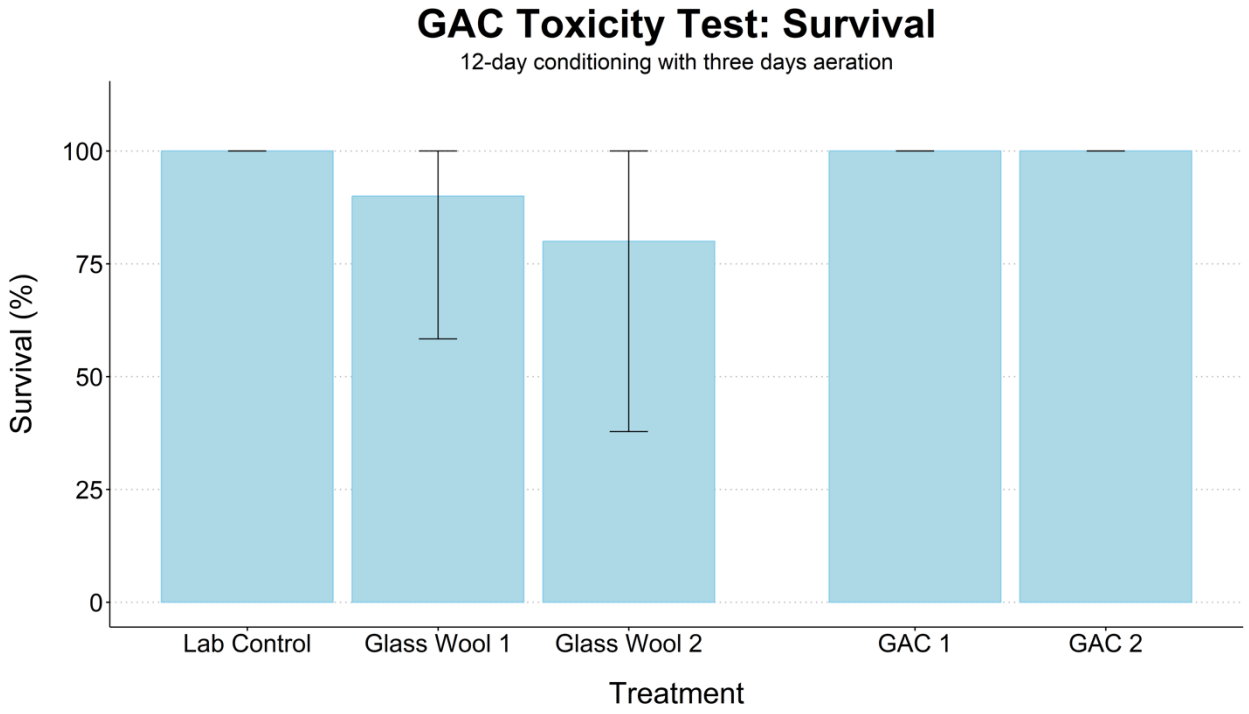

Figure 4.4.1: Total survival for the GAC extended conditioning *D. magna* toxicity test. Survival is listed out of 10 total organisms present at the start of the test. Note that GAC and glass wool each had two replicate groups. The error bars indicate standard deviations within each treatment (n=10 organisms), truncated between 0 and 100%.

Table 4.4.1: Water quality metrics measured at the 48-hour mark of the GAC extended conditioning toxicity test. Asterisk (*) denotes a rough estimate from water quality measurements that were taken with test strips. All other metrics were measured with water quality sensors.

Water Quality Metric	Source Water	Glass Wool 1	Glass Wool 2	GAC 1	GAC 2
Temperature (°C)	22.8	24.1	24.1	24.1	24.0
DO (mg/L)	8.26	7.82	7.80	7.31	7.33
pH	8.26	7.79	7.74	7.93	7.99
Conductivity (µS/cm)	545	590	574	562	556
Hardness (mg/L $CaCO_3$) *	150	150	150	150	150
Alkalinity (mg/L) *	80	90	90	90	90
Ammonia (mg/L) *	0	0	0	0	0

For the number of neonates per brood, no significant difference was found between conditioned GAC groups and control groups (Figure 4.4.2). For AFDW, only one significant difference was detected among groups, between Glass Wool 1 and GAC 1 (Figure 4.4.3). However, with this difference, organisms in GAC 1 were found to have significantly higher average AFDWs than organisms in Glass Wool 1, so this difference does not translate to a meaningful negative impact caused by GAC.

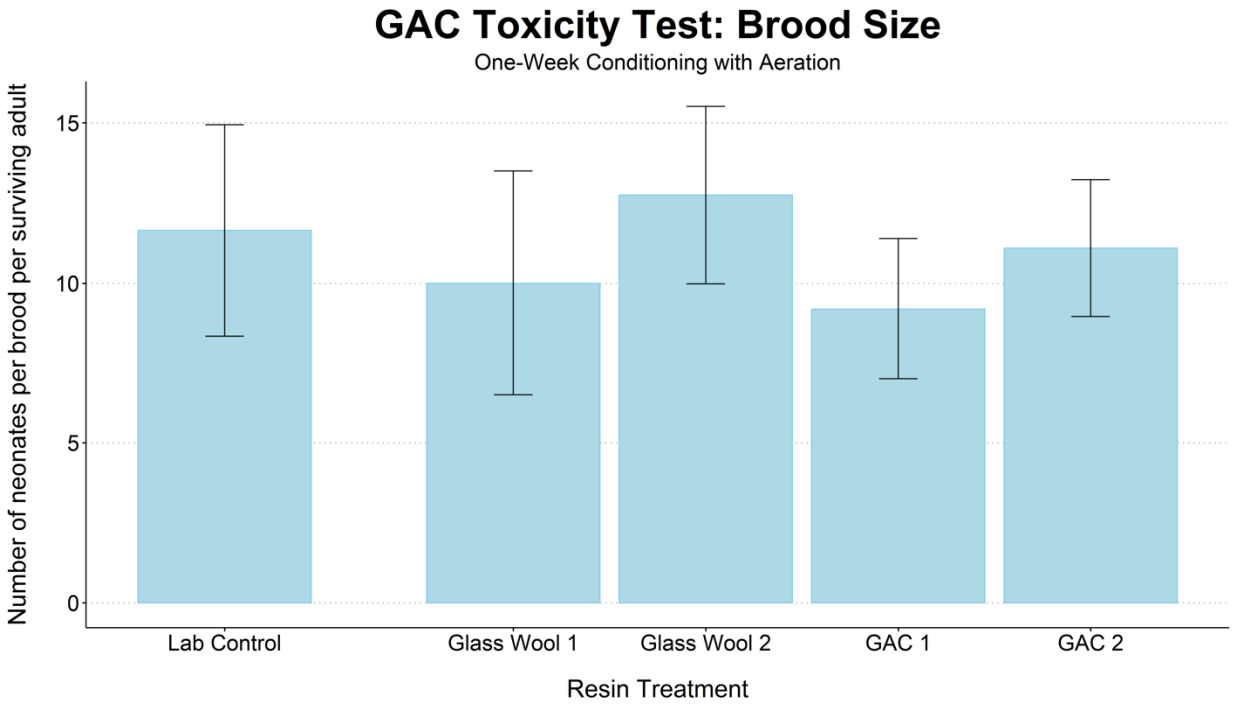

Figure 4.4.2: Average number of neonates per brood for the GAC extended conditioning *D. magna* chronic toxicity test. The error bars indicate standard deviations within each treatment (n=10 organisms). No significant difference was found between groups.

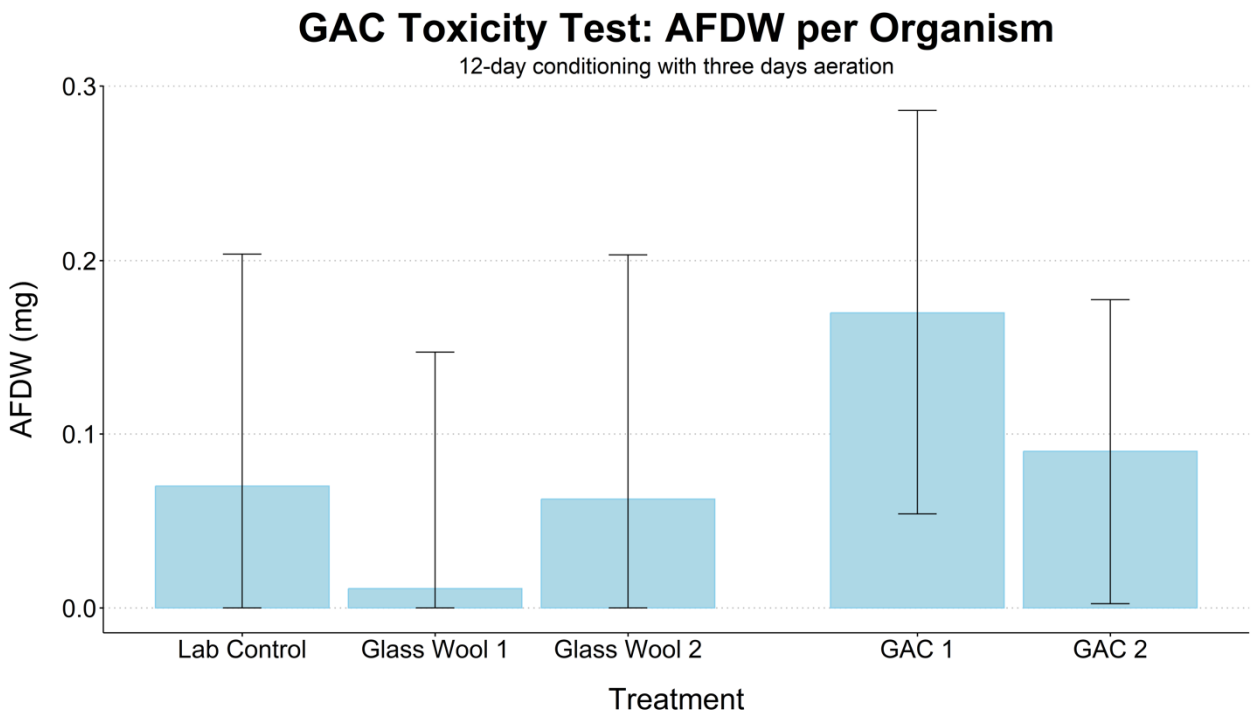

Figure 4.4.3: Mean ash-free dry weight, measured at the termination of the GAC extended conditioning D. magna toxicity test. The error bars indicate standard deviations within each treatment (n=10 organisms), truncated to not extend below zero.

For time to first brood, a significant difference can be detected between lab controls and iTIE groups, including both conditioned GAC groups and glass wool control groups (Figure 4.4.4). The lab control replicates, on average, brooded multiple days earlier than every iTIE group. However, no differences were found between the iTIE groups themselves, which may indicate that the iTIE procedure induced stress on organisms. The results of the Chelex toxicity tests exhibited a similar pattern, indicating a need for further investigation (Section 4.5).

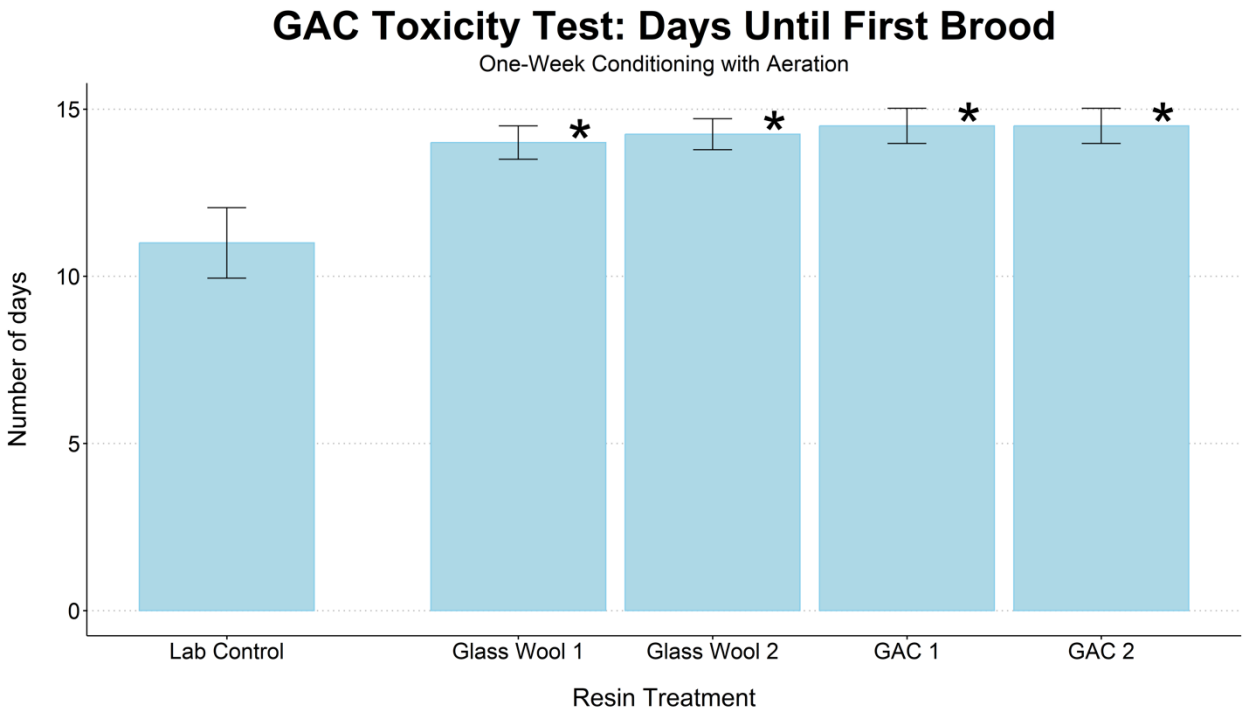

Figure 4.4.4: Average number of days until first brood from the GAC extended conditioning toxicity test. The number of days is measured from the end of the iTIE exposure portion of the test. Error bars display standard deviation. An asterisk (*) denotes a statistically significant difference from the lab control group ($p<.01$).

It can be concluded from the first test that GAC, when properly conditioned, can be used in iTIE experiments without causing stress to organisms. However, the research team was interested in whether GAC could be conditioned using an expedited procedure. A second test aimed to address this question.

The results of the second experiment evaluating a shortened conditioning period were stark (Figure 4.4.5). The GAC-Aerated treatment group had complete survival after 24 hours of exposure. Meanwhile, both the GAC-Unaerated and GAC-Unconditioned groups had no surviving organisms. Water quality was measured for the effluent water of each treatment group at the 24-hour mark (Table 4.4.2). Of the seven metrics, only pH appeared to be significantly impacted by unaerated and unconditioned GAC, while pH appeared to be unaffected by aerated GAC. This phenomenon was also noted during the conditioning process for both toxicity tests, with equilibration water pH typically spiking above 9.

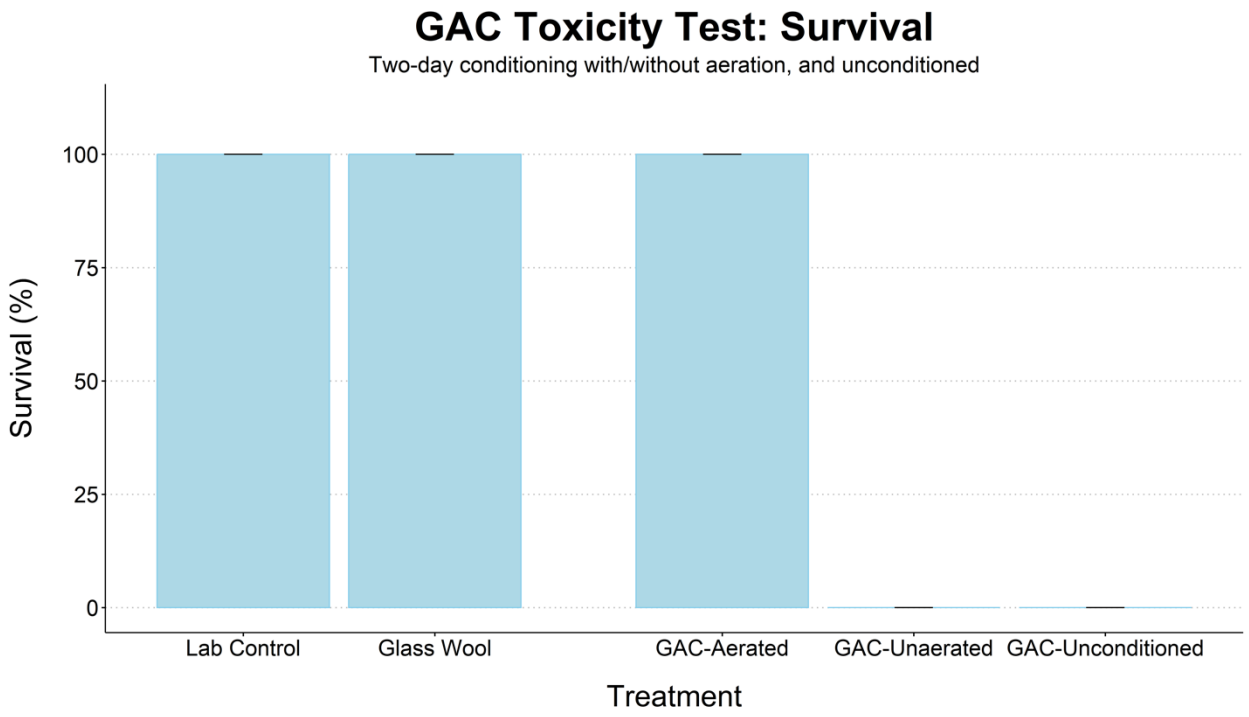

Figure 4.4.5: Total survival for GAC shortened conditioning toxicity test. The aerated and conditioned GAC treatment is denoted as "GAC-Aerated". The unaerated and conditioned GAC treatment is denoted as "GAC-Unaerated". The unconditioned GAC treatment is denoted as "GAC-Unconditioned". The error bars indicate standard deviations within each treatment (n=10 organisms), truncated between 0 and 100%.

Table 4.4.2: Water quality metrics measured at the 24-hour mark of the GAC shortened conditioning toxicity test. Underlined measurements denote the ones that are explicitly discussed. Asterisk (*) denotes a rough estimate from water quality measurements that were taken with test strips. All other metrics were measured with meters.

Water Quality Metric	Source Water	GAC- Aerated, Conditioned	GAC- Unaerated, Conditioned	GAC- Unconditioned	Glass Wool Control
Temperature (°C)	22.3	23.3	23.4	23.3	23.5
DO (mg/L)	6.73	6.13	6.39	5.96	6.07
pH	8.07	8.12	9.03	8.48	7.88
Conductivity (µS/cm)	532	507	503	511	544
Hardness (mg/L $CaCO_3$) *	125	125	125	125	125
Alkalinity (mg/L) *	120	120	120	120	120
Ammonia (mg/L) *	0	0	0	0	0

While it remains unclear how aeration could have counteracted this phenomenon, the results of this test demonstrate its clear effectiveness in preparing the GAC medium. Activated carbon's alkalizing impact on effluent water appears to be a common phenomenon (Fanner et al., 1996). Suneetha et al. (2017) provide a hypothetical explanation for this phenomenon, noting that carbon activation produces oxygen-containing and nitrogen-containing functional groups that can cause the surface of GAC and its particulates to have an innately acidic or basic nature. It remains unclear whether aeration was effective during conditioning due to physical disturbance or due to increased dissolved oxygen concentrations, but it is hypothesized that the

physical disturbance caused by aeration allowed the equilibration water to better interact with the surface of the GAC, in a process that could have simulated additional continuous rinsing.

With the above information, it is important to note that each source of GAC might have different impacts on effluent water quality. For the bituminous GAC used in this investigation, it can be concluded that the GAC does not cause toxicity to test organisms when conditioned properly. Proper conditioning can be accomplished by rinsing and equilibration in continuously aerated DI water for as little as two days. It is possible for other activated carbons to have different effects on pH, or other water quality characteristics.

5.0 ORGANISM ENDPOINTS

5.1 FISH EARLY LIFE STAGE TERATOGENICITY

This section details efforts to address Project Task 2: The testing of early life-stage fish in iTIES studies. Early life-stage fish are crucial to ecosystem structure and highly sensitive to CoCs that cause sub-chronic and chronic toxicity. Teratogenicity, defined as morphological defects in a developing embryo, is the primary sub-chronic toxicity endpoint for embryo-larval fish given its ease of detection. Observable teratogenic effects include scoliosis, abnormal tail curvature, skull deformities, pericardial edemata, and deflated yolk sacs. Their sensitivity makes embryo-larval fish ideal for inclusion in iTIE studies.

The research team sought to verify embryo-larval fish toxicity test protocols with the iTIES in laboratory and field settings. First, several in-lab toxicity tests were conducted using newly spawned fathead minnow (*Pimephales promelas*) embryos to detect sub-chronic toxicity. Exposures were completed using three compounds: copper, fluoranthene, and perfluorooctane sulfonate (PFOS) with concentrations derived from effective concentrations in literature (Besser et al, 2001; Farr 1995; Pandelides et al., 2024). In each test, three groups of ten *P. promelas* embryos (48 hours post-spawn) were exposed to a toxicant for 24 hours, while another three groups of ten embryos were exposed to clean water as controls. After exposure, all six groups were transferred to clean water and cultured for an additional seven days. Water changes were completed every two days. After embryos began hatching to larval fish, feedings with freshly hatched *Artemia* spp. nauplii were completed twice daily. Survival and teratogenicity were monitored throughout the experiment; any organism observed with terata were euthanized, preserved, and recorded as "dead" in survival logs. At the termination of each experiment, all organisms were euthanized.

While exposure groups in all three experiments experienced slightly lower average survival, no exposure groups had significantly ($p \leq 0.05$) different survival from control groups (Table 5.1.1). However, multiple individuals exposed to fluoranthene developed terata (Figure 5.1.1). Observed teratogenic effects include pericardial edema and other gross malformations like amorphous body shape. This confirms teratogenicity in embryo-larval fish as a feasible toxicity endpoint for 24-hour iTIE exposures. Additional tests should be conducted with a range of toxicant concentrations to better understand the potential for teratogenic effects of short-term exposures to copper and PFOS.

Table 5.1.1: Results from 24-hour iTIE exposures of P. promelas (48 days post-hatch) to various toxicants.

Toxicant	Concentration (ug/L)	Control Group Survival (%; mean ± s.d.)	Exposed Group Survival (%; mean ± s.d.)	P-value from unpaired one-tail Student's T-test
Copper	50	100 ± 0	83.33 ± 9.43	0.117
Fluoranthene	10	90 ± 8.16	76.67 ± 12.47	0.142
PFOS	120	100 ± 0	90 ± 8.16	0.272

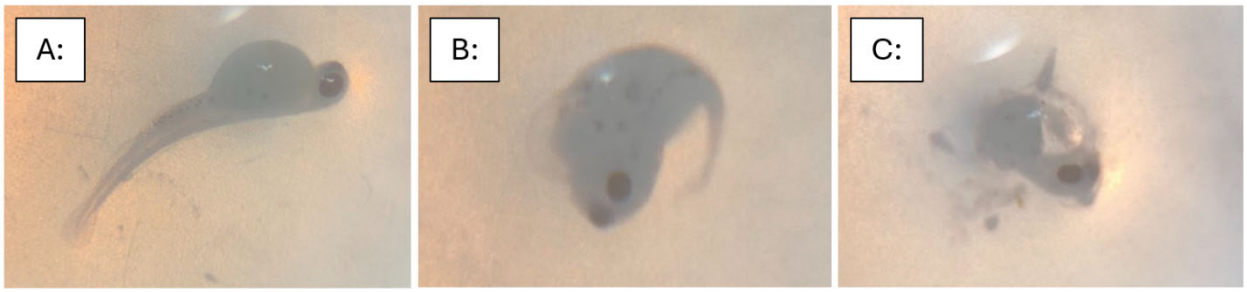

Figure 5.1.1: P. promelas larvae with developmental terata after exposure to fluoranthene. Larvae were exposed for 24 hours to fluoranthene at a concentration of 10 ug/L. A: pericardial edema, B: gross malformation, C: gross malformation.

After confirming the potential for observable teratogenic effects following a 24-hour exposure to low levels of toxicants, the research team completed a series of field-based iTIE exposures using embryo-larval fish. One experimental run using embryo-larval fish was conducted in a marine-estuarine environment at the mouth of Paleta Creek, National City, CA. This run utilized *Atherinops affinis* (topsmelt silverside). All other embryo-larval fish exposures were conducted in freshwater environments using *P. promelas*. These field exposures are described in greater detail in Sections 7 and 8.

Teratogenicity was observed in every field deployment at a site with toxicants present. One run, conducted near the mouth of the Rouge River, Detroit, MI, in October 2024, can be used as an example to showcase terata used effectively as a toxicity endpoint. An array of photographs of *P. promelas* larvae from the exposure are shown in Figure 5.1.2. Two healthy individuals are shown; an individual was deemed healthy if it developed at a typical rate with no spine curvature, no swelling around the heart, an inflated swim bladder, and a non-deformed skull.

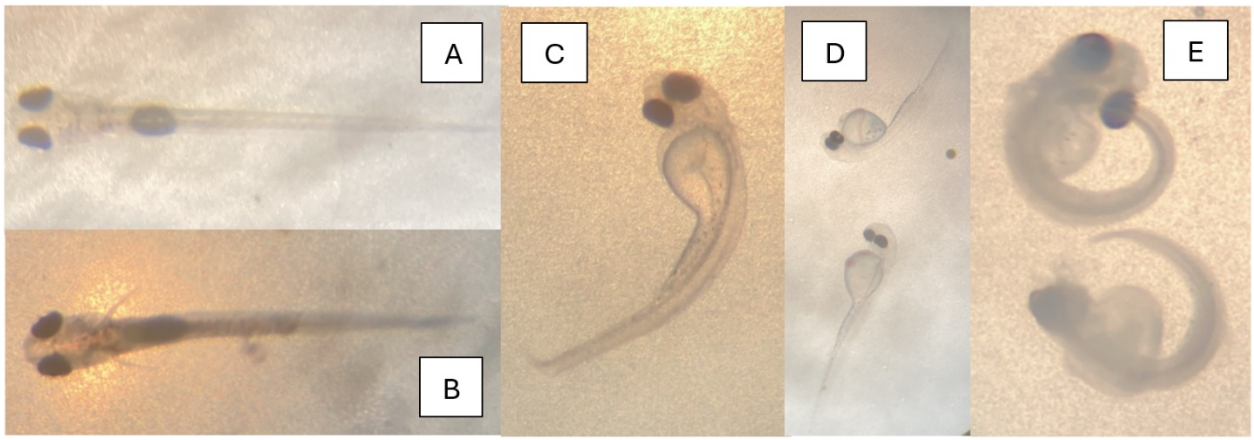

Figure 5.1.2: P. promelas larvae exposed for 48 hours in an iTIE deployment near the mouth of the Rouge River, Detroit, MI. A) lab control group, healthy development; B) WAX resin group, healthy development; C) HLB resin group, curved spine and pericardial edema; D) HLB resin group, pericardial edema; E) WAX resin group, gross malformations, stunted growth.

5.2 INVERTEBRATE SUB-CHRONIC ENDPOINTS: ACETYLCHOLINESTERASE

This section contains an overview of a study addressing Project Task 3: an expansion of available sublethal chronic endpoints in invertebrate test organisms. The full study is included in Appendix B.

Pesticides are a common CoC class found in human-dominated waterways, posing unique ecological threats (Schulz, 2004). Some common pesticides, including organophosphates like chlorpyrifos, cause toxicity through the inhibition of AChE, an enzyme necessary for brain function in many animal species (Julien et al 2008). AChE activity is quantifiable through bioassays and has been used as a chronic endpoint correlated with organophosphate exposure in aquatic species (Bartlett et al., 2015; Day & Scott, 1990; Laetz et al., 2020; Naddy et al., 2000). This section details efforts to integrate an AChE bioassay protocol for use with the iTIES, as well as using AChE quantification to evaluate the effectiveness of resins for adsorbing organophosphates.

Adult *Hyalella azteca* (approximately 4-6 weeks old) were obtained from an in-house mass culture for use as test organisms. Groups of ten adults were collected using a 600 um mesh sieve and placed into separate beakers for holding. Several experimental runs were conducted, some using clean culture water to measure baseline AChE levels, and others using chlorpyrifos-spiked water.

A baseline run was conducted using six iTIE units, all containing glass wool in their resin chambers. Groups of ten *H. azteca* adults were placed in each iTIE organism chamber. The tops of each unit were each connected to a designated Versa peristaltic pump to control water movement. All iTIE units were placed in a 10-L aquarium tank filled with clean culture water, and pumps were operated at 25 mL/hour for 24 hours. The resulting water was collected in sample bottles.

Several chlorpyrifos exposure runs were conducted, each utilizing a different sorptive resin. Resins evaluated included Oasis HLB (Waters), Amberlyst-15 (Sigma-Aldrich), GAC (Marineland), and C18 SPE (Waters), conditioned with Milli-Q and/or methanol and rinsed with Milli-Q as needed. Every run included three control iTIE units containing glass wool and three treatment iTIE units containing 5 grams of resin padded between small pieces of glass wool. Chlorpyrifos-spiked water was prepared by adding chlorpyrifos in acetone to clean culture water to a final concentration of 1 ug chlorpyrifos per liter. A 10-L aquarium tank was filled with spiked water, and iTIE units were situated upright in the tank. Peristaltic pumps were operated at 25 mL/hour for 24 hours, with the resulting water collected.

At the end of each run, surviving *H. azteca* in each group were counted and divided into centrifuge tubes containing 5 organisms (2 tubes per iTIE treatment of 10 organisms). AChE activity was quantified using a methodology adapted from Bartlett et al. (2015). The resulting AChE specific activity rates (in umol/min/g protein) were compared to the baseline AChE rates. Student's T-tests were used to compare specific activity between groups.

AChE bioassay results indicated that C18 and HLB were able to reduce the neurotoxicity experienced by test organisms, indicating effective removal of organophosphates (Figure 5.2.1). AChE activity is considered inhibited when it is <80% of baseline levels (Bartlett et al., 2015).

Glass wool-exposed groups in the C18 run had AChE specific activity at 70% (±16% (s.d.)) of baseline activity, indicating inhibition due to chlorpyrifos exposure. Conversely, C18-exposed groups in the C18 run had AChE specific activity at 94% (±24% (s.d.)) of baseline activity and did not significantly differ from average baseline activity (p=0.47).

Organisms in HLB-fractionated chambers had an average specific activity of 98% (±19% (s.d.)) of baseline, while organisms in glass wool chambers had an average specific activity of 80% (±22% (s.d.)) of baseline. HLB-exposed groups did not significantly differ from average baseline activity (p=0.82), but they did differ significantly from glass wool groups (p=0.03).

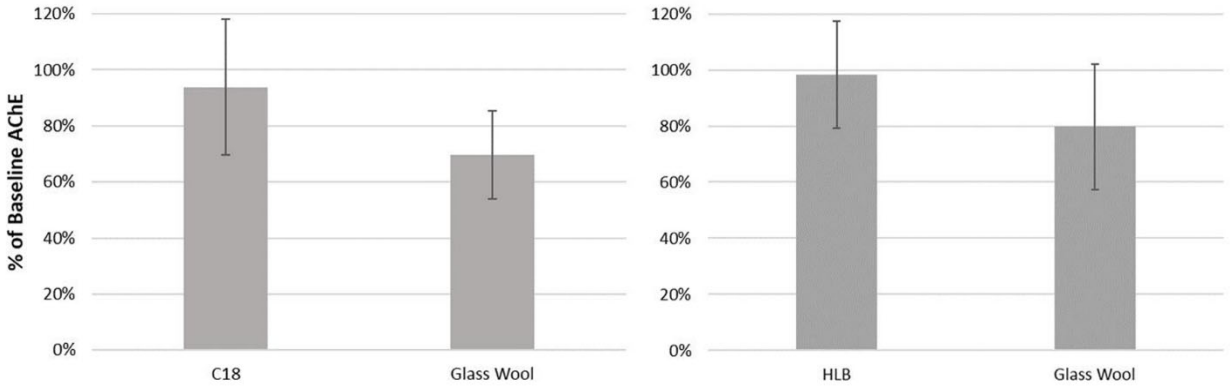

Figure 5.2.1: Average AChE specific activity in H. azteca after exposure to chlorpyrifos-spiked water separated by C18 and Oasis HLB. A) Average specific activity (expressed as % of baseline) for C18 run B) Average specific activity for HLB run. Error bars represent standard deviation, and asterisk denotes significant difference between treatment and baseline activity ($p \leq 0.05$).

Both GAC and Amberlyst-15 negatively impacted organism survival. GAC de-oxygenated culturing water, leading to complete mortality in several organism groups. Amberlyst-15 caused acidic conditions in water (pH 2.27-2.32), likely due to the resin being used in its manufactured hydrogen ion form. Additional conditioning of the two resins may have mitigated harmful water quality impacts. These exposures were completed prior to the identification of aeration as a necessary component of GAC conditioning. A follow-up study utilizing aerated GAC would be required to definitively assess GAC's ability to mitigate the effects of organophosphates on AChE specific activity.

Overall, this study demonstrated compatibility between AChE bioassay protocols and iTIES. AChE specific activity is a viable sub-chronic toxicity endpoint with iTIE testing. Bioassay protocols can be expanded to include other test organisms that utilize AChE as a neurotransmission enzyme. These results also indicate the potential for additional enzyme bioassays to be applied to iTIE research.

5.3 THE ITIE AS A STRESSOR INVESTIGATION

In both the Chelex and GAC conditioning investigations, chronic endpoints have suggested that the iTIE protocol itself may be a source of stress for test organisms. Specifically, lab control groups had a significantly lower time to first brood in both tests, and significantly more neonates per brood during the Chelex toxicity test. This is a topic that warrants its own

investigation. If the iTIE protocol is found to impact chronic toxicity endpoints, then it may complicate iTIE results and interpretation. Additionally, all iTIE experiments thus far have utilized glass wool in resin beds. It has been assumed that glass wool itself does not confer toxicity to organisms; however, this assumption should be evaluated. The research team conducted an iTIE toxicity test to determine: 1) whether the iTIE procedure causes detectable differences in chronic toxicity endpoints, regardless of resin treatment, and 2) whether glass wool or polyester wool, an alternative inert substance, causes toxicity to organisms. This test discerns which chronic endpoints may be viable for an iTIE deployment. Additionally, it provides a framework to test similar endpoints for test organisms other than *D. magna*.

Methods

For this experiment, the same 8-day toxicity test schedule as the GACand Chelex sections was used to assess the iTIE's role as a stressor (Section 4.1). As in previous resin investigations, the culturing portion was extended to 10 days to collect adequate reproduction data. Along with lab controls, the test contained three iTIE treatments: glass wool, polyester wool, and an empty resin chamber. Triplicate groups were established for each control and treatment, and each group contained ten organisms. No conditioning was required prior to test initialization.

Data collected throughout and at the end of the test included total survival, neonates per brood, time to first brood, and growth via AFDW. It should be noted that across all treatment groups, the average number of neonates per brood was uncharacteristically low compared to previous tests. As a result, the 20 neonates per brood threshold recommended in the ASTM method was not reached. In addition to this, the test was terminated before some organisms brooded due to time constraints. For the replicates that had not brooded at the conclusion of the test, time to first brood was assumed to be 11 days, the day following test conclusion. This assumption was made because in previous experiments, all organisms within groups typically released their first broods within 48 hours of one another.

For each toxicity endpoint, treatment groups were compared using ANOVA tests, followed by Tukey multiple comparisons of means tests with 95% family-wise confidence intervals. None of the data satisfied the normalization assumption. Despite this, it was determined that these statistical tests would provide valuable insight.

Results and Discussion

All treatment groups had perfect or near-perfect survival, with only one death occurring in a polyester wool group (Figure 5.3.1). The recommended 80% survival threshold was easily satisfied in each group, and this endpoint appeared to be unaffected by inert resin chamber substances and the iTIE procedure. In addition, there were no noticeable differences in any of the standard seven water quality metrics during the exposure and culturing portion of the test.

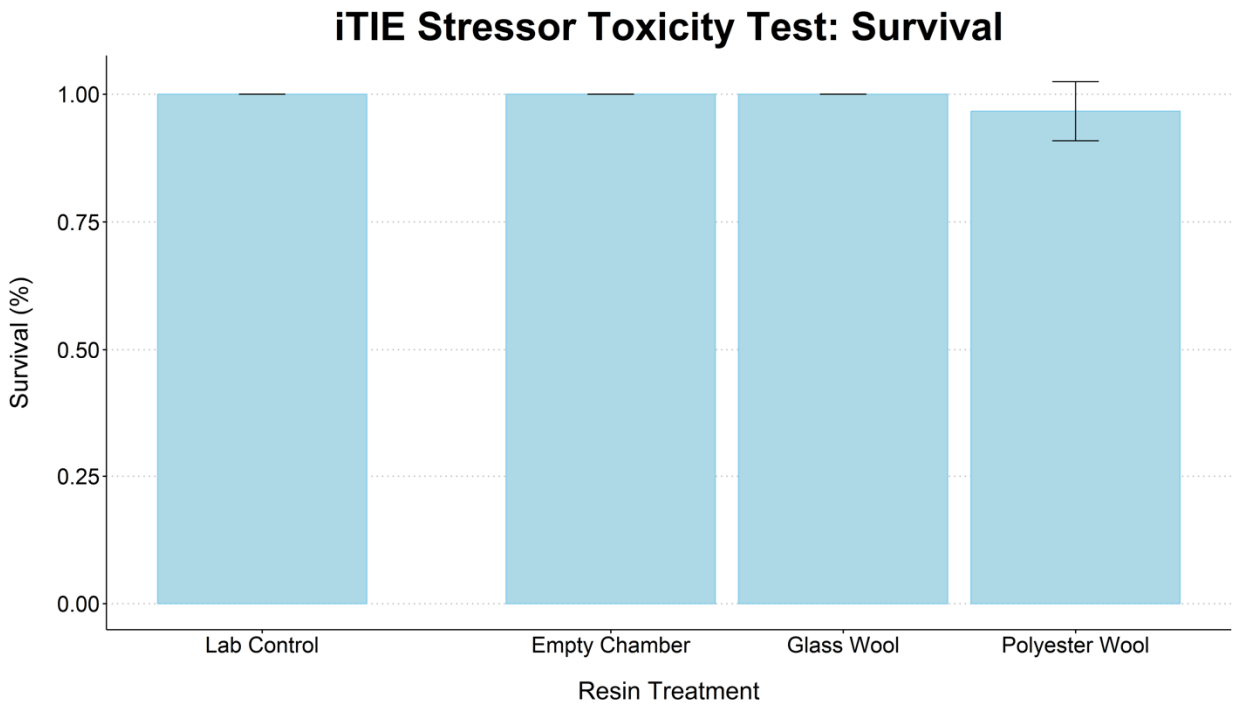

Figure 5.3.1: Total survival for iTIE stress toxicity test. Survival is listed as a percentage of the 30 organisms in each group at the start of the test. For all graphs in this section, the triplicates of each test group have been combined into one result.

The chronic reproduction and growth endpoints convey similar trends but with some important caveats. Much like the GAC and Chelex investigations, there were significant differences in the days to first brood between each iTIE group and the lab control group. On average, the lab control organisms brooded roughly three days prior to the iTIE groups (Figure 5.3.2: Days until first brood during the iTIE stress toxicity test.). The other measured chronic endpoints showed significantly weaker trends. For number of neonates per brood, a significant difference was found only between the lab control group and the empty chamber group. The other iTIE groups were not significantly different from lab controls (Figure 5.3.3). Additionally, no significant differences were found in AFDW (Figure 5.3.4). While there were no significant differences in the latter chronic endpoints, the lab control organisms appeared to perform slightly better overall.

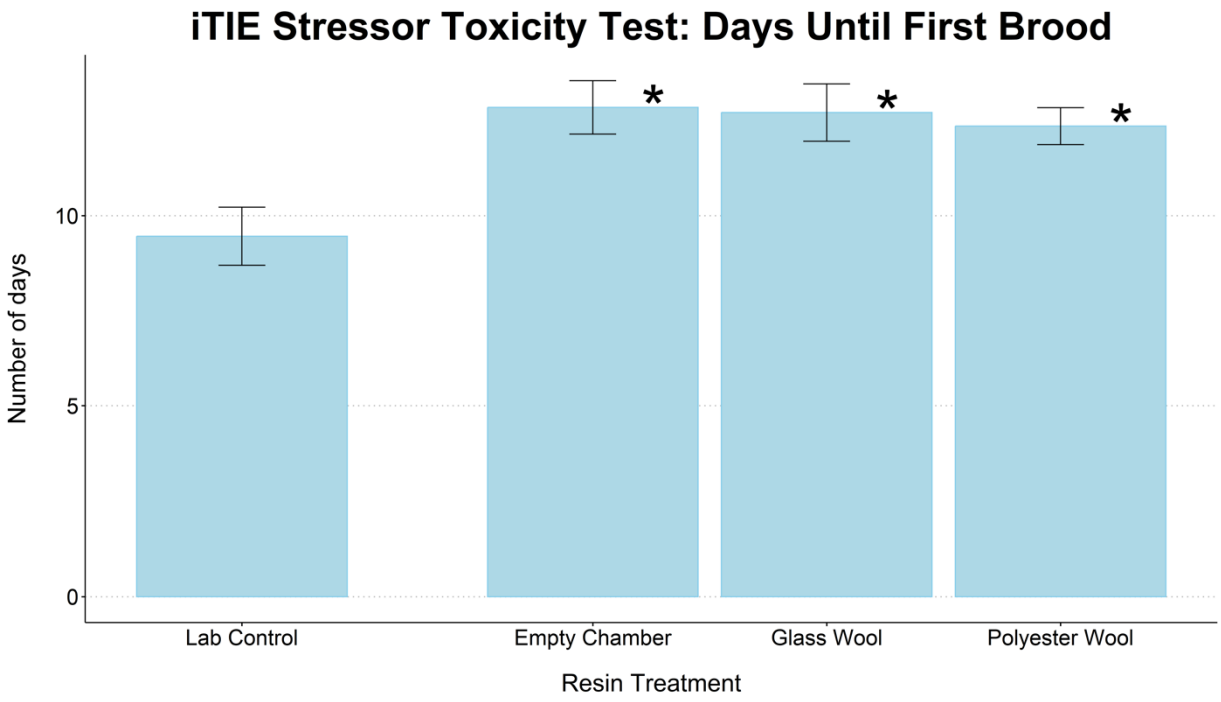

Figure 5.3.2: Days until first brood during the iTIE stress toxicity test. Day 1 is the collection date of organisms (<24 hours old). Error bars are the standard deviation within each *treatment*. An asterisk (*) denotes a statistically significant difference from the Lab Control group (p <.01).

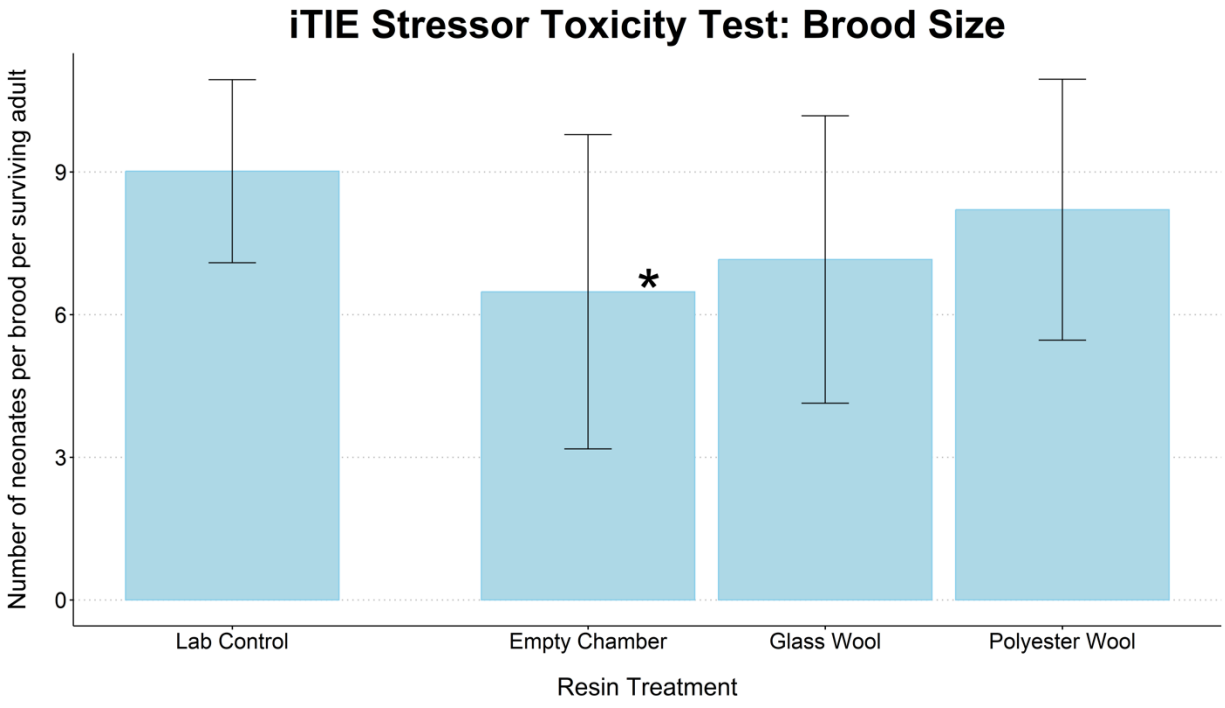

Figure 5.3.3: Number of neonates per brood during the iTIE stress toxicity test. Brood sizes were measured during the culturing portion of the test. Error bars are the standard deviation within each *treatment*. An asterisk (*) denotes a statistically significant difference from the Lab Control group (p <.01).

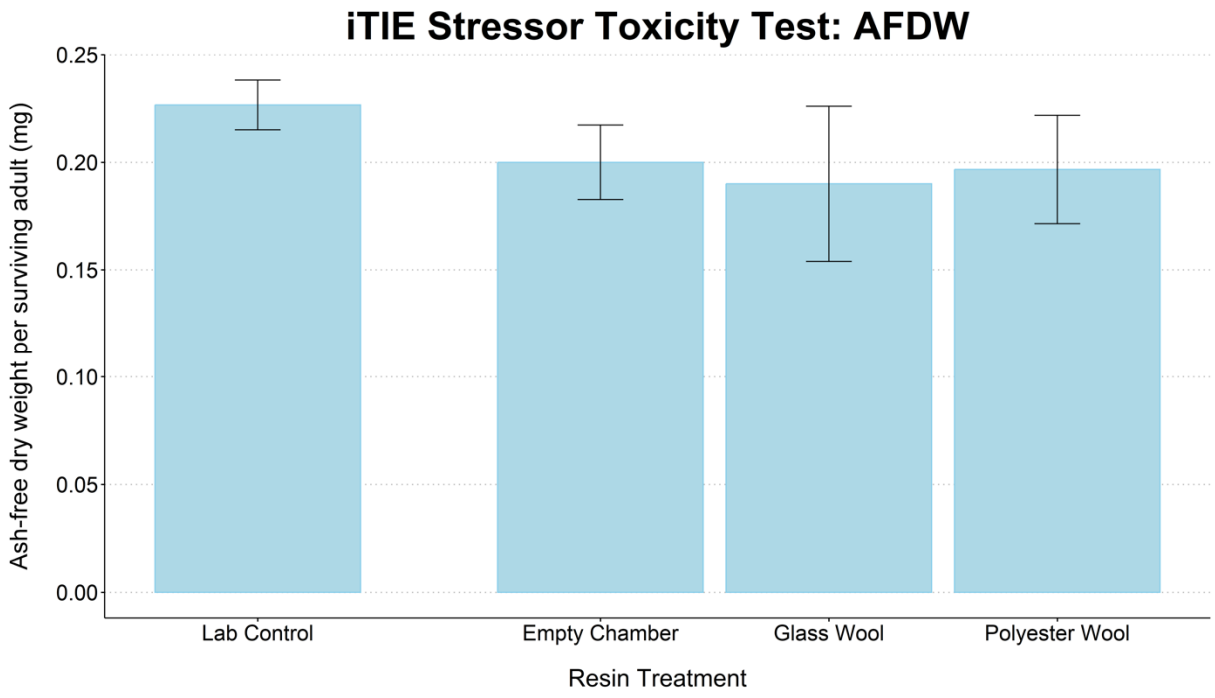

Figure 5.3.4: Ash-free dry weight (AFDW, in mg) for the iTIE stress toxicity test. AFDW was measured at the conclusion of the test. No significant differences were found between groups.

Based on these results, it appears that the iTIE protocol induces a small but detectable degree of stress to test organisms. However, this stress is not necessarily quantifiable for all chronic endpoints, nor does it invalidate the system's utility. Time to first brood data showed a clear trend in line with previous toxicity tests, and the iTIE appears to negatively impact this chronic endpoint for *D. magna*. For other chronic sublethal endpoints such as AFDW and average brood size, the iTIE does not have a significant impact. While the data for average neonates did include one significant difference, this trend is still largely inconclusive as the other iTIE groups were not impacted. The results of this test suggest that the iTIE protocol is viable to measure both acute and chronic endpoints, but each endpoint should be carefully investigated prior to usage. This test provides a framework for three common chronic endpoints with the organism *D. magna*, and any new endpoints should be similarly evaluated.

The results also suggest that neither glass wool nor polyester wool seem to cause any observable toxicity in addition to the iTIE protocol. As a result, both materials are viable as inert substances in the iTIE system. These substances are not intended to adsorb toxicants, but there may be potential for it. This test provides an alternative in the case of incidental adsorption.

6.0 MARINE FIELD VERIFICATIONS

6.1 PALETA CREEK, NATIONAL CITY, CA (2024)

The Paleta Creek watershed in National City, CA, is widely developed, with most of its area occupied by residential, commercial, and military land uses (Reible et al., 2018; SWRCB, 1999). The mouth of the creek, which flows into San Diego Bay on Naval Base San Diego, has been identified as a recontaminated area due to frequent stormwater runoff in the winter, delivering residential and industrial wastewater into the mouth. This area was classified by the State of California as an "impaired waterbody" due to high total maximum daily loads detected at the site. Total suspended solid, trace metal, and PAH concentrations at the mouth of Paleta Creek were found to change drastically before and after storm events, generally with a peak concentration in the first strong flood (Schiff & Carter, 2007; Drygiannaki et al., 2020). Additionally, pyrethroids in the surficial sediment layer were found to correlate with seasonal toxicity to amphipods (Hayman et al., 2019). Based on these findings, the impact of CoCs on benthic communities at the mouth of Paleta Creek remains a critical research priority. The site was chosen to evaluate the iTIE system's performance at marine and estuarine sites impacted by multiple chemical stressors.

The research team first conducted an iTIES deployment at the mouth of Paleta Creek in 2023. This deployment utilized a version of the iTIES with an oxygenation coil constructed from FEP tubing. The deployment utilized *Eohaustorius estuarius*, an ideal test organism for marine/estuarine settings. Resin treatments included glass wool, Chelex, Oasis HLB, and Oasis WAX. Approximately five hours into the exposure, widespread mortality was observed in all treatment groups, leading to the early termination of the test. Low DO levels were measured in all sampled water, despite exposure to the pressurized oxygenation coil. Additionally, sulfide odors were noticeable to personnel, though sulfide content could not be quantified at the time. From this, the research team concluded that an oxygenation coil constructed from FEP tubing may not be effective in aerating water with high dissolved sulfide content.

Subsequently, several alternate tubing materials and specifications were assessed in lab-based apparatus tests. These tests are detailed in Section 4.2 and suggest that to silicone is the optimal tubing material for oxygenation in the presence of high dissolved sulfide content. An upgraded iTIES prototype was constructed with a new oxygenation coil, as well as a drip chamber, to account for excessive gas bubble formation.

6.1.1 Methods

Test Organism Exposures

After prototype adjustments were made and evaluated in lab, a second iTIE deployment was completed at Paleta Creek in August 2024. This deployment utilized two test organisms: topsmelt silverside (*Atherinops affinis*) embryos (2-3 days post-spawn) and mysid shrimp (*Americamysis bahia*) larvae (3-4 days post-hatch). Topsmelt embryos are commonly used for chronic toxicity tests due to their sensitivity to chemical contaminants and short incubation period (typically five to fourteen days). Mysids are commonly used for short-term exposures to determine acute toxicity. The team procured 240 topsmelt embryos and 240 mysids from Aquatic

Biosystems on August 5, 2024 (Day -2) and received both species on August 6, 2024 (Day -1). Organisms were kept in temperature-controlled water baths throughout the experiment. The culture water used was filtered seawater (FSW), processed through 0.45-µm mesh. For each test organism species, four lab control groups, four travel control groups, and four treatment groups (one per treatment) were established, Each group contained ten organisms. Resin treatments included glass wool, Chelex, Oasis HLB, and Oasis WAX. Oasis HLB and Oasis WAX were conditioned by adding a small amount (<0.1 mL) of methanol, followed by repeated rinses and soaks with DI water. Chelex was conditioned with repeated rinses and soaks with DI water.

On the morning of Day 0 of the deployment, all organisms were acclimated to 23°C to match field temperature. At the site, a Trident was installed with a sampling depth of 3 inches below the sediment surface. Resin beds were prepared, and organisms were gently added to each iTIE unit along with a small amount of mysid shrimp feed (0.5 mL of concentrated, freshly hatched *Artemia* spp. nauplii). The oxygenation system was pressurized to 20 psi. The iTIE run was initiated, with iTIE units receiving water at a rate of 10 mL per hour.

Approximately 6.5 hours into the run, widespread morbidity and mortality of mysids was observed across all four resin treatment groups. Organisms were observed becoming immobile and whitish-opaque. The field exposure ended after eight hours. All mysid shrimp groups were terminated. Topsmelt embryo groups were collected, placed into centrifuge tubes, and transferred to beakers of clean culture water in a temperature-controlled water bath. Topsmelt were cultured following recommendations from standard methods, including water changes every two days and twice-daily feedings with freshly hatched Artemia spp. nauplii after topsmelt have hatched to larvae (Anderson et al., 1996). Survival was observed daily. Any individual exhibiting teratogenicity was photo-documented and euthanized. Topsmelt groups were cultured until all viable organisms hatched to larvae.

On Day 2 of the experiment, after the field deployment was terminated, the team conducted an additional *ex-situ* exposure to rule sulfides out as a dominant stressor at the site. Topsmelt silverside embryos (4-5 days post-spawn; n=10 per treatment) were used in this exposure. This experiment utilized fractionated water sub-samples from each of the four treatments of the iTIE deployment: glass wool, Chelex, Oasis HLB, and Oasis WAX. These water sub-samples were stored at 4ºC and not amended by any preservation reagent. Sub-samples (approximately 50 mL) of the fractionated water were placed in centrifuge tubes, brought to 23ºC, and aerated vigorously for 15 minutes while uncovered. This was done to oxygenate the water and to volatilize dissolved hydrogen sulfide content. Then, organism groups were exposed to each of the four sub-samples for 48 hours.

Water Sample Chemistry Analysis

The research team analyzed water samples for sulfide content using an iodometric titration approach based on standard methods (Eaton & Franson, 2005). Some of the reagents used, including iodine solution, sodium thiosulfate solution, and zinc acetate, were made at the University of Michigan in Ann Arbor, MI, and shipped to San Diego prior to use. Sodium thiosulfate solution was standardized (0.0247N) at the University of Michigan prior to shipment. The concentration of the iodine solution used was verified at the field laboratory in San Diego, CA. Sodium hydroxide and zinc acetate solutions were prepared in the field lab.

Prior to the iTIE deployment, bottles used to collect water samples were pre-loaded with zinc acetate and sodium hydroxide solutions for preservation of sulfides. The following water samples were collected for sulfide analysis: (1) unprocessed porewater; (2) aerated, unfractionated porewater; and (3-6) treatment water samples fractionated by a diagnostic resin. Approximately 100 mL of each water sample was collected for sulfide iodometry. Dissolved oxygen content was measured using a YSI ProODO meter. All water samples were stored at 4°C prior to analysis.

After samples for sulfide analysis were collected, iTIE pumps were operated to collect additional water for further analytical chemistry. Water samples were stored at 4°C and shipped to an analytical laboratory following the end of the deployment. A full list of chemical analytes is shown in Table 6.1.1. Due to the limited sample volume available, detection limits were increased proportionally.

Table 6.1.1: Analytes list for the following porewater samples collected during the 2024 Paleta Creek iTIE Deployment. 1) unprocessed porewater, 2) glass wool iTIE treatment, 3) Chelex iTIE treatment, 4) Oasis HLB iTIE treatment, 5) Oasis WAX iTIE treatment, and 6) an equipment blank. Analyses were completed by Eurofins Environment Testing.

Analyte Class	Analytical Method	Specific Analytes	Minimum Detection Limit	Reporting Limit	Unit
Metals	6020B	Copper	3.4	40	µg/L
		Nickel	5.3	40	µg/L
		Zinc	95	400	µg/L
Organophosphates	8141B	Chlorpyrifos	0.017 - 0.019	0.045 - 0.050	mg/L
PAHs	8270C SIM	Acenaphthene	0.92 - 0.94	1.9	µg/L
		Acenaphthylene	0.65 - 0.67	1.9	µg/L
		Anthracene	0.56 - 0.58	1.9	µg/L
		Benzo[g,h,i]perylene	0.95 - 0.98	1.9	µg/L
		Benzo[k]fluoranthene	1.4 - 1.5	1.9	µg/L
		Benzo[a]anthracene	0.81 - 0.83	1.9	µg/L
		Benzo[a]pyrene	0.59 - 0.61	1.9	µg/L
		Benzo[b]fluoranthene	1.7	1.9	µg/L
		Chrysene	0.56 - 0.58	1.9	µg/L
		Dibenz(a,h)anthracene	1.1	1.9	µg/L
		Fluoranthene	1.3	1.9	µg/L
		Fluorene	0.71 - 0.73	1.9	µg/L
		Indeno[1,2,3-cd]pyrene	1.0	1.9	µg/L
		Naphthalene	1.4	1.9	µg/L
		Phenanthrene	0.69 - 0.71	1.9	µg/L
		Pyrene	0.63 - 0.64	1.9	µg/L
PCBs	8082A	Aroclor-1016	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1221	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1232	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1242	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1248	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1254	2.1 - 2.7	3.2 - 4.2	µg/L
		Aroclor-1260	2.1 - 2.7	3.2 - 4.2	µg/L
PFAS	1633	PFOS	5.0	20	ng/L
Pyrethroids	8270D TQ	Permethrins (cis/trans)	0.083 - 0.088	0.093 - 0.098	µg/L
Other	LCMS	6PPD-Quinone	6.1	20	ng/L

6.1.2 Results

Water Sample Chemistry Analysis

Dissolved oxygen and sulfide content are shown in Figure 6.1.1 and Figure 6.1.2, respectively. Silicone tubing was effective in oxygenating sulfide-saturated porewater compared to FEP tubing. DO levels in porewater were raised from hypoxia (0.36 mg/L) to near-saturation (8.5-8.94 mg/L). Additionally, exposure to the silicone oxygenation coil decreased dissolved sulfide content in porewater samples. Porewater samples preserved immediately after extraction contained 65.2 mg S^{2-}/L. After exposure to the oxygenation coil, sulfide concentration dropped to 10.3 mg S^{2-}/L. Levels declined even further following resin fractionation, ending between 2.5-4.3 mg S^{2-}/L in iTIE treatments.

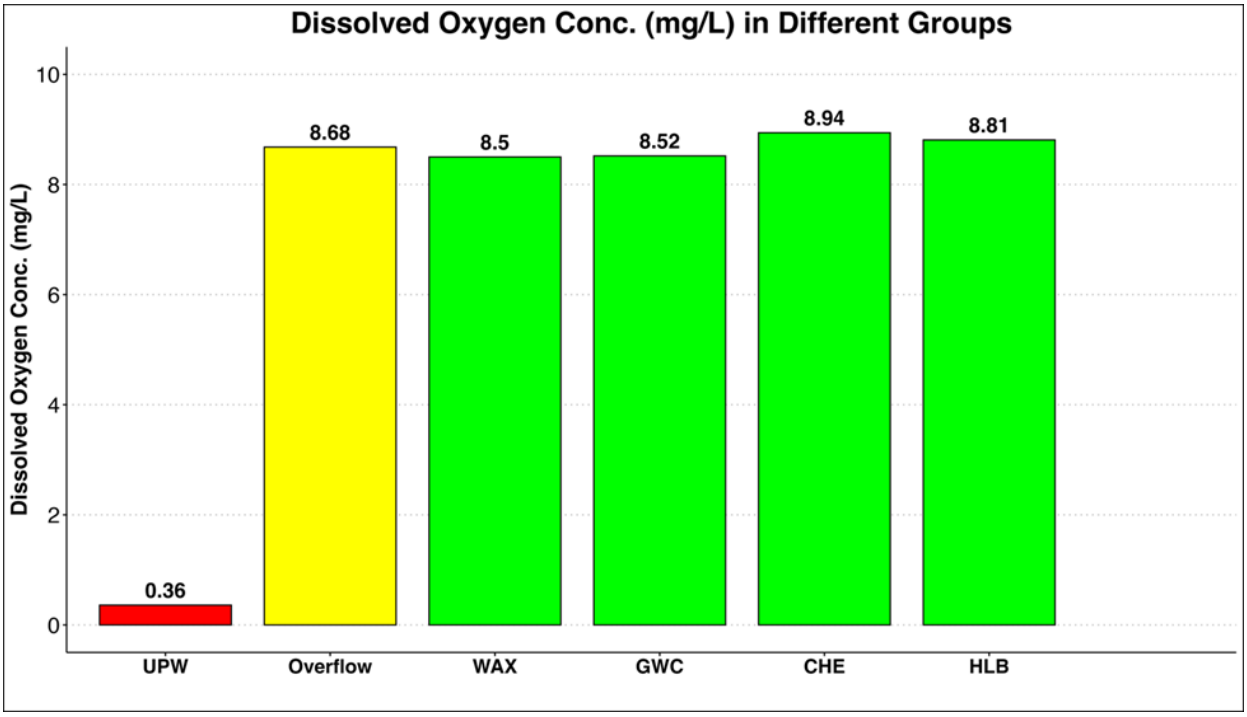

Figure 6.1.1: Dissolved oxygen concentration measurements in porewater samples collected during the 2024 Paleta Creek iTIE deployment. The abbreviations are defined as follows: UPW = unprocessed porewater; Overflow = aerated, unfractionated porewater collected from the overflow system; WAX = Oasis WAX; GWC = glass wool; CHE = Chelex; and HLB = Oasis HLB.

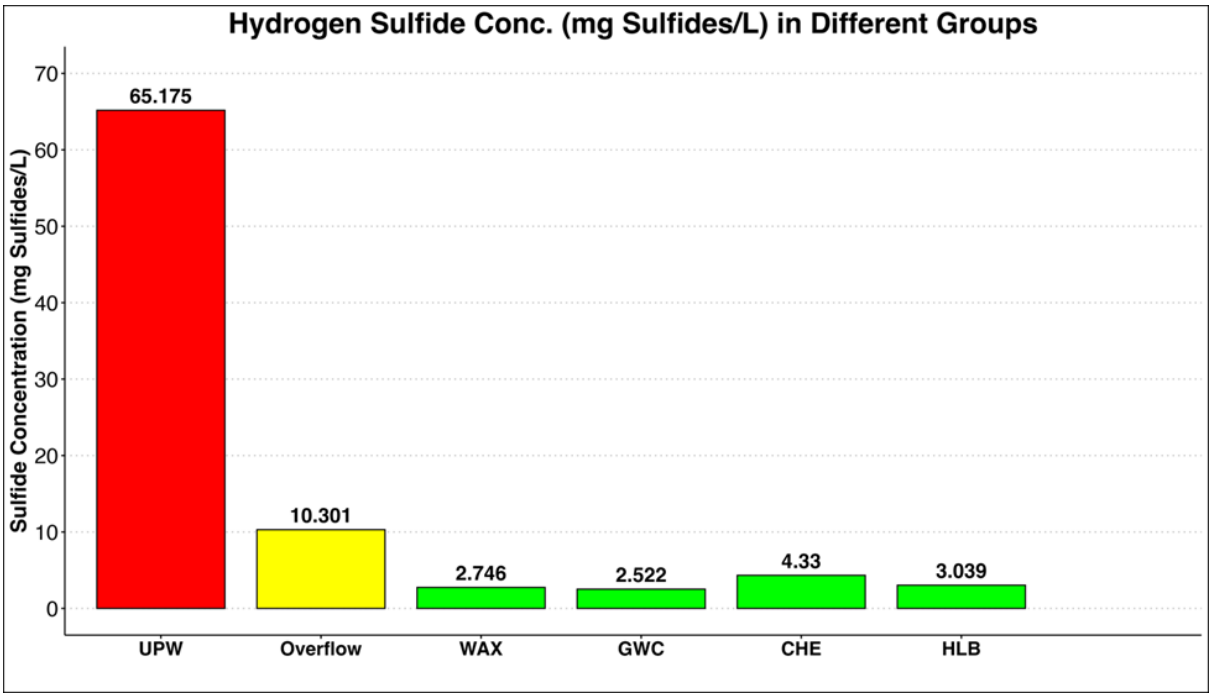

Figure 6.1.2: Dissolved sulfide concentration measurements in porewater samples collected during the 2024 Paleta Creek iTIE deployment. The abbreviations are defined as follows: UPW = unprocessed porewater; Overflow = aerated, unfractionated porewater collected from the overflow system; WAX = Oasis WAX; GWC = glass wool; CHE = Chelex; and HLB = Oasis HLB.

Analytical chemistry results were mostly returned with non-detections. The only analyte detected in samples was PFOS, detected at a concentration of 6.0 ng/L in the unprocessed porewater sample. This detection was qualified as being below the reporting limit of 20 ng/L, but above the minimum detection limit of 5.0 ng/L. Non-detections likely occurred because water sample volumes were limited and thus required dilution. These dilutions increased minimum detection and reporting limits accordingly.

Biological Results

Mysid larvae survival following an 8-hour iTIES exposure at Paleta Creek is shown in Figure 6.1.3. Lab and travel control groups had high survival, meeting minimum test acceptability thresholds. All iTIE treatment mysid groups experienced widespread mortality.

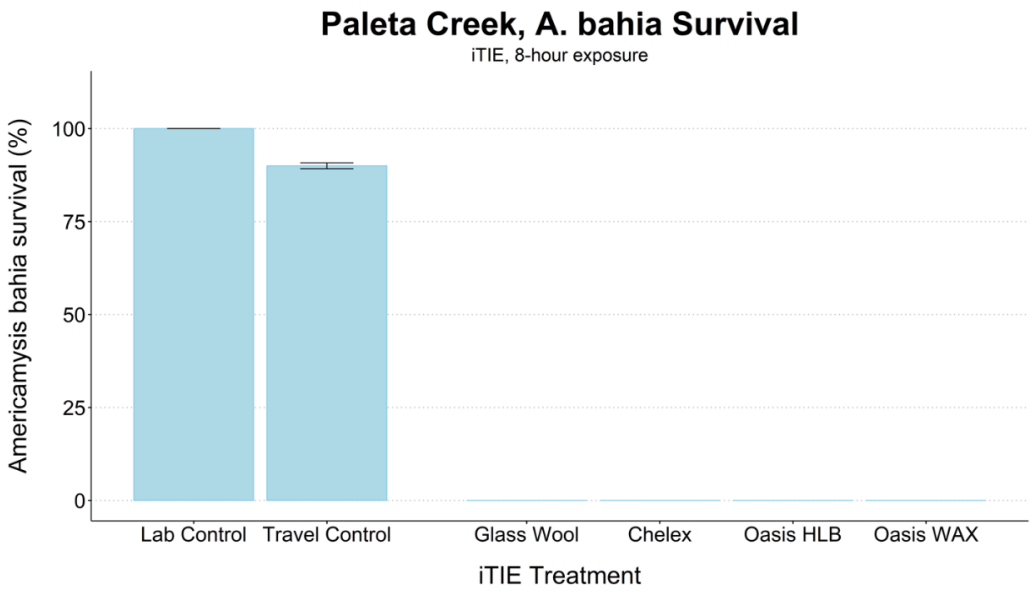

Figure 6.1.3: Survival in A. bahia larvae following an 8-hour iTIE exposure at Paleta Creek.

Topsmelt embryo-larvae survival and healthy development proportions following various iTIES exposures at Paleta are shown in Figure 6.1.4. Lab and travel control groups had high proportions of healthy development, meeting minimum test acceptability criteria. All topsmelt embryos exposed to fractionated water *ex-situ* died after 48 hours, suggesting the presence of chemical classes at concentrations causing acute toxicity. Because sulfides were volatilized from water samples prior to exposure, these results also support the conclusion that sulfides are not a dominant stressor at the site.

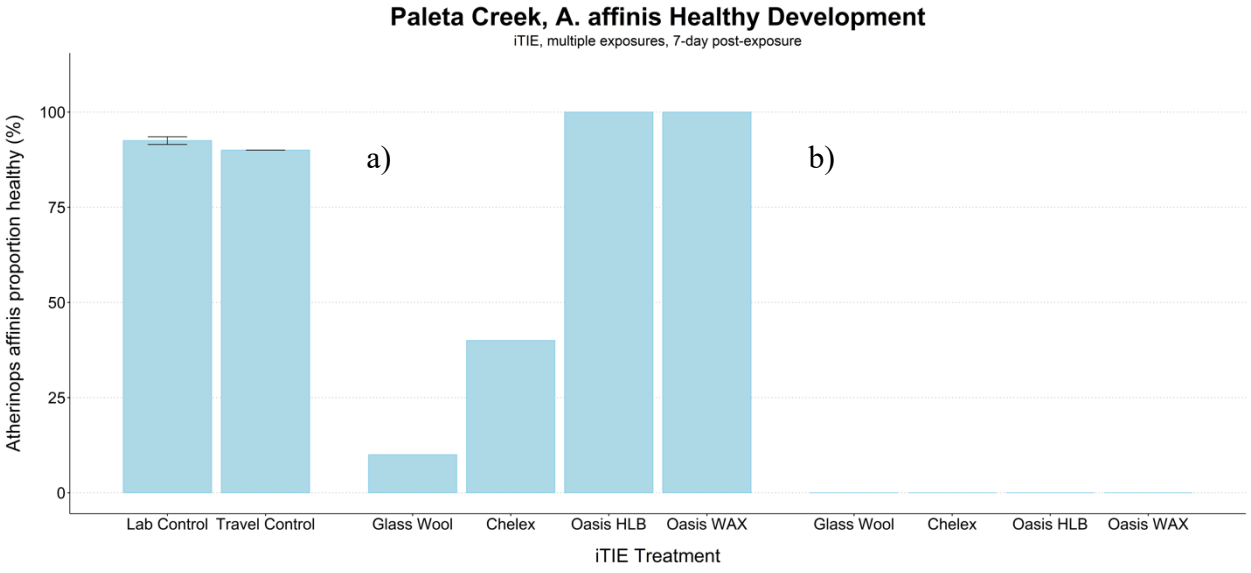

Figure 6.1.4: Survival and healthy development in A. affinis embryos and larvae following the 2024 Paleta Creek iTIE deployment. a) an 8-hour iTIE exposure at Paleta Creek; and b) a 48-hour *ex-situ* exposure to water samples collected from the Paleta Creek iTIE deployment and vigorously aerated for 15 minutes.

Topsmelt embryos exposed *in-situ* for 8 hours yielded additional insight. Following the 7-day post-exposure period, the glass wool treatment group had considerably lower survival (10%) than other groups. The Oasis HLB and Oasis WAX treatment groups both had full survival, which is a strong signal that an organic toxicant is the dominant stressor at the site. The Chelex treatment group had an improved but still lowered proportion of survival (40%), indicating that metals are a cause of toxicity at Paleta Creek, though they may not be the dominant stressor.

6.1.3 Discussion

These results affirm that the iTIES prototype upgrades made, including a silicone oxygenation coil and drip chamber, were effective in mitigating low DO and high dissolved sulfide levels in marine porewater. The newest iTIES prototype was able to increase DO levels in Paleta Creek porewater from hypoxia to saturation, despite high concentrations of dissolved sulfides in the water. Additionally, dissolved sulfide content decreased by 84.2% after traveling through the oxygenation coil, and decreased by an average of 95.2% after traveling through both the oxygenation coil and a diagnostic resin bed. It is likely that sulfides were oxidized by oxygen into less harmful forms of sulfur in the oxygenation coil. It is also possible that sulfides remaining after exposure to the oxygenation coil were physicochemically filtered out by the diagnostic resin beds. Furthermore, while sulfide is present in the porewater, it is not the dominant cause of toxicity at the site, as evidenced by the broad mortality observed in topsmelt embryos exposed *ex-situ* to aerated water samples.

CoCs are present in the sediment at the mouth of Paleta Creek at concentrations causing acute toxicity. The biological results of the 8-hour iTIE deployment indicate that the dominant stressor at the site is likely an organic CoC, because resins targeting general organics (Oasis HLB, Oasis WAX) corresponded with complete survival and healthy development in topsmelt groups. Metals are a secondary stressor at the site, illustrated by the improved survival in the Chelex treatment group compared with the glass wool control.

Although analytical chemistry results are unavailable to confirm these relationships, the findings are consistent with previous studies conducted at the mouth of Paleta Creek. Pyrethroids were found in abundance in the sediment at Paleta Creek, and were found to be highly correlated with amphipod toxicity (Hayman et al., 2020; Reible et al., 2018). Hayman et al. (2020) found evidence that elevated zinc levels are linked with toxicity at the site, though pyrethroids had significantly higher effects. Additionally, PAHs were identified as a primary stressor at the site, though modeled toxicity effects were determined to be chronic rather than acute (Greenstein et al., 2011).

The duration of exposure is an important factor when designing an iTIE experiment. At the mouth of Paleta Creek, a 48-hour exposure led to complete mortality in topsmelt embryos, while an 8-hour exposure provided more detailed results that allowed for stressor ranking. Practitioners should use prior site knowledge to determine whether a shorter or longer iTIE exposure duration is most appropriate to produce desired data outcomes. Ideally, toxicity is detectable in iTIE results without resulting in complete mortality.

It is also important to utilize multiple test organism species when possible. Mysids were more susceptible to acute toxicity than topsmelt embryos, potentially due to protective features present in fish embryos, such as the chorion layer and yolk sac. Multiple test species can provide more comprehensive toxicity data and helps ensure reliability in case one species fails.

7.0 FRESHWATER FIELD VERIFICATIONS

The iTIES deployments in this section are detailed chronologically.

7.1 THIRD SISTER LAKE, ANN ARBOR, MI (2023)

In October 2023, the research team completed an iTIES deployment at Third Sister Lake in Ann Arbor, MI to assess the iTIES prototype. At the time, the pumping sub-system had just been expanded to include a booster pump, and the new configuration needed to be tested in the field. Third Sister Lake is a 3.8-hectare lake located in Saginaw Forest, a property managed by the University of Michigan School for Environment and Sustainability (Bridgeman et al., 2000). The lake has a relatively protected watershed; however, the area's groundwater was discovered in 1985 to be impacted by the industrial solvent 1,4-dioxane (Loch-Caruso et al., 2022). A previous study suggests that 1,4-dioxane has an LC50 of approximately 2.3 g/L in *Gammarus pseudolimnaeus*, a freshwater amphipod (Brooke, 1987). A secondary goal of the deployment was to evaluate acute toxicity at the site to another sensitive freshwater amphipod species.

Porewater was sampled for iTIES testing directly adjacent to the dock on the south side of the lake (Figure 7.1.1). The deployment utilized *Hyalella azteca* (0-1 week old neonates, n=10 per treatment). Resin treatments included glass wool, Ambersorb 560 (DuPont), Oasis HLB (Waters), and zeolite, as well as duplicate lab and travel controls. Ambersorb 560 is a polymeric resin intended to adsorb 1,4-dioxane, among other organic CoCs. All resins were conditioned by rinsing and soaking in DI water. Organisms were exposed to site porewater, collected using a Trident with a sampling depth of 3 inches, for 24 hours. Following exposure, survival was noted as the primary endpoint for acute toxicity.

Figure 7.1.1: A satellite image showing the site of the iTIE deployment at Third Sister Lake in Saginaw Forest, Ann Arbor, MI. The tip of the red pin signifies the location of the site along the southern edge of the lake. Imagery ©2025 Google, Imagery ©2025 Airbus, CNES / Airbus, Maxar Technologies.

Results from the deployment are shown in Figure 7.1.2. The Oasis HLB treatment was confounded due to a setup error, which led to complete mortality in the group. High survival was observed in all other treatments. The iTIES prototype generally performed well during this deployment, with effective water sampling and movement. Full survival in the unfractionated glass wool control indicates that 1,4-dioxane is not present at levels causing acute toxicity. Further iTIE studies may be conducted at Third Sister Lake to evaluate potential chronic toxicity caused by 1,4-dioxane.

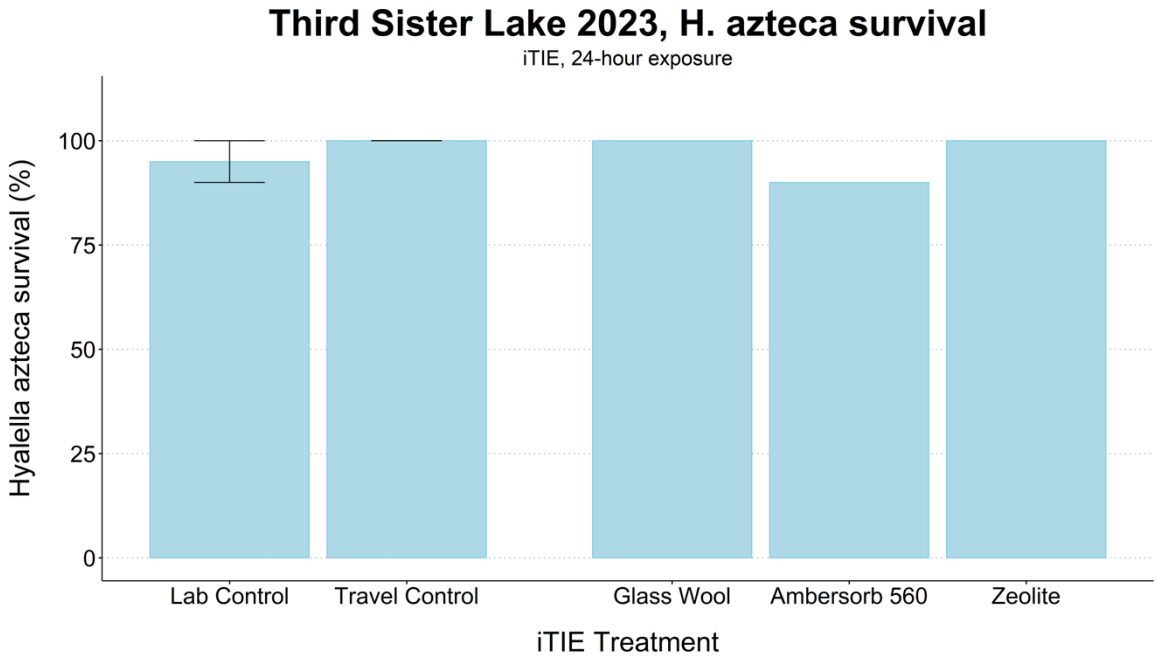

Figure 7.1.2: Survival of *H. azteca* neonates after an iTIE exposure at Third Sister Lake, Ann Arbor, MI. Organisms were exposed to site porewater for 24 hours. Error bars display standard deviation intervals.

7.2 SEXTON & KILFOIL DRAIN, TAYLOR, MI (2023)

The Sexton & Kilfoil Drain in Taylor, MI, is a flashy 13-mile stream used by the Detroit Metropolitan Wayne County Airport during storm events to alleviate flooding (Ecorse Creek Watershed Advisory Group, 2012). The drain is located within the Ecorse Creek Watershed, an urbanized watershed characterized by high anthropogenic inputs and proportions of impervious area. Airport drainage streams have been under scrutiny due to increased awareness of associations with PFAS (de Solla et al., 2011). The Sexton & Kilfoil Drain is an example of this as evident by a 2019 study done by the Michigan Department of Environment, Great Lakes, and Energy (EGLE) which determined that the drain had the highest levels of PFOS in southeast Michigan at 344 ppt (Armstrong, 2020). In addition to PFAS, the site is suspected to contain other CoCs associated with airport drainages, including ammonia from de-icing chemicals and PAHs from oil leaks. Due to these attributes, the research team opted to conduct an iTIES deployment at the site.

The research team completed a 24-hour iTIE deployment in a wadable area of the drainage in October 2023. The deployment location is shown in Figure 7.2.1. Water temperatures

averaged at 12.5°C during the time of study. Fathead minnow embryos (*Pimephales promelas*) were used as the sole test organism for this deployment due to their geographic relevance and high sensitivity to PFAS (Suski, 2023). Resin treatments included glass wool (control), Oasis HLB (general organics sorption; Waters), zeolite (ammonia), and Oasis WAX (PFAS sorption; Waters). All treatment groups contained ten individuals. For QA/QC, three lab control groups (n=10 per group) were maintained concurrently with the deployment and post-exposure period. Three travel control groups (n=10 per group) were transported to the site with other treatment groups, transferred back to the lab, and maintained concurrently with the deployment and post-exposure period. Organisms were cultured in accordance with recommendations from the U.S. EPA (2002).

Figure 7.2.1: A satellite image showing the site of the Sexton & Kilfoil Drain iTIE deployment. The image shows the eastern edge of Detroit Metropolitan Wayne County Airport, between Hildebrandt St. and Northline Rd. The tip of the red pin signifies the location of the site in the drainage. Imagery ©2025 Google, Imagery ©2025 Airbus, CNES / Airbus, Maxar Technologies.

The iTIES technology performed effectively in the Sexton & Kilfoil Drain deployment. The biological results of the Sexton & Kilfoil Drain deployment are shown in Figure 7.2.2. The glass wool treatment group had a survival proportion of 60%, indicating the potential presence of CoCs in the Sexton and Kilfoil Drain. An equal proportion of mortality was observed in the WAX treatment (60% survival), suggesting that PFAS, while present in the drainage, is not the dominant stressor. Survival was comparatively higher in both the HLB and zeolite treatments (90% and 80%, respectively). Given zeolite's affinity for ammonia and HLB's sorption of general organics, these results indicate that toxicity in the Sexton & Kilfoil Drain may be due to those chemical classes.

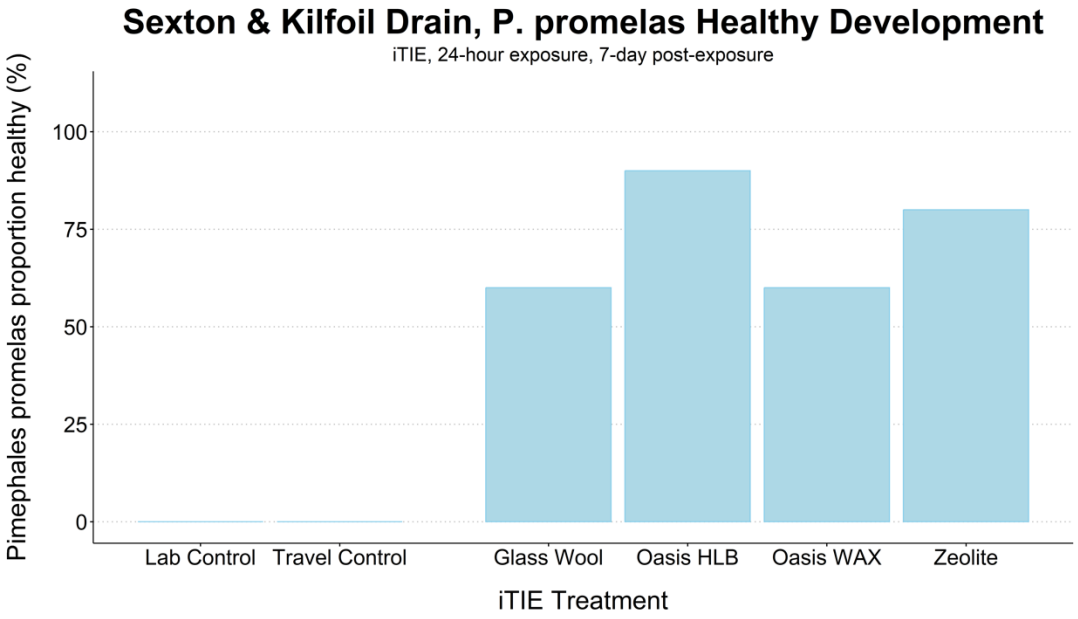

Figure 7.2.2: Survival and healthy development for *P. promelas* larvae after an iTIE exposure at the Sexton & Kilfoil Drain, Taylor, MI. Organisms were exposed as embryos to site porewater for 24 hours and cultured post-exposure for an additional 6 days.

The results of this deployment were confounded due to low control group survival. In the original concurrent exposure, both lab controls and travel controls had complete mortality by the end of the 7-day post-exposure period. The low survival of these control groups contrasted with high iTIE group survival. Following the termination of the treatment group post-exposure period, the research team attempted a second lab and travel control culture; this attempt similarly resulted in low survival rates.

It was suspected that this widespread mortality occurred due to a combination of factors related to culturing procedures: 1) the reconstituted culturing water formulation, and/or 2) the feeding regime. This experiment utilized a soft reconstituted water known as Ion Enriched Water (IEW), comprised of decarbonized water fortified with NaCl (40 mg/L), NaBr (2 mg/L), KCl (2 mg/L), and $CaCl_2$ (40 mg/L). Additionally, organisms were not fed in this experiment, as recommended by EPA Method 1001.0 (2002). Both elements of the lab's fish culturing procedure were overhauled due to these results. Subsequent experiments utilized MHRW. Additionally, fish larvae began to be fed freshly hatched *Artemia* spp. nauplii twice a day. These alterations improved survival and health in future experiments.

7.3 FLEMING CREEK, ANN ARBOR, MI (2024)

Fleming Creek is a tributary of the Huron River in southeastern Michigan with some documented anthropogenic impacts to its watershed (Hay-Chmielewski et al., 1995; Huron River Watershed Council, 2024). Much of the creek and its watershed is contained within Matthaei Botanical Gardens, a natural area owned and managed by the University of Michigan since 1907. The creek has a natural stream form and fair habitat diversity. Impervious surfaces comprise 25-50% of the watershed, comparatively lower than other tributaries to the Huron River. Previous monitoring efforts have detected moderate phosphorus levels and low nitrogen levels, indicating

low to moderate nutrient enrichment. Additionally, small amounts of *E. coli* have been detected. The stream represents a relatively unimpacted representative freshwater stream and is an appropriate candidate for a reference site for southeastern Michigan iTIE studies.

The research team conducted an iTIE deployment at Fleming Creek directly east of Matthaei Botanical Gardens in Ann Arbor, MI (Figure 7.3.1). The deployment utilized fathead minnow (*P. promelas*) embryos (2-3 days post-spawn; n=10 per treatment). Resin treatments included glass wool, Oasis HLB, Oasis WAX, and zeolite, as well as triplicate lab and travel controls. Oasis HLB and Oasis WAX were conditioned by adding a small amount of methanol (<0.1 mL), followed by rinsing and suspension in DI water. Zeolite was conditioned by rinsing and suspension in DI water. Organisms were exposed in the iTIES for 24 hours. After exposure, they were transferred to a lab setting and cultured in accordance with EPA Method 1001.0 for five additional days.

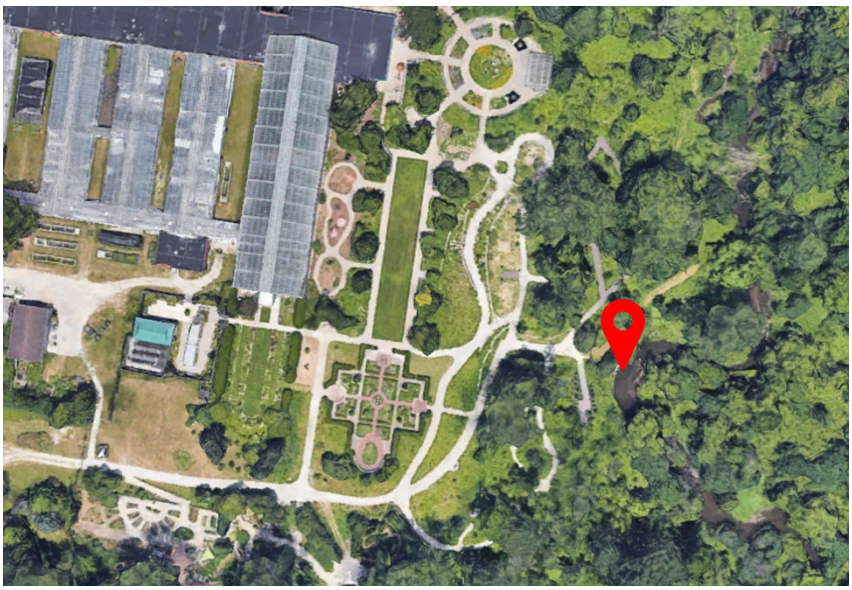

Figure 7.3.1: A satellite image showing the site of the 2024 iTIE deployment in Fleming Creek, Ann Arbor, MI. The map shows Matthaei Botanical Gardens, with stretches of Fleming Creek visible along the right. The tip of the red pin signifies the location of the site along the creek. Imagery ©2025 Google, Imagery ©2025 Airbus, CNES / Airbus, Maxar Technologies.

Survival and healthy development results are shown in Figure 7.3.2. Lab and travel control groups both had average survival proportions of 96.7 ± 4.7% (mean ± s.d.), exceeding the minimum test acceptability criteria threshold of 80%. The glass wool group had full survival and healthy development, indicating an absence of toxicants at levels that cause acute or sub-chronic toxicity. Other treatment groups had healthy development proportions exceeding 90%. These results affirm that the iTIES prototype configuration, specifically the upgraded pumping sub-system, was effective in sampling porewater, transporting water to and through the system interior, and exposing the water to resins and organisms. Additionally, these results support the designation of Fleming Creek as an appropriate reference site for environmental risk assessments in southeast Michigan.

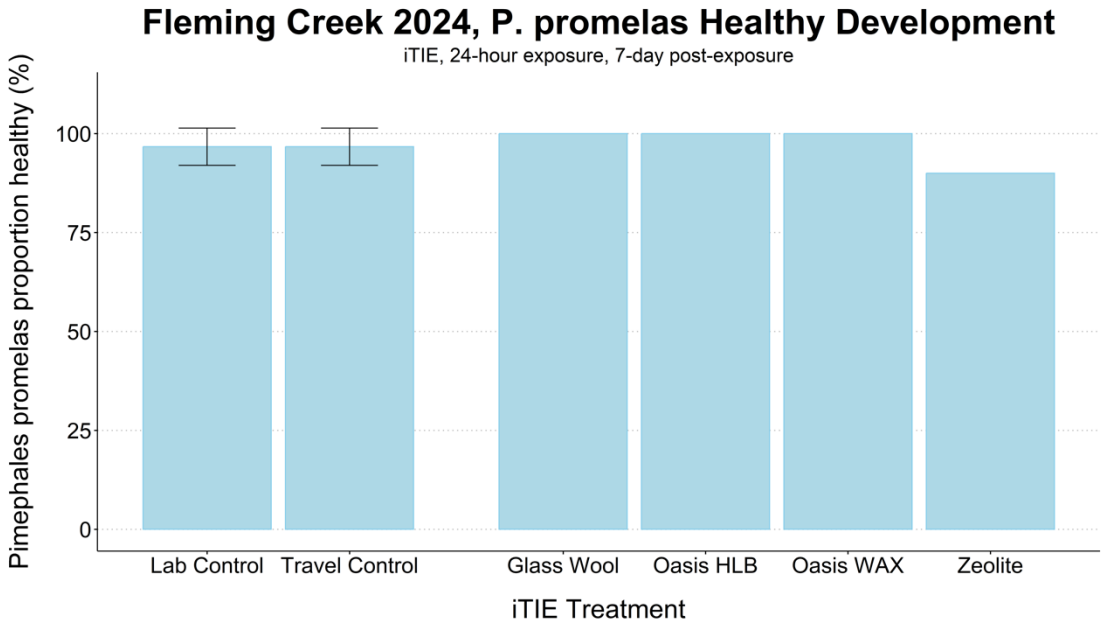

Figure 7.3.2: Survival and healthy development of *P. promelas* larvae after an iTIE exposure at Fleming Creek, Ann Arbor, MI. Organisms were exposed to site porewater as embryos for 24 hours and cultured post-exposure for an additional 6 days. Error bars display standard deviation.

7.4 CLARK'S MARSH, OSCODA, MI (2024)

Clark's Marsh is a wetland that drains into the lower Au Sable River near Oscoda, MI. The marsh is located immediately south of the former Wurtsmith Air Force Base and receives water inflows from the base. Firefighting foams containing PFAS were used regularly in training exercises at the former base until its closure in 1993 (East et al., 2020). Since then, elevated PFAS levels have been found in surface water, porewater, and biota in Clark's Marsh (Custer et al., 2019; Hoskins et al., 2023). In 2015, a GAC system was installed at Wurtsmith Air Force Base to remediate the PFAS plume in surrounding groundwaters (Matheny, 2020). Further interventions are being studied but have not yet been implemented (Birnstingl & Wilson, 2024). The research team completed iTIES studies at Clark's Marsh to assess the technology's ability to detect PFAS, and to verify test organisms for detecting PFAS in freshwater settings.

7.4.1 First iTIES run: *in-situ*

Methods

The first iTIES run at Clark's Marsh was completed *in-situ* in August 2024. The location of the deployment is shown in Figure 7.4.1. The 48-hour deployment utilized *P. promelas* embryos (3-4 days post-hatch; n=10 per treatment) and second instar *C. dilutus* (n=10 per treatment). *C. dilutus* larvae was chosen due to its relative sensitivity to PFAS (Krupa et al., 2022). Organisms of both species were acquired from Aquatic Biosystems the day prior to deployment. Beakers containing lab control groups in clean culture water were established in the main lab space in Ann Arbor, MI. *P. promelas* groups were placed in 1-L borosilicate glass beakers, while *C. dilutus* groups were placed in aerated 250-mL borosilicate glass beakers with fresh blended paper towel substrate. The beakers were situated in a temperature-controlled water

bath at 23°C. All other organisms were transported by car to a field laboratory in Oscoda, MI, one day prior to deployment. Upon arrival at the remote lab, three travel control groups and four treatment groups of each organism were established and acclimated to the field water temperature. All control groups were cultured with a 16-hour light/8-hour dark photoperiod. *C. dilutus* control groups were fed roughly 0.3 g of Tetrafin per day.

Figure 7.4.1: A satellite image showing the site of the in-situ iTIES deployment at Clark's Marsh, Oscoda, MI. The image shows Clark's Marsh, as well as the southern extent of the former Wurtsmith Air Force Base. The tip of the red pin signifies the location of the site along the shoreline of the marsh. Imagery ©2025 Google, Imagery ©2025 Airbus, CNES / Airbus, Maxar Technologies.

Resin treatments included glass wool, Oasis HLB, Oasis WAX, and zeolite. All resins were conditioned by repeated rinsing and soaking with DI water. Oasis HLB and Oasis WAX were pre-conditioned with a small amount of methanol (<0.1 mL) prior to rinsing.

Organisms were exposed at Clark's Marsh within the iTIES for 48 hours. Porewater was continuously sampled at a rate of 50 mL/hr using the Trident system with a sampling depth of 3 inches. *P. promelas* embryos and *C. dilutus* larvae for each treatment were placed in a single iTIE organism chamber. Each chamber contained fresh blended paper towel substrate for *C. dilutus*. Each chamber was fed approximately 0.3 g of Tetrafin in DI water at 0 hr, 24 hr, and 48 hr into the exposure (U.S. EPA, 2000). Each iTIE unit received water at a rate of 10 mL/hr.

Upon termination of the deployment, organisms were transferred to HDPE cups containing clean culture water and placed in a temperature-controlled cooler. All organisms, samples, and equipment were transported back to the main lab space in Ann Arbor, MI. There, organisms were enumerated, temperature-acclimated, and placed in beakers in the temperature-controlled water bath. *P. promelas* were placed in 1-L beakers, while *C. dilutus* were placed in 250-mL beakers with aeration. Organisms were cultured for five days, following EPA care recommendations (U.S. EPA, 2000; U.S. EPA, 2002).

Additionally, at the end of the deployment, water samples were compiled and stored at 4°C. These water samples included unfractionated porewater and water from the four iTIE treatment sample bottles. Approximately 480 mL of each sample was collected. A portion of each sample was preserved using nitric acid for metals analyses. All water samples were analyzed by Eurofins Environment Testing. A full analytes list is shown in Table 7.4.1.

Table 7.4.1: Analytes list for the following porewater samples collected during the Clark's Marsh iTIE Deployment. 1) unprocessed porewater, 2) glass wool iTIE treatment, 3) Chelex iTIE treatment, 4) Oasis HLB iTIE treatment, 5) Oasis WAX iTIE treatment, and 6) an equipment blank. Analyses were completed by Eurofins Environment Testing in Sacramento, CA.

Analyte Class	Analytical Method	Specific Analytes	Minimum Detection Limit	Reporting Limit	Unit
Metals	6020B	Copper	0.17 - 0.34	2.0 - 4.0	µg/L
		Nickel	0.27 - 0.53	2.0 - 4.0	µg/L
		Zinc	4.8 - 9.5	20 - 40	µg/L
Organophosphates	8141B	Chlorpyrifos	0.0079 - 0.019	0.020 - 0.049	mg/L
PAHs	8270C SIM	Acenaphthene	0.79 - 0.98	1.6 - 2.0	µg/L
		Acenaphthylene	0.56 - 0.70	1.6 - 2.0	µg/L
		Anthracene	0.48 - 0.60	1.6 - 2.0	µg/L
		Benzo[g,h,i]perylene	0.28 - 1.00	1.6 - 2.0	µg/L
		Benzo[k]fluoranthene	1.2 - 1.6	1.6 - 2.0	µg/L
		Benzo[a]anthracene	0.69 - 0.87	1.6 - 2.0	µg/L
		Benzo[a]pyrene	0.50 - 0.63	1.6 - 2.0	µg/L
		Benzo[b]fluoranthene	1.4 - 1.8	1.6 - 2.0	µg/L
		Chrysene	0.48 - 0.60	1.6 - 2.0	µg/L
		Dibenz(a,h)anthracene	0.93 - 1.2	1.6 - 2.0	µg/L
		Fluoranthene	1.1 - 1.4	1.6 - 2.0	µg/L
		Fluorene	0.60 - 0.76	1.6 - 2.0	µg/L
		Indeno[1,2,3-cd]pyrene	0.86 - 1.1	1.6 - 2.0	µg/L
		Naphthalene	1.2 - 1.5	1.6 - 2.0	µg/L
		Phenanthrene	0.59 - 0.74	1.6 - 2.0	µg/L
		Pyrene	0.54 - 0.67	1.6 - 2.0	µg/L
PCBs	8082A	Aroclor-1016	1.7 - 2.6	1.7 - 4.0	µg/L
		Aroclor-1221	1.1 - 2.6	1.7 - 4.0	µg/L
		Aroclor-1232	1.1 - 2.6	1.7 - 4.0	µg/L
		Aroclor-1242	1.1 - 2.6	1.7 - 4.0	µg/L
		Aroclor-1248	1.1 - 2.6	1.7 - 4.0	µg/L
		Aroclor-1254	1.3 - 3.1	1.7 - 4.0	µg/L
		Aroclor-1260	1.3 - 3.1	1.7 - 4.0	µg/L
PFAS	1633	PFOS	2.7 - 5.1	11 - 20	ng/L
Pyrethroids	8270D TQ	Permethrins (cis/trans)	0.074 - 0.090	0.082 - 0.10	µg/L

Results and Discussion

Fathead minnow survival and healthy development data from the first Clark's Marsh iTIES deployment are shown in Figure 7.4.2. Midge larvae survival data are shown in Figure 7.4.3. Results from the first Clark's Marsh deployment were confounded due to inadequate travel control group survival. As a result, a second organism exposure was needed to verify site hypotheses.

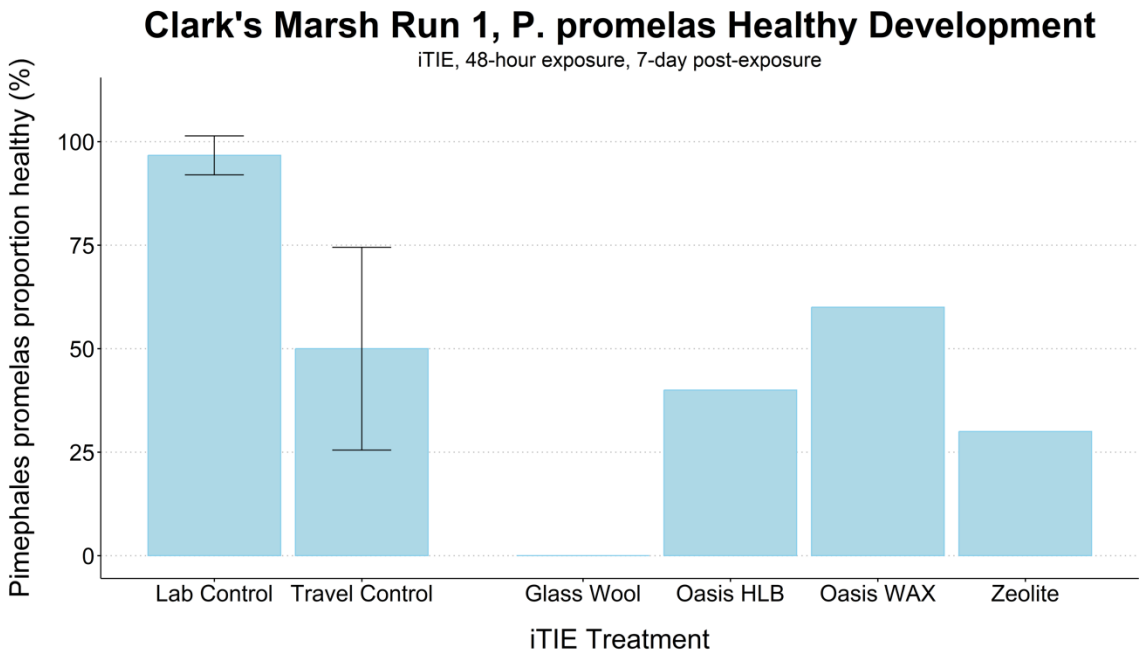

Figure 7.4.2: Survival and healthy development of *P. promelas* larvae after an iTIE exposure at Clark's Marsh, Oscoda, MI. Organisms were exposed to site porewater for 48 hours and cultured post-exposure for an additional 5 days. Error bars display standard deviation intervals.

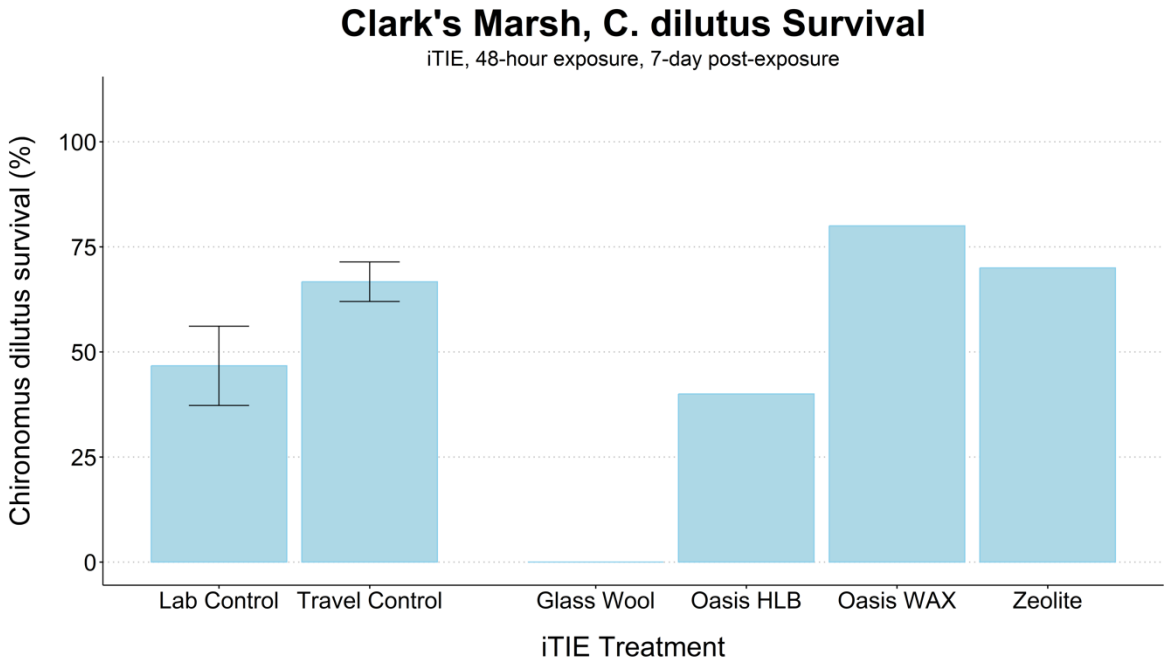

Figure 7.4.3: Survival and healthy development of second instar C. dilutus larvae after an iTIE exposure at Clark's Marsh, Oscoda, MI. Organisms were exposed to site porewater for 48 hours and cultured post-exposure for an additional 5 days. Error bars display standard deviation intervals.

Relevant chemistry analyses of water samples are shown in Table 7.4.2. As expected, elevated levels of PFOS were detected at Clark's Marsh, with a concentration of 630 ng/L in the unfractionated porewater sample. This level is below effective concentration thresholds for *P. promelas* and *C. dilutus* for longer exposure durations of PFOS as a singular stressor (Suski et al., 2023; Krupa et al., 2022). However, it can inferred that numerous other unquantified PFAS compounds are present at Clark's Marsh (Barzen-Hanson et al., 2017; Hu et al., 2016). Oasis HLB and Oasis WAX resin treatments were effective in adsorbing PFOS, lowering concentrations by two orders of magnitude. These resins also likely reduced concentrations for other PFAS. Glass wool and zeolite also lowered PFAS concentrations to lesser degrees, perhaps due to the physical filtration of suspended solids by the two media.

Heavy metals were also detected in all water samples from Clark's Marsh. All metal detections were below concentrations reported to impact *P. promelas* and *C. dilutus* in chronic exposures (Benoit & Holcombe, 1978; Hoang et al., 2004; Schubauer-Berigan et al., 1993; Servia et al., 2006; Shuhaimi-Othman et al., 2011). However, low concentrations of heavy metals were also detected in the iTIE equipment blank sample. No other analytes were detected in water samples collected from Clark's Marsh.

Table 7.4.2: Chemistry analysis of water samples collected during the first Clark's Marsh iTIES deployment in August 2024. Non-detect values were omitted from this table. Asterisks denote concentrations below the reporting limit (RL), but above the minimum detection limit (MDL).

Water Sample	Analyte	Concentration	Unit
Unfractionated Pore Water	Copper	2.4*	ug/L
	Nickel	6.6*	ug/L
	Zinc	15*	ug/L
	PFOS	**630**	ng/L
Glass Wool	Copper	3.9*	ug/L
	Nickel	2.1*	ug/L
	Zinc	23*	ug/L
	PFOS	**340**	ng/L
Oasis HLB	Copper	2.1	ug/L
	Nickel	2.2	ug/L
	Zinc	28	ug/L
	PFOS	3.9*	ng/L
Oasis WAX	Copper	3.1	ug/L
	Nickel	0.6*	ug/L
	Zinc	9.9*	ug/L
	PFOS	3.8*	ng/L
Zeolite	Copper	7.4	ug/L
	Nickel	2.3	ug/L
	Zinc	24	ug/L
	PFOS	**390**	ng/L
Equipment blank	Copper	2.9*	ug/L
	Nickel	1.1*	ug/L
	Zinc	22*	ug/L
	PFOS	11*	ng/L

7.4.2 Second iTIES run: *ex-situ*

Methods

A second iTIES run for Clark's Marsh was completed in November 2024 because the first run was confounded by low control group survival. The second run was completed *ex-situ* due to time and logistical constraints. Carboys of site water and 5-gallon buckets of sediment were collected from Clark's Marsh and transported to the central lab space in Ann Arbor, MI. The iTIE exposure occurred the day after sample collection by inserting the Trident into a 5-gallon bucket filled with sediment and saturating the bucket with site water. The Trident's sampling depth was set to 3 inches. *P. promelas* embryos (n=30 per treatment) and second-instar *C. dilutus* larvae (n=15 per treatment) were utilized during this exposure, as well as lab controls. Resin treatments included glass wool, Oasis HLB, Oasis WAX, and zeolite. All resins were conditioned as in the first Clark's Marsh exposure. Each iTIE unit received water at a rate of 10 mL/hour.

After the 48-hour exposure, organisms were cultured in clean water for five days, following culturing recommendations (U.S. EPA, 2000; U.S. EPA, 2002). At the termination of the post-exposure culturing period, organisms were counted, euthanized, and photographed. Ash-free dry weight was measured in organisms that lived until the final day of the experiment.

Other analyses for the *ex-situ* Clark's Marsh iTIE run were completed using chemistry data from the first *in-situ* deployment. Nonparametric Spearman's rank correlation analysis tests were completed comparing various biological endpoints (acute survival, chronic survival, deformity, and AFDW) with analytical chemistry results. These tests allowed for the quantification of relationships between CoC presence and organism stress. Spearman's rank correlations were calculated for each species separately and both species together. CoC concentrations for lab control groups were assumed to be zero.

Results and Discussion

Deformity was observed in three *P. promelas* groups (Figure 7.4.4). Two individuals in a lab control group were observed to have bent spines. Six individuals in the zeolite group and five individuals in the glass wool group exhibited an array of terata. The Oasis WAX group had one grossly deformed individual. Individuals with observed deformity were treated as dead in subsequent data analyses.

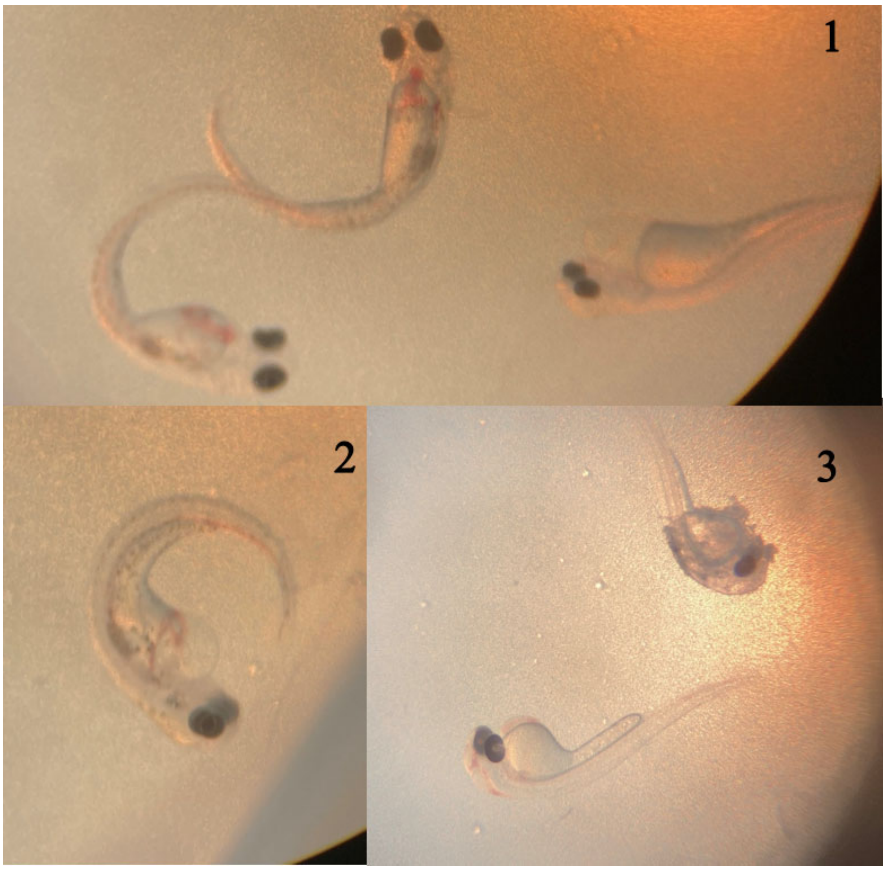

Figure 7.4.4: *P. promelas* individuals exhibiting various terata following an ex-situ iTIES exposure to Clark's Marsh porewater. 1) Three larvae in the glass wool treatment group with a variety of terata, including bent spines. 2) One larvae in the glass wool group with a bent spine. 3) Two larvae in the zeolite group with pericardial edemata.

P. promelas larvae survival and healthy development data are visualized in Figure 7.4.5. Control group survival exceeded minimum test acceptability criteria. The highest Day 7 survival for the resin groups was Zeolite at 60%, then Oasis WAX at 47%, then glass wool at 13%, and Oasis HLB at 0%. The high degree of toxicity observed in the glass wool group indicates the presence of CoCs at acute toxicity levels. Furthermore, no fathead minnow iTIE group had a higher survival rate than 60%. This indicates that Clark's Marsh contains multiple dominant chemical classes, none of which are entirely captured by any single resin. Fathead minnows were dominantly impacted by a CoC targeted by zeolite, likely ammonia, or to a lesser degree, PFAS (Booker et al., 1996; Ponge et al., 2024). Fathead minnows were also impacted by a CoC targeted by Oasis WAX, likely PFAS (Iannone et al., 2024).

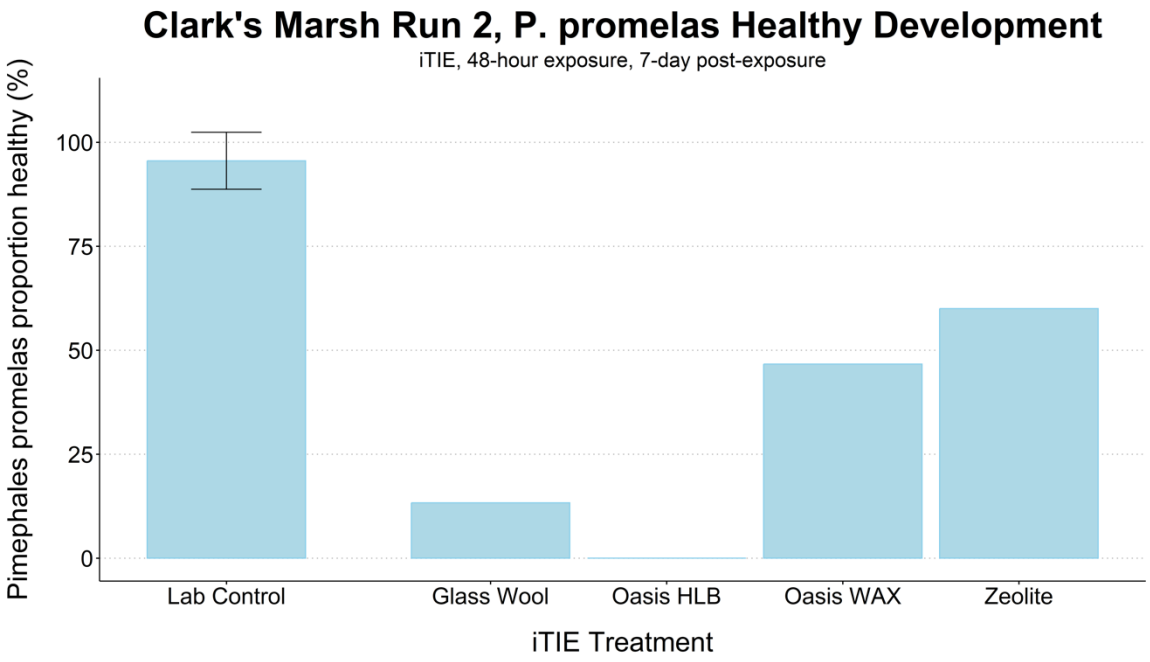

Figure 7.4.5: Survival and healthy development of *P. promelas* larvae after an *ex-situ* exposure to Clark's Marsh porewater in the iTIES. Organisms were exposed to site porewater as larvae for 48 hours and cultured post-exposure for an additional 5 days. Error bars display standard deviation intervals.

C. dilutus survival data is visualized in Figure 7.4.6. Control group survival met minimum test acceptability criteria. The Oasis WAX treatment had the highest survival at 87%, exceeding control group survival. This result aligns with expectations for an aqueous film forming foam (AFFF)-impacted site, given that chironomid larvae have been found to have a high sensitivity to some PFAS substances (Kadlec et al., 2024). The zeolite treatment group had moderate survival at 46.6%. Both species also experienced complete mortality in the Oasis HLB treatment. The cause of this anomaly is undetermined.

Mean AFDW measurements per surviving organism are shown in Table 7.4.3. AFDW measurements for iTIE treatment groups were not markedly lower than lab control groups, indicating no significant impact to growth rates among surviving organisms.

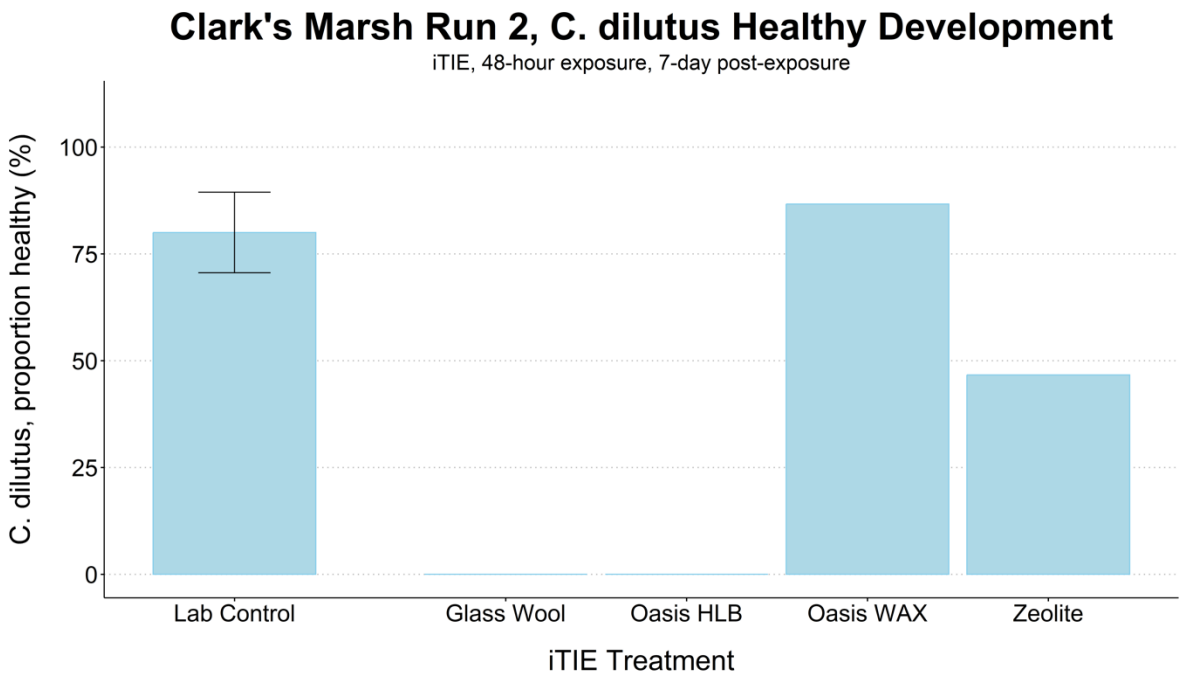

Figure 7.4.6: Survival and healthy development of second instar C. dilutus larvae following an ex-situ exposure to Clark's Marsh porewater in the iTIES. Organisms were exposed to site porewater for 48 hours and cultured post-exposure for an additional 5 days. Error bars display standard deviation intervals.

Table 7.4.3: Clark's Marsh ex-situ iTIES ash-free dry weight results. Blank cells indicate that no organism survived from that group to the end of the experiment.

Organism	Resin Treatment	AFDW (ug per organism)
P. promelas	Lab Control	117.3
P. promelas	Lab Control	103.2
P. promelas	Lab Control	87.93
P. promelas	Glass Wool	137.50
P. promelas	Zeolite	91.67
P. promelas	HLB	
P. promelas	WAX	146.4
C. dilutus	Lab Control	409.1
C. dilutus	Lab Control	500.0
C. dilutus	Lab Control	682.1
C. dilutus	Glass Wool	
C. dilutus	Zeolite	378.6
C. dilutus	HLB	
C. dilutus	WAX	419.2

Spearman's rank analysis revealed multiple statistically significant correlations between CoCs and biological endpoints. Figure 7.4.7 shows the strength, directionality, and significance

of the calculated Spearman's ranked correlations. Spearman Rho correlations for *C. dilutus* and *P. promelas* are visualized in Figure 7.4.8 and Figure 7.4.9, respectively.

For each species separately, ranked chronic (Day 7) survival proportions are significantly correlated with ranked zinc concentrations. This suggests causality between zinc and chronic toxicity in both species. When both species are analyzed together, ranked acute (Day 2) survival is significantly correlated with ranked nickel and zinc concentrations, further indicating causality between heavy metals and toxicity at the site. Additionally, ranked chronic (Day 7) survival is significantly correlated with PFOS, copper, nickel, and zinc concentration rankings. This suggests causal linkages between PFAS and chronic toxicity, as well as between heavy metals and chronic toxicity.

While neither deformity of *P. promelas* larvae nor AFDW of either species were found to be significantly correlated with any CoC, negative relationships can be seen between both endpoints and each of the four detected CoCs at Clark's Marsh.

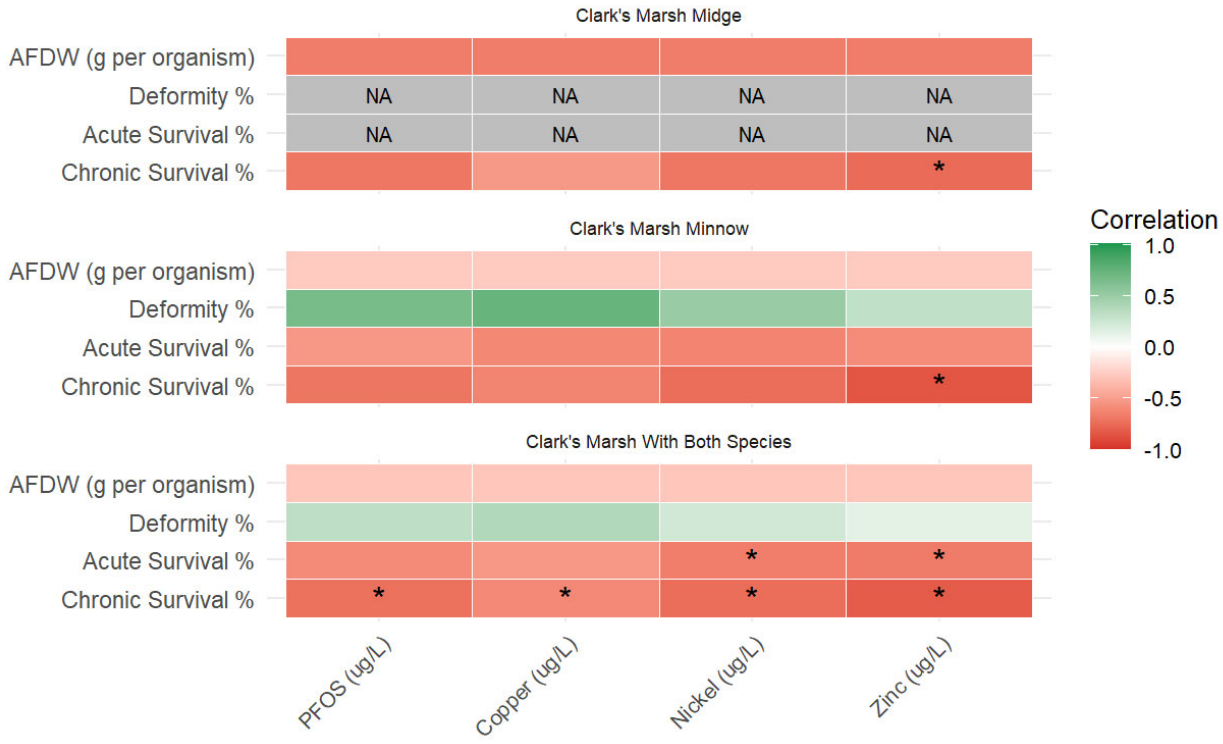

Figure 7.4.7: Table of the Spearman's rank correlations for the Clark's Marsh iTIES deployment. Statistical significance ($p < 0.05$) was calculated for all possible combinations of biological endpoint and detected CoC concentrations for both organisms separately and combined. Boxes with asterisks signify statistical significance. NA values are present for *C. dilutus* deformity and acute survival because these endpoints were not evaluated during testing.

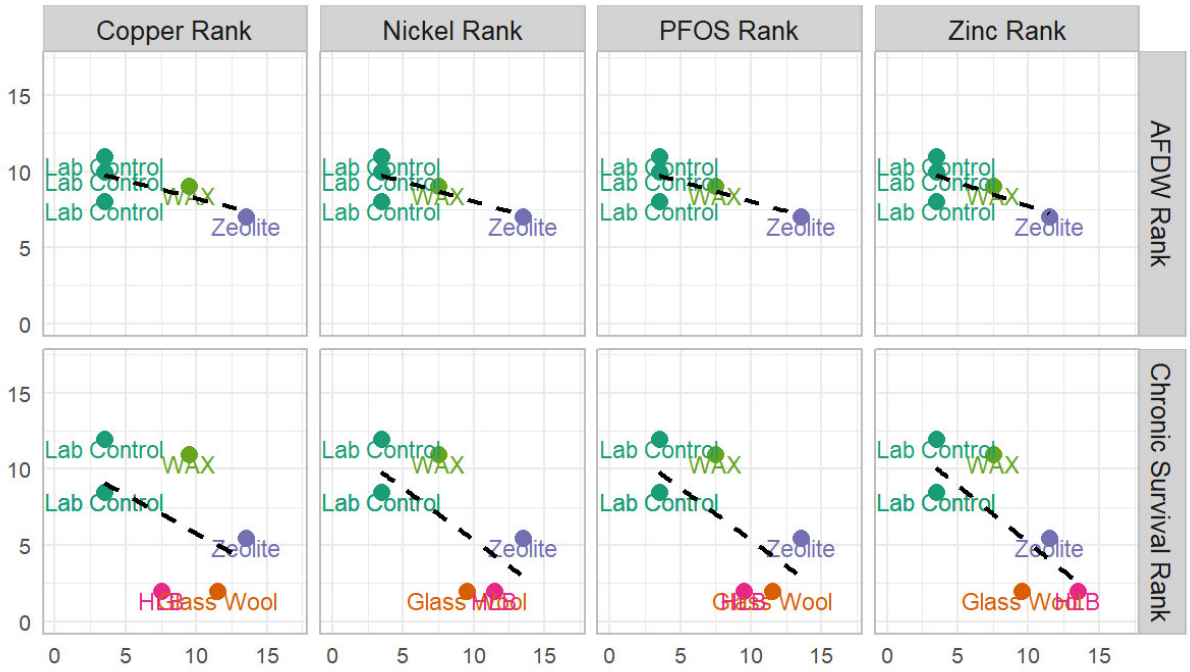

Figure 7.4.8: Plots depicting relationships between C. dilutus biological endpoints and chemistry data from the Spearman's rank analyses for the iTIE experiments at Clark's Marsh. The dotted lines show correlation coefficients. Groups with lower ranks on the x-axis were exposed to lower concentrations of contaminants. Higher ranks for AFDW indicate higher mean weights of surviving organisms. Higher chronic survival ranks indicate higher survival. Oasis HLB and glass wool do not appear on the AFDW plots because no organisms from those groups survived to the end of the experiment.

Figure 7.4.9: Plots depicting relationships between P. promelas biological endpoints and chemistry data from the Spearman's rank analyses for the iTIE experiments at Clark's Marsh. The dotted lines show correlation coefficients. Groups with lower ranks on the x-axis were exposed to lower concentrations of contaminants. Higher ranks for AFDW indicate higher mean weights of surviving organisms. Higher acute and chronic survival ranks indicate higher survival. Higher ranks in deformity indicate more teratogenicity occurred in the group. Oasis HLB does not appear on the AFDW plots because no organisms from the groups survived to the end of the experiment.

In summary, the dominant stressor at Clark's Marsh was found to be PFAS. The porewater at Clark's Marsh was found to contain 630 ng/L of PFOS. PFOS levels were reduced significantly by the Oasis WAX treatment, which was associated with high Day 7 survival of *C. dilutus* larvae and moderate survival of *P. promelas* larvae. The zeolite group also saw moderate survival of both test species, indicating that a chemical class targeted by the resin, such as ammonia, is another dominant stressor at the site. Through Spearman's rank correlation analyses, relationships were established between chronic toxicity at the site and PFOS, copper, nickel, and zinc, indicating that heavy metals are another potential source of stress at the site. Clark's Marsh served as an important site to test the iTIES capabilities to detect PFAS compounds, which it was successfully able to do along with other CoC classes. This deployment also made clear the value of midge larvae as a test species at PFAS-impacted sites.

7.5 ROUGE RIVER, DETROIT, MI (2024)

The Rouge River watershed in Detroit, MI, has a long history of industrial use, with impacts from a legacy of chemical dumping, channelization, damming, and urban runoff (Ridgway et al., 2018). This has led to degraded environmental conditions near the river's mouth, including pollution from heavy metals, PCBs, and PAHs. An iTIES deployment was conducted on a small side channel of the Rouge River surrounding Fordson Island, approximately two miles from the river's mouth (Figure 7.5.1). The channel stems from the main river just downstream from several industrial facilities including the Ford Rouge River Industrial Complex. This channel was identified as an ideal iTIES deployment site for logistical, access, and security reasons.

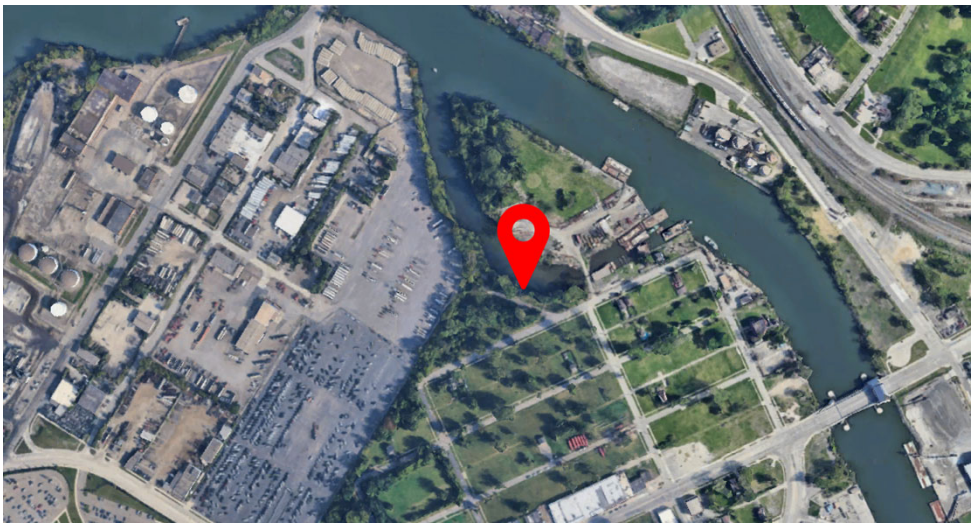

Figure 7.5.1: A satellite image showing the site of the iTIES deployment in the lower Rouge River in Detroit, MI. The image shows a side channel diverging from the main river surrounding Fordson Island. The tip of the red pin signifies the location of the site along the creek. Imagery ©2025 Google, Imagery ©2025 Airbus, CNES / Airbus, Maxar Technologies.

7.5.1 Methods

A 48-hour iTIES deployment was completed in October 2024. The deployment utilized *P. promelas* embryos (3-4 days post-hatch; n=30 per treatment) and second-instar *C. dilutus* larvae (n=15 per treatment). Organisms were procured from Aquatic Biosystems the day prior to exposure and acclimated to 15°C, the approximate field temperature. Resin treatments included glass wool, Chelex, Oasis HLB, and Oasis WAX, as well as lab and travel control groups. Chelex was conditioned by soaking in a calcium chloride solution (1M) for three days, converting the resin to Ca^{2+} form.

At the site, the Trident was installed with a sampling depth of 3 inches. Organism groups of each treatment were housed in the same iTIE exposure chambers, each with fresh blended paper towel substrate. At the initiation of exposure, each iTIE unit was fed 0.3 g of Tetrafin. Porewater was sampled at a rate of 105 mL/hour, while each iTIE unit received fractionated porewater at a flow rate of 20 mL/hour. Organisms were exposed *in-situ* for 48 hours. Following exposure, organisms were transported back to the central lab in Ann Arbor, MI for post-exposure

culturing. Organisms were cultured following recommended procedures for five additional days (U.S. EPA, 2000; U.S. EPA, 2002). At the end of the post-exposure period, organisms were counted, euthanized, and photographed. Ash free dry weight was measured for all organisms that survived until Day 7 of the experiment.

Water samples were also collected during the deployment, including an unfractionated porewater sample and four fractionated samples from each resin treatment. Approximately 480 mL of each sample was collected. Samples were stored at 4°C until analyses. A portion of each sample was preserved with nitric acid for metals analysis. Samples were shipped to and analyzed by Eurofins Environment Testing. A full analyte list is shown in Table 7.5.1. Previously, porewater collected from the Rouge River was analyzed for an array of CoCs described previously in Table 7.4.1, and only heavy metals and PFOS were detected. Thus, only heavy metals and PFOS were targeted in chemistry analyses for this iTIES deployment.

Table 7.5.1: Analytes list for the following porewater samples collected during the 2024 Paleta Creek iTIE Deployment. 1) Unprocessed porewater, 2) glass wool iTIE treatment, 3) Chelex iTIE treatment, 4) Oasis HLB iTIE treatment, 5) Oasis WAX iTIE treatment, and 6) an equipment blank. Analyses were completed by Eurofins Environment Testing in Sacramento, CA.

Analyte Class	Analytical Method	Specific Analytes	Minimum Detection Limit	Reporting Limit	Unit
Metals	6020B	Copper	0.17	2.0	µg/L
		Nickel	0.27	2.0	µg/L
		Zinc	4.8	20	µg/L
		Lead	0.16	1.0	µg/L
		Chromium	0.17	2.0	µg/L
PFAS	1633	PFOS	0.39 - 0.41	1.5 – 1.7	ng/L

Nonparametric Spearman's rank correlation analyses were completed comparing different CoCs with biological endpoints (Day 2 survival, Day 7 survival, deformity, and AFDW). Spearman's rank correlations were calculated for each species separately and both species together. CoC concentrations for lab control groups were assumed to be zero.

7.5.2 Results and Discussion

Water chemistry results are shown in Table 7.5.2. The unfractionated porewater collected at the Rouge River contained elevated concentrations of heavy metals and PFOS. No other analytes were detected. Significant decreases in heavy metal and PFOS concentrations were observed in all iTIE treatments including glass wool, indicating that some toxicant removal may have occurred due to physical filtration.

Table 7.5.2: Chemistry analyses of water samples collected during the Rouge River iTIE deployment. Non-detect values were omitted from this table. Asterisks denote concentrations below the reporting limit (RL), but above the minimum detection limit.

Water Sample	Analyte	Concentration	Unit
Unfractionated Pore Water	Chromium	5.5	ug/L
	Copper	7.8	ug/L
	Nickel	4.1	ug/L
	Lead	8.9	ug/L
	Zinc	35	ug/L
	PFOS	30	ng/L
Glass Wool	Chromium	0.64	ug/L
	Copper	2.1*	ug/L
	Nickel	1.9*	ug/L
	Lead	0.54*	ug/L
	Zinc	9.6*	ug/L
	PFOS	21	ng/L
Chelex	Chromium	0.68*	ug/L
	Copper	2.2	ug/L
	Nickel	0.31*	ug/L
	Lead	0.46*	ug/L
	Zinc	5.6*	ug/L
	PFOS	0.78*	ng/L
Oasis HLB	Chromium	0.52*	ug/L
	Copper	2.5	ug/L
	Nickel	3.0	ug/L
	Lead	0.21*	ug/L
	Zinc	13*	ug/L
	PFOS	0.61*	ng/L
Oasis WAX	Copper	1.3*	ug/L
	Nickel	1.3*	ug/L
	Zinc	5.5*	ug/L
	PFOS	0.56*	ng/L
Equipment blank	Chromium	0.91*	ug/L
	Copper	1.2*	ug/L
	Nickel	2.1	ug/L
	Lead	0.40*	ug/L
	Zinc	15*	ug/L
	PFOS	1.1*	ng/L

Survival data for *C. dilutus* larvae and *P. promelas* larvae are visualized in Figure 7.5.2 and Figure 7.5.3, respectively. Control groups for both organisms met minimum test acceptability criteria. For *C. dilutus,* the Oasis WAX treatment group had the highest proportion of survival (90%), followed by the Oasis HLB and Chelex treatments (70% each), and finally the glass wool treatment (50%). Oasis WAX was the most effective resin in removing PFOS from sampled porewater. It can be inferred that it most effectively adsorbed other PFAS compounds. Given the susceptibility of *C. dilutus* larvae to PFAS, it can be concluded that PFAS is a dominant stressor at the site.

For *P. promelas*, the highest proportion of healthy development was observed in the Chelex treatment (76.7%), followed by glass wool and Oasis HLB (56.7% each). The Chelex

resin was effective in adsorbing heavy metals as intended, resulting in low concentrations for all tested metals. Interestingly, the Oasis WAX group had the lowest survival (46.7%), although it contained the lowest levels of most tested CoCs.

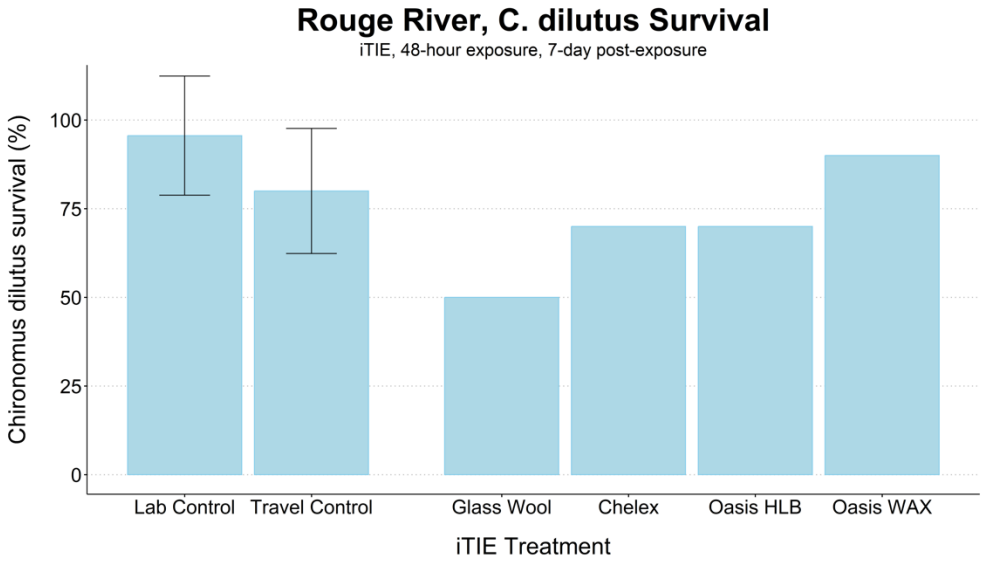

Figure 7.5.2: Survival of C. dilutus larvae after an iTIE exposure near the mouth of the Rouge River. Organisms were exposed to site porewater for 48 hours and cultured post-exposure for an additional 5 days. Error bars show standard deviation.

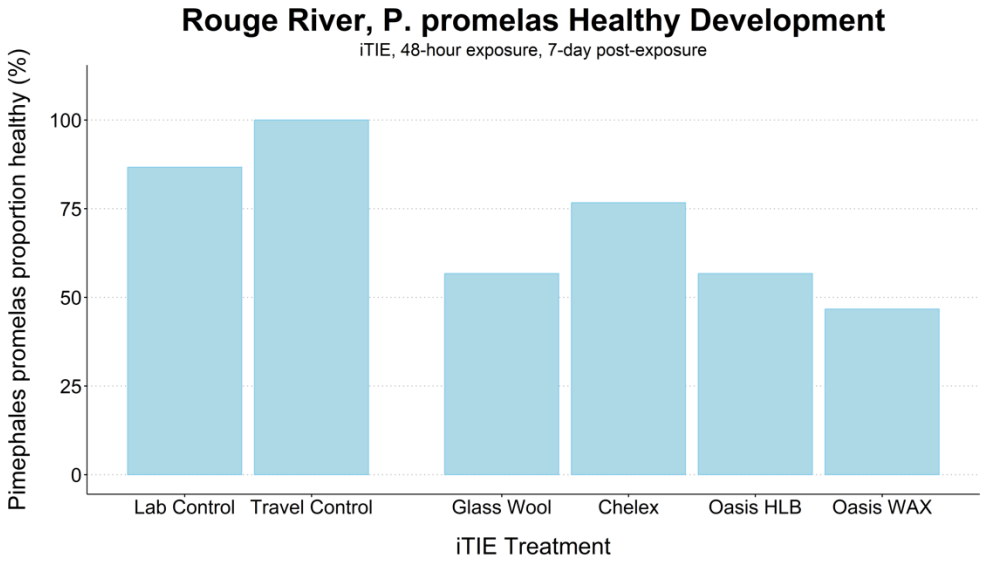

Figure 7.5.3: Survival and healthy development of P. promelas embryos and larvae following a 48-hour iTIE exposure near the mouth of the Rouge River. Organisms were exposed to site porewater as embryos for 48 hours and cultured post-exposure for an additional 5 days. Error bars show standard deviation.

Rates of deformity were high in iTIE treatment groups but low in control groups. Only two of the thirty *P. promelas* larvae in the lab control group exhibited any teratogenicity, while zero deformity was observed in the travel control group. The Chelex treatment also had low rates of teratogenicity, with only two deformed individuals. All other resin groups showed higher rates of teratogenicity, with the glass wool group containing eight deformities and both Oasis HLB and Oasis WAX containing nine. Some examples of observed terata are shown in Figure 7.5.4.

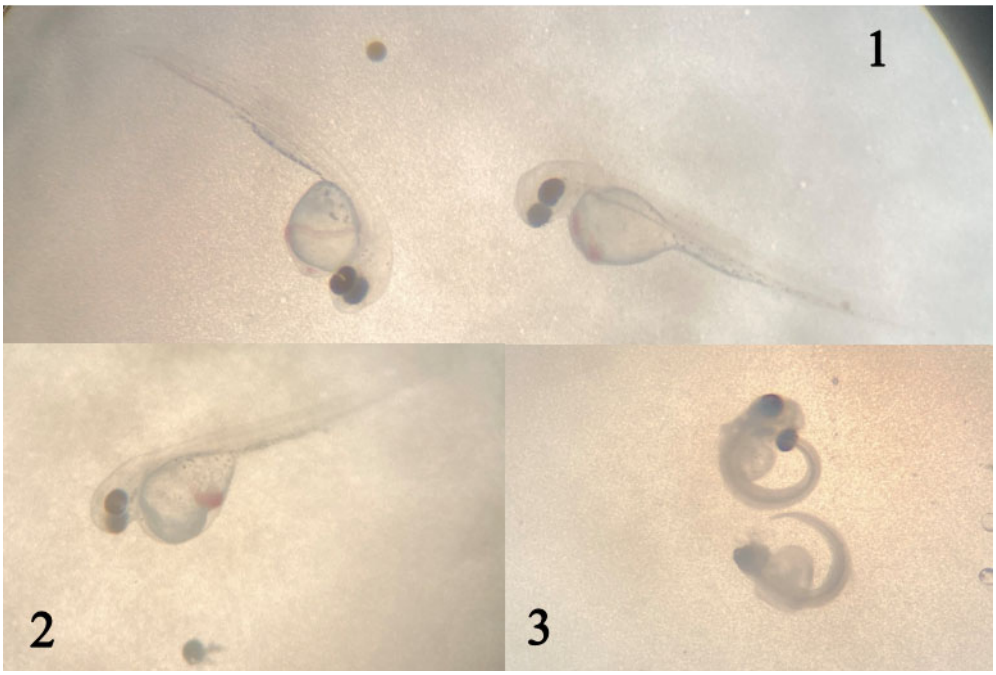

Figure 7.5.4: *P. promelas* larvae with terata following at 48-hour iTIES deployment in the Rouge River, Detroit, MI. 1) Two larvae in the Oasis HLB treatment with pericardial edemata. 2) One larvae in the glass wool group with pericardial edema. 3) Two larvae in the lab control group with bent spines and stunted development.

Mean AFDW measurements per surviving organism are shown in Table 7.5.3. For both test species, the glass wool groups contained the lightest individuals on average. Mean AFDW of *P. promelas* larvae in the glass wool group was 76.45 µg, less than half the mean AFDW of travel control individuals. Mean AFDW of *C. dilutus* larvae in the glass wool group was 90.00 µg, only 57.1% of the mean AFDW of travel control individuals. No other iTIE treatment groups had AFDW measurements discernably different than lab or travel controls.

Table 7.5.3: Rouge River iTIE ash-free dry weight results

Organism	Resin Treatment	AFDW (µg per organism)
P. promelas	Lab Control	121.2
P. promelas	Travel Control	155.0
P. promelas	Glass Wool	76.47
P. promelas	Oasis HLB	161.8
P. promelas	Oasis WAX	125.0
P. promelas	Chelex	108.7
C. dilutus	Lab Control	146.4
C. dilutus	Lab Control	175.0
C. dilutus	Lab Control	176.5
C. dilutus	Travel Control	180.8
C. dilutus	Travel Control	100.0
C. dilutus	Travel Control	192.3
C. dilutus	Glass Wool	90.00
C. dilutus	Oasis HLB	142.9
C. dilutus	Oasis WAX	144.4
C. dilutus	Chelex	135.7

Organism endpoints (chronic survival, acute survival, deformity, and AFDW) and CoC concentrations (PFOS, Copper, Nickel, and Zinc) for all Rouge River iTIE groups were compared in nonparametric Spearman's rank correlation analysis. These analyses are visualized in Figure 7.5.5 through Figure 7.5.7.

For *C. dilutus* larvae separately, several statistically significant correlations were found between CoC levels and organism endpoints. Ranked Day 2 survival and Day 7 survival were found to be significantly negatively correlated with all tested CoC rankings, including PFOS and all heavy metals. Ranked AFDW was significantly negatively correlated with PFOS and zinc. These correlations suggest causal relationships between heavy metals and toxicity, as well as between PFAS and toxicity within Rouge River iTIE treatments.

Several relationships can be seen when looking at *P. promelas* separately. However, only one correlation was found to be statistically significant: the relationship between nickel and deformity rankings. This indicates a causal linkage between iTIE treatments with more nickel removal, particularly Chelex, and reduced instances of deformity.

When examining data from both test species combined, all CoCs were significantly negatively correlated with ranked Day 2 and Day 7 survival. Additionally, ranked AFDW was significantly linked with PFOS. This indicates that PFAS and heavy metals are both important stressors in Rouge River sediment.

Figure 7.5.5: Table of the Spearman's rank correlations for the Rouge River iTIES deployment. Statistical significance thresholds ($p < 0.05$) were calculated for all possible combinations of biological endpoint and detected CoC concentrations for both organisms separately and combined. Boxes with asterisks signify statistical significance. NA values are present for *C. dilutus* deformity because this endpoint was not evaluated during testing.

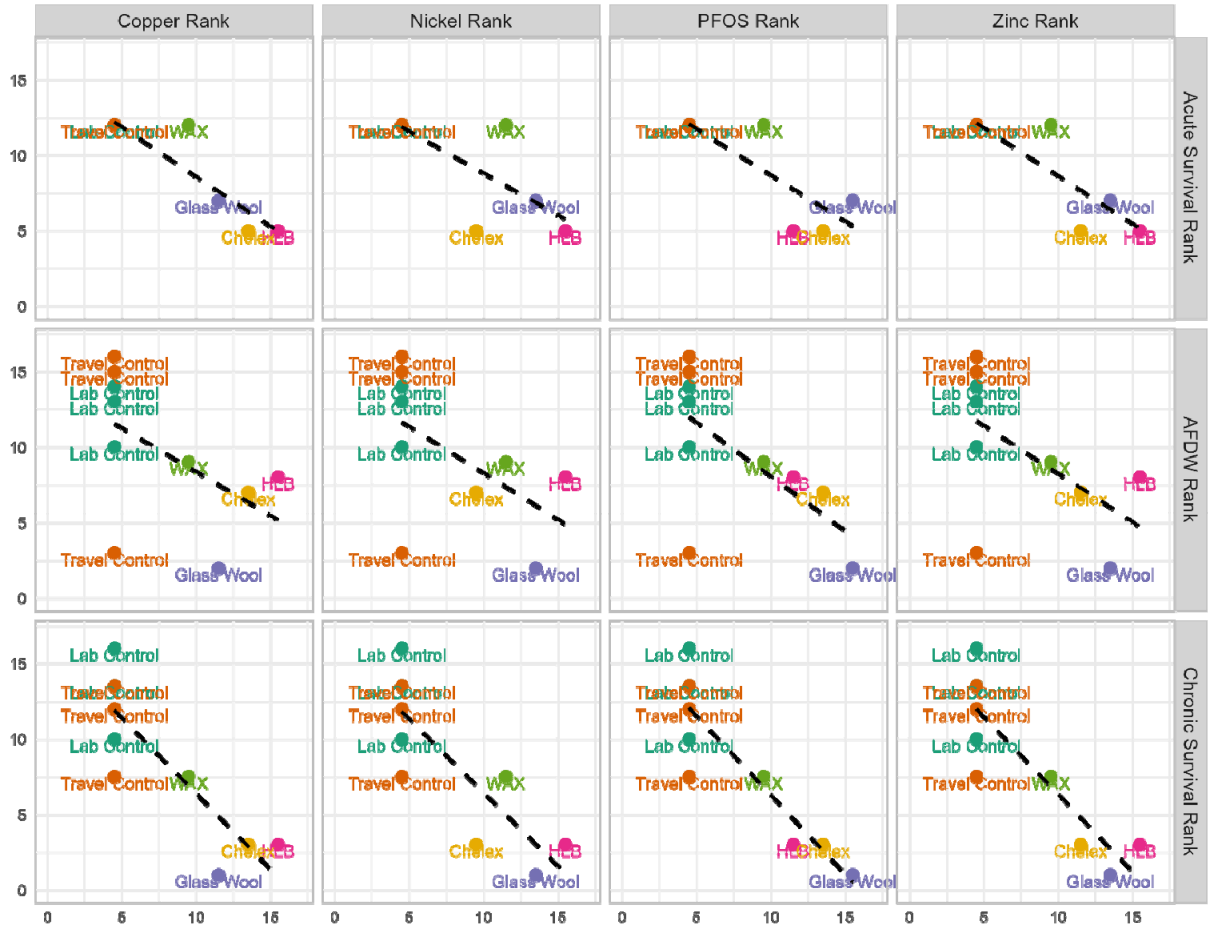

Figure 7.5.6: Plots depicting relationships between C. dilutus biological endpoints and chemistry data from Spearman's rank analyses for the iTIE experiments at the Rouge River. The dotted lines show correlation coefficients. Groups with lower ranks on the x-axis were exposed to lower concentrations of contaminants. Higher ranks for AFDW indicate higher mean weights of surviving organisms. Higher acute and chronic survival ranks indicate higher survival.

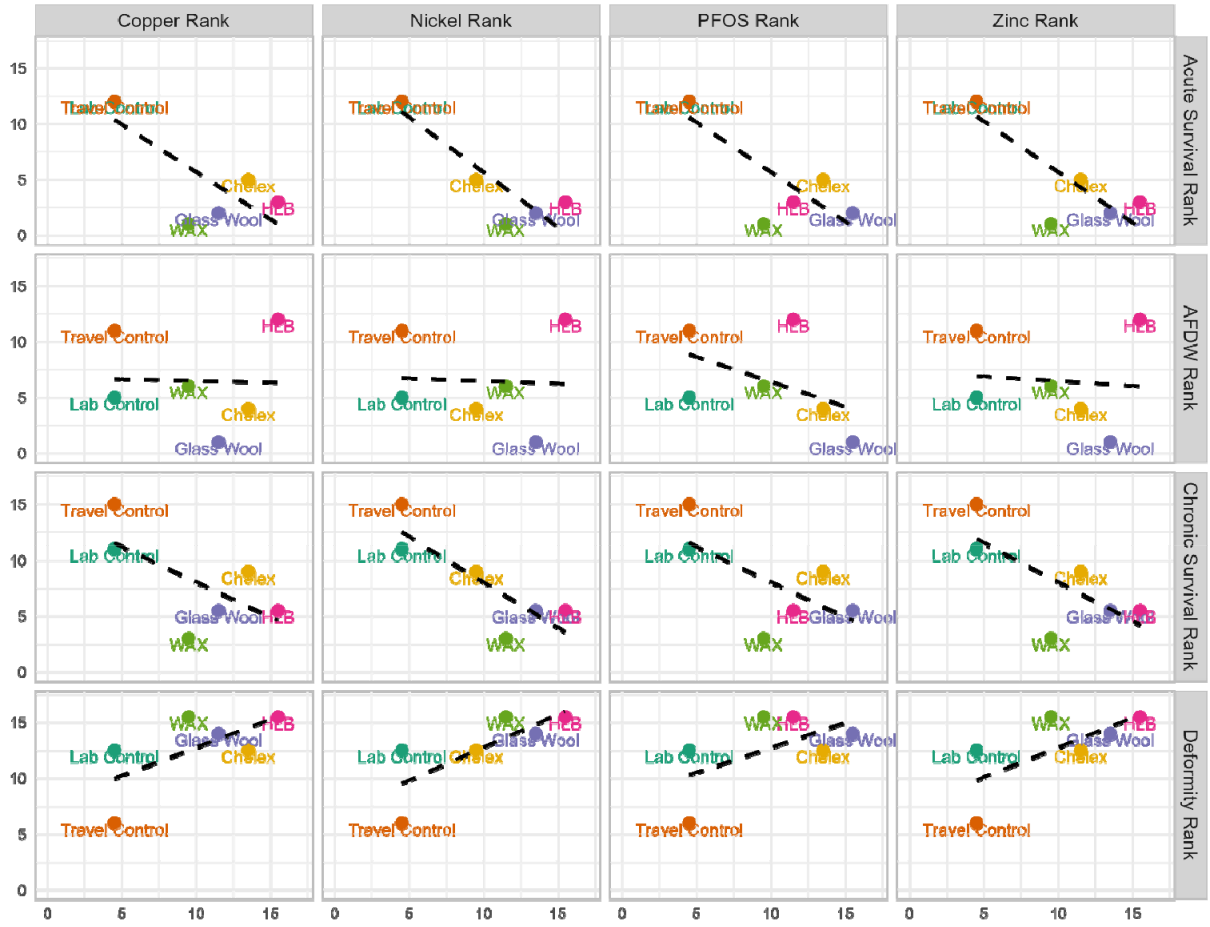

Figure 7.5.7: Plots depicting relationships between P. promelas biological endpoints and chemistry data from Spearman's rank analyses for the iTIE experiments at the Rouge River. The dotted lines show correlation coefficients. Groups with lower ranks on the x-axis were exposed to lower concentrations of contaminants. Higher ranks for AFDW indicate higher mean weights of surviving organisms. Higher acute and chronic survival ranks indicate higher survival. Higher ranks in deformity indicate more teratogenicity occurred in the group.

In summary, heavy metals and PFAS were found to be the dominant stressors at the Rouge River. *C. dilutus* larvae, a test organism especially sensitive to PFAS, had the highest survival rate in the Oasis WAX treatment. *P. promelas* larvae, which are less sensitive to PFAS but more sensitive to heavy metals, had the highest survival rate in the Chelex treatment. Furthermore, Spearman's ranked correlation analyses could be used to establish causal linkages between the two CoC classes and various toxicity endpoints.

The water quality has improved greatly at the mouth of the Rouge River since the initialization of remediation efforts by local and federal stakeholders. Some CoC classes including PAHs and PCBs were not detected at the site, contrary to expectations. However, some CoC classes remain, including PFAS and heavy metals, and those classes continue to cause toxicity to the benthic community.

8.0 DECISION-MAKING FRAMEWORK

The iTIE technology is best suited for use in higher tiered assessments, rather than Tier 1 surveys. Its primary purpose is to determine which class of chemicals is primarily responsible for site toxicity. This implies two things: 1) there is a mixture of CoCs present and 2) site toxicity exists due to impacted sediments and possibly overlying waters and/or outfalls. The presence of high levels of CoCs (exceeding known toxicity thresholds, such as criteria, guidelines, or benchmarks), is adequate support for use of the iTIE if site-specific toxicity data is unavailable. Other key requirements for use of the iTIE are ease of access, expertise for site deployment, and resources ensuring adequate QA/QC.

8.1 TIERED APPROACH FOR DECISION MAKING

The following Decision-Making Framework describes five tiered steps to follow to ensure data is produced that will aid decision-making for remediation, recovery or site status projects.

Tier 1. Set appropriate Reference Condition

Most impacted sediment sites reside in urban-dominated watersheds (i.e., catchments). This means that the impacted site is likely receiving a mixture of off-site CoCs, such as metals, nutrients, pesticides, oils, greases, and PAHs. Most urban waterways suffer from "urban stream syndrome", which has been recognized for decades (Burton and Pitt 2002). This term states that the great majority of urban streams are biologically impaired and have degraded habitat due to runoff from surrounding urban activities and impervious areas. These realities confound site assessments focused on a particular source (e.g., marina) or activity (e.g., shipping), since other off-site stressors are contributing to aquatic impairments.

Given this reality, it is challenging to select appropriate reference sites in human dominated systems. Certainly, pristine sites with little human activity are not appropriate, as attaining those conditions is unrealistic. The reference site selection is often controversial and should be done with input from the relevant regulatory authorities. It is well-recognized that more than one reference site is needed as all sites differ from each other, even when they are pristine. It is most appropriate to select multiple local sites that are free from the CoCs at the study site. By using multiple sites, a "Reference Condition" can be established which thereby acknowledges natural ecosystem variation as a point for statistical comparisons of site data. For example, if the study site is a Navy pier, then reference sites should have a similar hydrology, salinity, temperature with limited boating or impervious area runoff.

Tier 2. Do non-chemical stressors dominate?

It is common for impacted sediment sites surrounded by urban and industrial land uses to have degraded habitat conditions. In harbor areas, this is usually dominated by clayey depositional sediments, often mixed with silt and sand grain sizes. These sediments may be subject to resuspension from ship propellers, enhanced wave action, and hardscape (e.g., rip-rap, concrete banks), all resulting in turbid conditions. These sediment and overlying water conditions tend to accentuate diurnal flux of dissolved oxygen, which may drop to zero during

night-time hours. These conditions also result in physical stressors for aquatic biota and will limit the ability of pollution-sensitive species to reside there. Diminished riparian (vegetated banks) areas reduce inputs of terrestrial organic matter and insects, serving as food for aquatic biota at multiple trophic levels. Finally, all marine harbors have invasive invertebrates that have been introduced via ship hulls. Invasive benthic species tend to out-compete indigenous species for food and habitat; and are typically more resistant to CoCs (Havel et al., 2015; Karatayev et al., 2009). In summary, some non-chemical stressors may drive aquatic impairments at the project site and must be identified. The role of non-chemical stessors is best described by comparing to appropriate reference conditions or conducting reciprocal transplant studies where reference and site sediments are exchanged to show which benthic species dominate colonization exposures.

Tier 3. Is the site toxic? Are food-chain impacts a risk?

Measure *in-situ* chronic toxicity and bioaccumulation (key species in caged water/sediment exposures and colonization/transplants). If chronic toxicity or bioaccumulation are detected, characterize relevant chemicals in tissues, sediments, upwellings, surface and storm waters.

Tier 4. Which chemical(s) drive toxicity and or bioaccumulation?

Conduct *in-situ* TIEs to identify CoCs that contribute the most to site toxicity. Consider whether pulse exposures to sediment resuspension or stormwater runoff events may also contribute to site toxicity.

Tier 5. Identify and rank stressors driving biological integrity/impairments

If successful Weight-of-Evidence evaluations have been conducted, identify the likely dominant stressors at the test and reference sites, and the role that site toxicity has in biological impairments.

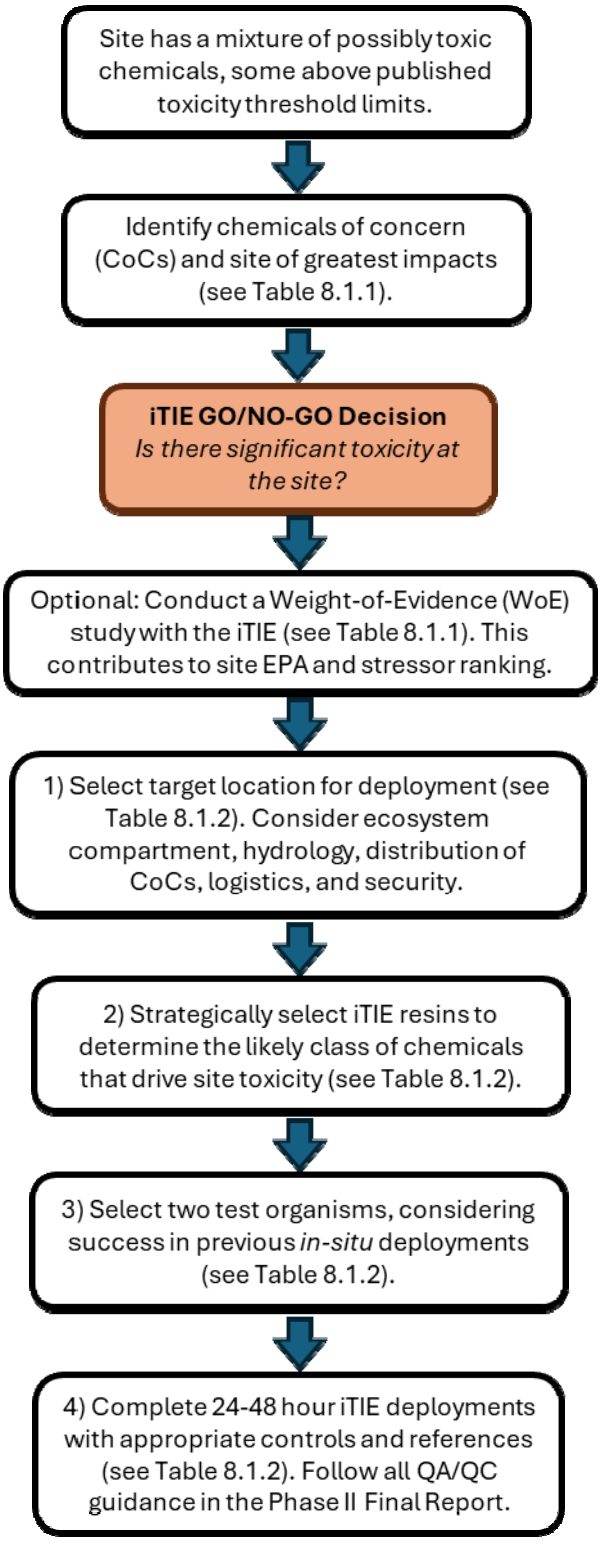

Figure 8.1.1: iTIE Decision-Making Flowchart

Table 8.1.1: Weight-of-Evidence Assessment Design.

Weight-of-Evidence Procedure	Considerations
1) Characterize the site.	• Site characterization should include salinity, hydrology, groundwater upwelling, and available habitat.
2) Select nearby Reference Sites.	• Consider regulatory approval. • Choose sites with minimal CoC or site chemical concentrations.
3) Characterize non-chemical stressors.	• Habitat-related stressors may include flashy flows, excess physical disturbance (e.g., hardscape energy, propeller resuspension), excess turbidity, elevated temperatures and sun exposure, changes to food availability, sediment grain size, and sediment transport. • Other natural stressors may include dips in dissolved oxygen, excess sulfides or nutrients, and salinity flux.
4) Characterize benthic macroinvertebrate communities.	• Determine the role of any present invasive species: competition for habitat or food, or excessive predation. • Conduct reciprocal sediment transplants for benthic macroinvertebrate colonization.

Table 8.1.2: iTIE Deployment Design Parameters.

Study Design Parameter	Options
1) Ecosystem compartment	• Surface water: use weighted intake tube configuration • Surficial sediment porewater (3-8 cm depth recommended for biologically active zone and recent CoC deposition): use Trident probe configuration
2) Diagnostic resin treatment	**Strategically select resins based on known chemicals present, to determine the chemical class likely driving toxicity.** • Glass or polyethylene wool; unfractionated control treatment. • C18 for nonpolar organics (i.e., PAH, organophosphates) removal • Chelex for metal removal • Oasis WAX for PFAS removal • Oasis HLB for organics (i.e., PAH, pesticides) removal • Zeolite for ammonia removal
3) Test organisms	**Consider successful use in previous iTIE deployments, toxicant sensitivity, ambient water temperature and salinity sensitivity, ecological relevance, benthic vs. pelagic species, and short-term chronic toxicity (via teratogenicity and post-exposure testing.** • For Pacific shoreline areas, use mysid shrimp larvae (i.e. *Americamysis bahia*) and topsmelt silverside embryos/larvae (*Atherinops affinis*) • For Gulf Coast areas, use mysid shrimp larvae and sheepshead minnow embryos/larvae (*Cyprinodon variegatus*) • For freshwater areas, use early-life stage daphnids (i.e. *Daphnia magna*) and amphipods (i.e. *Hyalella azteca*), midge larvae (*Chironomus dilutus*), or fathead minnow embryos/larvae (*Pimephales promelas*)
4) Controls and References	• Laboratory control groups: exposed in-lab to culture water • Site reference groups: exposed *in-situ* to site surface water at test and reference sites • Travel control groups: transported to the test site and exposed in-lab to culture water during deployment at the site ambient temperature

9.0 CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

In summary, this report details Phase II of SEED Project No. ER18-1181. This project focused on the development and proof-of-concept demonstration of the iTIES, which is a novel *in-situ* tool that systematically removes toxicity-causing chemical classes from site water and directly exposes test organisms to the water. The tool can be used to strengthen stressor-causality linkages at sites impacted by multiple stressors, through realistic, cost-effective exposures. The technology improves upon previously developed weight-of-evidence methodologies like the USEPA laboratory TIE method. The iTIES is intended for incorporation into Tier 2 or 3 of weight-of-evidence studies. The technology can be used in characterizations of impacted sediments, remediation, recontaminated sites, and CoC source identification investigations.

This project addressed several SERDP priorities, including the need for "innovative approaches for both monitoring and implementing *in-situ* remediation of impacted aquatic sediments" that ultimately "reduce costs associated with monitoring and treating contaminated aquatic sediments, while still being protective of human health and the environment". Additionally, this project produced an "innovative new monitoring and remediation technology for contaminated aquatic sediments", that will "improve the ability of site managers to manage contaminated sediment sites". Phase II of this project consists of six Tasks which were completed through a series of laboratory- and field-based studies.

Task 1 (the refinement of the iTIE prototype to allow for porewater sampling and diverless deployment) was addressed in several studies during Phase II. First, the prototype iTIES was expanded to include the Trident probe, a robust porewater sampling system previously developed by Chadwick et al. (2003) and widely used at DoD sites. The Trident probe allows for porewater extraction at a variety of sediment depths. In waters up to 10 meters deep, the Trident can be deployed diverlessly from a pier or boat using a direct push pole system. The Trident includes a stopper plate that helps control sampling depth below the sediment surface, as well as limiting surface water infiltration. A lab-based study completed by the research team confirmed that no surface water infiltration occurs when using the Trident at sampling depths of >7.5 cm below the sediment surface (Section 4.1).

As part of Task 1, it was necessary for the iTIES to include the capacity to aerate anoxic porewater samples. The iTIES was expanded to include an oxygenation system, comprised of a passive pressurized oxygen canister that supplies oxygen gas to a length of gas-permeable tubing. The pressurized tubing is threaded within larger tubing, through which sampled porewater is conveyed and is gently exposed to oxygenation. The nested tubing system is coiled within the central iTIES cooler for ease of transportation. Several tubing materials were assessed in a lab-based study, particularly in exposures to anoxic water containing high concentrations of dissolved sulfide (Section 4.2).

Immediately after exiting the oxygenation coil, aerated sample water enters a drip chamber, where gas bubbles that had formed within the oxygenation coil, as well as excess sampled water, are routed from the system to an overflow collection bottle. Water is then split to different iTIE units, each containing different diagnostic resin treatments and test organism groups. The water is systemically cleaned of various CoC classes, exposed to organisms, and collected in sample bottles contained within the iTIES cooler. Water movement through the iTIES is driven by a series of peristaltic pumps, housed in two easily transportable waterproof cases.

The remaining project tasks were completed using the expanded iTIES prototype. Task 2 (the testing of early life stage fish) was completed in an array of lab- and field-based tests (Sections 6.1, 7, and 8). For freshwater applications, the U.S. EPA Method 1001.0, a method utilizing *P. promelas* larval survival and teratogenicity as chronic toxicity endpoints, was adapted for iTIES use. This test method was successfully used at several impacted sites, including at Clark's Marsh in Oscoda, MI, and the Rouge River in Detroit, MI. Teratogenicity was observed following exposure to a variety of CoCs including heavy metals. For marine applications, embryo-larval *A. affinis* was successfully used as a model organism, with survival and teratogenicity as chronic toxicity endpoints.

Task 3 (an expansion of available sublethal chronic endpoints in invertebrate test organisms) was also addressed in several lab- and field-based investigations. In a lab-based study, changes in the AChE activity of adult *H. azteca* were used to detect and quantify chronic toxicity due to exposure to organophosphate pesticides (Section 6.2). This chronic endpoint suggests the potential application of other enzymatic bioassays for iTIES use. Additional chronic toxicity endpoints were used in various iTIES studies, including growth rates of early life stage fish and macroinvertebrates and reproduction rates of daphnids (Sections 5, 7, and 8).

To address Task 4 (a continuation of resin optimization efforts), several resins were investigated with a focus on resin conditioning procedures and toxicity caused by the resin itself (Section 5). These efforts yielded several important findings. 1) Of the numerous resins evaluated by the research team which are marketed as margetting PFAS, Oasis WAX appeared to cause the least acute and chronic toxicity. 2) Chelex, a chelating resin effective in adsorbing heavy metals, can cause stress to freshwater organisms due to hardness and pH shock when improperly conditioned, and must be converted from sodium-form to calcium-form prior to use. 3) GAC, a class of resins that can target a wide array of CoCs including organics, metals, and sulfide, can itself induce stress to organisms through release of suspended solids and acidifying or alkalizing substances, and to preclude this, GAC conditioning must include suspension in continuously aerated deionized water, as well as repeated rinsing.

Task 5 (additional field verifications in marine and freshwater environments) were completed at a variety of sites. Marine deployments were completed at Paleta Creek in National City, CA, where high degrees of acute toxicity were linked to the presence of organic toxicants (Section 7.1). Though analytical chemistry results were unavailable, the results of the iTIES deployments aligned with previous studies at the mouth of Paleta Creek. These studies identified pyrethroids as the dominant stressor class at the site, with heavy metals and PAHs as secondary and tertiary stressors.

Freshwater deployments were completed at several sites throughout the state of Michigan. The iTIES prototype was field-verified at relatively clean sites including Third Sister Lake and Fleming Creek in Ann Arbor, MI. Moderate toxicity was detected at the Sexton and Kilfoil Drain in Taylor, MI, though procedure improvements were identified and required implementation as a result of the deployment.

One key freshwater iTIES deployment occurred at Clark's Marsh in Oscoda, MI (Section 8.4). Clark's Marsh is located downstream of an AFFF-impacted site and has a history of PFAS impacts. Due to logistical constraints, Clark's Marsh sediment and site water were collected, and the iTIES was deployed within the sediment in an *ex-situ* setting with early life stage fish and

chironomid larvae. PFAS was found to be highly correlated with toxicity responses at the site and was thus identified as the dominant stressor class.

A second key freshwater iTIES deployment was completed at the Rouge River in Detroit, MI (Section 8.5). The Rouge River drains a heavily industrialized, urbanized watershed, with numerous CoCs previously detected in its sediment. Significant remediation efforts completed by local and federal stakeholders have improved site conditions, though concerns remain regarding legacy CoCs. Through an iTIES exposure at the site including early life stage fish and chironomid larvae, the research team found that heavy metals and PFAS remain as dominant stressors in the Rouge River.

Finally, Task 6 (development of a decision-making framework to guide iTIES implementation) was addressed in Section 9. A flowchart and several decision tables are included to aid future users of the iTIES to incorporate the technology and testing protocol into weight-of-evidence studies.

The iTIES is an effective, durable, user-friendly, and broadly applicable diagnostic tool that can be used to strengthen stressor-causality linkages and rank CoC classes at sites impacted by multiple stressors. After Tier 1 assessments suggest that chemicals may be causing toxicity at a site, the iTIES should be integrated as a Tier 2 or 3 level methodology to determine which CoC classes should be targeted in further study or remediation efforts.

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APPENDIX A: PALETA CREEK ITIE DEPLOYMENT CHEAT SHEET

A.1 SUMMARY OF RESEARCH ACTIVITIES

The Burton Ecotoxicology Lab from the University of Michigan, in collaboration with Coastal Monitoring Associates and the Naval Warfare Information Center, plans to conduct a test deployment of the in-situ Toxicity Identification Evaluation (iTIE) technology at Paleta Creek in San Diego, CA between August 4th and 17th, 2024. This test deployment is aimed to address the following research goals: 1) demonstrating a successful iTIE deployment in a marine/estuarine setting; 2) validating the updated oxygenation coil's performance in the presence of anoxic pore water with high dissolved sulfide content; and 3) verifying the applicability of a draft topsmelt (*Atherinops affinis*) embryo-larval toxicity test to the iTIE system and testing protocol. The results of this deployment may also be useful in future site characterization efforts.

The iTIE prototype will be deployed at Paleta Creek for 48 hours. The following diagnostic resin treatments will be used: Oasis HLB for the detection of organic contaminants; Chelex for the detection of metals; and KDF-85 or granular activated carbon for the detection of toxicity due to dissolved sulfides, pending upcoming lab experiments. A fourth treatment will contain glass wool as a control. The primary organism to be used in this deployment is topsmelt embryos (4 dpf). The test will use 100 embryos: three lab control groups, three travel control groups, and four treatment groups, with 10 embryos per group. Other potential organisms include mysid larvae (*Americamysis bahia*) and juvenile amphipods (*Eohaustorius estuarius*). If any additional test species are added, the test will include 100 individuals of that species.

Site water samples will be collected and preserved for chemical analysis. Six water samples will be collected, including an equipment blank prior to the deployment, unprocessed site water at the initiation of the deployment, and site water from each of the four diagnostic treatments. Water subsamples will be preserved for later dissolved sulfide analysis using zinc acetate and sodium hydroxide. This analysis will be completed by the Burton Ecotoxicology Lab. Other chemical analyses, including metals (copper, nickel, and zinc), organophosphates (chlorpyrifos), per- and polyfluoroalkyl substances (PFOS), polycyclic aromatic hydrocarbons (EPA's 16 High Priority PAHs), and pyrethroids (permethrin), will be conducted by a commercial analytical lab. Water subsamples will be preserved, stored, and shipped appropriately.

Following the completion of the 24-hour field deployment, surviving topsmelt embryos will be cultured in clean seawater until 4-days post-hatch. This should take approximately 12 days. At the end of the culturing period, surviving individuals will be preserved using ethanol, visually assessed under a microscope, and photographed. The following results will be recorded: embryo and larval mortality, hatch rate, and gross morphological deformities. Any other species used during the deployment will only be assessed for survival rates.

A.2 TASK LIST AND TIMELINE

Table A.1: A timeline and list of tasks for the Paleta Creek iTIE Deployment completed in August 2024.

Day	Activities
Prior to arrival	• Begin resin conditioning • Ship equipment to San Diego in pallet crate • Receive equipment at CMA/NIWC
(Day -2)	• Verify site water temperature – pore water and water at depth • Continue resin conditioning • Make starch solution for sulfide iodometry • Standardize sodium thiosulfate solution, iodine solution for iodometry • Prep Trident – clean, check tubing connections, glass beads • Set up water bath – heater or chiller • Compile data sheets, cheat sheets, SOPs, packing list • Charge first pump case overnight
(Day -1)	• Receive organisms • Acclimate test organisms, hold them at field temp overnight • Finish resin conditioning • Collect and preserve equipment blank • Sulfide iodometry of equipment blank • Charge second pump case overnight • Charge backup battery overnight • Prepare ice / heat packs • Assemble supplies to be packed
(Day 0)	• Sort organisms into control, treatment groups • Set up lab controls • Initiate iTIE deployment at site • Collect and preserve unprocessed site water sample • Return to lab, set up travel controls in water bath • Sulfide iodometry of unprocessed site water
(Day 1)	• Complete iTIE deployment at site • Return to lab, set up treatment groups in water bath • Acclimate organisms to 21 +/- 1C • Begin cleaning equipment • Sulfide iodometry of treatment water samples
(Day 2)	• Ship water samples to Eurofins • Continue cleaning equipment if needed • Culture organisms (water quality checks, feeding, euthanasia)
(Day 3-7)	• Culture organisms (water quality checks, feeding, euthanasia)
(Day 7-9)	• Examine and photo-document organisms under microscope

A.3 MATERIALS LIST

Organisms:

- Topsmelt silverside embryos (arrival on Day -1 at 4 dpf)
- Mysid shrimp (arrival on Day -1
- Brine shrimp (*Artemia*) cysts

Reagents:

- Culture water (Filtered Seawater)
- Deionized water
- Bleach (for *Artemia* decapsulation)
- pH calibration standard (4, 7, 10)
- Resins
    - Glass wool
    - Oasis HLB
    - Oasis WAX
    - Granular activated carbon
    - Chelex
- Resin conditioning reagents
    - Calcium chloride dihydrate, solid
    - Methanol (also for decontamination)
- Sample preservation reagents
    - Zinc acetate solution, 2M
    - Sodium hydroxide solution, 6N
    - Nitric acid
- Sulfide iodometry reagents
    - Hydrochloric acid, 6N
    - Iodine solution, 0.025N
    - Sodium thiosulfate solution, 0.025N
    - Standard potassium bi-iodate solution, 0.025N
    - Soluble starch, solid
    - Salicylic acid, solid
- Ethanol, 70% (for fish preservation)
- Liquinox (for decontamination)

iTIE Equipment:

- iTIE cooler
    - iTIE units (4x; including bottom fittings, resin chambers, middle fittings w/ mesh, organism chambers, and top fittings w/ mesh)
    - Overflow bag
    - Pressurized gas regulator
    - Oxygen canister (at least 2x)
    - Sample bottles (4x)
- iTIE pump cases
    - iTIE booster pump case
    - iTIE regulation pump case
    - Pump case charger
- Trident
    - Trident w/ stopper plate

    - Push poles
    - Locking pins
    - Stopper plate stabilizer and nut
    - Fence post driver
    - Trident probe tips (6x; including wire screen inserts, outer sheaths, and pointed tips)
    - Glass beads
- Ice packs/bags

iTIE Toolboxes:

- Thermometers
- Tube cutting tool
- Tube groovers (3, different sizes)
- Phillips head screwdriver
- Flat head screwdriver
- Hex screwdriver for oxygen canister
- Scissors
- Pliers
- Dissecting needle
- Zip ties & zip tie cutter
- Plumber tape
- Electrical tape
- Lab labeling tape, different colors
- Field notebook
- Sharpies

Water Quality Meters:

- YSI ProODO, calibrated
- Thermo A121 Orion Star pH meter and probe
- YSI ProDSS meter, calibrated

Field Kit & Other Field Supplies:

- Roll of 1/4” OD tubing for booster pump
- Roll of 1/18” OD, 1/16” ID tubing for regulation pumps
- Nitrile gloves
- Squirt bottles of DI and culture water (4x)
- Centrifuge tubes (50x; for resin equilibration/transport and organism transport)
- Thermometers (at least 3)
- Pen lights (2x)
- Plastic weigh boats for resin loading
- Plastic disposable scoopulas
- Falcon pipettes
- Sorting trays (2x)
- Funnels
- Nalgene bottle with lid for waste, 1L
- Gallon Ziploc bag for waste
- First Aid Kit

Sample Processing and Shipping Equipment:

- Clean amber glass sample bottles, labeled

- Cooler with ice packs, maintained at <4C, available to ship to Eurofins
- Bubble wrap pouches

Sulfide Iodometry Equipment:

- Vacuum pump system with trap
- Vacuum flasks
- Vacuum filtration funnel
- Glass filters
- Conical flasks, 500 mL
- Squirt bottles
- Burettes (2x)
- Burette stand (1x or 2x) w/ clamps (2x)
- Funnels
- Serological pipette pump (50 mL capacity
- Serological pipettes and (50 mL)
- Hotplate

Fish care equipment:

- Carboys for FSW and DI water
- HDPE basin for water bath
- Aquarium controller
- Aquarium heater
- Aquarium chiller and pump
- Timed portable light (fluorescent or LED)
- Light table
- Aeration system
- Centrifuge tubes, 50 mL
- Refrigerator
- Microscope for organism observation

Brine shrimp hatching equipment:

- Separatory funnel (1 L)
- Separatory funnel ring stand
- Aeration system w/ glass serological pipette
- Heat lamp
- Aluminum foil or cloth (to block light when collecting brine shrimp)
- Mesh cup, 100 um size (for brine shrimp collection)

Other equipment:

- Sieve and/or mesh, 75 um size (for resin rinsing and conditioning)
- 5 gallon bucket (for equipment blank collection, decontamination)
- Graduated cylinder, 50 mL (for pump calibration)

Smartphone or tablet with Mobius app

A.4 DEPLOYMENT CHEAT SHEET

This section will describe iTIE system preparation, deployment, and deconstruction.

I. Prior to Day -2:
   1. Make a **Go/No Go decision** for deployment.
   2. Select **site-specific resin treatments**. Begin **conditioning resins**.
   3. Select organism species and age(s) to be used. **Place order for organisms** if needed, for arrival on Day -1.

II. Day -2:
   1. Measure site water quality parameters, including temperature, pH, conductivity, salinity and hardness.
   2. Continue **conditioning resins**, by rinsing with DI on a sieve and equilibrating in DI.
   3. Prep the **Trident**, including adding glass beads and orienting the stopper plate.
   4. Plug in the **booster pump** case to charge overnight.
   5. Plug in the **quad pump** case to charge overnight.
   ▪

III. Day -1
   1. **Receive organisms**. Remove dead organisms, unhealthy organisms, and debris.
   2. **Acclimate organisms** to site water temperature and quality parameters.
   3. Finish **conditioning resins**, by rinsing with DI on a fine sieve and placing into 50 mL centrifuge tubes with a small amount of culture water.
   4. **Calibrate all pumps** using the Mobius app. Select each pump, navigate to "Calibrate", and calibrate using DI and a graduated cylinder.
   5. **Clean all iTIE equipment**, using a Liquinox and DI triple-rinse. If needed, clean with a stronger solvent, such as methanol.
   6. Collect an **equipment blank** by running DI water through the entire system, including the Trident, iTIE coil, iTIE units, and sample bottles. The Trident can be positioned in a bucket filled with DI water. Preserve the equipment blank in accordance with Section A.5: Water Sample Preservation and Shipping Cheat Sheet.
   7. Install a new oxygen canister into the iTIE cooler bottom. Check that the outlet pressure is adequate (above 100 psi).
   8. Place ice packs in the freezer if needed on Day 0.
   9. Gather supplies, as listed in Section 3, for ease of packing on Day 0.
   ▪

IV. Day 0
   1. Count and sort healthy organisms into treatment groups.
      i. Set up lab controls in a clean mesh beaker.
   2. Travel to site.
   3. Install Trident.
      i. Groove one end of the ¼" tubing.

ii. Attach grooved tubing end to the Trident. Use plumber's tape if needed to make connection watertight. **Do not cut the tubing yet.**
iii. Adjust the **stopper plate** to a **3-inch sampling depth** to prevent surface water infiltration. Make sure the holes on the stopper plate stabilizer align with the holes on the top of the Trident.
iv. Attach push poles to the Trident, locking the stopper plate at the correct position.
v. Lower the Trident into the water and insert it into the sediment. Use the fence post driver to hammer the Trident firmly into the sediment. Minimize lateral movement when installing.
vi. Cut the ¼" tubing, leaving enough length so the tubing can be connected to the booster pump. **However, do not connect it to the pump yet.** Instead, secure the tubing so it doesn't fall into the water. It will be connected to the pump later.

4. Prime the oxygenation coil using surface water.
   i. Cut another length of ¼" tubing, long enough to collect site surface water.
   ii. Groove one end of the ¼" tubing and connect it to the "IN" port of the booster pump. Use plumber's tape to make the connection watertight if necessary.
   iii. Cut a 2-meter-long length of ¼" tubing. Groove both ends of the tubing. Connect one end to the booster pump "OUT" port and the other end to the oxygenation coil inlet, threading through the port in the cooler. Use plumber's tape to make the connections watertight, especially on the oxygenation coil inlet connection.
   iv. Adjust the T-valve to connect the oxygen coil port and the drip chamber port (T pointing to the right).
   v. Attach Masterflex silicone tubing to the drip chamber outflow port and route the tubing into an overflow bottle.
   vi. Tip the cooler forward, so the oxygenation coil is vertically oriented.
   vii. Pump site surface water quickly into the oxygenation coil. Tap/wiggle the coil tubing to dislodge air bubbles. Catch water exiting the overflow tube in a waste bottle. Continue pumping until all air bubbles are cleared.
   viii. Close the manifold stopcock valve.
   ix. Tilt the cooler back to the correct orientation, and raise the overflow tube above the oxygenation coil so water does not leak out.
5. Prime the drip chamber, manifold, and manifold tubes.
   i. Install iTIE unit bottoms and resin chambers in the iTIE cooler rack. Connect the iTIE unit bottoms to the manifold tubes.
   ii. Open the manifold stopcock valve.
   iii. Run the booster pump to prime the drip chamber, manifold and manifold tubes. Aim to fill the drip chamber with as much water as possible. Close the manifold stopcock valve once complete.
6. Prime the Trident tubing, collect an unprocessed site water sample, and connect to the iTIE cooler.

i. Disconnect the surface water tubing from the booster pump "IN" port. Groove the end of the tubing connected to the Trident and connect it to the booster pump "IN" port.
ii. Disconnect the tubing attached to the oxygenation coil inlet. Place the end of that tubing in a waste bottle.
iii. Run the booster pump to prime the Trident tubing. The water may be notably cloudy/dirty at first. Run the pump until the initial cloudiness has passed.
iv. Divert the tubing end into a sample bottle. Run the booster pump until an adequate volume of water is collected (around 500 mL). Preserve the water as needed and bubble-wrap and store in a cooler at 4C.
v. Reconnect the booster pump "OUT" tubing to the oxygenation coil. Plumber's tape will once again be necessary to ensure this connection's watertightness.

7. Load resin chambers.
    i. Place a small amount of glass wool at the bottom of each resin chamber.
    ii. Record which resin will go into each iTIE unit in the field notebook.
    iii. Prep each resin by scooping it into a clean weigh boat.
    iv. Use a scoopula to load each resin into the appropriate resin chamber. Add culture water dropwise using a squirt bottle or Falcon pipette until resins are settled and saturated with water.
    v. Place a small amount of glass wool on top of the resin chamber, leaving room to connect iTIE middle pieces to the tops of each resin chamber.
    vi. Saturate all resins with clean culture water.
    vii. Place iTIE middle pieces and organism chambers on top of all resin chambers.
8. Load organisms and complete construction.
    i. Squirt a small amount of culture water into each organism chamber.
    ii. Load each organism group into an organism chamber. Rinse all organisms into the chambers.
    iii. Gently fill all organism chambers with culture water or clean surface water.
    iv. Attach iTIE top fittings on top of all organism chambers.
    v. Attach regulation pump "IN" tubes to each iTIE unit. Make sure the correct tubes are attached to each iTIE unit using color-code guides.
    vi. Attach regulation pump "OUT" tubes to sample bottles, using color-codes to ensure the tubes are connected to the correct bottles.
    vii. Place sulfide preservation reagents into each sample bottle.
        1. 1 mL of the zinc acetate solution
        2. 0.2 mL of the sodium hydroxide solution (4 drops)
9. Complete the final setup steps.
    i. Make sure all stopcock valves are set to the correct position.
        1. The vent valve (top of the iTIE carriage) should be closed.
        2. The valve adjacent to the oxygen canister regulator should be open.

3. The T-valve should be set so all three ports are connected (bottom of the T pointing to the left).
4. The manifold stopcock valve should be open.

ii. Open the master regulator valve (directly adjacent to the canister).
iii. Check that the regulator gauges are reading the correct pressure.
1. The cylinder pressure gauge (right) should read above 100 psi.
2. The delivery pressure gauge (left) should read at 20 psi. Adjust the regulator dial if needed, turning clockwise to increase pressure.
iv. If needed, place ice/heat packs in the iTIE cooler to moderate temperatures.

10. Program and operate the pumps.
i. Reset the date/time on the Mobius app to a new date, to reset daily pump volumes.
ii. Schedule all pumps to begin at the same start time, and to run for 23:59:59.
iii. Set total daily volumes (TDV) for the booster pump and each regulation pump.
1. Each regulation pump should have the same TDV.
2. The booster pump should have a TDV that is greater than the sum of the TDVs of the regulation pumps. For example, if each regulation pump has a TDV of 480 mL (20 mL/hr), then the booster pump should have a TDV greater than 1920 mL (80 mL/hr). Typically, in this scenario, the booster pump would be set to have a TDV of 2400 mL (100 mL/hr).
iv. Once the start time is reached, press Play (▶) on all pumps in Mobius app.
v. Wait for the pump programs to begin. When the pumps begin running, the LED indicator light will turn blue and begin slowly pulsing. You should also see the pumps begin to move.
1. If the LED indicator light is yellow and blinking, the pumps are not set to run. Press Play to run the pumping program.
2. If the LED indicator light is blue and not pulsing, the pumps are scheduled to run but not currently running. Press Stop and Play to reset and run the pumping program.
vi. After 10 minutes, check that the pumps are operating correctly. Sometimes the pumps glitch and stop running.

11. Secure all equipment.
i. Make sure equipment is on solid ground and secure from tipping over.
ii. If leaving the site, zip-tie cases and coolers closed to discourage tampering. Attach laminated signage to the equipment with contact information in case passersby have questions or concerns.

12. Travel back to the lab and set up travel controls.
i. At the lab, set up travel controls in mesh beakers of clean culture water in the water bath. Feed if needed.
ii. Place unprocessed site water sample in the fridge.

13. Periodically check the equipment through the run duration. Check that:
i. Pumps are still operating correctly.

ii. Oxygen bubbles have not disrupted the flow of water through the system.
iii. The drip chamber still contains water.
iv. Organism chambers are full of water.
v. No widespread organism mortality across all treatments.
vi. Sample bottles have roughly equal volumes of water.
vii. Water quality parameters in overflow water are within bounds.

-

V. Day 1

1. Continue to monitor equipment.
2. After 200 mL of samples have been collected in each sample bottle, switch in new sample bottles pre-loaded with 1 mL nitric acid to preserve for Eurofins chemical analysis.
    i. Preserve samples for sulfide analysis in amber glass bottles, wrap in bubble wrap, and place in a cooler at <4C for future sulfide analysis.
3. Measure sulfide content of the water samples with the procedure in Section 6: Sulfide Iodometry Cheat Sheet.

-

VI. Day 2

1. Verify successful iTIE operation on site.
    i. Measure and record the temperature and DO content each sample bottle.
    ii. Check that the iTIE organism chambers are still full of water.
    iii. Check that the sample bottles have all collected approximately the expected volume of water, with minimal volume discrepancies.
2. Transfer preserved water samples into labeled amber glass bottles. Bubble-wrap the bottles and place them in a cooler at 4C.
3. Close the oxygenation canister's master valve and depressurize the oxygenation coil by opening the vent valve.
4. Observe organisms and log survival.
    i. Count living organisms.
    ii. Transfer all live organisms to clean centrifuge tubes and fill the centrifuge tubes halfway with clean culture water.
5. Travel to lab and set up treatment groups in the water bath.
    i. Acclimate organisms up to the appropriate culturing temperature at a rate of 1C per hour. Feed organisms if needed.
6. Place preserved sample bottles in the fridge. These will be shipped with the procedure in Section A.5: Water Sample Preservation and Shipping Cheat Sheet.
7. Decontaminate and dry all iTIE equipment.
8. Culture fish using the procedure in Section 7: Fish Care Cheat Sheet.

A.5 WATER SAMPLE PRESERVATION AND HANDLING CHEAT SHEET

This section will describe preservation and handling of water samples. Sulfide content analysis will be conducted in-lab using the sulfide iodometry method detailed in Section A.6. Eurofins Analytical Labs will provide other water analysis services for this deployment.

Preserving samples for analysis of sulfides:

1) This analysis requires 200 mL of each sample.
2) Zinc acetate solution: Dissolve 200 grams of zinc acetate dihydrate in 870 mL of water. This makes 1 liter of solution.
3) Sodium hydroxide solution, 6N.
4) The sulfide iodometric method requires 200 mL of sample. Prior to iTIE run initiation, place approximately 1 mL of the zinc acetate solution and 0.2 mL (4 drops) of the sodium hydroxide solution into each sample bottle.
5) Once 100 mL of sample water has been collected in each treatment, add 0.2 mL (4 more drops) of the sodium hydroxide solution to the sample. The pH should be elevated to >9.
6) Collect 200 mL of sample water from each treatment. Seal each bottle closed, shake vigorously, and transfer each sample into a labeled amber glass bottle. Labels should include date, time, run name, treatment name, preservatives added, and initials. Place each bottle into a bubble wrap pouch, and place in a cooler at < 4C.

Preserving samples for analysis of metals, PAHs, pesticides, and PFAS:

1) These analyses require 250 mL of each sample.
2) Nitric acid solution, 6N.
3) Once 100 mL of sample water has been collected in each treatment, add 0.5 mL of the nitric acid solution to the sample.
4) Collect 250 mL of sample water from each treatment. Transfer each sample into a labeled amber glass bottle. Labels should each include a unique sample ID number including date, time, run name, and treatment name. Also record the preservatives used and initials. Place each bottle into a bubble wrap pouch, and place in a cooler at < 4C.

Handling samples after run conclusion:

1) As soon as possible, place samples into a fridge at < 4C.
2) Package the samples in a cooler with ice packs. Include a packing list with "Burton Ecotoxicology Lab", a list of samples, and a list of analyses needed.
3) Thoroughly tape the cooler closed, and ship overnight to Eurofins.

A.6 SULFIDE IODOMETRY CHEAT SHEET

Reagents needed:

1) Hydrochloric acid, 6N.
2) Standard iodine solution, 0.025N: Dissolve 25 g of KI in a small amount of DI water. Add 3.2 g of elemental iodine and dissolve. Dilute to 1000 mL with DI water. Standardize against 0.025N sodium thiosulfate solution with starch as an indicator.
3) Standard sodium thiosulfate solution, 0.025N: Dissolve 6.205 g of sodium thiosulfate pentahydrate in DI water. Add 0.4 g of solid sodium hydroxide and dissolve. Dilute to 1000 mL with DI water. Standardize with potassium bi-iodate solution.
4) Starch solution: Heat 100 mL of DI water. Add 2 g of laboratory-grade soluble starch and 0.2 g of salicylic acid, and dissolve.
5) Potassium bi-iodate solution, 0.025N.

Standardizing reagents:

1. Standardize the sodium thiosulfate solution using potassium bi-iodate standard.
2. Standardize the iodine solution using the standardized sodium thiosulfate solution.
    -

Collecting preserved sulfide using vacuum filtration:

1) Label 500 mL conical flasks.
2) Vacuum-filter each sample, collecting all precipitation on a glass filter. Rinse the sample bottle thoroughly with pH 9 water to ensure all precipitates are collected.
3) Use tweezers to transfer the glass filter into the corresponding conical flask. Fill the flask with 100 mL of water.
4) Add 2 mL of the hydrochloric acid solution. Swirl the flask.
5) From a burette, add iodine to the flask until the iodine color remains in the solution.
6) Back-titrate with the sodium thiosulfate solution. As the endpoint is approached, add 3 drops of the starch solution, turning the sample dark blue. Complete the titration by adding 1 drop at a time, swirling the flask each time, until the blue color disappears.

Calculations:

$$mg\frac{S^{2-}}{L} = \frac{[(A * B) - (C * D)] * 16000}{mL\ sample}$$

- A = mL iodine solution
- B = normality of iodine solution
- C = mL sodium thiosulfate solution
- D = normality of sodium thiosulfate solution

A.7 FISH CARE CHEAT SHEET

This section will describe fish care procedures.

Receiving and acclimating organisms:

1) Receive organisms on Day -1 of the test. Place in a beaker in a temperature-controlled room or water bath in the original shipping water.
2) Acclimate the fish to site water temperature at a rate of 1C per hour. If needed, acclimate the fish to site water quality conditions (conductivity, salinity, pH) by dropwise adding reconstituted water to the beaker. Once site water temperature is reached, maintain the water bath at that temperature until the conclusion of the iTIE deployment.
    -

Fish care during iTIE deployment:

1) Count and sort organism groups. Set up lab controls in a 250 mL beaker.
2) Conduct iTIE run. After setting up the run, set up travel controls in a 250 mL beaker.
3) After the iTIE run is completed, set up each treatment group in a 250 mL beaker.
4) Acclimate the fish to 21C by adjusting the water bath temperature at a rate of 1C per hour. Once reached, maintain the water bath at 21 +/- 1C for the duration of the test.
    -

Daily fish care:

1) Record survival daily. Individuals with gross morphological deformities are considered dead for statistical purposes. Remove dead embryos, fungus, and debris using a Falcon pipette.
    a. If survival for any lab or travel control group drops below 30%, the test is invalid and must be terminated.
2) Remove any individuals with gross morphological deformities, euthanize, and preserve in 70% ethanol or 10% buffered formalin.
3) Record DO and pH in each mesh beaker, as well as in the beaker of temperature-equilibrated clean culture water.
4) Water change: Pour 75% of the water from each beaker into a waste container. Gently refill each beaker with clean, temperature-equilibrated (21C) culture water.
5) Feed each beaker 3 drops of concentrated, newly hatched (<24 hours) Artemia nauplii in culture water.
6) After an hour, collect debris from the bottom of each mesh beaker using a Falcon pipette.
    ▪

End of culturing period:

1) Once the end of the culturing period is reached (4 days post-hatch), record final survival numbers. Euthanize all fish and preserve them in 70% ethanol or 10% buffered formalin.

APPENDIX B: ACHE AS A CHRONIC TOXICITY ENDPOINT FOR THE ITIE

Excerpt from Nichols, E. (2023). *Methods for Identification and Prioritization of Stressors at Impaired Sites* (thesis). *University of Michigan Deep Blue Documents*. Retrieved from https://dx.doi.org/10.7302/7081.

Introduction

Pesticides are frequently infiltrating waterways through agricultural runoff and spray-drift, which poses a threat to the survival of organisms that inhabit these contaminated ecosystems (Schulz, 2004). Aquatic invertebrates are of particular concern, as they are often more vulnerable to the effects of pesticides due to sharing a similar morphology to typical target pesticide species (Bartlett et al., 2016). The heightened sensitivity of aquatic invertebrates to pesticides can lead to high rates of mortality being observed at extremely low concentrations (Maggio et al., 2021; Rasmussen et al., 2013).

Chlorpyrifos is particularly well known for lethal effects observed at low doses, with *Hyalella azteca* demonstrating a 10-d LC50 of 0.0086 µg/L and 48-hour LC50 of 0.1 µg/L (Phipp et al., 1995; Moore et al., 1998). This highly toxic pesticide is classified as an organophosphate, which is a commonly used class of pesticides that also includes malathion, diazinon, and parathion (Ganie et al., 2022). Organophosphates are grouped by their mechanism of lethality, which is inducing neurotoxicity through the inhibition of acetylcholinesterase, an enzyme that aids in the degradation of the neurotransmitter acetylthiocholine (Julien et al., 2008). Acetylcholinesterase (AChE) activity of organisms can be established through bioassays, and this has been utilized as a chronic endpoint for quantification of organophosphate exposure for aquatic species (Naddy et al., 2000; Day & Scott, 1990; Laetz et al., 2020).

The main goals of this study were to 1) establish a protocol allowing for integration of AChE activity as a short-term chronic toxicity endpoint for future iTIE deployments and 2) use AChE to evaluate resin effectiveness for absorption of chlorpyrifos. Incorporating AChE activity into the iTIE protocol would improve the overall robustness of the system by providing insight into the presence of contaminants below the levels required to induce mortality within a 24–48-hour period. On the other hand, if concentrations of chlorpyrifos are at levels where lethality can be observed, employment of a resin with the capability of isolating this toxic insecticide would provide more clarity concerning the primary stressors at that site. These objectives were accomplished through laboratory iTIE runs with chlorpyrifos-spiked water that organisms were exposed to after the water was fractionated by various resins, and the effectiveness of the resins were determined through comparison of AChE activity of the exposed organisms.

Methods

Test Organisms

Freshwater benthic macroinvertebrate *H. azteca* were obtained from an existing laboratory mass culture at the University of Michigan. This culture was kept within a 5-gal aquarium tank filled halfway with ion-enriched water and was fed 2 rabbit pellets on M and F.

Adult *H. azteca* were utilized in this study to maximize tissue for AChE analysis. Adults were collected by filtering through a 600 µm mesh sieve (Plomp et al., 2020), and groups of 10 each were transferred into 30 mL centrifuge tubes prior to exposure.

Resins and Chemicals

Resins tested in this study included Oasis HLB (Waters™), Amberlyst-15 (Sigma Aldrich), activated carbon (Marineland), and C18 SPE (Waters™) (Figure B.1). Oasis HLB and C18 are both designed for solid phase extraction, with Oasis HLB being preferred for the extraction of polar compounds (Dias & Poole, 2002), and C18 using hydrophobic interactions to adsorb non-polar compounds. Chlorpyrifos is relatively non-polar, however, HLB was still considered as a candidate as it also has non-polar compound adsorption capabilities. Activated carbon, as previously mentioned, can adsorb a wide range of compounds. Amberlyst-15 was chosen due to its demonstrated ability to adsorb malathion, an organophosphate sharing structural similarity to chlorpyrifos (Dias & Poole, 2002).

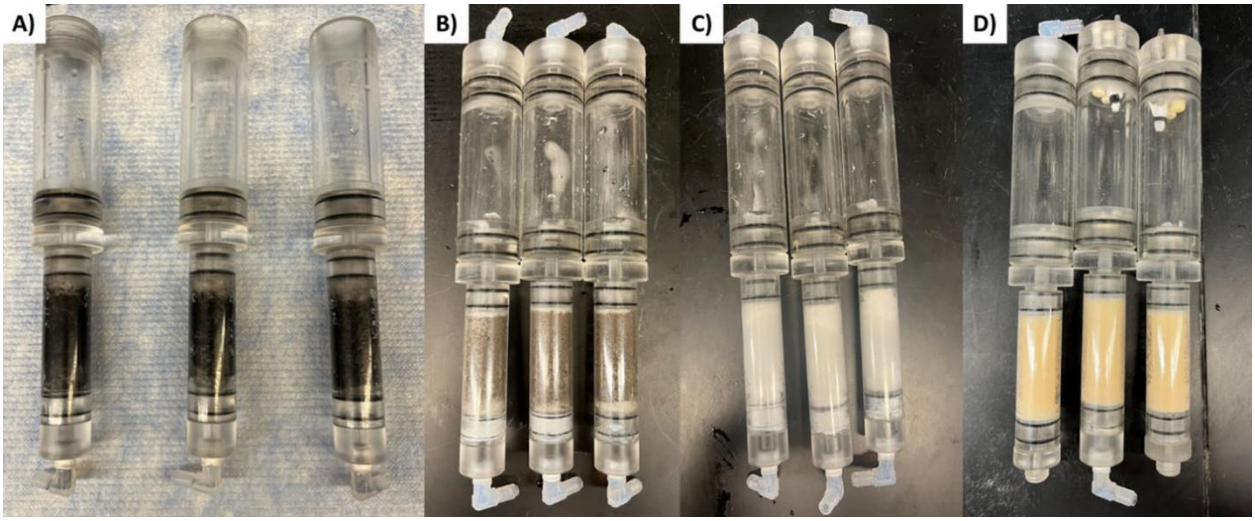

Figure B.1: Resins tested for adsorption of chlorpyrifos. A) Activated carbon B) Amberlyst-15 C) C18 D) Oasis HLB.

Chlorpyrifos and chemicals used for AChE analysis were obtained from Sigma Aldrich. 10 mg of chlorpyrifos was mixed with 100 mL of acetone to yield a stock solution of 0.1 µg/mL, which kept in an amber bottle stored in the refrigerator to prevent degradation. The quality control enzyme standard was prepared daily at a concentration of 0.2 units/mL of electric eel acetylcholinesterase and homogenizing buffer. The homogenizing buffer (pH=7.4) consisted of 1% v/v Triton X-100/Tris buffer (0.05 M), with the tris buffer being prepared at pH 8. Ellman's reagent was prepared by diluting 0.025 g of Ellman's powder with homogenizing buffer and adjusted the pH of the solution to 7.4. Acetylthiocholine iodide (0.156 M) and bicinchoninic acid working reagent were both prepared daily. Bicinchoninic acid working reagent was prepared by making a 50:1 volumetric ratio of bicinchoninic acid solution with 4% (w/v) copper (II) sulfate pentahydrate. BSA protein standards were made at concentrations of 0 µg/mL, 200 µg/mL, 400

µg/mL, 600 µg/mL, 800 µg/mL, and 1000 µg/mL by dilution of bovine serum albumin with homogenizing buffer.

Chlorpyrifos Exposure

To establish a streamlined protocol for exposure, preliminary tests with granular activated carbon (GAC) were conducted due to its availability, low cost, and ability to adsorb a wide range of contaminants (Burton et al., 2020). The results from the GAC trials led to the following exposure protocol to be established.

A total of 3 chlorpyrifos exposure runs were conducted, and each run was associated with a different resin. 6 iTIE units were used per run, with 3 treatment iTIE units containing 5 grams of resin (sandwiched between glass wool) and 3 control units containing glass wool. Prior to the initiation of the exposure, Versa pump tubing was purged with ethanol, followed by a rinse with Liquinox and Milli-Q. iTIE units and sample bottles were rinsed with Liquinox and soaked in a 10% HCL solution. Quality control samples of 500 mL were taken to establish if any chlorpyrifos was leaching off equipment. Versa pump tubing was then primed with ion-enriched water (IEW).

A chlorpyrifos-spiked water solution was prepared by adding 60 µL of 0.1 µg/mL stock solution to 6000 mL of IEW, for a final concentration of 1.0 µg/L. This solution was kept within a 2.5-gallon aquarium tank, rinsed with ethanol in between runs, and iTIE units were placed in this aquarium for the duration of the exposure (Figure B.2A).

Resins were conditioned with either Milli-Q (HLB, Amberlyst-15) or methanol (C18) before being added into iTIE units. Once resins were added, the units and sample bottles were attached to their respective Versa pump tubing. Chambers were filled with IEW, and 10 adult *H. azteca* were added to each unit (Figure B.2B). The run was then initiated, with each Versa pump running at 25 mL/hour for a 24-hour exposure. Water quality parameters (DO, temperature, pH) were recorded for both the IEW and chlorpyrifos-spiked water.

At the end of the exposure, *H. azteca* were removed from iTIE units and counted. These organisms were then divided into respective centrifuge tubes, with each centrifuge tube sample containing 5 organisms (2 samples of 5 organisms each per iTIE unit). The exception for this was cases where mortality was observed, resulting in the remaining organisms within an iTIE unit being split between two centrifuge tubes. Centrifuge tubes were place in a -80℃ freezer until AChE analysis. Water quality parameters were recorded for both water within the iTIE unit and sample bottles, and volume of sample bottles was also recorded. Sample bottle volume was then transferred into amber bottles to be stored in the refrigerator until analysis. Additionally, a 500 mL sample of the remaining volume of chlorpyrifos-spiked water was transferred into an amber bottle for analysis. The samples were shipped off within 24 hours of the exposure to Eurofins, Canton for analysis of chlorpyrifos.

An additional 24-hour test was conducted in the absence of chlorpyrifos in order to obtain baseline levels of AChE activity. The baseline run followed the aforementioned steps, with the exception of chlorpyrifos being added to the water, and all 6 iTIE units contained glass wool.

Figure B.2: A) Setup for chlorpyrifos exposure, with aquarium tank containing water spiked with chlorpyrifos to a concentration of 1.0 µg/L B) Addition of organisms to iTIE units.

AChE Activity Quantification

The following protocol for the acetylcholinesterase analysis utilized for quantifying toxicity was adopted from Bartlett et al. (2016). *H. azteca* were thawed on ice before being homogenized in 500µL of homogenizing buffer and spun down in a centrifuge for 10 minutes. The supernatant from centrifuged samples was then transferred into a new centrifuge tube to be used for analysis.

Acetylcholinesterase determination was carried out by addition of 40µL of homogenizing buffer, sample supernatant, or quality control standard into separate wells of a 96-well round bottom plate, with samples being run in triplicate (Figure B.3A). This was followed by the addition of 250µL of 5,5'-dithiobis{2-nitrobenzoic acid]) to each well, and the reaction was initiated by adding 10 µL of acetylthiocholine iodide (Ellman et al., 1961). After addition of acetylthiocholine iodide, absorbances were immediately read on a microplate spectrometer every 2 minutes for 30 minutes at 405nm. It is worth noting that the quality control sample was degraded due to exposure to suboptimal temperature during trial runs and could not be included in AChE analysis of samples examined for this study. However, the quality control absorbance was within expected bounds before degradation occurred, and the same reagents were used for exposure run AChE analysis.

Protein concentration was measured using a BSA standard curve. This was done by adding 25µL of supernatant in triplicate and 25µL of each of the BSA protein standards. 200µL of bicinchoninic acid solution was then added to each well, and the plate was incubated at 25°C for 2 hours before reading absorbances at 562 nm (Figure B.3B). The standard curve was then used to calculate protein concentrations of the sample supernatant. Specific activity for AChE was then calculated using the equation shown in Figure B.4.

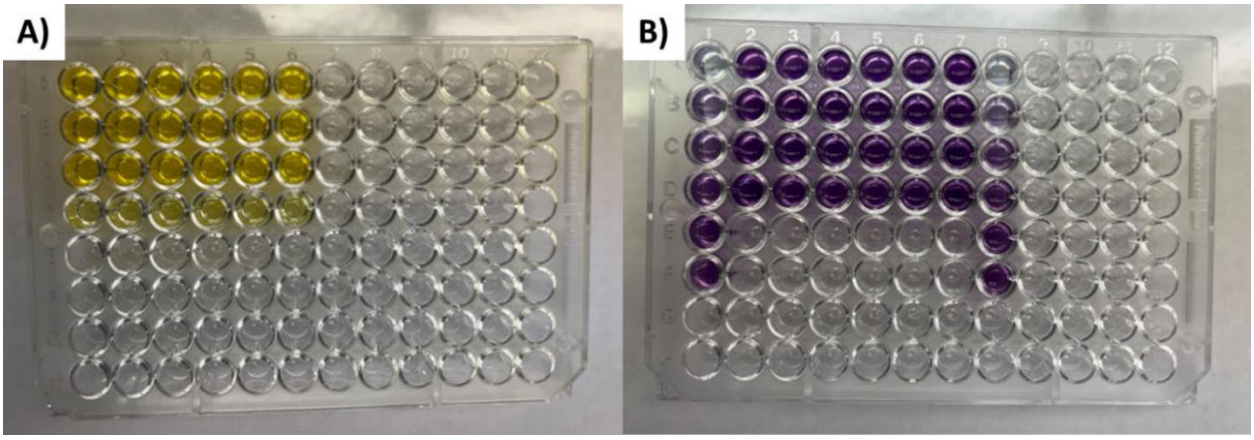

Figure B.3: A) AChE activity plate. B) BSA protein plate after 2-hour incubation.

$$Specific\ activity = \frac{A \times Vol_R \times 1000}{E \times PL \times Vol_H \times PR}$$

Figure B.4: Equation used to calculate specific activity for AChE (in µmol/min/g protein), where A= change in absorbance/min, Vol_R= reaction volume (0.3 mL), E=extinction coefficient for 5,5'-dithiobis{2-nitrobenzoic acid]) (1.36 × 104 M-1 cm-1), PL= pathlength (0.875 cm), Vol_H= sample volume (0.04 mL), and PR= protein in the homogenate calculated from standard curve (mg/mL).

Statistical Analysis

Average specific activity for *H. azteca* for resin and glass wool chambers in chlorpyrifos exposures were divided by average baseline activity to express specific activity as a percentage of baseline. Standard deviation was calculated through propagation of error.

Student's T-tests were used to compare average specific activity of *H. azteca* associated with resin and glass wool iTIE units in chlorpyrifos exposures to average specific activity of baseline organisms. A p-value of ≤0.05 was used to determine statistical significance.

Results & Discussion

Granular Activated Carbon

As previously mentioned, GAC was used in preliminary trials to establish standard operating procedure for chlorpyrifos exposure. These tests were originally carried out using the iTIE rack, which was changed due to the rack being able to only contain 4 units at a time. All units were removed from the rack and transferred into the aquarium tank to keep treatment consistent across all units.

During the tests in which the iTIE rack was used, increased mortality was observed in units containing GAC, with units having survival rates of 0%. Further investigation showed DO levels reaching levels of concern that likely resulted in observed mortality (Figure B.5). However, not all units shared the same low levels of DO. The unit containing GAC that was positioned further down on the manifold had the lowest DO, and DO increased with positioning on the manifold. This is related to the trend concerning uneven water distribution and sample

bottle volume observed during previous deployments; while gravity pulls down water to the bottom of the manifold, air can escape at the top of the manifold, resulting in unit 1 receiving more oxygen. Although the manifold likely plays a role in observed DO levels, glass wool was positioned furthest down on the manifold and demonstrated higher DO levels than GAC counterparts, suggesting GAC contributed to lower DO content.

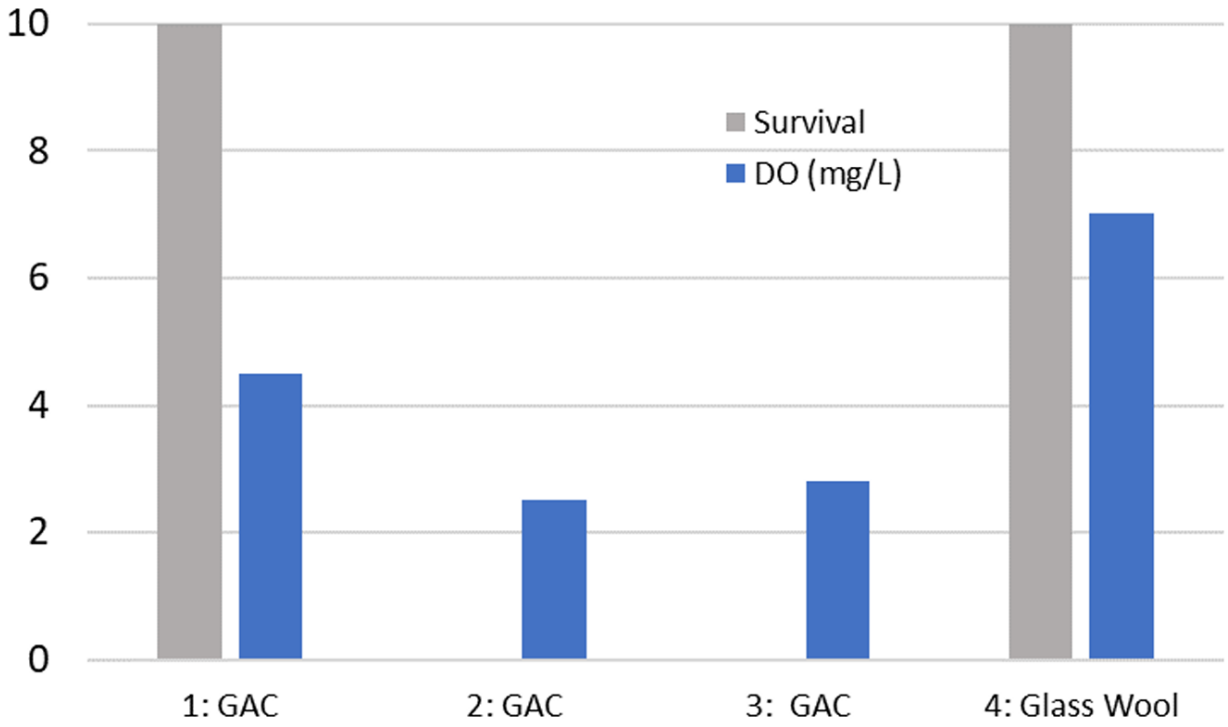

Figure B.5: Survival (maximum survival = 10) and observed DO content within respective resin chambers. Number in front of resin chamber denotes position on manifold, with 1 being at the top and 4 at the bottom.

While previous iTIE prototype exposures were successful when using GAC (Burton et al., 2020), the closed nature of the updated iTIE prototype can result in uneven distribution of oxygen and limits the amount of oxygen that can enter the system. This appears to be less of a concern in runs where the oxygen coil is in operation (Fleming Creek), however, use of future exposures GAC should take DO concerns into account to avoid falsely attributing mortality to a stressor present at the site.

Amberlyst-15

Amberlyst-15 also encountered issues with regards to adverse impacts on organism survival. Less than 2 hours into the run, mortality within iTIE units containing Amberlyst-15 was observed, and complete mortality in these units occurred within 5 hours. The run was terminated, and water quality measurements revealed pH levels ranging from 2.27 – 2.32. Glass wool pH ranged from 7.6-7.7, and no mortality occurred within these units. This provides evidence supporting acidic conditions within Amberlyst-15 units being attributed to the resin, ultimately

leading to the complete mortality of *H. azteca*. The low pH in these chambers can likely be explained by Amberlyst-15 being a hydrogen form resin, and the exchange of water led to an excess of hydrogen ions entering the organism chamber, lowering the pH.

C18 & Oasis HLB

AChE activity of organisms in units with C18 (Figure B.6A) and Oasis HLB (Figure B.6B) alludes to both resins having the capacity for adsorption of chlorpyrifos. Average specific activity for organisms in glass wool chambers for the C18 run was 70% (± 16% (s.d.)) of baseline activity; AChE activity is considered to be inhibited when it is ≤80% of baseline activity, proving organisms within glass wool chambers were exposed to chlorpyrifos (Bartlett et al., 2016). In addition, average glass wool specific activity was proven to significantly differ from mean baseline activity ($p = 0.001$). On the other hand, C18 average specific activity was 94% (± 24% (s.d.)) of baseline activity and did not significantly differ from average baseline activity ($p = 0.47$).

Organisms in chambers where water was fractionated by HLB also had a higher average specific activity as a percent of baseline (98% ± 19% (s.d.)) than organisms in glass wool (80% ± 22% (s.d.)). Mean specific activity for HLB was not significantly different from mean baseline activity ($p = 0.82$), but a significant difference was observed for glass wool ($p = 0.03$).

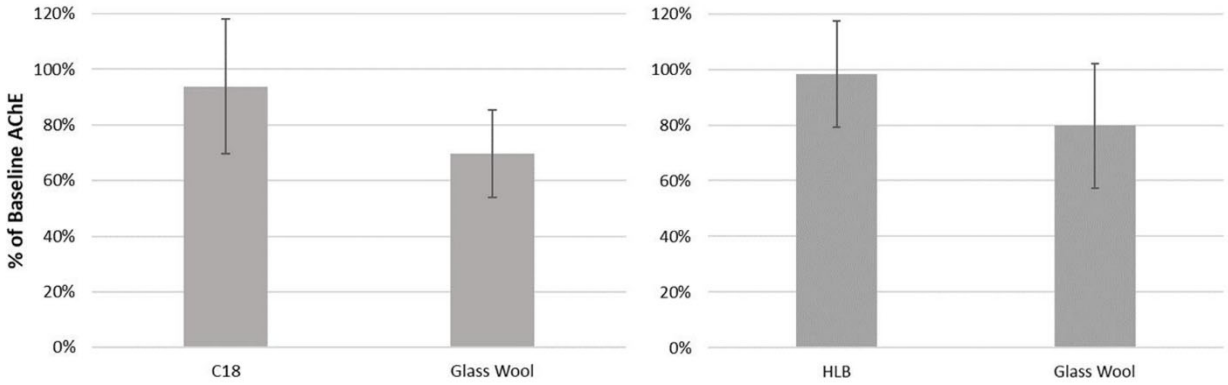

Figure B.6: A) Average specific activity (expressed as % of baseline) for C18 run B) Average specific activity for HLB run. Error bars represent standard deviation, and asterisk denotes significant difference between treatment and baseline activity ($p \leq 0.05$).

Laboratory Analysis

Analysis of samples fell below Eurofins' minimum detection limit of 0.50 µg/L, therefore no chlorpyrifos was detected in any of the samples. However, laboratory quality control (QC) samples ran alongside samples showed losses of up to 12.0 ug/L when comparing QC sample concentration to concentration detected, suggesting that chlorpyrifos was likely present within the samples shipped off, but was unable to be detected by the instruments. This highlights the need for specialty laboratories when analyzing compounds with concentrations in the parts per billion (ug/L) range.

Conclusions

While adsorption capabilities were unable to be confirmed due to the inadequate detection limit by Eurofins, AChE specific activity suggests C18 and HLB were successful in removal of chlorpyrifos. In addition, methods for quantification of AChE activity as a chronic toxicity endpoint were successfully established for use in future iTIE deployments.

This study highlighted the need to consider the role resins can play in alteration of critical water quality parameters, which can lead to increased stress and mortality. Failure to account for effects of resins on organisms can lead to falsely attributing these adverse impacts to site conditions, as previously mentioned.

Future resin optimization should also consider cost, availability, and specificity of resins. Expensive resins that are not widely available are unrealistic for use in the iTIE system, and increased specificity allows for the effects of targeted contaminants to be isolated. While Oasis HLB and C18 demonstrated successful adsorption of chlorpyrifos, these resins have been proven to adsorb other contaminants that could be also be present at a site (Burton et al., 2020). This lack of specificity has implications for the accuracy of linkage between exposure and effects; improved organism health within chambers containing these resins cannot be directly attributed to a single contaminant, and definitive conclusions cannot be made without laboratory analysis of sample bottle concentrations. While the aforementioned factors are important to consider, finding a resin that meets these criteria can be a difficult task, and resins lacking one of these qualities can still be utilized in future iTIE deployments.

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