The in situ Toxicity Identification and Evaluation (TIE) Device:

A Novel Method for Assessing the Source of Toxicity in Aquatic Systems

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Abstract

Thousands of unregulated contaminants are broadly distributed in our natural waters and have either gone undetected until recently, or are now being detected in greater concentrations. Contaminants of Emerging Concern (CECs) are trace chemicals that may pose serious ecological and human health risks. The exact sources and prevalence of these compounds are largely unknown and difficult to assess. Some known CECs are components of pharmaceuticals, anti-biotics, and other personal care products, which are ubiquitous and commonly discharged, untreated, from wastewater management facilities. It is usually not fiscally or technologically feasible to filter, extract, or degrade all these chemicals, so individual targeting of specific compounds is the most viable treatment option. Finding a causal link between observed toxicity and a specific compound or group of compounds is difficult when thousands exist in wastewater effluent, with significant variations in spatial and temporal concentrations. Toxicity Identification and Evaluation (TIE) is an EPA-developed experimental approach to take a complicated matrix with established toxicity and partition the components to identify the exact compound(s) responsible. Though TIE methods have been applied to wastewater effluent before, most tests are conducted in a laboratory environment, in which contamination and other artifacts can significantly affect the accuracy of final results. This research aimed to develop a device capable of autonomous *in situ* TIE experiments, providing unparalleled accuracy in the identification of toxicity sources. Deployed directly in the aquatic environment of concern, the device can continuously collect the source water, fractionate its complex chemical mixture with sorbent resins, and conduct bioassay exposures. The first field version deployed in environments with observed biological impairment successfully targeted specific compounds for extraction, reducing their concentration by 100% in some treatments. Through a series of selective CEC extractions, the possible source(s) of toxicity in a complex solution become clearer. After addressing mechanical issues with the first model, a second iTIE system was designed and tested in a series of laboratory fractionation tests, which demonstrated its ability to reliably conduct autonomous TIE experiments. These lab results also demonstrated that genetic methods could be used in conjunction with the iTIE system to identify sub-lethal toxicity, which can be difficult to assess amidst an intricate web of natural and anthropogenic variables. The in situ TIE System can begin to fractionate and isolate confounding variables in a complex system, and help identify indistinct biological threats in the environment.

Acknowledgements

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I. Contaminants of Emerging Concern

Thousands of unregulated contaminants are broadly distributed in our natural waters and have either gone undetected until recently, or are now being detected in greater concentrations [26]. What the U.S. Environmental Protection Agency (EPA) refers to as "Contaminants of Emerging Concern" (CECs) primarily consist of pharmaceuticals/personal care products (PPCPs), fluorocarbon compounds, and other trace chemicals that may pose serious ecological and human health risks. What constitutes a CEC is still controversial, with some experts arguing that known, but untreated chemical discharges pose the greatest threat, while others believe that unidentified chemicals and their sources should be targeted [29]. Disagreement over where to focus studies and management efforts is not the only complication for CEC research. The exact source, toxicity, concentration, and prevalence of these compounds are largely unknown and difficult to assess.

1.1 Sources and Identification

There are multiple point and non-point sources of CECs, each of which often releases a complex mixture of compounds in spatially and temporally varying concentrations. Many pharmaceuticals and compounds from PPCPs, such as antibiotics, are knowingly discharged from wastewater treatment plants, unfiltered, in relatively low concentrations [29]. Runoff from farmland can introduce synthetic hormones, pesticides, herbicides, disinfectants, and similar compounds into groundwater and nearby waterways [27,30]. Industrial waste, urban stormwater overflow, rural septic systems, and atmospheric deposition also act as unregulated CEC sources [2,33]. Many CECs are polar and non-volatile so they tend to persist in the environment, though many factors can affect their degradation, bioavailability, and toxicity [31]. Toxicity thresholds for aquatic life, especially with respect to chronic toxicity, have only been established for a few CECs and the tendency of some to bioaccumulate may lead to genetic disorders associated with endocrine disruption [32].

1.2 Chronic Toxicity and Other Indistinguishable Effects

One of the major concerns with respect to trace organic compounds (TOrCs) is endocrine disruption, especially for chemicals, such at perfluorinared compounds, that bioaccumulate in tissue and blood [20]. Human and animal exposure to endocrine disrupters can result in developmental, reproductive, and neurological impairments and negatively affect immune systems. Many compounds found in common products are thought to act as endocrine disruptors. Triclosan, commonly found in antibacterial products, and atrazine, a popular herbicide, are known to suppress the expression of important genes and are often found in aquatic systems [34]. Bisphenol A (BPA), especially, has been a major concern for both ecological and human health due to its bioaccumulation and chronic toxicity [2,15, 21]. The nature and potential threat of the thousands of CECs discharged daily into our waterways is not fully understood. A better framework, built on specific site screening tools and risk assessment protocol is needed to identify and target the greatest threats of biological impairment.

A current project, led by the Water Environment Research Foundation (WERF), is working to evaluate the risks associated with TOrCs to help guide management decisions for water quality agencies. Phase I developed site screening tools that will help establish any links between TOrCs discharged from wastewater treatment plants (WWTP) and ecological or biological impairments in effluent-receiving waters [22]. Phase II identifies regions where research will be focused, specifically aquatic systems downstream of WWTPs that already have documented biological impairment. This will help develop a causal link between TOrCs and biological issues.

1.3 Establishing Individual Toxicity

The causal link between a particular stressor and negative ecological effects is sometimes difficult to ascertain with so many confounding variables. Areas with a high influx of TOrCs and other CECs may also be affected by high impact stressors such as siltation, habitat disruption, invasive species, pathogens, legacy metals and chemical contamination, among others.

The toxicity of the TOrCs themselves can be affected by their bioavailability and the characteristics of their surrounding environment [23,28]. The fraction of TOrCs that are bioavaible or become so is unknown. Hardness, pH, temperature, the presence of sulfides and DOC can all affect the amount of a chemical to which organisms can be exposed. The trophic structure of the environment will determine amplification pathways and the spread of toxicity to other areas.

The National Pollutant Discharge Elimination System (NPDES) faced many of these issues while attempting to establish permit limits for wastewater treatment plant effluent [28]. In response, the EPA established a series of physical and chemical fractionation tests to partition chemical matrices and perform toxicity assessments on individual analytes.

II. Toxicity Identification and Evaluation (TIE)

2.1 History and Advantages

Toxicity Identification and Evaluation (TIE) is an experimental approach developed to take a complicated matrix with established toxicity and partition the components to identify the exact compound(s) responsible. USEPA established TIE protocol in the 1980s to understand the source(s) of toxicity in wastewater discharges and better inform management decisions [35,36]. Bioassays have been used since the early 20th century to identify the presence of ecological risks, but traditional exposure methods typically integrate a variety of compounds and stressors, making if difficult to isolate variables [37]. In complex systems, simply demonstrating an incidence of organism stress response does not necessarily identify the cause. TIE combined bioassays with a series of fractionation steps to build a weight-of-evidence case against specific chemicals (Table 1).

Table 1. USEPA TIE Process (Reproduced from EPA/600/R-07/080 [28])

Phase 1	A suite of physical/chemical manipulations is used to build a general "profile" of the causative toxicant(s), with the goal of determining the general category or type of toxicant involved (e.g., metals, nonpolar organics, volatiles, ammonia).
Phase II	More refined procedures are used to focus on the specific category of chemical implicated in Phase I, with the goal of isolating the causative toxicant(s) from other chemicals in the sample, thereby simplifying the sample for chemical analysis. This process generally culminates in the analytical identification of the suspected toxicant.
Phase III	The investigator collects the corroborating data to build a weight-of-evidence case that the suspect toxicant is in fact the cause of toxicity, an important step before initiating management actions to control the problem chemicals.

The EPA originally designed TIE manipulations for effluent water, but has since outlined methods for interstitial sediment waters and whole sediments [35]. Solid-Phase Extraction (SPE) techniques combined with bioassays have been successful at identifying estrogenic activity in wastewater, with subsequent Phase II GC-MS analyses identifying the specific organic compound responsible among the dozen present [38]. Mixing test sediments with sorbent resins and chelating agents during Phase I has been very effective in removing specific classes of compounds prior to exposure tests [39,42,43,44]. Manipulating environmental factors, such as pH and UV radiation, have revealed condition-dependent toxicity for some chemicals [37,41,42,44]. Sometimes, the complexity of these matrices and their environments, as well as limitations on analytical chemistry, has prohibited precise identification. A 2006 study on toxic agricultural stream sediment used carbonaceous resins to remove organic chemicals from the sediment samples [43]. Phillips et al. observed reduced toxicity from sediment treated with Ambersorb, so the resin particles were separated from the sediment and eluted with methanol to remove the adsorbed compounds. The resulting solution remained toxic to *Hyalella azteca*, but chemical analyses revealed the presence of only four pesticides, all in concentrations lower than established LC50 values. The authors were unable to establish if the four pesticides were additively toxic or if the sediment, which received discharges from many non-point sources, contained another organic compound that was not detected by their chemical analyses. The limitations of current TIE protocol are more apparent in increasingly complex systems, sometimes resulting in misleading laboratory results.

2.1 The Accuracy of *In Situ* over Laboratory Experiments

Semi-controlled field experiments allow for more realistic exposure conditions and analyses of variables that cannot be replicated in the lab, while lowering the impact of artifacts. During laboratory exposures it is difficult to maintain natural fluctuations, including temporal variation in dissolved organic carbon, temperature and pH, which can affect the toxicity and bioavailability of particular compounds [3,6,7,44,45]. Variations in environmental conditions can influence toxicity enough than organism survival is significantly lower in field exposures, compared to lab tests using the same sediment

[33,46,47]. Overlaying water has been cited as a contributor to toxicity during sediment exposures and is especially susceptible to variations [47].

Concerns over contaminant fluctuations have led some researchers to question the accuracy of some habitat risk assessments. Artifacts associated with transport, storage and manipulation of the sample can alter concentrations in lab exposures, [41,56]. Many organic compounds can adhere to collection or exposure vessels, artificially lowering their concentration [35]. The test organisms can also consume or absorb chemicals and, without a natural replenishment, the total uptake and bioaccumulation could vary significantly compared to field organisms. Added environmental drivers such as toxicant pulses and interactions between natural variable and anthropogenic factors may also create fluctuations [56].

Continuous spatial and temporal variation in contaminant inputs and flows is perhaps the most important factor lost in lab exposures when considering the impact of CECs. EPA chronic toxicity test protocol requires three fresh samples over a seven-day test period, but with CECs and other toxicants entering the environment from a variety of sources, each impacted by geologic and atmospheric conditions, the choice of sampling times could drastically affect the composition of the sample [2,28]. Static renewal tests essentially measure single events, exposing test organisms only to stream conditions at the moment of each sampling [3]. Chemicals that exist in trace concentrations with significant spatial and temporal variation may not be accurately represented in a random sampling effort, especially if the exposure test focuses on open water.

2.2 Laboratory Limitations of TIE Tests

The current TIE protocol for Phase I fractionation utilizes either of the standard static or renewal laboratory exposure tests [5,28], which may not provide the most accurate results. Accuracy of current TIE methods depends on knowledge of the exact compounds present in a system, the complexity of the system (physically and chemically), and the ability of laboratory tests to replicate or otherwise account for *in situ* conditions. In most cases, study sites have to be pre-screened and tested for the presence of metals, ammonia, organic compounds, and other sources of toxicity. Selecting the proper test organism relies on knowing what compounds will elicit a known response. If metals are the source of toxicity and the TIE organisms are not sensitive to the metals present at *in situ* concentrations, then a false negative result can occur [35]. False negative results can also occur if organisms are not tested for sub-lethal effects. Most bioassays use mortality to determine toxicity, which ignores the threats of chronic toxicity, bioaccumulation, and genomic disruption [28,36]. Methods do exist for identifying or predicting genetic problems, such as identifying genetic subunit patterns with known biomakers and phenotypic changes in cells [11,42]. The database of response biomarkers for test organisms is, however, very limited [35]. Likewise, the identity and persistence of trace organic compounds that could cause genome disruption are not fully understood.

Identifying contaminants of emerging concern, both in preliminary habitat assessments and in subsequent TIE experiments, remains a challenge. There are hundreds, possibly thousands, of anthropogenic compounds entering streams from dozens of point and non-point sources. Some of these compounds, or their degraded forms and

metabolites, have yet to be identified. Those that are known may be difficult to fractionate or detect in TIE Phase II tests. Some TOrCs, like pesticides and pharmaceuticals, were manufactured to target specific binding sites or enzymes, so using cells with custom manipulated receptors in a TIE test could identify those compounds [37,40,42]. For most compounds, especially byproducts of industrial manufacturing, will not be so easily targeted. Analytical chemistry, including GC-MS and HPLC, usually requires standards to identify chemicals, so if the machine is not looking for something, it likely will not be found [43]. Even for known chemicals, the concentration measurements could be affected by laboratory conditions and may not accurately reflect *in situ* toxicity.

Some studies have found no significant variation between *in situ* and lab survival, but chemical simplicity in the chosen field environment is usually the reason, further highlighting the importance of in situ exposures for complex systems. A 2009 study by Ho et al. related TIE laboratory methods to observed field effects, using agricultural sediment toxicity to *M. merceneria*. TIE methods in the field and lab both accurately identified the same compounds as the source of toxicity, and bioassays demonstrated similar organism responses [42]. The system chosen for the study, however, had no-known point pollution sources and PAH compounds dominated the sediments. The PAH concentrations were so high that test organisms stopped feeding during the exposure. The PAHs also elicited a very obvious physical response, unique to those compounds, by altering the phenotype of the clams' cells. Essentially this was a best-case scenario for a TIE test since there was one dominant type of compound in an otherwise uncontaminated site with little influence from other stressors. When addressing sites that are constantly influenced by complex, diverse, and ambiguous slurries of chemicals, the most accurate toxicity evaluation will be *in situ*.

2.4 In Situ Toxicity Identification and Evaluation (iTIE)

In situ TIE has been attempted using caged bioassays, but controlling for other stressors while conducting chemical modifications is difficult [42]. A new device designed for SPE chemical fractionation was developed by Burton and Nordstrom in 2004. Their goal was improve the accuracy of TIE by conducting Phase I manipulations and bioassays entirely within the target environment [1,17]. Greater sensitivity, realistic environmental conditions, and minimal sample manipulation made this method more accurate than traditional TIE.

The iTIE used slow suction to pull pore water from the sediment into a series of two-chamber cylinders. Each cylinder contained a different sorbent resin in the first chamber, designed to target one of the major classes of contaminants (nonpolar chemicals, metals, and ammonia-type groups). As one of these classes was removed, the water would pass into a second chamber where test organisms were exposed to the modified solution. The results showed significantly higher toxicity in iTIE tests compared to lab TIE. The field validation study showed strong support for in situ Phase I manipulations with this or a similar device.

III. Building an iTIE System for Field Application

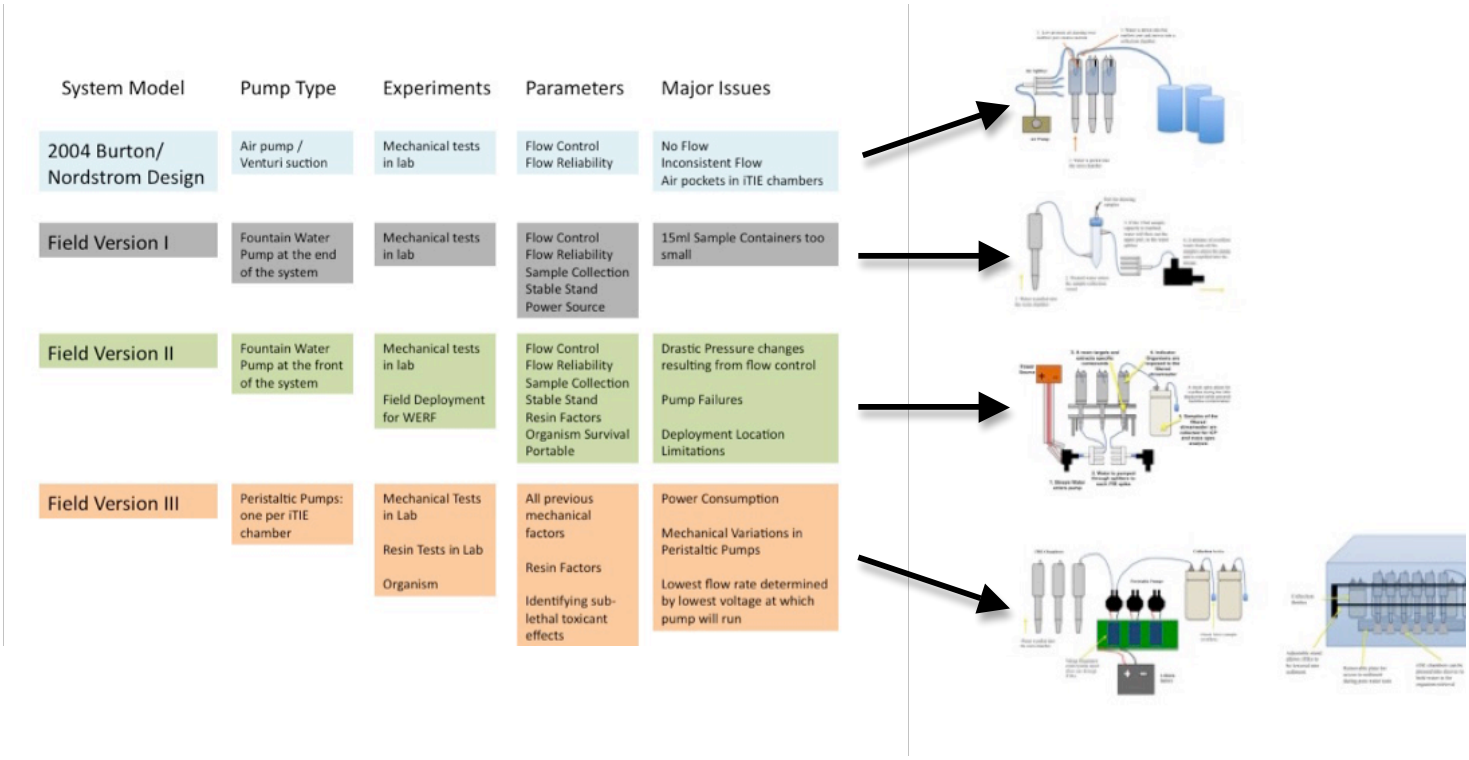

Fig. 1 Outline of the four iTIE Systems tested throughout this study. There is a chronological and technological progression from top to bottom in the chart as various mechanical and experimental factors were tested. System diagrams on the right are explained in detail in their respective sections. See Table 2 for complete list of parameter goals in this study.

Four iTIE system models were tested throughout this study (Fig. 1). Each model was designed and tested by considering the functional requirements and experiment goals outlined in section 3.1 and Table 2. The 2004 Burton/Nordstrom Model and Field Version I were only tested for mechanical functions in the lab and ultimately rejected for fieldwork due to serious design flaws. Field Version II was deployed at three field sites as part of the habitat risk assessment WERF study (Section IV). Following the field deployments, redesign efforts to address problems encountered with Version II, resulted in Field Version III (Section V). Field Version III was used in a laboratory validation study (Section VI) and is undergoing further development to incorporate the internal mechanisms into a field-ready version (Section VII). Color-coded headings throughout this paper correspond with Fig. 1, identifying the iTIE system model discussed in each respective section.

3.1 Goals for iTIE Development and Deployment

The primary goal of this study was to produce a field-ready system capable of performing *In Situ* Toxicity Identification and Evaluation (iTIE) for direct application in Phase II of the WERF project. The viability of the 2004 Burton/Nordstrom design would be assessed first, while repairing or redesigning any aspect necessary to meet field

demands. If the original 2004 version exhibited significant problems or could not be adapted to the WERF project, a new model would be constructed based on the same concept. The machine had to function properly for 24h and act as an effective TIE chamber for analysis of the possible toxicity sources. Various parameters were set to determine the effectiveness of a particular system model (Table 2). An effective system had to conduct autonomous *in situ* Phase I TIE fractionation with bioassays. The *in situ* nature of the device would provide more accurate TIE results and the autonomous operation would minimize artifacts.

Table 2. Functional Requirements for a field iTIE System

Parameter	*Description*
Slow Flow Rate	The ideal flow rate for effective resin sorption is 25ml/hr [1]
Consistent Flow Rate	Flow must be consistent and constant through each iTIE cylinder throughout the test, and the flow rate must be the same for all cylinders/treatments deployed
Sufficient Resin	There must be sufficient resin to continually extract target compounds without becoming saturated during the test.
Resin Coverage	The resin powders must be compact and fully cover the circumference of the resin chamber. There can be no pockets where water can seep through or around and never contact the resin.
Resin Held in Place	All resin particles must stay in the resin chamber and not enter the organism chamber
Organisms	Organism should be easily put in chamber without experiencing excessive stress.
Sample Collection	The system must store processed samples in individual containers, sealed to prevent contamination from other treatments or the open water.
Stable Stand	The stand holding the iTIE cylinders in place must be stable and easily submerged.
Portable System	The stand, iTIE cylinders, and other components must be portable. If some components cannot be waterproofed (like the power source, pumps, etc.) then all conduits must be long enough to allow for minimal restrictions on deployment.
Inconspicuous	The whole system should be as inconspicuous as possible to prevent vandalism
Power Consumption	The pump(s) used must consume as little power as possible. They must run on a portable battery that can power them for at least 24h. Ideally, the battery is as light as possible for shipping purposes.
Self-Sufficient	The system must continuously filter stream water for 24h without failing or requiring maintenance

3.2 Testing the 2004 Burton / Nordstrom Design

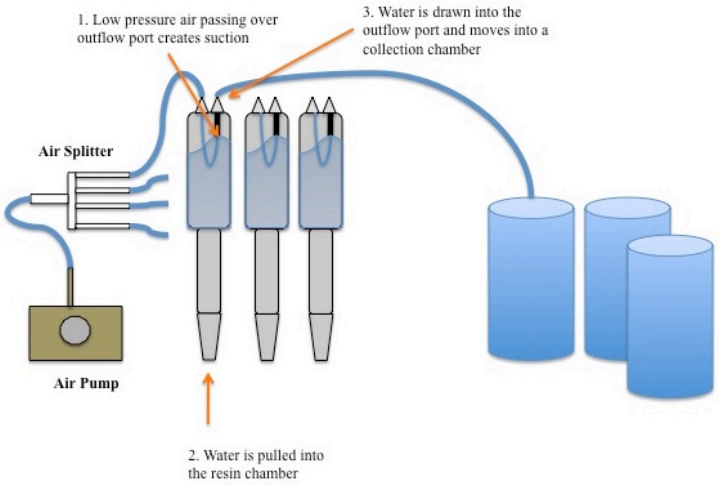

Fig. 2 Overview of the Burton/Nordstrom (air pump-based) design. This system used an air pump to create suction in the organism chambers via the Venturi Effect.

Introduction

Initial preparation for a field-ready iTIE system began with testing the 2004 Burton/Nordstrom configuration, as this model had proven to be effective for in situ TIE exposures [1,17]. The iTIEs were conceived for sediment pore water analysis, so the 2004 versions required a very slow flow rate (25ml/h) that would allow sufficient contact time with the resins for targeted compound extraction [1]. To achieve this flow, Burton and Nordstrom relied on the Venturi effect – suction that occurs when air passes through an increasingly confined space, leading to an increase in speed and drop in pressure. This concept was incorporated into a system where the flow through multiple iTIEs was powered by a single air pump drawing water into the chambers and pulling it out into collection bottles (Fig. 2).

The original test tube iTIE chambers used by Burton and Nordstrom (Fig. 3) were not used while testing this model. Instead, sturdier acrylic versions with better seals and more easily assembled components were utilized (Fig. 4). Water would enter the bottom port of the iTIE, pass through a resin chamber where Phase I fractionation would occur, and into the organism chamber for the bioassay, finally passing through the outflow port. Before any other parameters could be tested, the mechanical functionality of an air pump based system had to be established using the acrylic iTIE chamber (Fig. 4). The first goal in assessing the feasibility of this model was meeting the water flow criteria (Table 2). If flow parameters could not be met, a new approach would be necessary.

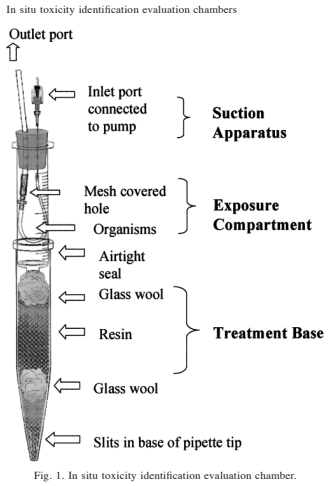

Fig. 3 The iTIE cylinder design used in Burton and Nordstrom (2004). Water flow through their model was achieved with suction, created by air flow through the tubing in the organism chamber. This chamber design was not tested in any of the iTIE system models used for this study.

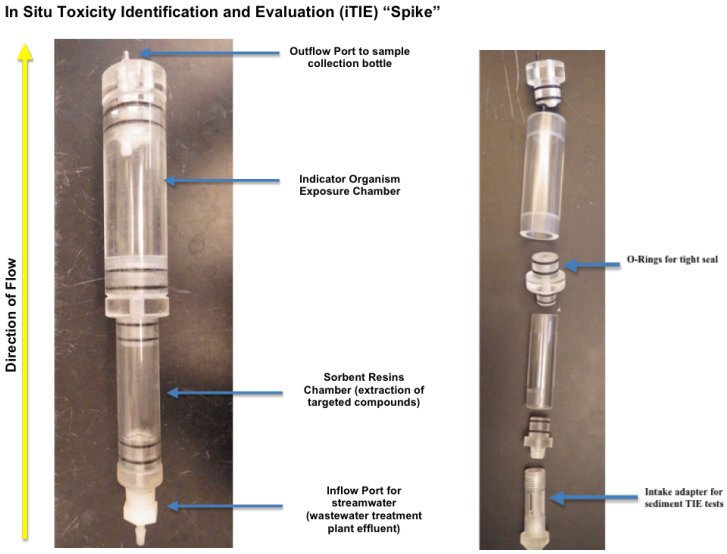

Fig. 4 The iTIE cylinder design used for all iTIE system models in this study. Each piece is manufactured from acrylic. Rubber o-rings on the connectors maintain a seal throughout. The cap on the organism chamber has three holes that can be used as flow ports or sealed with plugs. The overall volume of this acrylic chamber is larger than the 2004 test tube model (Fig. 1).

Methods for Testing Flow Rate

The Venturi suction model was adapted for the acrylic iTIEs by fitting cap with 1/8" threaded hose barbs in two of its three ports (top and bottom) while the third port was sealed with a threaded plug. This created an inflow port for the air and outflow port for the air/water mixture. The outflow port inside the organism chamber was fitted with a hose barb containing a small, mesh-covered hole. A loop of 1/8" ID, 1/16" wall silicone tubing connected the inflow to the outflow, but did not cover the hole on the outflow port (Fig. 5)

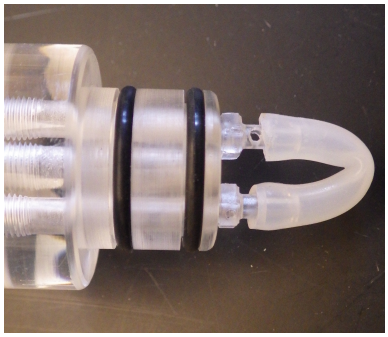

Fig. 5 The iTIE cap with inflow/outflow ports and tubing loop to create Venturi effect. Note: 25um nylon mesh (not pictured) covered the outflow hole to prevent organisms from leaving the chamber.

Air was pumped through the inflow port, through the tubing loop, and exited the outflow port into a length of silicone tubing. For the purposes of the flow rate test, the silicone tubing leading from the outflow port on the top of the cap simply deposited the water into a graduated cylinder for flow rate measurements. As air entered the tubing loop, its speed increased and the pressure dropped, creating a low-pressure area inside the outflow port. The low pressure created suction that would draw water up into the iTIE cylinder and pull it into outflow port hole (Fig. 5). To improve suction, 1/6" tubing was inserted into the tubing loop to further restrict the airway, increase airspeed, and drop the pressure.

Standard 12V DC aquarium air pumps were used to pump air through the tubing loop. Each pump had an adjustable airflow rate so various settings were tested to see their effect on iTIE water flow. Several pump/iTIE cylinder combinations were tested including one pump per iTIE and one pump for multiple iTIEs, using a 4-plex airsplitter to divide the airflow. Flow valves on the 4-plex splitter were used to try individual flow adjustments for each iTIE. Flow rate adjustments were tested both with empty chambers and with resins present in the chamber to account for varying water resistance.

The formation of air bubbles in the organism chamber was a concern. If too much air escaped from the outflow port hole, water flow might stop. Several methods were tested to provide air vents that would allow excess air in the organism chamber to exit. It was thought this could also help the iTIEs fill with water by providing an additional way to relieve pressure. The threaded plugs were removed from the third port on the iTIE cap and the port was fitted with either an air stone or a check valve (Fig. 6).

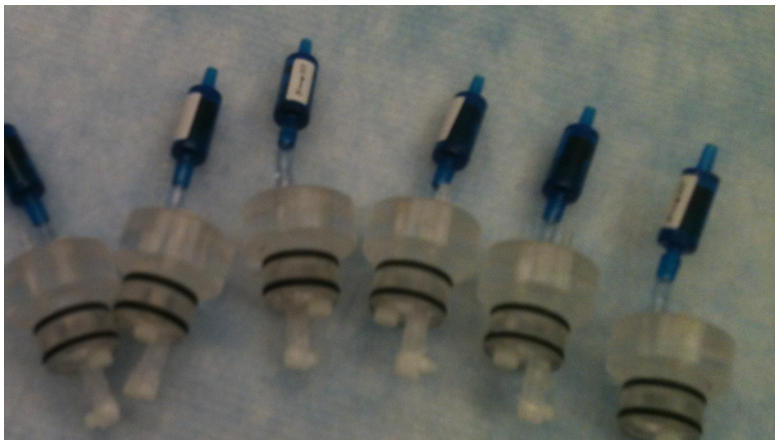

Fig. 6 Check valves on third iTIE cap port to provide air vent for organism chamber. These were tested as a method to prevent air bubble formation that could stop water flow through the 2004 Burton/Nordstrom system.

Resins

The resins selected for this study were designed to target the major classes of compounds most commonly observed in wastewater effluent: organics, metals, and ammonia [1,35]. Zeolite (Aquarium Pharmaceuticals) and Chelex (Sigma) were used for ammonia and metals, respectively. HLB (Waters), Sep-Pak (Waters), and Amberlite XAD-2 (Sigma Aldrich) designed to target organic compounds.

Activated Carbon (Marineland) was also chosen due to its affinity for organics, but it is also known to extract metals and ammonia so it was tested as a possible negative control option [39,43]. Due to time constraints, resin capacity was not tested before the field deployment. Since effluent concentrations were expected to be less than 100 ng/l, 5g of each resin was expected to be more than enough. Additional iTIE cylinders would be filled with glass wool only (no resin) as a positive control. Due to cost concerns, not all the resins were used for the Burton/Nordstrom model flow rate tests were used in the WERF project field tests (Section IV).

Results and Discussion: Burton/Nordstrom Model Flow Rate Tests

The primary difficulties with Venturi suction were inconsistent (or non-existent) flow rates and the introduction of air into a water-filled chamber. During initial flow-rate trials, between 5 and 8 iTIE spikes were connected to an air pump via air/water splitters with adjustable flow valves. The resin chambers were filled with different resin types (Activated Carbon, Chelex, XAD-2, and No Resin) and it became clear that the contents of the resin chamber would offer resistance that affects flow rate. Each chamber contained glass wool to hold the resins in place and one chamber (the control) had only glass wool. The No Resin iTIE was the only one flowing initially (Table 3). Flow increased in the No Resin treatments over time (48 ml/h to 160ml/h over 30 min), suggesting that water pressure combined with the siphon effect.

Table 3. iTIE flow rate trials (air pump system)

		ml of water pumped per time period		
Pump #	Treatment	10 min	20 min	30 min
1	Glass Wool (No Resin)	48 ml (4.8/min)	110ml (5.5/min)	160ml (5.3/min)
1	Activated Carbon	0	0	0
2	Glass Wool (No Resin)	88ml (8.8/min)	170ml (8.5/min)	250ml (8.3/min)
2	Zeolite	0	0	0

The Venturi suction was apparently too weak to initiate water flow through the resins, as zeolite and carbon treatments showed 0 ml/h flow rates throughout the 30 min trial. Tubing with a smaller inner diameter and pipette tips inserted into the tubing were utilized to further reduce pressure at the outflow port. While this approach succeeded in starting flows for all resin types, the rates were uneven (Table 4). Although carbon had previously been suspected of offering too much resistance compared to the No Resin treatments, the carbon treatment's 1440 ml pumped over 30 min was much higher than the 310 ml pumped through one No Resin iTIE (Table 4). These results suggested another possible factor in flow variation – air pump and pressure differences.

Table 4. iTIE flow rate trials – Resin Resistance

Pump #	Treatment	Water pumped over 30 min
1	Activated Carbon	1440 ml
1	Chelex	75 ml
1	XAD-2	190 ml
1	Glass Wool (No Resin)	310 ml
2	Activated Carbon	439 ml
2	Chelex	290 ml
2	XAD-2	200 ml
2	Glass Wool (No Resin)	680 ml

The venturi suction approach inherently creates a slow flow rate, but when multiple chambers utilize the same pump, pressure differences surge throughout the system as a whole. Each iTIE required custom flow rate adjustments to account for unique resin resistance, which could be done either with flow control valves on the air splitters or with the air pump itself. Adjusting pump speed led to changes in the flow rates, but the air distribution was uneven, regardless of the pump setting. The iTIE connected to the first port on the air splitter always experienced a higher air pressure and faster flow than iTIEs connected to the fourth position on the splitter. The drastically different rates for three iTIEs on pump 1, 0 ml/h compared to 720 ml/h, illustrated the uneven distribution (Table 5). The flow valves on the splitter were used to account for this variation, but fine adjustments were not possible. The system is interconnected so even a slight change in air flow at one port will cause a significant change in air pressure throughout, suddenly increasing flow rates for previously adjusted iTIEs. Even when the

perfect balance is achieved initially, the slow formation of an air bubble in the organism chamber would stop flow entirely in 1 to 3 hours (Table 5).

Table 5. iTIE flow rate trials (air pump system) – 24h
**** No resin in any chambers**

	ml of water pumped per time period			
Pump #	1h	18.5h	22h	Notes
1	0	0	0	Chamber partially filled, then stopped
1	0	0	0	Chamber partially filled, then stopped
2	0	0	0	Chamber partially filled, then stopped
1	720	0	0	Stopped after 1h
2	470	0	0	Stopped after 1h
2	1240	1390	2620	Ran for 24h

Air bubble formation seemed to be a natural side effect of the air pump based system so several modifications were made in an attempt to remove that air from the chamber. As the air passed over the outlet port, water would be drawn into the low-pressure corridor, but the pressure difference between the organism chamber and the tubing was not enough to keep an air pocket from gradually forming. A second outflow port was added to the iTIE cap to give the air a way to "vent" from the organism chamber. This approach created a contamination problem. When submerged, an open line for the air vent would fill with stream water unless the vent line ran to the surface. Air stones and one-way aquarium check valves were utilized to prevent stream backflow. Water leaked in through the air stones and there was not enough pressure in the chamber to force air through the check valve.

The new acrylic iTIE chambers had a larger total volume than those used in Burton and Nordstrom (2004), which possibly contributed to flow issues overall (Fig. 3, Fig. 4). The weak suction may not have been sufficient to draw the relatively larger volume of water into the chambers. The siphon effect helping to pull water out of the organism chamber was unable to overcome the force of gravity acting on the volume of water in that chamber. It is possible that higher water pressure at depth could prevent chamber draining, but this configuration was intended for shallow, nearshore deployment.

Due to the consistent difficulties associated with introducing air into a water filtration system, no other field parameters for the Burton/Nordstrom Model were tested. The Venturi-effect concept and air pumps were abandoned. Development began on a new system, powered by water pumps.

3.3 Field Version I

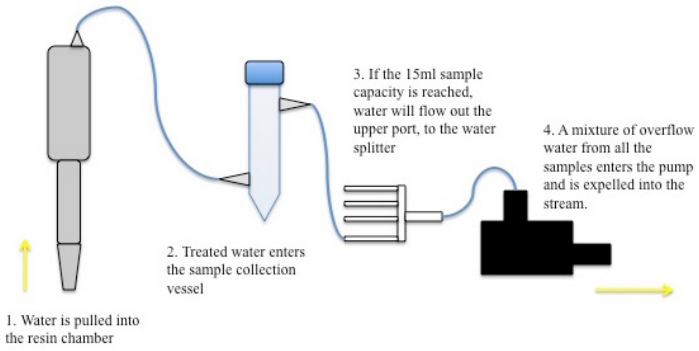

Fig. 7 Overview of Field Version I. After the failure of the air pump-based system, this water pump-based system was designed. Version I underwent mechanical tests in the lab but was never used for TIE experiments.

Introduction

The acrylic iTIE chambers were a sturdy component that could withstand heavy river flow rates and be easily disassembled for cleaning. A field system would have to meet all the iTIE parameters (Table 2) while incorporating this larger-volume chamber. A water pump was used for Field Version I (Fig. 7) to provide a stronger suction force without the risk of introducing air into the chamber. While a consistent 25ml/h flow rate was the first test priority of this configuration, the system as a whole needed to be designed with other field parameters in mind. Sample collection containers were essential for preserving water treated by each iTIE, to be collected and analyzed at the end of the exposure. During initial resin tests it would be important to understand how effective the resins were at removing some targeted compounds and allowing others to pass through. Eventually, when organisms were added, it would be important to know the concentrations of compounds the organisms were exposed to in each treatment.

Designing an easily deployed, autonomous system was essential for making Field Version I viable for the WERF tests. A portable power source for the new pump, and a sturdy deployment stand were all explored in this design phase. Resin parameters (Table 2) could only be tested once mechanical issues were solved to determine realistic resin responses within the system under field-like flow conditions. Following the design of each component, the entire system was assembled and submerged in a tank for flow and battery power tests.

Methods

Pump and Flow Control Design

The first pump choice was a 12V DC, 4.2W Magicfly Brushless Submersible Waterproof Pump. The pump did not have flow control, but it was a low-power model with an inherently slow pumping speed. To further slow the flow rate, the pump was put at the end of the system so it would have to pull water through all the components, increasing resistance and slowing overall flow rate. To keep power consumption low, only one pump was used. The pump's suction was distributed via two 4-plex splitters to 8 iTIE cylinders (Fig. 8).

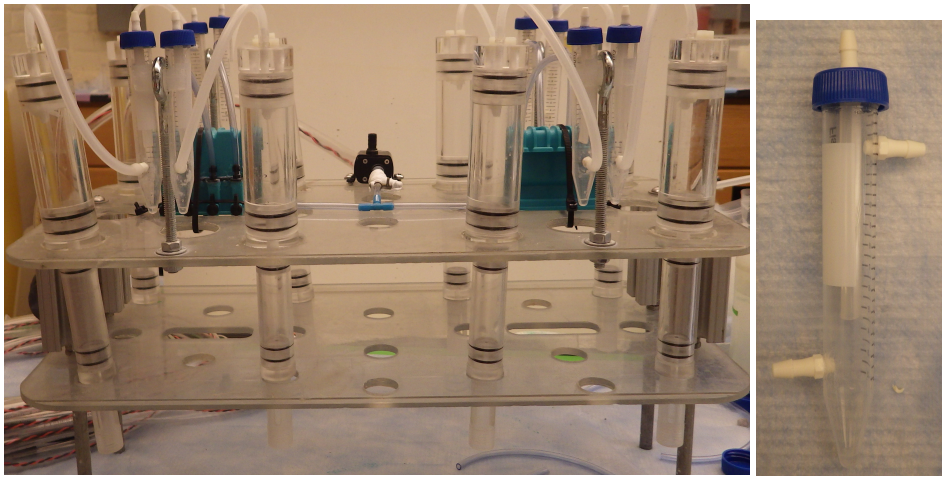

Fig. 8 Field prototype I (left) and the 15ml collection vessel (right)

Sample Collection Vessel Design

A 15ml sample collection vessel was designed to collect sufficient water for analysis after deployment, while keeping the total volume of air in the system as low as possible (Fig. 8, right). Water analysis was originally to be conducted on an Orbitrap LC-MS (Thermo Scientific), which would only require about 5ml of sample, so 15ml seemed sufficient. The 15ml limit was also a practical decision for Field Version I to avoid air volume-related flow problems, similar to those observed while testing the Burton/Nordstrom model. The iTIE chambers, collection vessels, tubing, and splitters would all contain air when initially submerged. Once the pump is switched on, it has to purge the entire volume of air in the interconnected system to initiate water flow. If there is too much air in the system, the pump may take too long or be unable to pull air out and draw water into the chambers.

Deployment Stand Selection and Modification

Due to time constraints with an approaching field season, the original iTIE stand from the Burton/Nordstrom system was utilized. The stand consisted of two parallel sheets of acrylic supported by metal spacers, resting on four thin, cylindrical legs (Fig. 8, left). The iTIE chambers fit through circular holes in the acrylic sheets. The thin legs

allowed the stand to be pushed into the sediment for pore water analysis. Zinc plated eyebolts (5/16" – 18) were added so the sample vessels could be secured to the stand.

Power Source Selection

Power consumption was a major concern, as this device would need a mobile power source for easy deployment in almost any aquatic system. Even with a small pump, meeting power needs for continuous operation over 24h could be difficult. The Duracell Power Pack 600 was selected for its versatile power outlets (AC Outlets, DC adapter input, etc.), clear power remaining indicator, and high amp hour (ah) capacity.

The Power Pack was not waterproof (and too large to be placed in a reasonably-priced waterproof container) so it was placed out of the water, protected and concealed in a camouflage dry bag, as it would be in the field. A waterproof conduit was constructed by threading electrical wires through a 9m length of acrylic tubing. The connection between the tubing and the pump was sealed with silicone. The "plug" end of this power cable consisted of a DC "cigarette lighter" adapter that could be plugged directly into the Power Pack.

Flow and Power Consumption Tests

The iTIE Field Version I was assembled in a field-ready configuration (Fig. 8) to test flow and power parameters (Table 2). Sample vessels were secured to the stand eye-bolts with zip ties and iTIE chambers were set in stand holes. Silicone tubing with 1/8" inner diameter (Fisher Scientific) was used to connect the sample vessels to the iTIEs and pump. Silicone was selected to minimize absorption of trace compounds and the 1/8" ID corresponded with the hose barbs on the iTIEs and sample vessels. Each collection vessel's upper outflow port (Fig. 8, right) was connected to a port on one of two aquarium 4-plex air splitters, which were each connected to a T-joint that funneled the water into the pump where it was expelled back into the water tank. As this test was simply for basic flow control, resins were not used. Flow rate was measured by the amount of time it took for each respective collection vessel to fill with water up to the 5 ml mark.

To determine if the power source was sufficient, the entire system ran with the pump drawing water through 8 iTIE chambers over 24h. The Duracell Powerpack would be considered sufficient if the pump was still running after 24h and the battery power indicator had at least one bar remaining. The water tank was filled so that the pump was submerged and water was pumped back into the tank so the water level would remain constant.

Results and Discussion: Field Version I

Flow rate was reasonably consistent and slow without direct control. The collection vessels filled at rates between 20 and 30 ml/h. With the pump at the end of the system, it had to overcome the resistance of the various chambers and tubing so an ideal flow rate was achieved without using water valves or a pump with adjustable speed.

The Duracell Powerpack was a sufficient power source, but the configuration left the iTIE stand tethered to the shoreline, limiting where it could be deployed. The pump was still operating efficiently after 24h and the battery had one power bar remaining on its indicator, which would support the planned 24h *in situ* TIE experiments. The battery would have to be left on the shoreline in a dry bag, though, which did not entirely meet the "inconspicuous" or "portable" goals (Table 2), but was deemed acceptable for a field prototype test. A submersible power system was not necessary for proof-of-concept field test, since it has no direct effect on flow rate, resin extraction, or bioassays. A more suitable power source could be utilized in future models if this configuration were successful.

Resin flow and field deployment were never tested with this system. It was decided that sample analysis on the Orbitrap would not be possible due primarily to a detection limit that was much higher than what was expected in field samples (cost of analysis and wait time for the machine were also factors). Consequently, sample analysis duties were handed over to the U.S. Environmental Protection Agency's Region 8 and Cincinnati Laboratories. Two-weeks before the first field deployment, a memo from EPA Cincinnati indicated that a 500ml sample would be needed from each treatment in order to test for CECs. The system had to be re-designed to support larger sample collection containers

3.4 Field Version II

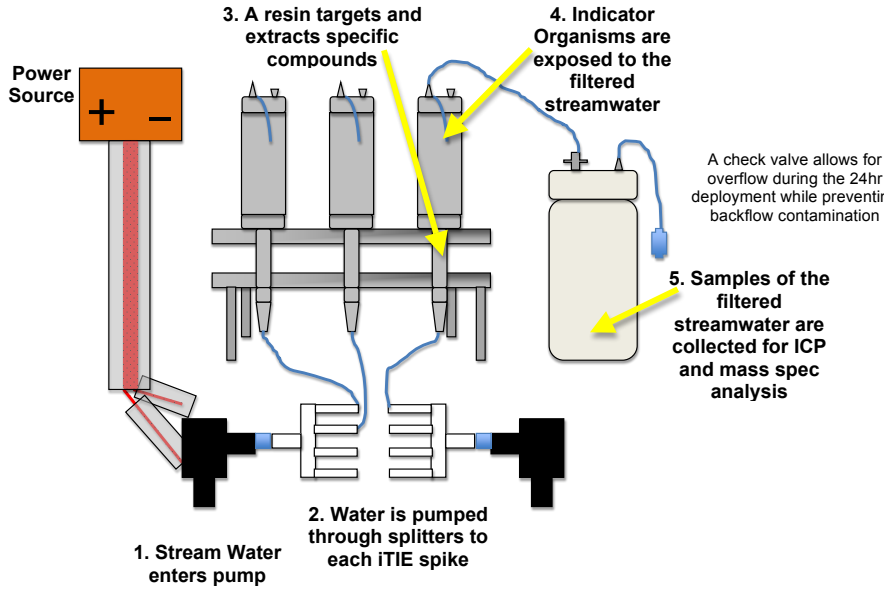

Fig. 9 Field Version II, used for WERF study *in situ* deployments. For Denver and Schaumberg field tests, each iTIE system had one pump driving 8 iTIE chambers. For Boise tests, each stand had 2 pumps (one pump per 4 iTIE chambers).

Introduction

The primary goal for developing and testing Field Version II in the lab was incorporating the working concepts from Version I into a similar model with larger sample collection containers. The power source, deployment stand, iTIE chambers, and tubing were considered ready for field trials. Adding larger volume containers to the model, however, raised new concerns about flow rate and consistency. To avoid the large volume problems encountered during the Burton/Nordstrom models tests, the pump was moved to the front of the system, pushing water into the iTIEs rather than pulling it through against resistance of an increased volume (Fig. 9).

Moving the pump to the front of the system put the collection bottles at the end, which necessitated design considerations for flow rate control and expulsion of overflow water from the system. The design and testing of Version II had to consider methods of reducing flow if the pump pressure was too high, in order to meet the project parameters (Table 2). The system also had to continue pumping continuously over 24h without backflow contamination from the different iTIE treatments or the river itself (Table 2), so the sample bottles needed a mechanism to discharge overflow and maintain an unimpeded flow rate.

Methods

Sample Collection Bottle Design

The 15ml centrifuge tubes were replaced with 500ml Nalgene bottles. The caps of the bottles were drilled and tapped to support one flow valve at the inflow port and one hose barb at the outflow port. A line of 1/8" ID silicone tubing connected the outflow port to a one-way aquarium check valve (Petco). Since the bottles were the last stage of the system, the overflow had to be discharged into the river through one-way aquarium check valves, preventing river water from entering the container contaminating the treated sample (Fig. 10). To ensure that the check valves would work at depth, against the exterior water pressure, the new configuration was setup in the lab with the check valve submerged at the bottom of a 1L graduated cylinder. The pump, iTIEs and collection bottles were connected in the field configuration (Fig. 14). The collection bottle contained dyed water, so if overflow water were exiting the check valve as designed, the colored water would be visible in the graduated cylinder. If, however, water pressure forced the backflow into the iTIE chamber, then the colored water would be visible there.

Fig. 10 Field Version II Sample Collection Bottle (left)
and cap with flow valve and outflow port (right)

Pump and Flow Control Design

The pump was connected to a T-splitter that directed water to two 4-plex splitters, connected to each iTIE chamber (Fig. 9). The sediment adapters were removed from the iTIE chambers (Fig. 4) since this was an open-water study, but the iTIE cylinders required a bottom fitting where water tubing could be attached. The bottom connectors on the iTIEs were threaded, allowing the sediment adapters to be unscrewed. They were replaced by ¼" threaded female adapters with 3/16" hose barbs. These adapters allowed a direct tubing connection between the splitters and the individual iTIE chambers (Fig. 11).

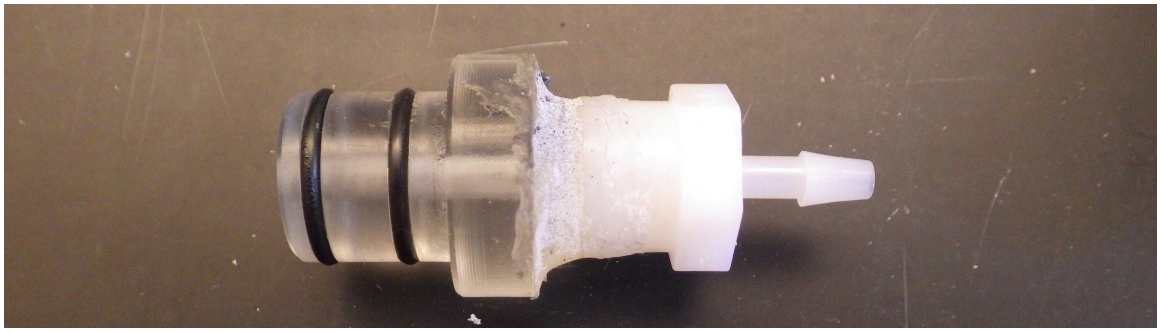

Fig. 11 Hose barb adapter for bottom of the iTIE cylinder

Two methods of flow control were tested, individual flow control using valves on the collection bottles and overall flow control using a valve near the pumps outflow port. The water valves on the sample collection chambers would slow flow through each of the

iTIE cylinders (Fig. 10). The valves were tested in various positions to control water speed for each individual iTIE chamber. Flow valve settings were calibrated based both on ideal flow rate and the unique resistance of each resin. Resins were added to the iTIEs and the valves were adjusted to achieve the slowest drip possible. A single flow valve was also added to the tubing circuit, between the outflow of the pump and the T-splitter to control water speed as it exited the pump (Fig. 12). Flow rate was determined by measuring the outflow water collected in a graduated cylinder over several minutes. For the flow tests, the entire stand in its field configuration (Fig. 14) was deployed in a water tank, filled so that the pump was submerged, but collection bottles remained mostly above water to avoid buoyancy problems and to better observe flow rate.

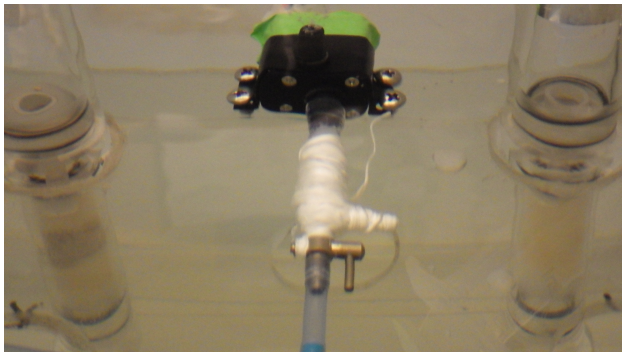

Fig. 12 Pump flow control with water valve

Deployment Stand Design

The same stand used for Version I was utilized in Version II, with a few minor changes. The collection bottles were clustered around the eye-bolts and secured with zip ties, since they would be buoyant until filled (Fig. 13). Keeping the whole system submerged during the lab tests was not an issue, since the collection bottles remained above water. In the field, cinder blocks would be chained to the system to keep it submerged throughout the *in situ* TIE experiment.

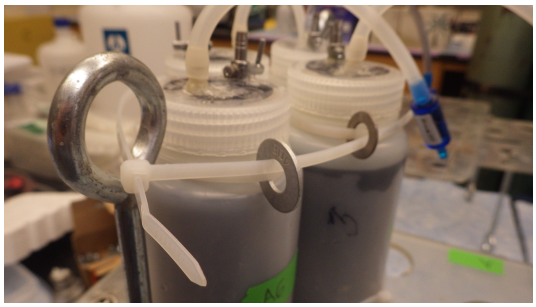

Fig. 13 Sample collection bottles secured to eye-bolts on the deployment stand for iTIE Field Version II.

Fig. 14 iTIE Field Version II (Complete). This version was used for the three WERF field studies as an in situ TIE prototype.

Discussion and Results: Field Version II Lab Tests

The main water valve near the pump did not effectively slow flow rates for the entire system. Closing this valve partially restricted the waterway at that point, increasing the water pressure as it entered the splitters and, subsequently, pushed resin particles out of their chambers, clouding the organism chamber. Flow rate was effectively slowed with the individual water valves, however, so the main valve was deemed unnecessary.

A flow rate of 60 ml/h was the lowest rate possible using the individual water valves on the collection bottles. This was not the ideal flow rate of 25 ml/h [1], so some chemicals could potentially break through into the organism chamber without contacting the resin. To mitigate this possibility, the amount of resin in the chambers during field tests would be doubled to 10g, which is enough to fill the iTIE chamber, while leaving room for glass wool above and below the resin to hold it in place.

The whole system pumped successfully as a circuit, with the check valves working as a one-way overflow port. The dyed water test showed that water was leaving through the check valve, at depth, as the capacity of the sample collection bottles was exceeded. The outside water could not enter the sample bottle through the check valve and the sample water did not backflow into the iTIE chamber. The Field Version II configuration allowed for a continuous flow of stream water through the system while maintaining the integrity of the individual processed samples.

IV. Experimental Field Deployment: iTIE Version II

4.1 Introduction

The field deployments were intended to provide both TIE results for the WERF study and test the iTIE parameters (Table 2) for Field Version II. Deployments occurred at three sites designated by WERF for Phase II ecological risk assessment. The sites had observed evidence of biological impairment and local research agencies could provide site data documented over many years [22].

The iTIE systems would conduct TIE Phase I fractionation and bioassay exposures, using acute toxicity as the parameter for biological impairment. All the iTIE System parameters (Table 2) were assessed, including general functionality of the machine, ease of deployment, and the ability of resins to extract compound classes.

4.2 Methods

Test Sites

The Denver, CO deployment occurred in the South Platte River, just downstream of the wastewater discharge point for the Metro Wastewater Reclamation District, Northern Treatment Plant (39.8131284324, -104.953360675). The immediate deployment site had thinly vegetated riparian zones, but was primarily surrounded by industrial sites. The upstream watershed was the City of Denver so there were various possible point and non-point sources of pollution from urban and suburban sites, in addition to the effluent discharge.

The study site in Schaumberg, IL was in Salt Creek (42.03196, -88.01123). This deployment site was farther downstream from the effluent discharge point than the site in Colorado and the effluent discharge point was not visible. Woods and prairie surrounded the exact deployment location, but the upstream watershed passed through urban and suburban areas.

State laws in Idaho, designed to prevent foreign species invasions, prohibited the deployment of the iTIE system *in situ*, due to the presence of test organisms in the chambers. To conduct the tests, water samples were collected downstream of the effluent (Boise River, near Glenwood Bridge: 43°39'37.01"N, 116°16'42.60"W), in a suburban area with minimal riparian vegetation. The samples were transported to large containers, in which the iTIE systems were deployed. Stream water was not renewed during the 24h exposure.

Resins

The resins used in the first were selected from the types tested in the laboratory flow rate tests. Three iTIE stands, each able to support 8 iTIE chambers, would be deployed. To ensure enough successful replicates, 6 resins were selected. Carbon, HLB,

and Sep-Pak would target organics for extraction, chelex would remove metals, and zeolite would absorb ammonia.

Test Organisms and Exposure Period

The organisms selected for the bioassay portion of the iTIE were *Daphnia magna* and *Hyalella azteca*. Both are commonly used in TIE bioassay experiments and are listed as recommended organisms in the USEPA TIE protocol [28, 48]. A 24h exposure period as it is an established time period for toxicity assessment and within the power capacity of the iTIE system [49,50].

Contamination and Ambient Toxicants Variables

Various sampling and control measures were taken to identify ambient chemicals and artifacts. River control samples were collected upstream of the effluent discharge using 24h autosamplers. An autosampler was also deployed downstream of the iTIE deployment site. iTIE chambers with only glass wool provided No Resin positive control treatments for each iTIE stand. In No Resin treatments, the bioassay in the organism chamber was conducted with unfiltered streamwater. Grab samples of the wastewater effluent itself were collected during the 24h iTIE deployment.

Some trace chemicals that have been observed in wastewater effluent are common in plastics and other materials similar to those used for various components on the iTIE system. To account for leaching from the equipment, pieces from Field Version II were soaked in MilliQ, which was analyzed using the same sample methods as all stream and iTIE samples.

Pre-Deployment

All components of the iTIE system underwent decontamination procedures to avoid introducing foreign concentrations of organic compounds into the testing sites and water samples. Non-metal pieces were washed and soaked in acid for 24h. Metal pieces were washed and briefly dipped in acid. Although an acid wash will not remove all trace organics, cleaning solvents such as Methanol risked damaging the acrylic components of the iTIE chamber. All components were stored and shipped in plastic tubs lined with Nalgene 74043-00 Super VERSI-DRY® Lab Soaker Bench Protector Mat. During preparation and deployment, equipment was handled with gloves.

The surface area of the granuatled resins, zeolite and carbon, was increased by grinding them up into a fine powder. Powdered resins were also more easily packed into the iTIE resin chamber, ensuring that no water could seep through air pockets without contacting the resin. To purge air from pores and interstitial spaces, all of the resins were soaked in milliq [39]. The soaked resins were vacuum filtered to remove fine particles that could pass into the organism chamber. The powders were then packed into their respective chambers between two clumps of glass wool. A circular piece of 60um vent filter (WEB) was added to the top of the resin chamber, flush with the upper connector, to capture any remaining resin particles. The iTIE chambers were then divided among three stands so that each stand had a replicate of each resin. Sample bottles, pump, and other

components were secured to the stand and connected according to the lab flow rate test methods for Version II (Fig. 14).

Deployment

The iTIE stands were first deployed, partially submerged, in shallow water so organisms could be added and flow rate could be calibrated. A short piece of rubber tubing with a mesh covering was fitted onto the pump intake to prevent large debris from clogging the system. The pump was switched on until each iTIE filled with streamwater up to the top of the organism chamber. Ten *Daphnia magna* and ten *Hyallela azteca* were added to each chamber. Once this was completed, the iTIE caps were fitted in place, and the stand was submerged in deeper water, where it was secured to cinder blocks (Fig. 15). The three stands of eight iTIE chambers each were arranged inline with the streamflow, just downstream of the effluent discharge point. The pumps were switched on and flow rate was estimated based on the air bubbles flowing out of the check valves. Flow control valves were adjusted until the bubbling rate was consistent among all treatments. The chemical fractionation and organism exposure test ran for 24h.

Fig. 15 Three iTIE Field Version II stands deployed in the South Platte River, Denver, CO. Each stand contained at least one replicate of the six resin treatments and the No Resin control. Power conduits ran to each system's respective battery onshore.

Retrieval

The mesh-covered tubing was removed from each pump intake and replaced with a plug to keep water in the system as it was removed from the stream and placed on the shoreline for organism retrieval and water sample collection. To collect the organisms, the tubing at the top and bottom of the iTIE was cut. While keeping the bottom tubing crimped, the iTIE cap was removed, and the water from the organism chamber was drained into a sample cup. The chamber was rinsed with culture water and inspected by two investigators to ensure that no test organisms remained. Water samples were transferred from the iTIE system collection bottles to 500ml glass sample bottles.

Due to low-light conditions onsite, additional sample processing was conducted in the lab immediately following retrieval. Organism survival was determined for each

treatment by placing the organisms in a clear dish and observing movement. The dish was shaken to initiate movement and a count of living and dead organisms was taken. Water samples from the effluent grab, upstream and downstream autosamplers, and iTIE chambers were stored in ice-filled coolers. The samples were shipped overnight to USEPA Region 8 and Cincinnati Laboratories.

Water Sample Analysis

Water samples from the stream, effluent, and iTIE collection containers were prepared, analyzed, and verified by the USEPA Region 8 Laboratory (Golden, CO) according to the requirements of the Laboratory Services Request (LSR) and procedures outlined in the laboratory Quality Assurance Manual (QSP-001). The samples were analyzed for the presence of various pesticides and PPCPs. Additional water samples were tested for TOrCs at USEPA Cincinnati using LC/MS/MS. Metal concentrations in the stream and iTIE samples were determined at the University of Michigan using inductively coupled plasma mass spectrometry.

4.3 Results and Discussion

Equipment Contamination

Several trace organic compounds were detected in MilliQ exposed to the various components of iTIE System Version II (Fig. 14). Leaching from exterior plastics (zip ties, deployment stand plates) and artifacts could contribute to iTIE sample concentrations. The 250 to 400 ng/l concentrations of BPA and nonylphenol compounds in the iTIE part and Collection Bottle water were concerning (Fig. 16), especially considering that many organic compounds detected in the streamwater samples were under 1000 ng/l (Fig. 17 – 20). If a constant flow through the iTIE system interior components was maintained, however, concentrations of these chemicals in the streamwater should overwhelm leached concentrations. Since no BPA or nonylphenol compounds were detected in the Colorado iTIE samples (Fig. 17-2-) and the pumps were still running when retrieval began, constant flow rates may make negate leaching.

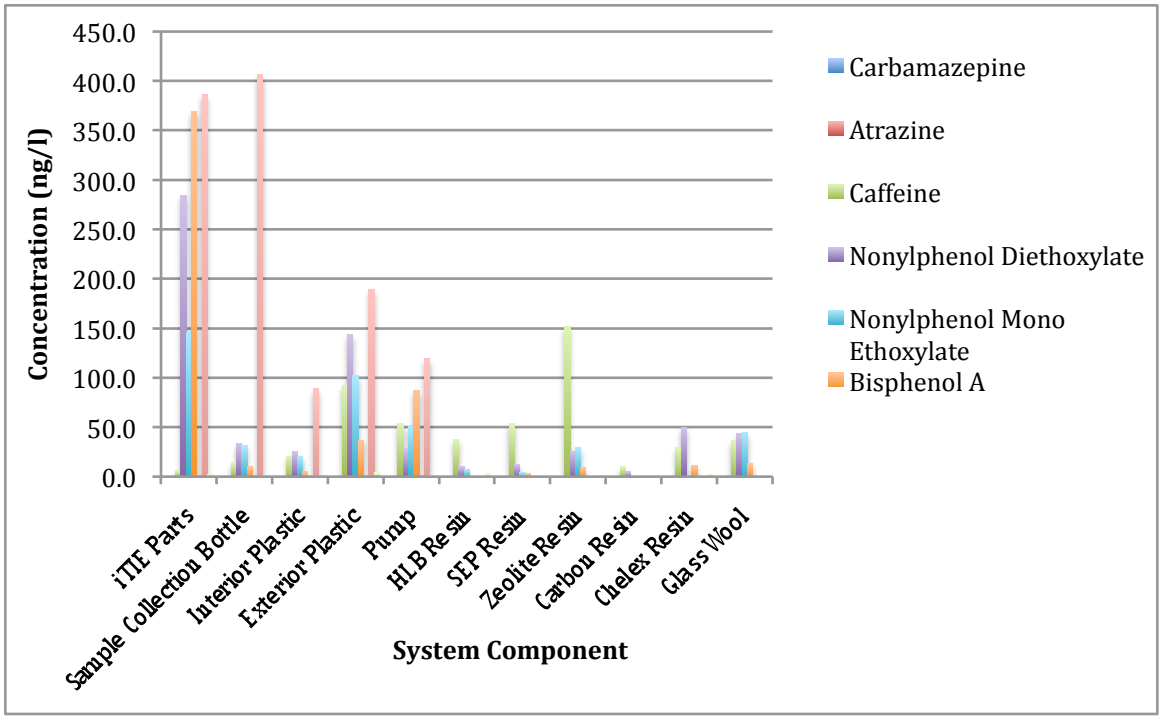

Fig. 16 Organic compounds leaching from iTIE System Version II components (laboratory MilliQ soak).

Denver, CO

Problems with Deployment

With eight interconnected iTIEs, a change in water pressure at one point will have repercussions throughout the system. Adjustments to flow often had a delayed reaction so when the system was fully submerged and left for the 24-hour exposure period, water would stop flowing through some iTIEs entirely when met with too much resistance. Concurrently, flow would sometimes increase in other chambers, resulting in several forms of contamination in the organism chamber.

Following retrieval of the system, it was immediately obvious that several factors had disrupted the test. First, in some exposures, the organism chamber was clouded with resin, indicating either a surge in flow that forced particles into the chamber or too many fine particles remained after vacuum filtration. This introduced another variable into the test that could have impacted organism survival. Second, we observed some collection bottles that were empty or only partially filled when, at the standard flow rate, they should have overflowed at some point during the 24hr test. None of the treatments collected 500ml of sample in all of their replicates, so replicate samples had to be combined to get a sufficient amount to conduct water chemistry. The combined samples from No Resin treatments for this deployment produced insufficient sample for detection of trace organic compounds so this positive control was only analyzed for metals.

CEC Concentrations and Organism Survival

Analysis of streamwater from the composite auto-sampler, iTIES, and grab samples revealed a complex mixture of herbicides, pesticides, pharmaceuticals, antibiotics, and various human metabolites. Concentrations ranged from about 10 ng/l to 10,000 ng/l. As expected, these trace organic chemicals were present in very low concentrations, no more than 10ppb. Though some of them do have an acute toxicity threshold, those that do were magnitudes below their LC50 values. As a result, there was no significant effect on organism survival, regardless of the treatment (Fig. 17).

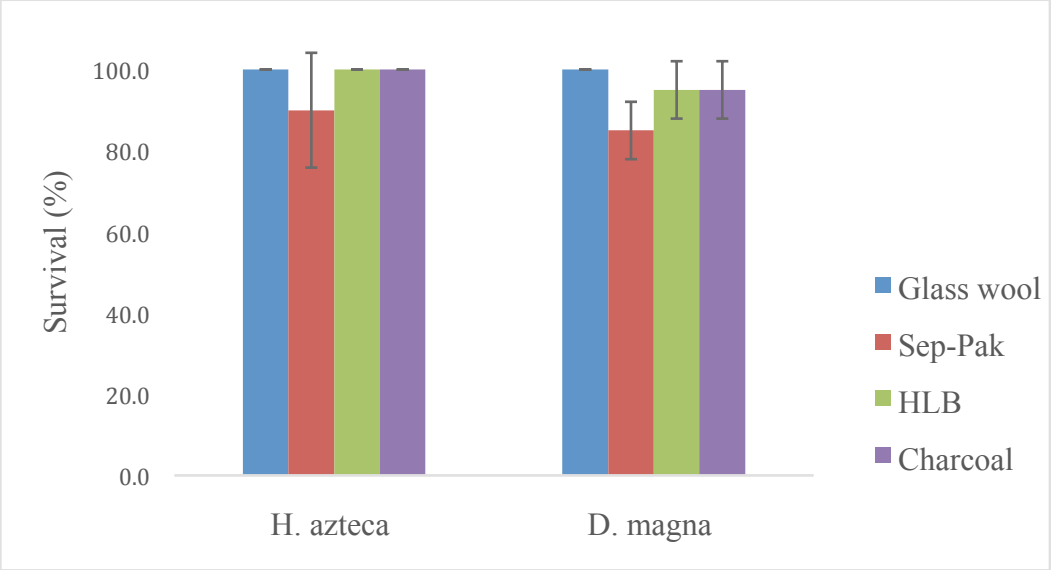

Fig. 17 Percent organism survival by iTIE treatment (Denver deployment)

Some compounds that initiate non-lethal effects were magnitudes lower than their EC50 values. For example, Acetaminophen, a pharmaceutical common in painkillers, was measured at 22.50 ng/l in the effluent and 29.6 mg/l in Sep-Pak iTIE samples. This compound is known to significantly reduce the heart rate of *D. magna* but only at a minimum concentration of 9.2 mg/l and over 48h [33]. Diclofenac, which was present in the carbon-treated sample at 1,510 ng/l (Fig. 18), has an acute toxicity after 48h of exposure at 40mg/l [57]. The relatively high concentration of Diclofenac in the carbon treatments, compared with the Effluent levels may be the result of contaminant fluxes that only an *in situ* test can address. The compound was not detected leaching from the equipment (Fig. 16) so the higher concentration in the iTIE sample likely represents a flux. When the effluent grab sample was taken, Diclofenac was present at 329.67 ng/l (Fig. 17), but this level must have increased at some point during the 24h TIE test. The Diclofenac flux was likely also spatial, as the downstream samples did not reflect this spike (Fig. 18). The trace nature of these compounds, combined with their spatial and temporal variation makes it difficult to establish causal links between any one compound and observed ecosystem disruption. The ability of the iTIE system to fractionate this complex mixture *in situ* will determine its feasibility as an effective TIE tool.

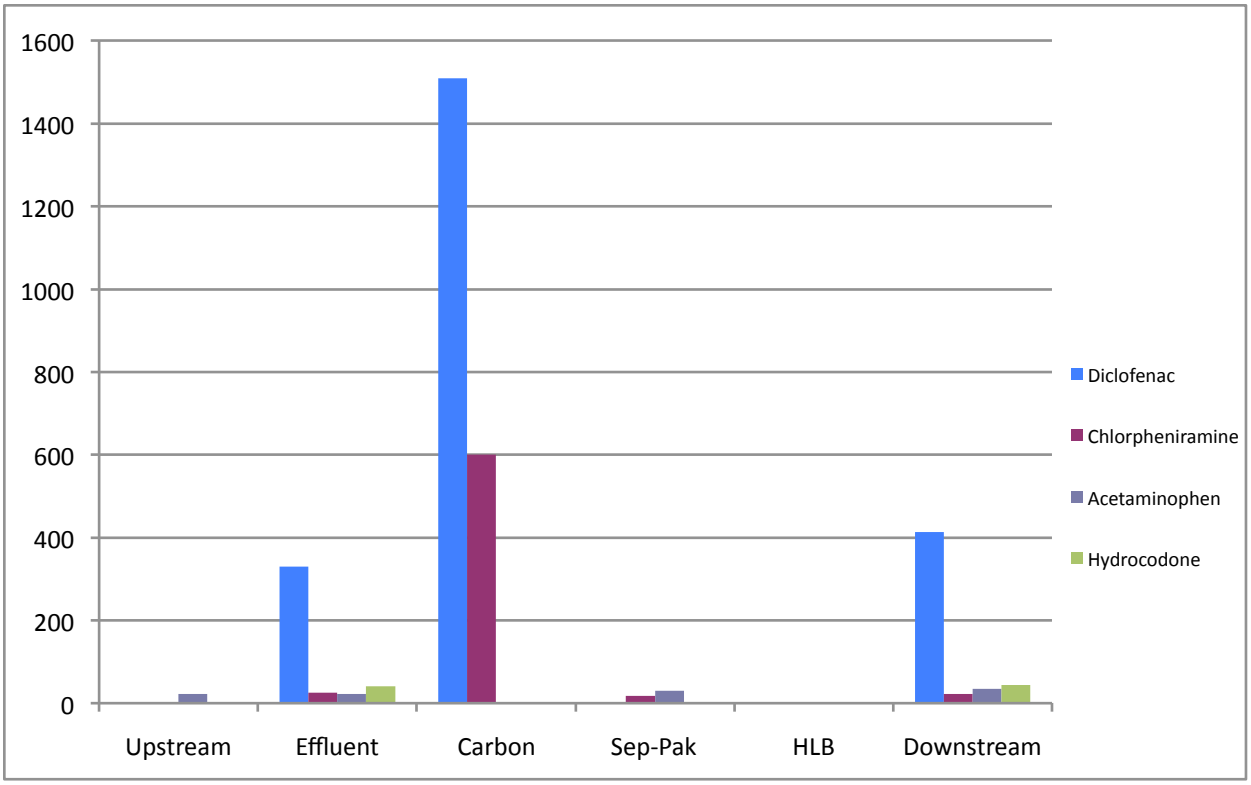

Fig. 18 Concentrations of pharmaceuticals in the South Platte River. Resin names indicate water samples processed by the associated iTIEs.

Assessing the chronic toxicity of trace compounds is essential to understanding long-term environmental and human-health risks posed by exposure to this effluent. Only by eliminating confounding variables and fractionating the CECs can we begin to understand their individual and additive effects. Though the iTIE systems experienced some mechanical difficulties, their ability to fractionate the complex slurry of compounds in the streamwater was apparent. By comparing the samples treated by the iTIEs to the effluent and downstream stream samples, there is a noticeable drop in CEC concentration for several treatments. Concentration of the antibiotic Sulfamethoxazole was reduced by 100% in the Carbon and HLB iTIEs compared to the 927.67 ng/l and 947.00 ng/l concentrations in the effluent and downstream samples, respectively (Fig. 19). Likewise, the herbicide Propachlor ESA was extracted completely from HLB, Carbon, and Sep-Pak treated water, so organisms in those chambers were not exposed to the compound (Fig. 20). The iTIE systems were able to draw in stream water, process it with organic-targeting resins, then expose organisms to the modified solutions. The resins did not, however, target entire categories of compounds, instead showing specificity for certain types while not extracting others.

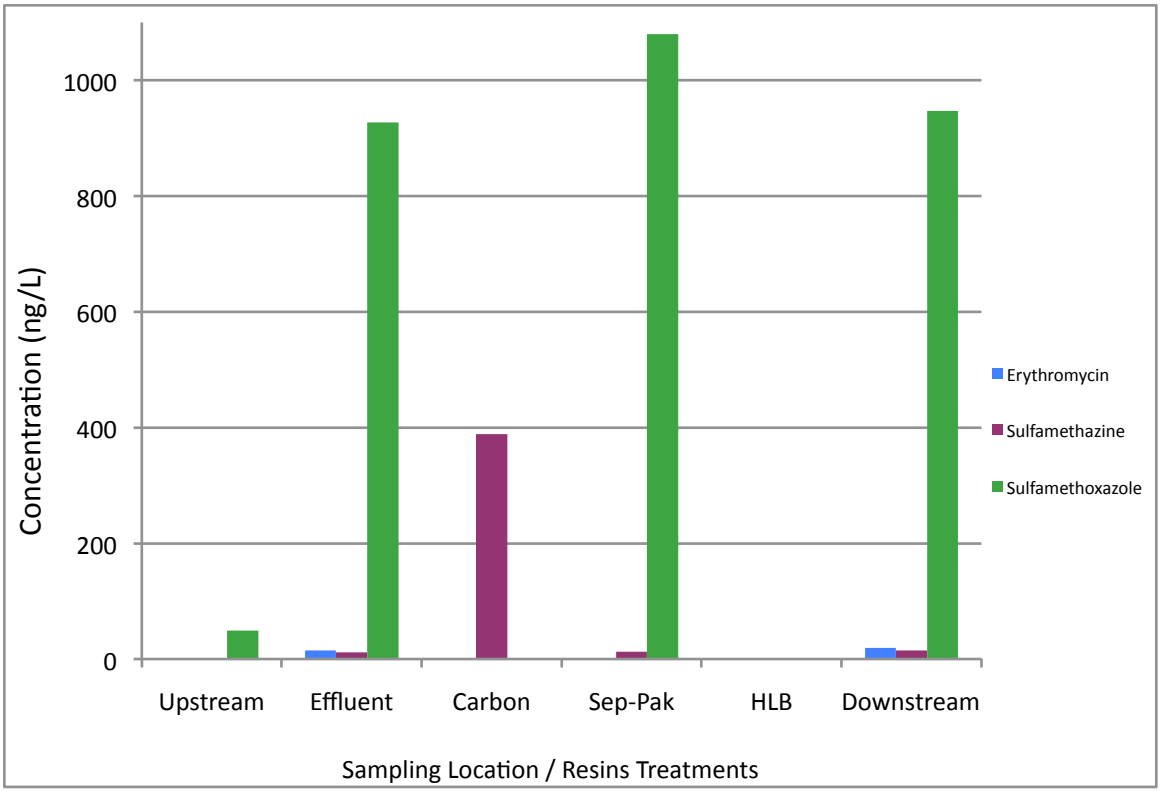

Fig. 19 Concentrations of antibiotics in the South Platte River. Resin names indicate water samples processed by the associated iTIEs.

Although all three resins shown in Fig. 19 are expected to adsorb organic compounds in general, they show variations in specificity. HLB was able to reduce the concentration of sulfamethazine below detectable limits, while carbon showed less affinity for the same compound with a 389 ng/l concentration in that treatment's sample water. Both Carbon and HLB completely removed the herbicide Diuron from the streamwater as it passed through, but Sep-Pak did not (Fig. 20). Assessing resin affinities at the compound level, instead of simply the family level, can help future studies that want to target specific contaminants for removal and potentially conduct *in situ* TIE Phase II analyses. By using Sep-Pak in a Phase II fractionation study, Propachlor ESA could be removed but Diuron would not, allowing for compound-specific bioassays (Fig. 20).

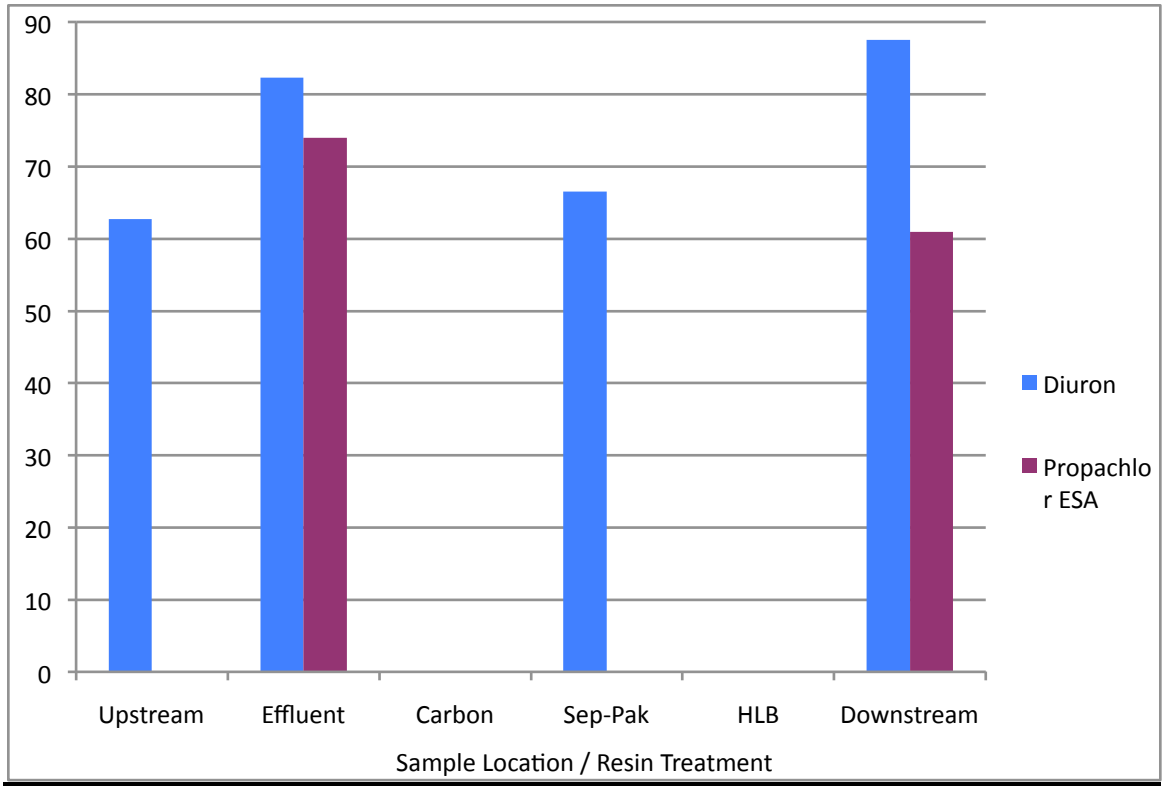

Fig. 20 Concentrations of herbicides in the South Platte River. Resin names indicate water samples processed by the associated iTIEs.

Phase I and Phase II in situ bioassay methods will likely have to be adapted to reflect more organism responses than mortality from acutely toxic concentrations. Linking sub-lethal effects to specific compounds can be difficult if behavioral, physical, or genetic responses of a particular organism to a particular compound are unknown. The pesticide DEET was present at 268.83 ng/l in the effluent and 541 ng/l in downstream water (Fig. 21). Though it was successfully removed from all three organic resin treatments, organism survival was not different between these treatments and the No Resin control (Fig. 17). Although the No Resin treatments did not produce enough sample to test for organics, it can be inferred that organisms in these iTIEs were exposed to streamwater that still contained DEET, yet the bioassay did not affect survival. DEET is suspected to cause forms of endocrine disruption and other genetic issues in several organisms, but the exact responses to this and other pesticides are widely unknown [58].

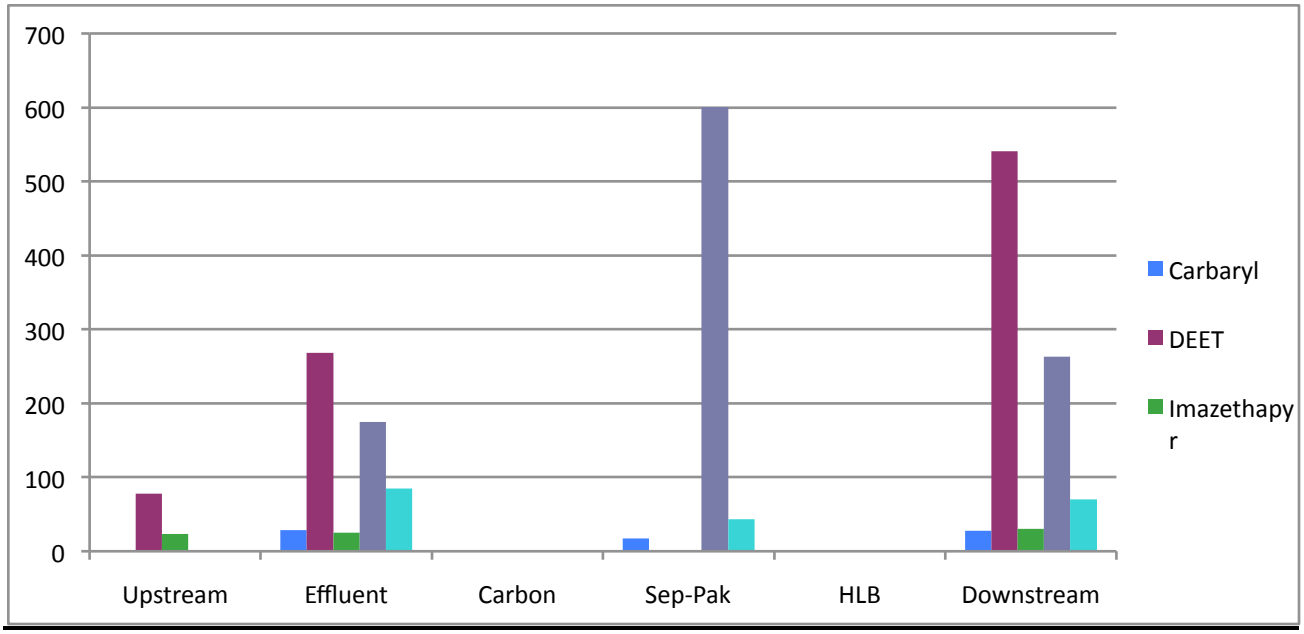

Fig. 21 Concentrations of pesticides in the South Platte River. Resin names indicate water samples processed by the associated iTIEs.

The chelex iTIEs did not appear to be as successful removing metals as the organic resins were for their target compounds (Fig. 22). Metal concentrations overall were much lower than organic compound concentrations, with most present at fewer than 8 ng/l. The chlex iTIEs reduced lead and manganese levels by 18.2% and 18.8%, respectively, and nickel was 22.2% lower compared to the No Resin iTIEs. Copper concentrations in the chelex iTIEs were actually higher than in the No Resin samples (1.125 ng/l compared to 0.879 ng/l). Without replicates it is not clear if these differences are significant. Since the concentrations are so low in the river, metal ions may have been able to pass through the iTIE resin chamber without directly contacting resin particles.

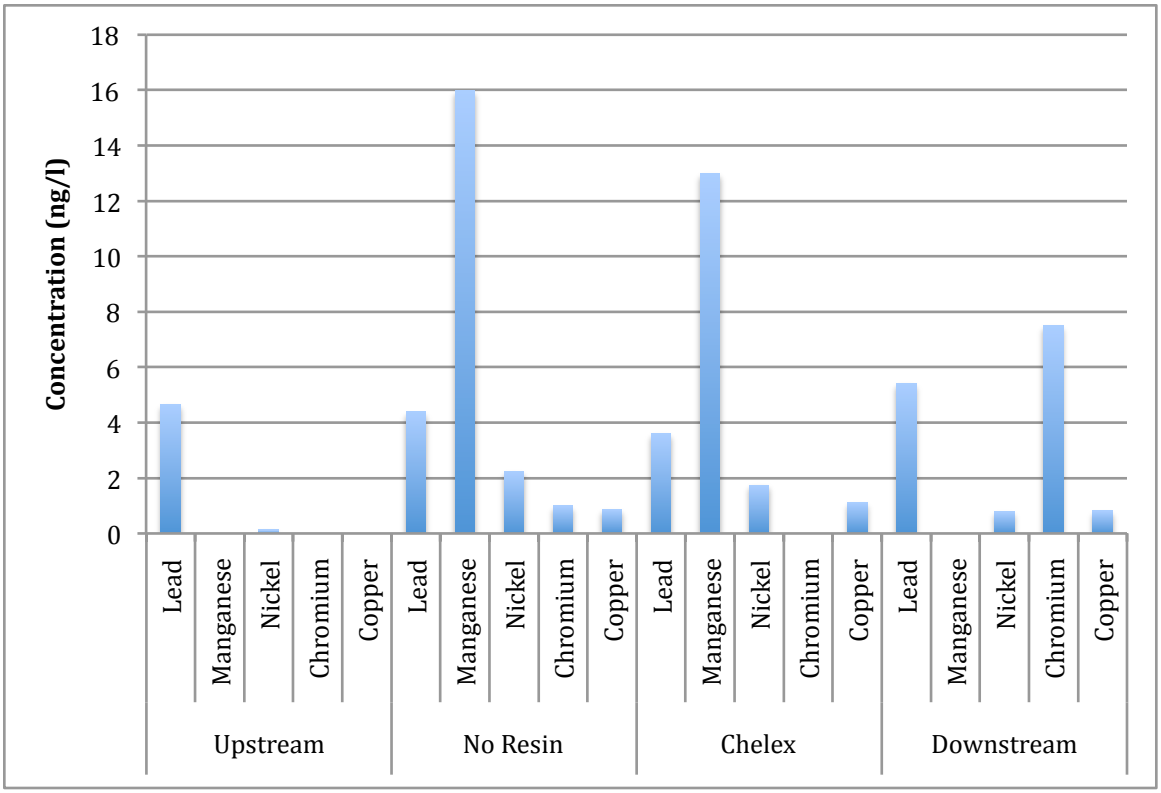

Fig. 22 Concentrations of metals in the South Platte River and in iTIE processed samples. Resin names indicate water samples processed by the associated iTIEs.

Schaumberg, IL

Mechanical Problems Encountered

The pump experienced mechanical failure for one stand, which led to the loss of several replicates and likely affected organism survival. The pump was not running at the 24h mark, even though the battery for that system still had power. The pump itself likely failed, probably early on in the exposure, so the water in these samples did not flow constantly throughout the 24h exposure. Some iTIE chambers on the associated stand were partially or entirely filled. One No Resin treatment was empty. The other two iTIE stands had functioning pumps but flow rates between iTIE chambers were once again uneven. As in Denver, replicate samples had to be combined to provide sufficient water for chemistry analysis. There was insufficient water for ICP-MS analysis for metals.

The deployment limitations of iTIE Field Version II were especially apparent during this field test. Since the stands were tethered to the shoreline by very visible conduits, the whole system was very conspicuous. The site was a very public area (just off a walking trail in a park) so the battery packs had to be set on a section of the shore that was relatively inaccessible. Ultimately only one spot was inconspicuous enough and had stable enough sediment to be a viable deployment site. The equipment was still partially visible and had to be covered with debris to avoid theft and vandalism.

Organism Survival

There was complete organism mortality in the empty No Resin iTIE chamber so this replicate was not included in the survival data. Across all treatments and stands, there was much more variation in survival for this deployment compared to Denver (Fig. 23).

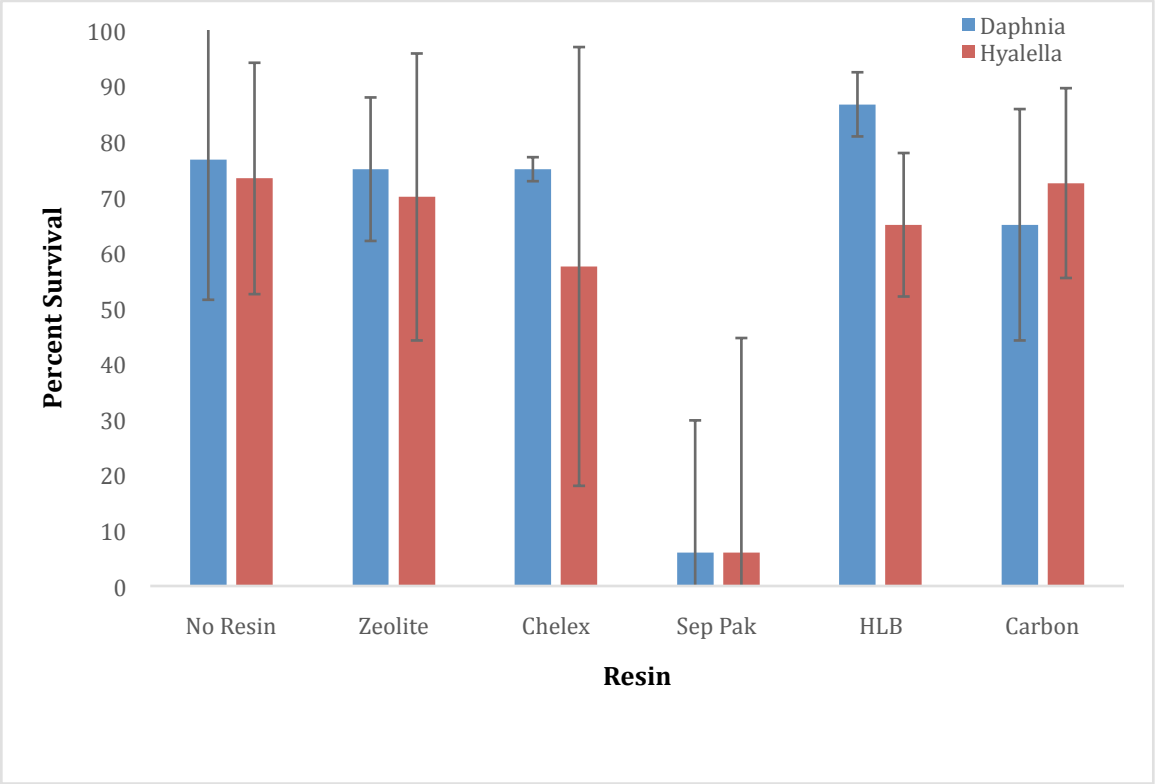

Fig. 23 Organism survival by iTIE treatment (Schaumberg deployment). Organism survival in one No Resin replicate was not included because the chamber had drained. Each chamber started with 10 individuals from each species.

The flow problems during this deployment were much more severe overall compared to Denver, primarily due to the mechanical failure. It is possible that survival was affected by dissolved oxygen depletion in organism chambers where flow stopped early in the test. Sep-Pak survival is significantly lower than other treatments, including HLB and No Resin (Fig. 23). Since survival was lower than in the positive control, it is unlikely than acute toxicity was the cause. In Denver, Sep-Pak survival was, on average, lower than the other resin treatments, though the difference was not significant (Fig. 17). During Denver and Boise retrieval, Sep-Pak powder was visible in the organism chamber, which could explain the decreased organism survival. The turbidity created by the resin particles could have induced stress or the resin itself could be toxic to the test organisms.

The severe system failures during this deployment seemed to lead to some less successfull compound extractions. Atrazine concentrations were 29.2 ng/l and 27.0 ng/l in the No Resin and Sep-Pak treatments, respectively (Fig. 24). Carbamazepine levels were 16.3% higher in the Sep-Pak samples, compared to the No Resin samples (Fig. 25).

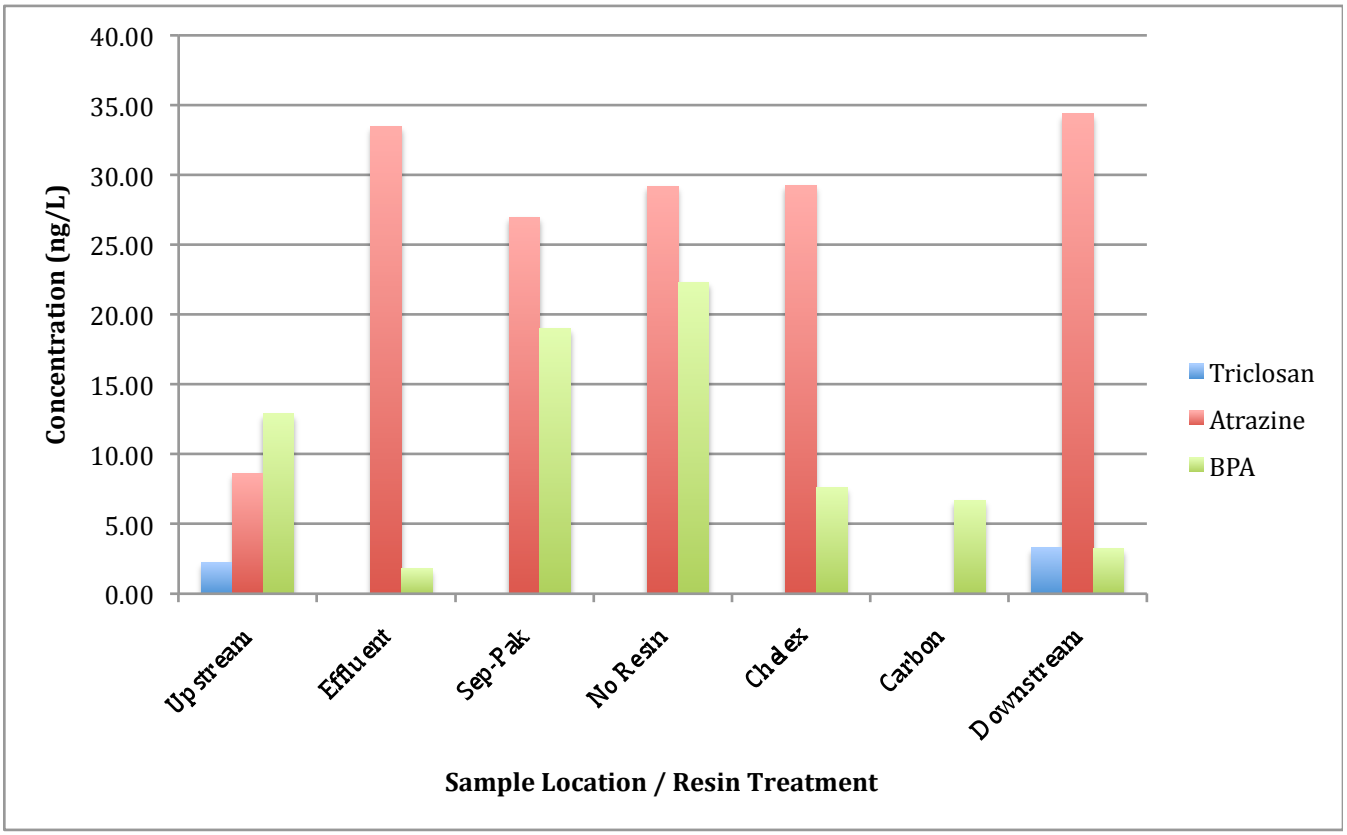

Fig. 24 Concentrations of CECs in Salt Creek. Resin names indicate water samples processed by the associated iTIEs.

Though these concentrations are very low and may not pose an immediate threat, it is important to consider the possibility of long-term accumulation. The organic carbon/water partition coefficient (K_{oc}) of BPA, for example, makes it highly sorptive to sediments and the compound has some capacity for bioaccumulation [2]. The BPA concentrations observed in the iTIE samples, however, may not be representative of stream concentrations at this site.

The most important result from the Schaumberg deployment may be evidence for leaching compound influence. BPA does leach from iTIE system components when the water is stagnant (Fig. 14). Although this was not an issue in Colorado when flow continued for most of the test in most of the treatments, many more iTIE treatments in Schaumberg slowed and/or stopped early in the deployment. The water in these samples remained in contact with the system components much longer than in Denver. This may

account for higher BPA and nonlyphenol concentrations in the iTIEs compared to the upstream and effluent samples (Fig. 24 and 25), since these two compounds were present in the equipment leaching experiment (Fig. 14). These data support the initial hypothesis that contamination from the iTIE system itself is minimal as long as a consistent flow rate is maintained.

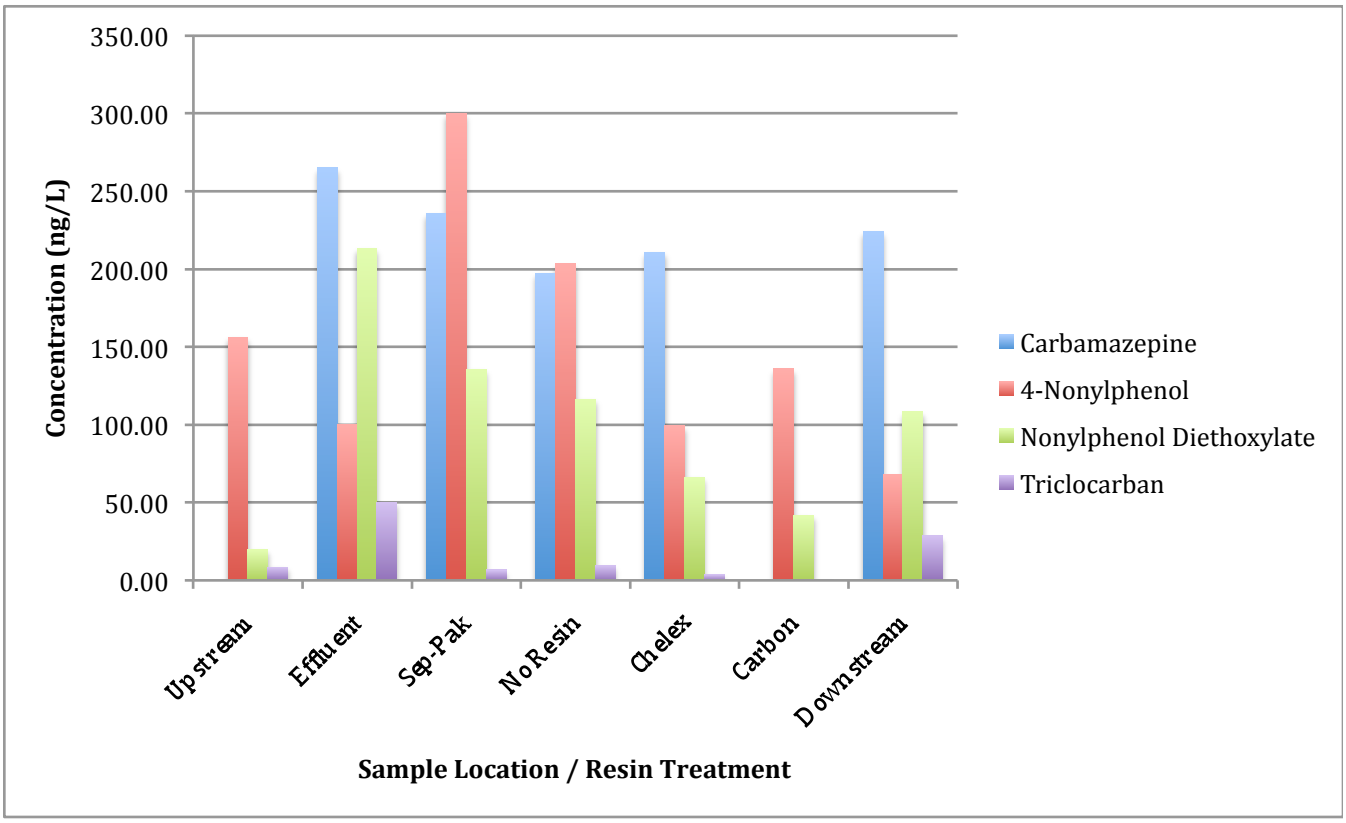

Fig. 25 Concentrations of pharmaceuticals and PPCPs in Salt Creek. Resin names indicate water samples processed by the associated iTIEs.

Boise, ID

System Modifications and Results

The most pressing mechanical issue in Colorado was addressed during the two-week period before the second WERF expedition. A second water pump was added to each iTIE stand to ensure each chamber received adequate water pressure. Two 4-plex splitters, each with its own pump, directed water to the eight iTIE chambers on each stand (Fig. 9). The pumps were wired in series so only one battery pack was required. Lab tests proved that the battery amp hours were sufficient to run both pumps continuously for 24h.

Flow variation between individual iTIE chambers was still significant. The same slowing, stopping, or high flow increases observed in the previous two field deployments

were once again apparent in Boise. Pressure shifts throughout the system caused some replicates to stop, necessitating another combination of replicate samples. The pressure shifts also lead to drastic increases in flow rate for other treatments, which may have impacted organism survival.

Organism Survival

As in Denver and Schaumberg, organism survival was not significantly affected by exposure to the streamwater, as evidenced by the 100% survival in positive control (No Resin) treatments (Fig. 26), but there is evidence of another stressor. The lower survival in Sep-Pak iTIEs and variation in survival for *D. magna* in the Carbon chambers suggests that resin particle movement does introduce a stressor into the iTIE organism chamber. Sep-Pak and Carbon particles were observed in the organism chambers. Their presence was likely a combination of too fine particles in the resin chamber and high flow surges caused by pressure changes in the system. Activated Carbon is known to be toxic to *H. azteca* after direct exposure in TIE tests [43], so the organism chamber contamination may have created some toxicity.

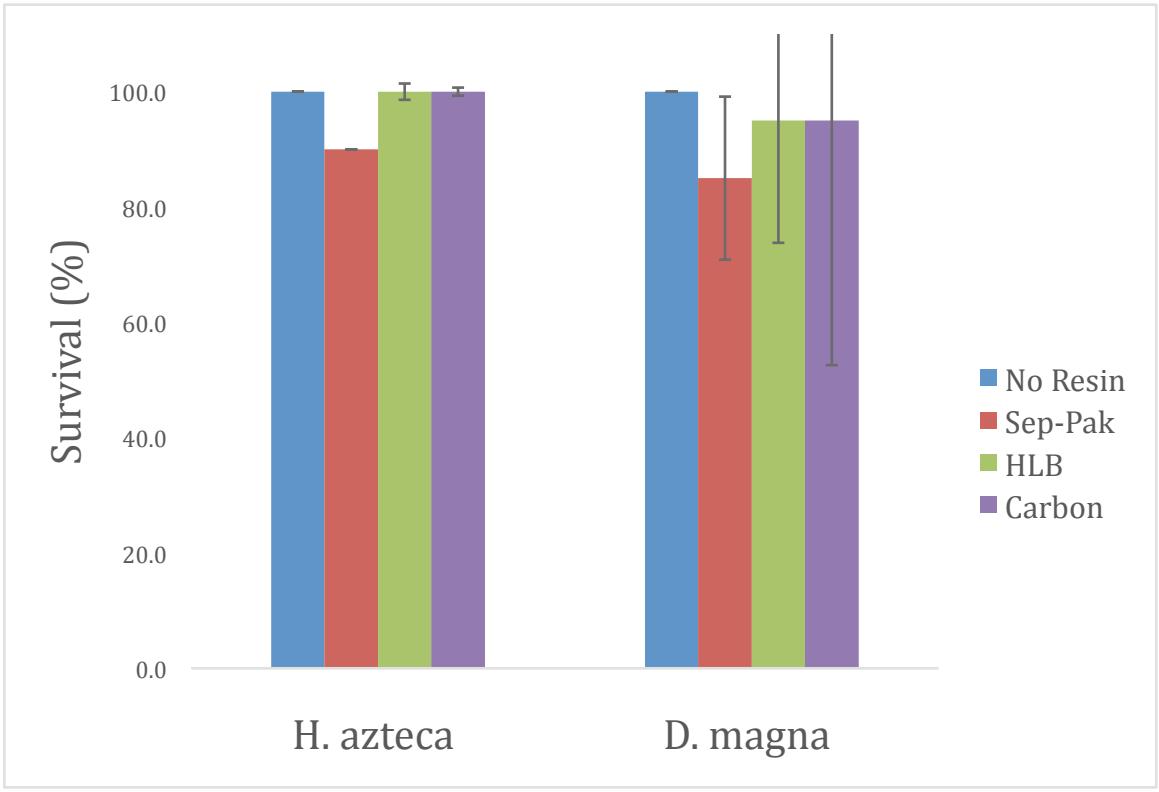

Fig. 26 Organism survival by iTIE treatment (Boise deployment)

Equipment Leaching

Results from the resin tests show higher CEC concentrations in iTIE samples, compared to the field levels. BPA was present in the effluent at 10.2 ng/l, but was as high as 1,118.8 ng/l in the Sep-Pak samples (Fig. 27). The Boise deployment was partially a static non-renewal exposure in the lab, since the iTIEs were submerged in a closed pool. Though the iTIEs system cycled water, the water surrounding the system was not renewed and the iTIE parts essentially soaked in a small volume of water. Leaching of BPA and Nonylphenol compounds from the equipment may have contributed to higher iTIE sample concentrations compared to river samples (Fig. 16, Fig. 27, Fig. 28). Without replenishment of contaminant concentrations in an open, natural system, the leaching contaminants contributed significantly to the overall concentration.

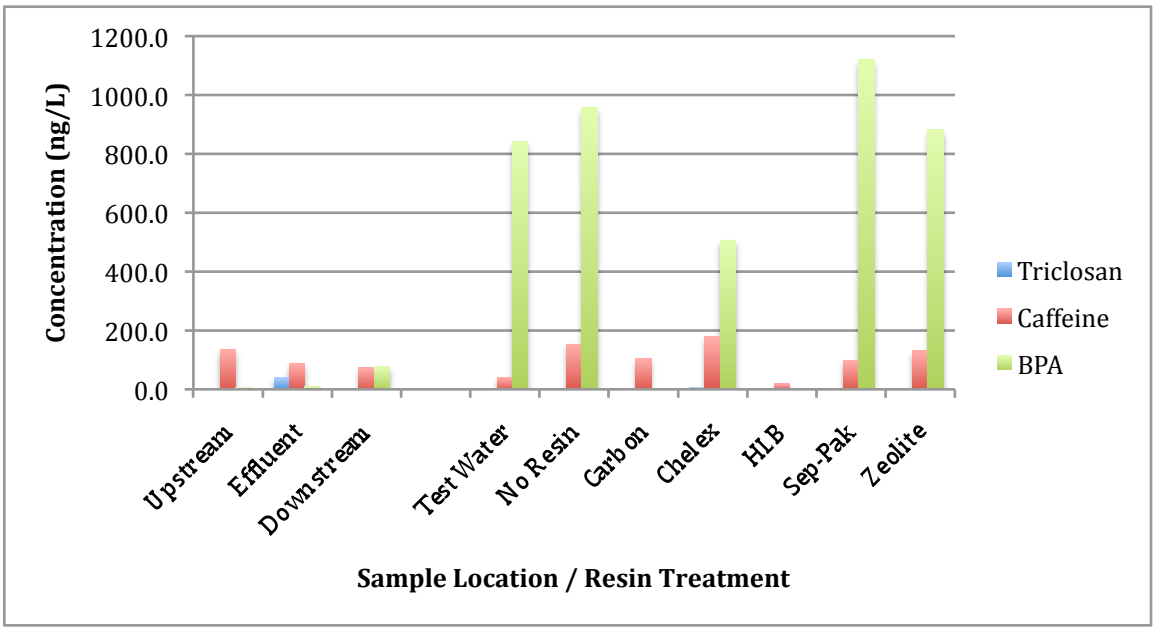

Fig. 27 Concentrations of CECs in the Boise River and laboratory exposure test. Resin names indicate water samples processed by the associated iTIEs, "Test Water" indicates the sample collected from the Boise River for iTIE test.

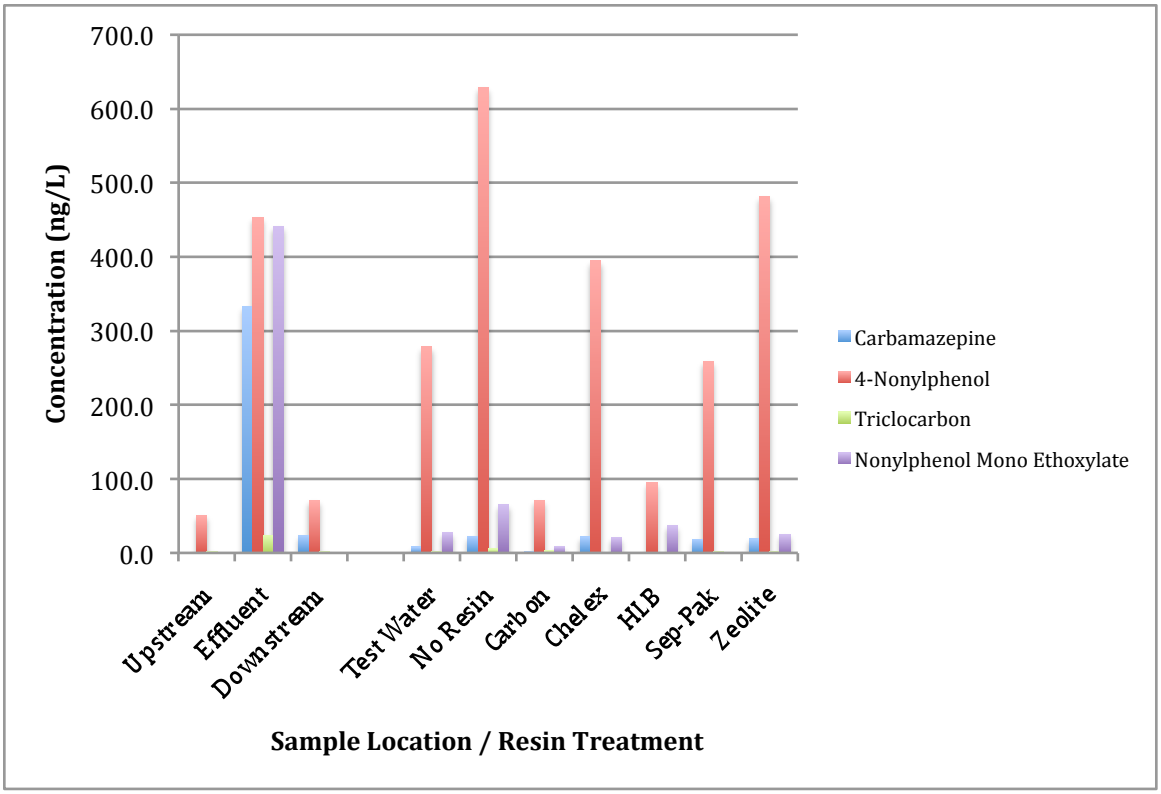

Fig. 28 Concentrations of select PPCPs in the Boise River and laboratory exposure test. Resin names indicate water samples processed by the associated iTIEs, "Test Water" indicates the sample collected from the Boise River for iTIE test.

Natural chemical changes could also explain some of the variation between field and lab compound levels. Nonylphenols, for example, can change form once they are discharged into the environment. These compounds, a broad category of isomeric chemicals typically exist as nonylphenol ethoxylates in pesticides, oil additives, and other similar products [2]. Nonylphenol ethoxylates breakdown into to 4-nonylphenol in aquatic environments [24]. The concentration of the commonly discharged ethoxylate form was higher in the effluent than in the iTIE samples, while the degraded form was higher in the lab samples (Fig. 28). Though lack of replicates makes statistical assessment of this difference difficult, these results may demonstrate one of the major issues with laboratory TIE tests. Chemical degradation and other artifacts can change the concentration and composition of streamwater samples during transport, handling, and manipulation. As a result, the conclusions for laboratory TIEs may not accurately reflect natural conditions.

General Field Results Discussion

The Fall 2014 field expeditions provided a preliminary assessment of the trace compounds present in wastewater effluent and demonstrated the iTIE system's ability to fractionate this complex mixture. Although the system suffered from serious mechanical problems and other inherent design flaws, the data showed promise for the concept.

There were some obvious differences in the ability of different types of resins to target particular compounds. HLB, a resin designed with an affinity for organic compounds in general, was more effective at removing organic compounds than chelex, a resin designed to remove metals (Fig. 22). These expected differences are important for general risk assessment studies to help narrow the field of focus. Narrowing the field even further, though, is the ultimate goal of this system and data from these tests suggests that Phase II TIE fractionation (Table 1) is possible. Activated Carbon and Sep-Pak both target organics in general, but Carbon seems to have a higher affinity for 4-Nonylphnol than Sep-Pak (Fig. 25). The removal of specific compounds during exposure tests will help identify those that pose the greatest risk and use that information to guide treatment protocol.

Identifying risks associated with these compounds will require a different approach than a simple 24h survival test. Compounds that lack acute toxicity, but which cause genetic effects that only manifest over time, will not necessarily be apparent in a general risk assessment. Incorporating a genetic analysis into the iTIE protocol was considered and later employed (Section VI), but fixing the iTIE system design flaws was the first priority.

V. Design of the iTIE System Version III (Winter 2014-15)

5.1 Introduction

The three deployments of Version II provided proof-of-concept support for the revised *in situ* TIE system, but the important parameters for a fully functional model (Table 2) were only partially achieved. The primary goal for designing Version III was to learn from each problem and success encountered during fieldwork, then incorporate the necessary changes into a new model. Version III would have to fully meet all the iTIE system parameters (Table 2) without compromise. The partially unstable deployment stand, non-submersible power system, and imprecise flow control were all compromises made for Version II that had to be targeted and revised.

A field-ready model would not be possible until the basic internal mechanisms functioned reliably. Development of Version III would, therefore, be conducted in two main stages: 1) design and laboratory validation of the basic mechanisms (Sections V and VI) and 2) Incorporation of fully functional iTIE mechanisms into a deployment housing that meets the Table 2 parameters (Section VIII). Stage 1 redesign would target parameters such as flow control and system reliability (Section V). Stage 1 would be complete once the iTIEs effectively and consistently fractionated chemical solutions through continuous resin extraction and organism exposure. The validation of stage 1 design parameters was a series of laboratory tests outlined in Section VI.

5.2 Version III Design Methods

Managing Flow Control

The most obvious flaw with the field-deployed prototype was lack of control over water flow through the iTIEs. Flow rates were difficult to set and frequently changed, sometimes stopping entirely. Flow control valves simply increased resistance to flow for a particular iTIE. With an interconnected system, an increase in resistance at one point will force the water to find an easier path, halting flow to one iTIE while significantly increasing it in another. Flow control should not have been conducted with changes in water pressure, as slight variations will have large responses – the flow of water needed to be controlled at the pump itself. An interconnected system also posed problems for flow control, with one adjustment inevitably affecting another, so a one pump per iTIE chamber approach was explored.

Low power peristaltic pumps allowed each iTIE to have its own pump without creating too large of a power demand. The peristaltic pumps also had the advantage of pumping the sample water through silicone tubing, not the pump itself, lowering the risk of leaching chemicals from the pump parts. These new pumps did not have a flow control built in, but were easily controlled with a circuit modification.

A new circuit was designed to support 6 pumps with one battery and offer flow control options. Peristaltic pumps are controlled by a spinning motor that drives one or more drum heads. The drum heads compress the silicone tubing, drawing fluids through. The speed of fluid movement is dependent on the speed of the drum heads. The pump motor runs on low-voltage DC, so the speed at which is turns can be controlled, with fine adjustment, by raising or lowering the amount of voltage delivered to the pump. A DROK LM2596 Voltage Switching Regulator was incorporated into the pump circuit. Each regulator came with a digital voltage display so pump speed could be set by dialing in a particular voltage, controlling water flow at the source (Fig. 29). Although changes in voltage will reverberate through an electrical circuit, the effects are miniscule compared to the effects of water pressure changes to an interconnected system.

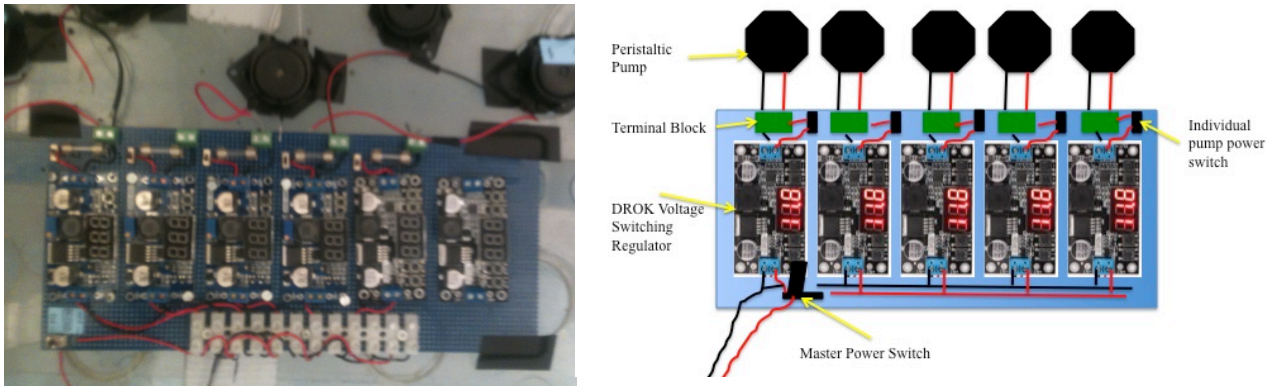

Fig. 29 Field Version III pump control circuitry for precise control of water flow rates through the iTIE chambers. Voltage regulators with digital displays allow for peristaltic pump speed control.

Preventing System Failures

The likelihood of losing multiple replicates also decreases with the multi-pump approach. Fuses incorporated into the circuit board will fail and cut power to one iTIE if its pump malfunctions. This safety measure will prevent a system-wide breakdown that

affects all iTIE treatments. Losing one pump could no longer result in the loss of multiple treatments, as happened in Schaumberg.

Starting with more battery power than necessary was also an essential consideration and difficult problem for this new system. Version III stands had 5 pumps, one for each iTIE, so power consumption was greater. Even at low flow rates, each pump drew roughly 0.25 amps so all the batteries on one circuit would draw 1.25 amps/hr over 24h. Assuming the amperage does not change, a 30 ah battery would be sufficient for 24h. The amperage does vary as the pumps encounter resistance, so at least 40ah of battery power would be needed for a safety margin and to ensure that the battery is not drained completely during the test, which is important for long-term battery life.

Streamlining the Design

Though field deployment parameters such as battery type and housing structure were not directly addressed as part of the first stage of redesign and testing, they had to be considered when selecting pumps and configuring the layout of the electronic circuit and the iTIE water circuit. The Version II Duracell Power Pack limited where the system could be deployed in the river and made the device more conspicuous and vulnerable to vandalism. The power source was changed to a rechargeable lithium battery with a 40-50 ah capacity. Lithium batteries are much more expensive than sealed lead acid batteries, but both can be sealed in a waterproof case and the lithium versions weigh significantly less. The initial purchase cost of a high-capacity lithium battery is offset by the funds saved shipping the light-weight battery to future study sites. The capacity needed was calculated based on the amps drawn by each pump individually and the additive power consumption of all the pumps in the circuit over a 24h period.

Planning began for a housing to give the system stability regardless of the substrate, make for easy deployment, and protect the system from large debris and heavy flow changes, while still maintaining the in situ nature of the device. Before investing in this custom build, however, the newly designed inner mechanisms of the machine had to be tested. The winter re-design of the iTIE system was tested for mechanical feasibility at the University of Nanjing, China (Section VI)

VI. Laboratory Validation Paper: iTIE Version III

This section summarizes the research conducted at the University of Nanjing, China, using the post-field work redesign of the iTIE system. The following is written in publication format as this work will constitute the majority of the journal-submission portion of the overall thesis. Figure numbers are re-started for this section as it is intended to be self-contained.

In Situ Toxicity Identification and Evaluation (iTIE) Water Analysis System:
Part I: Laboratory Validation

Introduction

Current methods for ecological risk assessment in aquatic systems are limited in their ability to replicate realistic conditions. With the rising threat posed by Contaminants of Emerging Concern (CECs), a new method of ecological risk assessment is needed to fractionate chemicals in test site water and eliminate confounding ecological variables, while providing unparalleled accuracy with *in situ* exposures.

Surrogate toxicity assays, including the static non-renewal and static renewal exposure tests the U.S. Environmental Protection Agency (EPA) recommends [5], contain inherent flaws that can affect their accuracy. Throughout sampling, transport, and exposure, study site water may undergo volatilization or degradation of contaminants, compound adsorption to containers, and other changes that could create concentration differences between the exposure water and the actual study site water [3, 5, 10]. During laboratory exposures it is also difficult to maintain natural conditions (D.O., temperature) and account for temporal variation in dissolved organic carbon, inorganic matter and turbidity [3,6,7]. Continuous spatial and temporal variation in contaminant inputs and flows is perhaps the most important factor lost in lab exposures. EPA chronic toxicity test protocol requires three fresh samples over a seven-day test period, but with CECs and other toxicants entering the environment from a variety of sources, each impacted by geologic and atmospheric conditions, the choice of sampling times could drastically affect the composition of the sample [2]. Static renewal tests essentially measure single events, exposing test organisms only to stream conditions at the moment of each sampling [3]. During in situ exposures, test organisms experience natural fluctuations.

While common methods of in situ exposure help increase the accuracy of ecological risk assessments, they also maintain confounding variables that can obfuscate the true cause of observed detrimental effects. In situ deployment of exposure cages is a common method for assessing water quality, but when multiple variables are involved it is difficult to determine what had the greatest impact on test organisms [6,9]. *Daphnia magna* exhibit a clear behavioral response to the presence of deltamethrin, but will also exhibit stress-induced behavior with changes in turbidity [11,12]. Likewise, confounding factors can inhibit the identification of genome disruptors in the water. Disinfectants can

be acutely toxic to *daphnia* within 24 hours, but the appearance of genomic biomarkers in response to an anti-ecdysteroidal fungicide might not occur for 48 hours, so one significant contaminant is identified, while another goes unnoticed [13, 14]. Natural stressors can mask the effects of anthropogenic contaminants and acutely toxic compounds can induce mortality before the effects of chronically toxic compounds become visible. A new approach of *in situ* fractionation is needed for the individual risk assessment of each stressor or toxicant in the environment of concern.

A novel in situ aquatic contaminant fractionation and exposure device was developed for this study. The concept for the machine is based on an EPA developed approach of fractionating toxicants within a water sample and conducting biological exposures with the modified solutions to assess the primary chemicals of concern [28]. The device in this study utilizes a two-chamber filter spike (Fig. 2), conceived by Burton and Nordstrom, which extracts specific compounds from water passing through, using sorptive resins, then directs the modified solution into an organism exposure chamber [1]. The in situ toxicity identification and evaluation (iTIE) chamber was originally designed for sediment pore water analysis. Using the two-chamber concept, a new system was developed to support both sediment and open-water experiments.

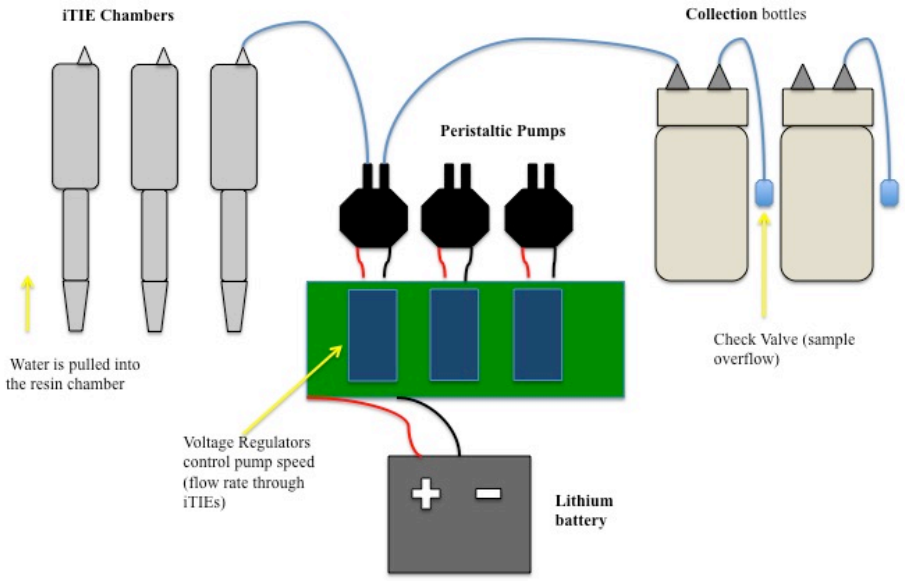

Fig. 1 Overview of the iTIE system design used for chemical fractionation and exposure tests.

The laboratory testing and design stage, described in this paper, determined the feasibility and effectiveness of the new machine's core mechanisms (Fig. 1). An assortment of commercially available sorptive resins, each designed to target a particular family of compounds, were tested in the iTIE device for selective removal capabilities. Solutions containing various metals, organic chemicals, and ammonia were prepared to simulate mixtures that could exist in the environment. Post-filtration water samples

collected by the machine were analyzed to assess the device's ability to collect a chemical slurry, fractionate the mixture, and expose indicator organisms to modified solutions.

Creating a system that could identify threats posed by trace chemicals, especially those with chronic toxicity, was one major goal with the fractionation approach. A chronic 21-day exposures tests followed by reproductive health assessments is a commonly recommended method for identifying chemicals with endocrine disruption properties [18]. Long exposures, however, are difficult to conduct *in situ* and do not support a rapid detection system [16]. Comparing variations in gene expression and the presence of biomarkers in organisms exposed to various treatments within the machine could provide a more sensitive way to identify endocrine disrupting compounds (EDCs) and other contaminants that lack acute toxicity, but which pose long-term threats.

Biomarkers, changes in gene expression, and other genetic responses to EDCs could provide early warning signs of dangerous trace organic chemicals present in the water. This study examined the potential usage of genetic data as part of the ecological risk assessment. Organism exposure tests were conducted using BPA, a known endocrine disruptor, even at concentrations below 1 $\mu g/m^3$ [2,19]. The iTIE device is designed to remove organic chemicals in some treatments and allow them to pass through in others. A comparison of genetic results from the different exposures could demonstrate early indicators of chronic toxicity and offer a faster and more accurate method for detecting sub-lethal effects of toxic trace compounds.

Methods

iTIE System Design

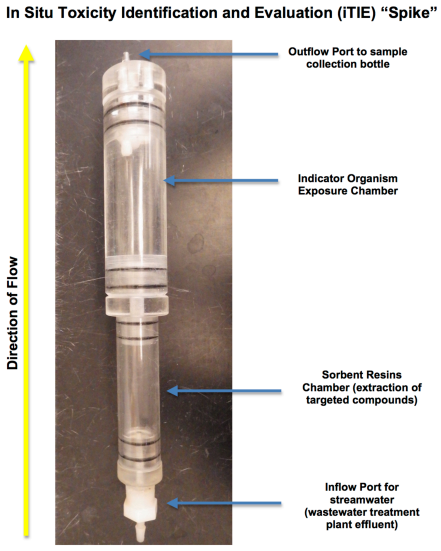

Fig. 2 The dual chamber acrylic iTIE "spike" used for chemical fractionation and subsequent bioassay exposure

The dual chamber spikes, for filtration and organism exposure, were constructed from acrylic, with rubber o-rings to seal the connections between pieces (Fig. 2). To accommodate the laboratory tests, the water intake port was extended with silicone tubing. Tubing was connected at the intake and outflow ports of the iTIE spikes using Nylon 1/8" Hose-to-Threaded Male Pipe adapters for ¼" ID Tubing (McMaster-Carr). The interior outflow port in the organism chamber was covered with 25 nm X-brand mesh.

Water was drawn through the chambers using 12V DC peristaltic dosing pump heads (ZjChao, China). The rotation of each pump head was regulated individually with a custom-made circuit board (Fig. 2). Using DROK LM2596 voltage switching regulators, the pump speed could be tightly controlled by raising or lowering the voltage delivered to each individual pump. The pump circuit was powered with an LS-DL12-40C12V lithium battery (Lishen Energy Company, Shezhen, China).

Samples drawn from each iTIE chamber were pumped into nalgene collection bottles. To address field conditions for when the system is submerged, the collection bottle caps contained both an inflow port (for treated water from the iTIE spike) and an outflow port. In the event that the water sample exceeded the capacity of the collection bottle during the test, overflow could escape through a line of silicone tubing fitted with an aquarium non-return air pump check valve (Petco). This check valve prevents backflow from the environment that would contaminate the filtered sample.

Resins

Commercially available resins used in this study were Zeolite for ammonia, NDA-88 and NDA-150 (Nanjing University Environmental Protection Company) for organic compounds, TP-207 (Bayer Company) and Chelex (Solarbio) for metals, and Activated Carbon, which has commonly been used for organics extraction but has an affinity for other types of compounds, including metals.

Calibration and Blank Run

The intake tubing for each iTIE chamber was submerged in a bucket of MilliQ for the pump rate calibration test. Flow rate was measured by filling the collection bottles with MilliQ and collecting outflow from the check valve in a graduated cylinder for one minute. Ideal flow rate was identified for each treatment by finding the lowest voltage setting at which the pump could still operate, slowing the pump and subsequent flow rate as much as possible. Some resins offered more resistance than others, so voltage was increased as needed to insure similar flow rates for each treatment. Due to the nature of the pumps, air pockets in the resin chambers, and similar factors, flow rate would vary from one minute to the next. Due to this common variation, an acceptable flow rate range was established: 5 to 9 ml/min.

Resins selected for the calibration and subsequent chemical test (Resin Test I) were zeolite, NDA-88, NDA-150, TP-207, and Activated Carbon, which would theoretically act as a negative control by targeting all types of compounds. Air was purged from interstitial space and pores in the resins with a 2h a MilliQ soak. Immediately prior to adding the resin into the iTIE chamber, excess water and fine dust

particles were drained off. Five grams of each resin were added to their respective chambers in triplicate. Two iTIE chambers contained no resin as a positive control (some iTIE chambers had damaged seals so only 17 were available for testing, hence the lack of a third "No Resin" replicate). Every iTIE chamber contained glass wool above and below the resin to prevent movement and ensure tight surface area and volume coverage. A vent filter (WEB) was placed at the top of the resin chamber to prevent movement of resin particles into the organism chamber.

Water was pumped through the system for two hours, after which 10ml water samples were taken from the collection bottles. These samples would be analyzed for the test compounds to establish a baseline concentration, if any, in the water or leaching from any of the equipment.

For all tests in this study, the pH and temperature of each water sample was measured at the end of the experiment. Flow rate measurements were taken for every treatment, throughout the test. Dissolved oxygen measurements were taken in the organism chamber following the daphnia exposure test.

Resin Effectiveness Test

A 21L spiked solution was used as the source water during Resin Test I. The volume of solution was determined based on the combined flow rates of the 17 iTIE chambers and the length of the experiment (2h). Cadmium (Alfa Aesar), cupric chloride (Nanjing Chemical Reagent Co., LTD), lead nitrate (Nanjing Chemical Reagent Co., LTD), and zinc sulfate heptahydrate (Sigma-Aldrich) were added to 21L of MilliQ in 2ppm concentrations. The water was also spiked to 2ppm for BPA (Aldritch), Atrazine (AccuStandard), Pyrene (AccuStandard), and ammonium chloride (Sigma).

The 17 TIE chambers processed the water for two hours. Flow rate was recorded using the outflow from the check valves. Due to variation between the pumps, some voltages were adjusted during the exposure to achieve minimal variation between individual treatment flow rates. Final samples from this test were collected from the organism chambers to represent the most recent flow rate.

A second resin test (Resin Test II) was conducted using three replicates each of Activated Carbon, Chelex (Solarbio) and NDA-150. The source water contained the same compounds as the first resins test, except ammonia, in 2 ppm concentrations.

A 15ml water sample was collected from each iTIE treatment replicate following 2h of constant filtration for both resin tests. The samples were stored in at 4°C for later analysis.

Test Organism

Daphnia magna have previously been used in TIE experiments looking for chemical identification based on gene expression factors [53]. The organisms were cultured in the lab for several years, fed daily with green alga, and kept at 24 ± 0.5°C with a light/dark cycle of 16h:8h [52].

Organisms selected for EDC Exposure Test I were 7-day-old juveniles. Those in EDC Exposure Test II were 14-day-old adults. During the organism tests, nutrient solutions of $CaCl_2$, $MgSO_4$, $NaHCO_3$, and KCl were added to the spiked source water.

EDC Exposure Test I and II

Source water for this test was spiked to 2ppm BPA. The iTIE chambers were pre-filled with culture water so the organisms could be added. The pumps for all treatments were run continuously for 12h, when they were stopped so that water samples and organisms could be collected. Three daphnia were taken from each treatment for RNA extraction. Three individuals were the minimum number needed to extract sufficient mRNA for sequencing. At the 12h mark and 24h, a maximum of three individuals were removed. If fewer than 3 remained alive at either time, the maximum possible number were collected and processed in case sufficient mRNA could be extracted. One 15ml water sample was collected from each replicate before the pumps were restarted. After an additional 12h, organism and water samples were again collected.

Sample Analysis

Samples were analyzed for the presence of BPA, atrazine, and pyrene using High Performance Liquid Chromatography (Waters 2414 Refractive Index Detector). Inductively coupled plasma mass spectrometry with a NEX10N300X (Perkin Elmer) was utilized to measure the concentration of metal ions in the iTIE-filtered samples.

Immediately following the 12h and 24h daphnia collections in the exposure test, RNA was extracted and converted to cDNA for long-term storage according to protocol devised for the Total RNA Extraxction with TRIZOL Reagent and Purification with QIAGEN RNeasy Mini Kit (© DGC, Indiana University, 2007). The quality of the RNA was assessed with a Synergy H4 hybrid reader (BioTek). The cDNA genes were sequenced using an Ion Torrent Proton Semiconductor Sequencer (Life Technologies).

Daphnia magna genes previously shown to exhibit EDC-related responses were searched for among the top 60 genes found in the sequencing [53]. Changes in gene expression were determined based on fold change in expression relative to the iTIE treatment with the lowest BPA concentration.

Data Analysis

An Analysis of Variance (ANOVA) test was used to analyze differences in concentration means between various treatments, with significance defined as $p<0.01$. If significant variation among and between groups was determined, a post-hoc Tukey test was used for multiple comparisons to identify significant differences between specific groups.

Results

HPLC analysis of the MilliQ sample water collected by the iTIEs during the calibration run yielded no peaks for the organic chemicals used in subsequent spike tests. No statistical tests were run on these data, as all treatments yielded concentrations below the detection limit.

Resins Test I: Flow Rate and pH

Mean flow rate during the 2h test did not differ significantly between treatments (ANOVA, F=3.695, p-0.0178). Zeolite treatments have the slowest flows while TP-207 had the fastest (Table 1).

Table 1. Mean flow rates through iTIEs by resin treatment	
Resin	Mean Flow Rate (ml/min)
Carbon	4.65 ± 3.68
NDA-150	6.12 ± 2.42
NDA-88	7.72 ± 4.01
No Resin	8.48 ± 3.05
TP-207	9.45 ± 1.22
Zeolite	3.93 ± 4.50

The pH varied between samples collected from different iTIE chambers. The average pH in NDA resin-treated water samples was lower, compared to the No Resin control, while the other resin treatment samples had higher pH (Table 2).

Table 2. Mean pH of sample water processed by the iTIE system	
Resin	pH of sample water
Carbon	7.98 ± 0.13
NDA-150	5.24 ± 0.53
NDA-88	4.03 ± 0.21
No Resin	6.03 ± 0.01
TP-207	10.10 ± 0.19
Zeolite	7.06 ± 0.65

Resins Test I: Metals Extraction

Only 17 iTIEs were functioning during Resins Test I so only two No Resin treatment samples were available. In the following statistical analyses for this Resins Test I, chemistry data for the spiked source water was used as a third No Resin replicate, as it was untreated like the iTIE control samples.

Following the 2h resin effectiveness test, there was a significant difference in the concentration of metals between iTIE treated samples (Fig. 3). The resin present in the iTIE chamber significantly affected the concentration of metals in the sample collected

(ANOVA, F=6.183, p<0.01). The lowest metal concentrations were detected in water samples processed by the TP-207 iTIEs. While the highest concentrations were observed in water passing through chambers without a resin (Fig. 3). There was a significant difference between the No Resin and Tp-207 groups (p<0.01). The mean concentration of copper, for example, in TP-207 treatments (15.12 ± 6.34 ppb) was 99.3% lower than in the samples processed by the No Resin iTIES (2184.76 ± 101.56 ppb). Zeolite treatments were also significantly different from the No Resin groups (p<0.01), with Cd, Cu, Pb, and Zn levels 78.0%, 90.1%, 99.6%, and 75.5% lower, respectively, than mean concentrations in the No Resin group. The concentrations of metals in the Zeolite groups were not significantly different from those in the TP-207 samples (p=0.51).

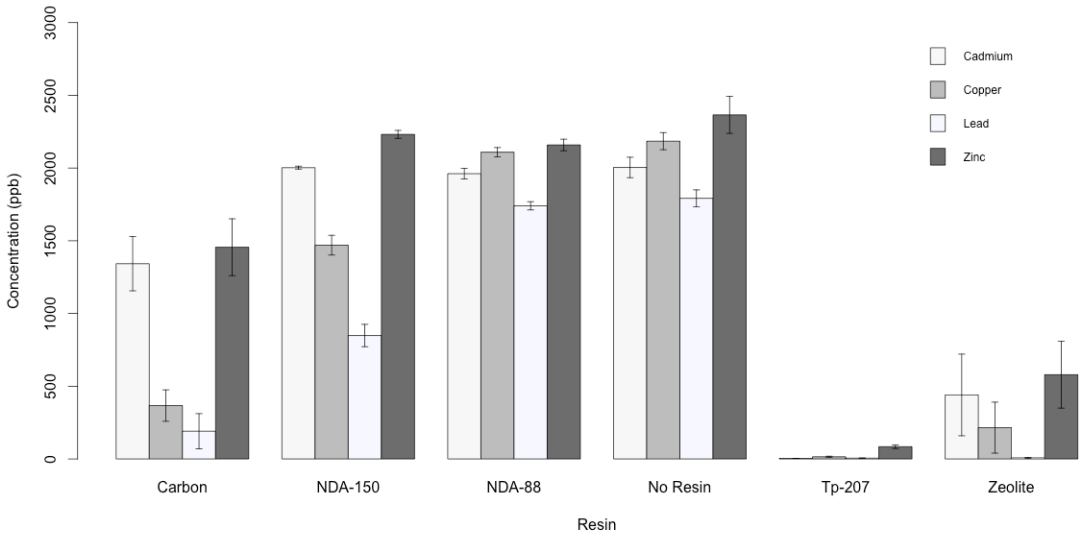

Fig. 3 Metal concentrations in water samples processed by various iTIE resin treatments. "No Resin" is the positive control

No metals in the NDA-88 treated samples were present in significantly different concentrations than those in the No Resin group (p=0.99). NDA-150 group concentrations of zinc were not different from the No Resin treatment (p=0.99) and levels of cadmium were also similar (p=0.99). Lead levels were significantly different (p<0.01) in the NDA-150 treatment, with a 52.6% lower average concentration than lead in No Resin treatments. The Carbon treatments showed a significant difference in the concentrations of copper (p<0.01) lead (p<0.01), and zinc (p<0.01) when compared to the No Resin samples, but cadmium concentrations did not differ significantly between the two groups (p=0.02).

Resins Test I: Organic Chemical Extraction

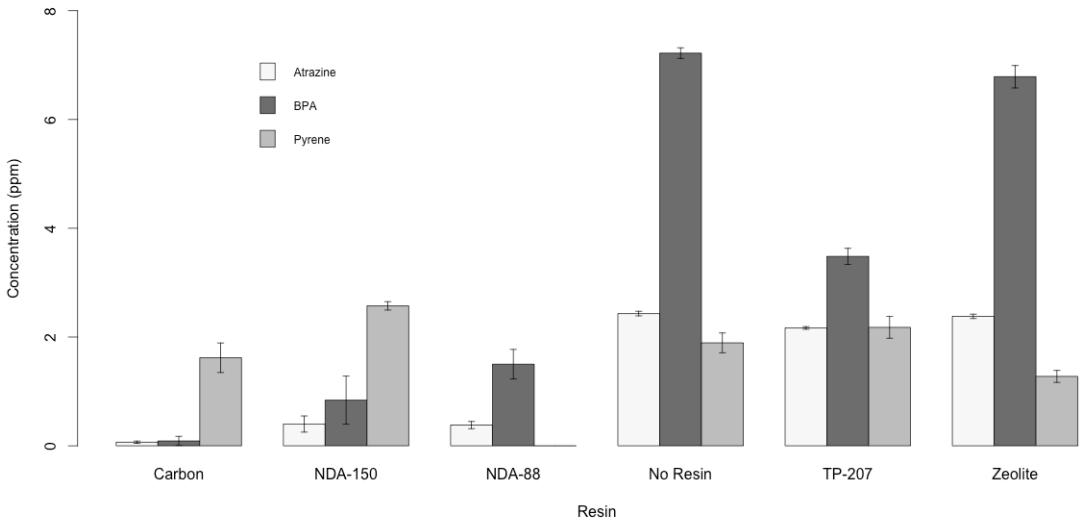

Fig. 4 – Organic compound extraction by iTIE treatment. No Resin is a positive control.

Water samples collected after the 2h resins test showed significant variation in the concentration of organic chemicals based on the resin used in the iTIE chamber (ANOVA, $F=52.41$, $p<0.01$). There was no significant difference in the concentrations of organic chemicals between the No Resin and TP-207 treatments samples (Fig. 4) Concentrations were also not significantly different between the zeolite and No Resin samples for all chemicals.

Carbon iTIE filtration resulted in significantly different concentrations of Atrazine ($p<0.01$) and BPA ($p<0.01$), compared to iTIEs with no resin, but the concentration of Pyrene between these two groups did not differ significantly ($p=0.99$). The mean BPA concentration in Carbon-treated water (0.13 ± 0.17 ppm) was 98.2% lower than in the No Resin samples, while the mean Atrazine concentration was 96.4% lower at 0.086 ± 0.013 ppm.

Comparing NDA-150 to No Resin, Atrazine and BPA levels were significantly different ($p<0.01$), but Pyrene concentrations were not ($p=0.27$). The NDA-150 sample levels of Atrazine and BPA were on average 78.0% and 82.7% lower.

Water collected from iTIEs containing NDA-88 had significantly different levels for all three contaminants compared to No Resin. Atrazine, BPA, and Pyrene concentrations were 81.6%, 75.8%, and 100% lower than in the unfiltered No Resin samples.

Resins Test I: Ammonia Extraction

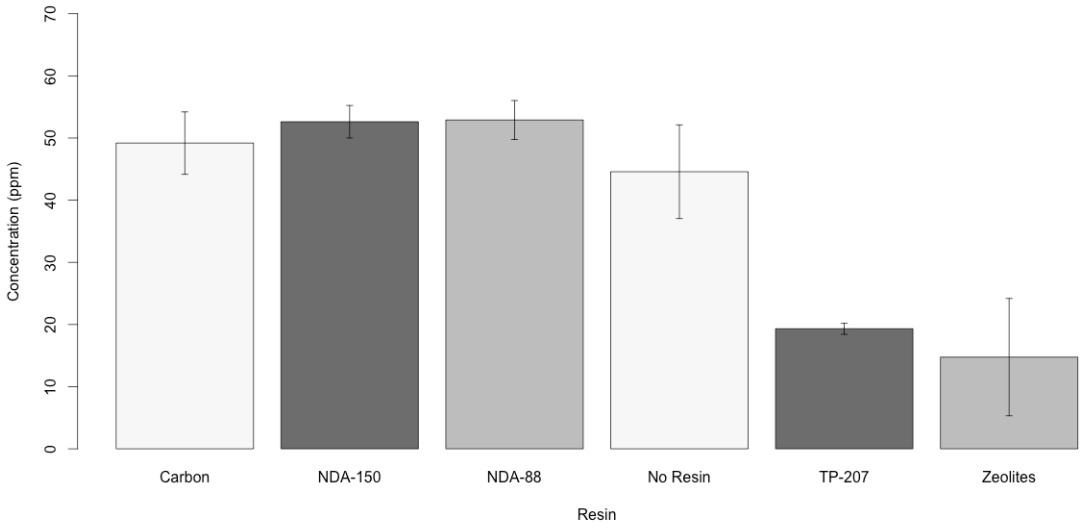

Fig. 5 – Ammonia extraction by iTIE treatment. No Resin is a positive control.

The concentration of ammonia in iTIE-processed water (Fig. 5) differed significantly based on the resin in the chamber (ANOVA, $F=9.478$, $p<0.01$). TP-207 and Zeolite treatments were significantly different from the Carbon and NDA treatments ($p<0.01$). Ammonia concentration in TP-207 iTIE water samples was not significantly different from the No Resin samples ($p=0.06$). Zeolite sample concentrations also did not differ from No Resin ($p=0.02$).

Resins Test II: Flow Rate and pH

Flow rate differed significantly between iTIE treatments during the second resins test (ANOVA, $F=7.624$, $p<0.01$). A post-hoc Tukey test showed so significant difference between the NDA-105 and Carbon iTIE flow rates ($p=0.97$). There was a significant difference between the Chelex and Carbon flow rates ($p<0.01$) but not between the Chelex and NDA-150 rates ($p=0.013$). The average water flow rate through the Chelex iTIEs was lower than the other two treatments (Table 3).

Table 3. Mean flow rates through iTIEs by resin treatment

Resin	Mean Flow Rate (ml/min)
Carbon	8.32 ± 0.95
Chelex	6.52 ± 0.53
NDA-150	8.20 ± 1.10

The pH of samples collected from the Chelex treatments were higher on average than the other two treatments. The pH for all samples collected during this test was basic (Table 4).

Table 4. Mean pH of sample water processed by the iTIE system (Resin Test II)	
Resin	pH of sample water
Carbon	9.50 ± 0.07
Chelex	10.49 ± 0.14
NDA-150	8.39 ± 0.62

Resins Test II: Organic Chemical Extraction

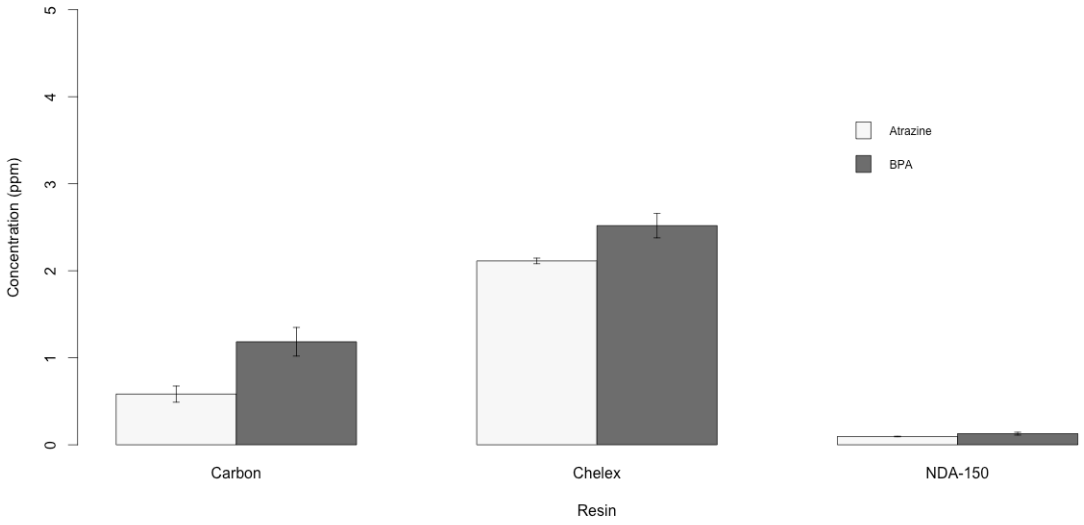

Fig. 6 – Organics extraction by iTIE treatment.

The concentration of Atrazine and BPA (Fig. 6) was affected significantly by the resin present in the iTIE Chamber (ANOVA, $F=266.008$, $p<0.01$). Atrazine concentrations were significantly different between Chelex and Carbon iTIE sample water ($p<0.01$) but not between the NDA-150 and Carbon treatments ($p=0.079$). BPA concentrations differed significantly between all treatments ($p<0.01$).

EDC Exposure Test I: pH, Dissolved Oxygen, and Organism Survival

Table 5. Mean pH of sample water and Dissolve Oxygen in organism chamber (EDC Exposure Test I)		
Resin	pH of sample water	D.O. in Organism Chamber (mg/L)
Carbon	5.9 ± 1.16	8.0 ± 0.04

NDA-88	7.4 ± 0.22	8.0 ± 0.26
NDA-150	7.9 ± 0.25	7.9 ± 0.32
No Resin	7.5 ± 0.20	8.1 ± 0.12

The pH of the solutions collected by the iTIE system ranged from 5.9 for Carbon treatments to 7.9 for the No Resin control treatments. Dissolved oxygen measured in the iTIE organism chambers was between 7.9 and 8.1 mg/l (Table 5).

Table 6. Mean *Daphnia magna juvenile survival after 24h*	
Resin	Percent Average Survival
Carbon	6
NDA-88	0.67
NDA-150	0.33
No Resin	0.66

Organism survival was less than 1% for the NDA resins and the No Resin treatment. In the Carbon treatments, there was an average of 6% survival for *D. magna*. Due to high organism mortality, mRNA extraction did not occur.

EDC Exposure Test II: BPA Removal

The resin, or lack of resin, used in the iTIE chamber significantly impacted the concentration of BPA in the water before it entered the organism exposure chamber (ANOVA, $F=21.48$, $p<0.01$). The mean BPA concentration for the No Resin treatments was 4.45 ± 0.28 ppm at 12 hr and 3.09 ± 2.55 ppm (Fig. 7). The concentration of BPA in these treatments did not change significantly from 12 to 24hr (t-test, $df=4$, $p=0.41$).

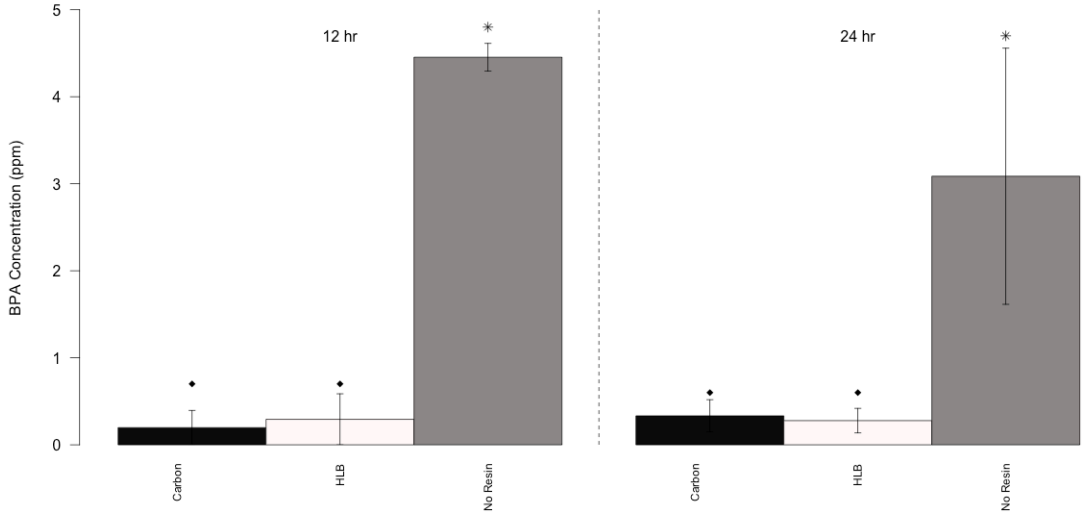

Fig. 7 – BPA chemical concentrations in water samples processed by various iTIE resin treatments. "No Resin" is the positive control.

At 12hr, the water filtered by Carbon iTIEs contained a mean concentration of 0.19 ± 0.34 ppm BPA,, which differed significantly from the No Resin treatments ($p<0.01$). The mean concentration at 24h (0.33 ± 0.32 ppm) was also significantly different from the untreated water samples ($p<0.01$). BPA levels in HLB-treated samples were significantly lower than the No Resin treatments at 12h ($p<0.01$) and at 24h ($p<0.01$). At both times, the BPA concentrations for Carbon and HLB treatments were not different from each other ($p=0.99$ at 12h and $p=0.99$ at 24h).

BPA levels in the Carbon and HLB treatments were 95.6% and 93.4% lower, respectively, than chemical levels in the No Resin chambers at 12h. After 24h, levels in the two resin chambers were 89.2% and 91.0% lower than the chambers with no resin, on average.

EDC Exposure Test II: Flow Rate, pH, and Organism Survival

The mean flow rate did not differ between iTIE treatments (ANOVA, $F=1.091$, $p=0.352$). Water flow through the iTIEs ranged from 7-11 ml/hr (Table 7).

Table 7. Mean flow rates through iTIEs by resin treatment (EDC Exposure Test)

Resin	Flow Rate (ml/min)
No Resin	8.63 ± 2.34
Carbon	8.44 ± 1.78
HLB	7.94 ± 1.75

The pH of water samples collected by the iTIE system ranged from 7.91 to 8.72 (Table 8). The variation was not significant.

Table 8. Mean pH of sample water processed by the iTIEs (EDC Exposure Test)

Resin	pH of sample water
No Resin	7.87 ± 0.12
Carbon	8.06 ± 0.07
HLB	7.98 ± 0.06

Organism survival over the 24h BPA exposure test did not vary significantly between treatments (ANOVA, $F=2.34$, $p=0.18$). Some mortality occurred (Appendix 12).

EDC Exposure Test: Gene Expression

Variations in gene expression at 12h and 24h are expressed through fold change comparisons with the Carbon treatment (designated as 1-fold). At 12hr, *daphnia* in the No Resin treatments showed a mean 1.82 fold increase for the DMEDC1 (C1) gene (Fig. 8). This gene, which codes for the alpha subunit of putative Na^+/K^+ ATPase, was expressed 0.85 fold, on average, among *daphnia* in HLB-treated water.

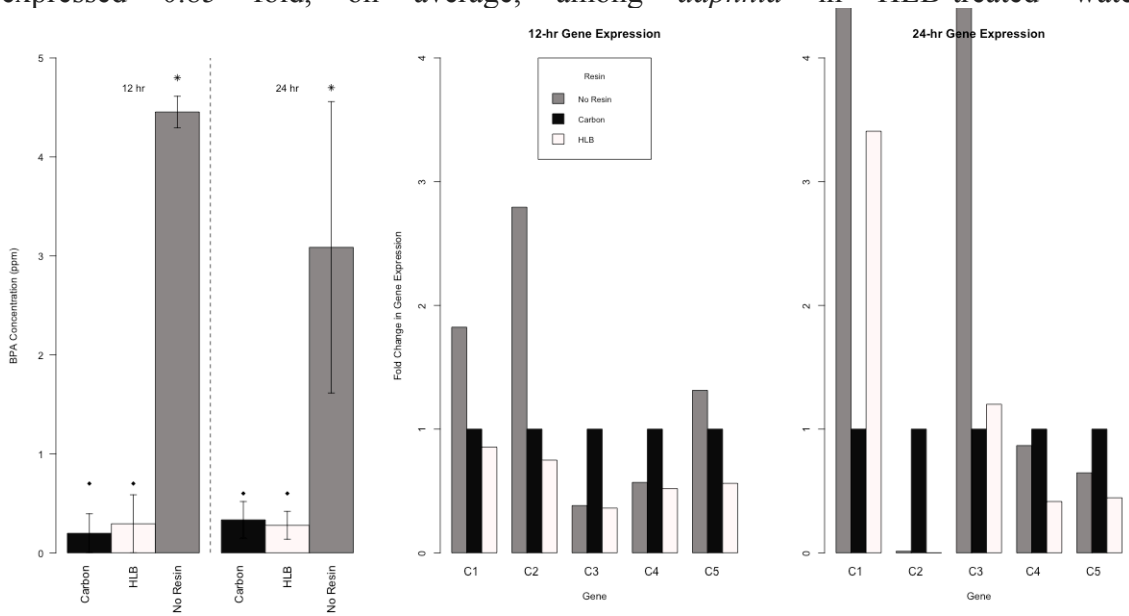

Fig. 8 – (Left) BPA concentrations in the different iTIE treatments. (Right) Genome expression frequencies for genes C1-C5 at 12h and 24h.

Expression of this same gene increased to 23.11 fold at 24h for the No Resin organism groups. HLB organism groups also increased expression of the C1 gene, with a 2.79 fold increase, compared to Carbon treatments.

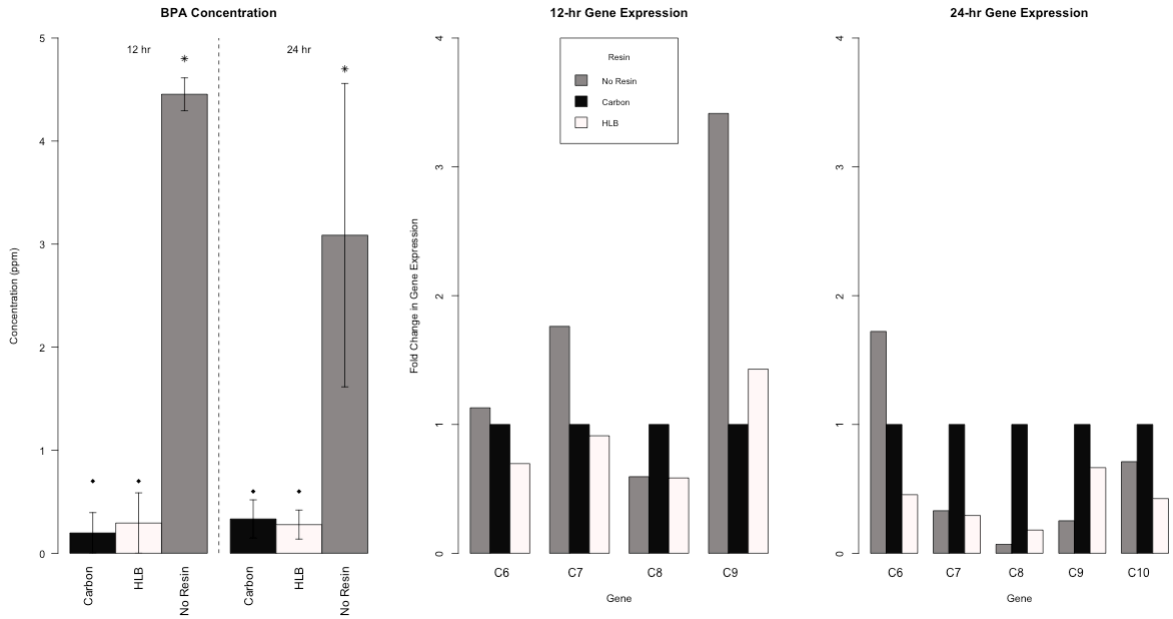

Fig. 9 – (Left) BPA concentrations in the different iTIE treatments.
(Right) Genome expression frequencies for genes C6-10 at 12h and 24h

The DMEDC5 (C5) gene, which codes for tRNA pseudouridine synthase, was expressed 1.31 fold in No Resin treatments at 12h. The same gene had a 0.56 fold decrease at 12h for organisms in HLB-treated water. At 24h, expression of C5 genes decreased to 0.65 and 0.44 fold for No Resin and HLB treatments, respectively.

The remaining genes observed (C2, C3, and C6-C9) are hypothetical proteins. One of these hypothetical proteins, C3, was expressed 4.87 times more in No Resin organisms than those in the Carbon treatments at 24h, while expression of C3 did not change significantly for HLB treatment *daphnia* over the testing period. The expressions of C7 and C9 genes were significantly different for the No Resin control compared to the other two treatments (Fig. 9).

Discussion

This laboratory test of the novel In Situ Toxicity Identification and Evaluation Risk Assessment Device has demonstrated that a diverse solution of compounds can be fractionated to facilitate targeted toxicity assessment studies. Resins succeeded in extracting broad families of contaminants as intended, while also showing specificity for particular compounds. Targeted removal in organism exposures combined with genomic analysis shows a more sensitive method is possible for identifying toxicants that other screening tools might miss, but which pose a significant long-term threat to the ecosystem and human health.

Concentrations of toxicants used for this study are magnitudes greater than those observed at most contaminated sites, but the significant reductions observed over the relatively short test period suggest that the iTIE filter chambers will be even more effective *in situ*. The effectiveness of the iTIE device in an aquatic system will depend on its ability to draw source water through the filter chambers continuously and the capacity of the resins for highly selective compound extraction.

The commercially produced resins selected for this study vary in their selectivity, which was apparent during the 2h resin test. The No Resin treatments acted as a positive control, allowing spike water to pass through the iTIEs completely unfiltered. Though ammonia was reduced in the zeolite and chelex treatments, the change was not significant compared to the No Resin control. Since the organic resin treatments showed ammonia levels in their samples that were significantly higher than the zeolite and chelex samples, contamination may have been an issue. Results from the organics and metals resins were more successful.

Treatments using NDA-88, a resin designed to target organic chemicals, show no significant difference in the concentration of metals compared to the No Resin iTIEs. Chambers with TP-207, however, successfully extracted 96-99.7% of metals from the spike water. During the same test, the concentrations of atrazine, BPA, and pyrene were reduced by 75-100% in the NDA-88 iTIEs, while these compounds easily passed through the TP-207 resin. Two distinct solutions were created from the same source water and continuously replenished in the exposure chamber. The use of these two resins during a stream deployment could provide a broad toxicity assessment for metals and organic

compounds. In comparing resins with similar design functions, the possibility of more specific exposure tests is apparent.

Several resins showed unique affinities for particular compounds. Carbon, for example, did not significantly reduce cadmium concentrations in the sample, but decreased copper and lead levels by 83.2% and 89.4%, respectively. The NDA-150 treatments were even more specific in their affinity, targeting lead for removal and leaving the other metals relatively untouched. Likewise, carbon was able to remove nearly all traces of BPA and atrazine from the source water, but did not alter pyrene levels. NDA-88 was able to lower pyrene concentrations below detectable limits. Understanding the characteristics of each resin will allow researchers to design very specific, self-regulating exposure tests in ecosystems of concern. By removing a different compound in each treatment, the system isolates each potential source of toxicity for individual organism risk assessment.

Some resins may have affected organism survival. The sample water collected during Resins Test I exhibited low pH for the NDA Resins. NDA-150 and NDA-88 processed water had pH values of 5.24 and 4.03, respectively (Table 2). When these resins were used during Exposure Test I, juvenile *D. magna* percent survival was below 1% (Table 8). These are ion exchange resins so it is possible that as various compound are adsorbed to the resin particles, H^+ ions are stripped off and released into the water, lowering pH. This acidic water could certainly contribute to organism mortality during bioassays, so these resins may not be ideal for future tests. The pH of treatment samples for these resins was relatively neutral, however, during Exposure Test I (Table 5). Since only BPA was in the source water, perhaps fewer H^+ ions were stripped off as fewer ion exchanges occurred, compared to the Resins Test, which had multiple compounds in the source water. Further testing is needed to determine if and how the NDA ion-exchange resins alter pH before they are used in organism toxicity assessments.

Negative organism responses to acute toxicity are well documented so the exposure tests in this study aimed to identify a more sensitive analysis approach for trace compounds with less apparent short-term effects. The EDC exposure test utilized a known endocrine disruptor in order to demonstrate the iTIE system's ability to prevent genetic defects in one treatment, through selective extraction, while allowing them to occur in another. In a river ecosystem, where the exact nature and concentration of compounds in the water is unknown, a comparison of organism gene expression between similar resin treatments could alert researchers to the presence of harmful organic compounds in the environment of concern and identify specific compounds if the proper biomarkers are known [53].

Observing differences in growth and reproduction is a common method for identifying genetic disruption [15], but this approach necessitates a longer experimental time, which makes *in situ* approaches difficult. The severe developmental, neurological, and reproductive effects associated with endocrine disruption can take weeks or months to manifest, but early signs of organism responses to these compounds can be identified with genetic biomarkers [16]. Since the current genomic database for *D. magna* is limited, it is not clear whether the genes identified in the BPA exposure tests are linked to BPA-derived endocrine disruption. BPA is an estrogenic compound that can cause severe hormone disruption in human infants and cause variable gene expression in *Daphnia magna* [2,53]. The significantly different fold changes in gene expression observed in the

iTIE exposure organisms may be an important indicator of similar effects. There was a significant increase in expression for several genes in the No Resin treatments, compared to limited change in the HLB treatments. This variation is especially apparent for the C1 and C3 genes at both the 12h and 24h sampling times. Since organisms in the No Resin treatments were exposed to significantly higher BPA concentrations, the change in gene expression may be a response mechanism.

Identifying and understanding more subtle response mechanisms will be essential as research continues on contaminants of emerging concern. The slurry of untreated chemicals discharged from wastewater treatment plants and other point sources may contain hundreds of unknown compounds. How these compounds react under varying conditions, such as turbidity, DOC and pH, is largely unknown. Assessing the threat these compound pose is further complicated by their low concentration and potential lack of acute toxicity. With so many environmental stressors potentially masking the subtle effects of trace compounds, a new method of risk assessment is needed.

This preliminary laboratory test of the iTIE device supports the in situ fractionation and exposure concept for ecological risk assessment. The success of the core components of the machine provide strong evidence for the feasibility of a self-contained, submersible version that could conduct in situ risk assessments with unparalleled accuracy. The earlier version of the iTIE concept showed promise in pore water field experiments, but a sturdier and more versatile machine is needed [17]. The field validation of this method and the final in situ TIE system design is presented in part II.

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VII. Continuing Development

7.1 iTIE System Version III

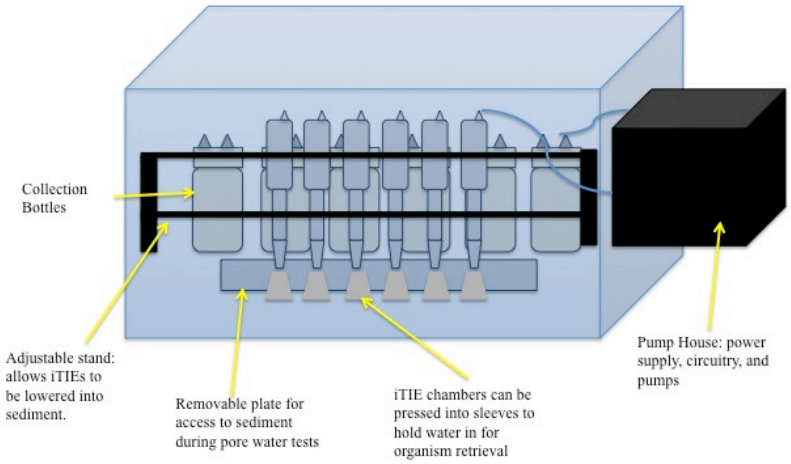


Fig. 30 Proposed Version III housing. Screened openings on the front and back broad sides (not pictured) allow for natural stream flow

With the successful laboratory test of the new internal mechanism design, construction of a final field version began (Fig. 30). The main testing components (iTIE chambers, collection bottles) will be housed in a clear acrylic case that protects the components from large debris, while stabilizing the system on any substrate with adjustable spikes. Screen on all sides of the case will allow streamwater to flow through naturally, maintaining the *in situ* nature of the device.

The new iTIE system will not be limited to open water tests. A removable base on the housing and adjustable iTIE holders will allow the chambers to be lowered into the sediment for pore water analysis. To simplify organism retrieval after any exposure test, the iTIE inflow ports can be pressed into watertight sheaths fixed to the base of the housing. These sheaths will keep water in the organism chamber as the system is removed from the river.

The battery, pumps and the flow control circuit are sealed in a waterproof case attached to the side of the main housing. Hose barb ports connecting iTIE tubing to the peristaltic pump tubing on the inside allow water to pass through the pumps while keeping the interior of the power box dry. A new printed circuit board was constructed, based on the prototype configuration. The board is smaller and eliminates the need for loose wires, which streamlines the design and reduces the risk of short-circuiting, burnouts, and fires (Fig. 31).

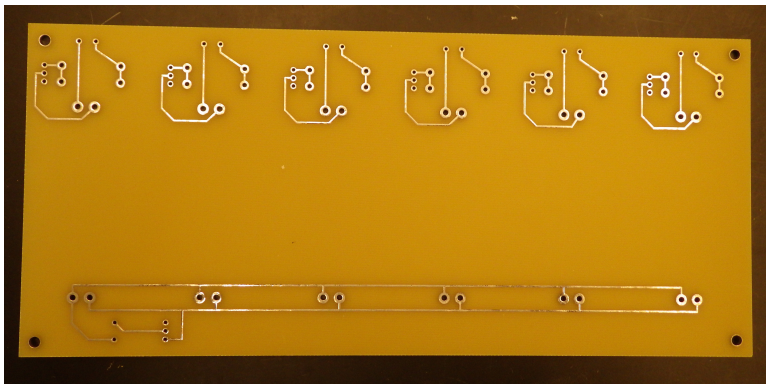


Fig. 31 Printed circuit board without circuitry components. This board, its components, and the battery constitute the flow control mechanisms for iTIE Version III. Pump power and speed is controlled with power switches and voltage regulators (not pictured).

The new field prototype is easy to deploy, protected from damage, and entirely self-contained so that it can be placed in almost any aquatic environment for Phase I and potentially Phase II TIE experiments.

7.2 Future Tests

Resin Effectiveness and Specificity

Both the 2014 field deployments and the 2015 lab tests showed that similar resins can have unique affinities for particular compounds in one family of contaminates. Though two resins may be designed to remove organic compounds, one may reduce BPA concentrations by 100%, while another fails to target any BPA molecules. Fractionating these variables as much as possible will be the key to assessing risks they may pose and subsequently guiding water treatment protocol. Determining how best to target specific compounds is an essential step and unintentional resin specificity may help conduct *in situ* TIE Phase II exposures by fractionating classes into individual compounds.

Direct targeting of compounds is also possible in some instances and could be adapted for iTIE use. Methods exist for Phase II fractionation of pesticides and pharmaceuticals [35]. Compounds in these families are often chemically designed by companies to target specific enzymes or binding sites. By introducing specific enzymes into exposure tests, they can act like resins, binding to the target compound [37]. Easily manipulated cells (e.g. yeast) can be engineered with specific binding sites with a high affinity for a particular organic compound [37]. The application of these methods will be limited, however, to chemicals designed with known affinities.

Adapting iTIE for Sediment Pore Water

The 2004 Burton/Nordstrom iTIE was designed for pore water analysis and further development of Version III could make that possible for this new system. It will have to be established whether the current system's pump speed is slow enough to prevent sediment particle intake and extract pore water only, not overlying water. Since

pore water is often low in dissolved oxygen, a mechanism may have to be added to the iTIE organism chamber to oxygenate the water.

Sediment tests with the iTIE system may risk inaccurate toxicity assessments. Some studies have show that organism toxicity is much higher when exposed to extracted interstitial water than during exposures to the unaltered sediment [42]. The sediment can bind to many of these compounds, reducing their bioavailability to organisms. If the iTIEs were to remove that sediment variable by conducting bioassays with the water alone, the compound concentrations may be much higher than those organisms are exposed to *in situ*.

Determination of Sub-Lethal Organism Responses

Fish intersex has been observed in streams and linked to endocrine disrupting chemicals in nearby effluent discharge [54]. This ability to find a causal link between EDCs and observed intersex can be difficult when multiple natural factors and phenomenon may also be contributing or entirely responsible [54,55].

Refining genetic analyses is an important step that will ultimately determine the iTIE system's ability to quickly identify severe risks to environmental and human health by differentiating between natural phenomena and EDC-derived effects. Trace organic chemicals can change form in the environment, accumulate in blood and tissue, and undergo biomagnification in a complex trophic structure. Tracking these compounds as they change and accumulate is difficult, making it harder to determine their long-term effects. By continuing to identify gene responses to trace organic chemical exposure and associating those responses with future developmental or reproductive issues, we can begin to build a database of early warning signs. These genetic signals, determined with in situ exposures, can be more accurate and offer a faster identification than traditional exposure / reproduction studies.

The in situ Toxicity Identification and Evaluation device offers an approach and accuracy that will be invaluable as researchers begin to explore the complex mixture of untreated chemicals continuously discharged into our waterways. As we single out the hidden threats in our aquatic systems, we can begin to refine water treatment methods to ensure the safety and health of our ecosystems and population.

VIII. References

1. Burton GA Jr. and Nordstrom JF. 2004. An in situ Toxicity Identification Evaluation Method Part I: Laboratory Validation. *Environmental Toxicology and Chemistry* 12: 2844-2850.
2. Careghini, AC, AF Mastorgio, S Saponaro, E Sezenna. 2015. Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: a review . *Environmental Science and Pollution Research* 22: 5711-5741.
3. Phillips, Bryn M., et al. 2004. "In situ water and sediment toxicity in an agricultural watershed." *Environmental toxicology and chemistry* 23.2: 435-442.
4. U.S. Environmental Protection Agency. 2007. Freshwater Chronic Toxicity Test Procedure and Protocol. USEPA Region 1, Boston, MA.
5. U.S. Environmental Protection Agency. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. EPA-821-R-02-013. Office of Water, Washington, DC.
6. Burton Jr GA. 1999. Realistic assessments of ecotoxicity using traditional and novel approaches. *Aquatic Ecosystem Health and Management*, 2(1):1-8.

7. Farag, AM, DD Harper, D Skaar. 2014. In Situ Laboratory Toxicity of Coalbed Natural Gas Produced Waters with Elevated Sodium Bicarbonate. Environmental Toxicology and Chemistry. 33.9: 2086-2093.
8. Chappie, D. J., & Burton Jr, G. A. 2000. Applications of aquatic and sediment toxicity testing in situ. *Soil & Sediment Contamination*, *9*(3), 219-245.
9. Kelley, M. A., Gillespie, A., Zhou, G. D., Zhang, S., Meador, J. P., Duncan, B., ... & McDonald, T. J. 2011. In situ biomonitoring of caged, juvenile Chinook salmon (Oncorhynchus tshawytscha) in the Lower Duwamish Waterway. *Marine pollution bulletin*, *62*(11), 2520-2532.
10. Tucker, KA, GA Burton, Jr. 1999. Assessment of nonpoint‐source runoff in a stream using in situ and laboratory approaches. *Environmental Toxicology and Chemistry*, *18*(12), 2797-2803.
11. Chen, L., X. Fu, G. Zhang, Y. Zeng, Z. Ren. 2012. Influences of Temperature, pH and Turbidity on the Behavioral Responses of Daphnia magna and Japanese Medaka (Oryzias latipes) in the Biomonitor. *Procedia Environmental Sciences* 13: 80-86.
12. Ren, Z., Li, Z., Ma, M., Wang, Z., & Fu, R. 2009. Behavioral responses of Daphnia magna to stresses of chemicals with different toxic characteristics. *Bulletin of environmental contamination and toxicology*, *82*(3), 310-316.
13. Ton, S. S., Chang, S. H., Hsu, L. Y., Wang, M. H., & Wang, K. S. 2012. Evaluation of acute toxicity and teratogenic effects of disinfectants by Daphnia magna embryo assay. *Environmental Pollution*, *168*, 54-61.
14. Soetaert, A., van der Ven, K., Moens, L. N., Vandenbrouck, T., van Remortel, P., & De Coen, W. M. 2007. Daphnia magna and ecotoxicogenomics: gene expression profiles of the anti-ecdysteroidal fungicide fenarimol using energy-, molting-and life stage-related cDNA libraries. *Chemosphere*, *67*(1), 60-71.
15. Collard, H. R. J., Ji, K., Lee, S., Liu, X., Kang, S., Kho, Y., ... & Choi, K. 2013. Toxicity and endocrine disruption in zebrafish (Danio rerio) and two freshwater invertebrates (Daphnia magna and Moina macrocopa) after chronic exposure to mefenamic acid. *Ecotoxicology and environmental safety*. *94*, 80-86.
16. Kim, J., Kim, Y., Lee, S., Kwak, K., Chung, W. J., & Choi, K. 2011. Determination of mRNA expression of DMRT93B, vitellogenin, and cuticle 12 in Daphnia magna and their biomarker potential for endocrine disruption. *Ecotoxicology*, *20*(8):1741-1748.
17. Burton, G. A., & Nordstrom, J. F. 2004. An in situ toxicity identification evaluation method part II: Field validation. *Environmental toxicology and chemistry*, *23*(12), 2851-2855.
18. Gourmelon, A., & Ahtiainen, J. 2007. Developing Test Guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances—The OECD perspective. *Ecotoxicology*, *16*(1), 161-167.
19. Arslan-Alaton, I., Aytac, E., & Kusk, K. O. 2014. Effect of Fenton treatment on the aquatic toxicity of bisphenol A in different water matrices. *Environmental Science and Pollution Research*, *21*(21), 12122-12128.
20. U.S. Environmental Protection Agency. 2013. Contaminants of Emerging Concern (CECs in Fish: Perfluroinated Compounds (PFCs). EPA-820-F-13-005. Office of Water. Washington, D.C.
21. Onesios-Barry, K. M., Berry, D., Proescher, J. B., Sivakumar, I. A., & Bouwer, E. J. 2014. Removal of pharmaceuticals and personal care products during water recycling: microbial community structure and effects of substrate concentration. *Applied and environmental microbiology*, *80*(8), 2440-2450.
22. LimnoTech. 2012. Request for Proposal in Application of Site Screening Tools to Evaluate Ecological Effects from Trace Organics in the Aquatic Environment (WERF CEC6R12).
23. Fairbairn, D. J., Karpuzcu, M. E., Arnold, W. A., Barber, B. L., Kaufenberg, E. F., Koskinen, W. C., ... & Swackhamer, D. L. 2015. Sediment–water distribution of contaminants of emerging concern in a mixed use watershed. *Science of the Total Environment*, *505*, 896-904.
24. Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., & Lester, J. N. 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment international*, *34*(7), 1033-1049.
25. Sun, H., & Gu, X. 2005. Comprehensive toxicity study of nonylphenol and short-chain nonylphenol polyethoxylates on Daphnia magna. *Bulletin of environmental contamination and toxicology*, *75*(4), 677-683.

26. U.S. Environmental Protection Agency. 2014. "Contaminants of Emerging Concern." http://water.epa.gov/scitech/cec/.
27. Wegst-Uhrich, S. R., Navarro, D. A., Zimmerman, L., & Aga, D. S. 2014. Assessing antibiotic sorption in soil: a literature review and new case studies on sulfonamides and macrolides. *Chem Cent J*, *8*(5).
28. U.S. Environmental Protection Agency. 2007. Sediment Toxicity Identification Evaluation (TIE): Phases I,II, and III Guidance Document. EPA/600/R-07/080. Office of Research and Development, Washington, D.C.
29. Diamond, J. M., Latimer, H. A., Munkittrick, K. R., Thornton, K. W., Bartell, S. M., & Kidd, K. A. 2011. Prioritizing contaminants of emerging concern for ecological screening assessments. *Environmental Toxicology and Chemistry*, *30*(11), 2385-2394.
30. U.S. Environmental Protection Agency. 2013. Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality. EPA 820-R-13-002. Office of Water, Washington, D.C.
31. Ismail, N. S., Müller, C. E., Morgan, R. R., & Luthy, R. G. 2014. Uptake of Contaminants of Emerging Concern by the Bivalves Anodonta californiensis and Corbicula fluminea. *Environmental science & technology*, *48*(16), 9211-9219.
32. Vidal‐Dorsch, D. E., Bay, S. M., Maruya, K., Snyder, S. A., Trenholm, R. A., & Vanderford, B. J. 2012. Contaminants of emerging concern in municipal wastewater effluents and marine receiving water. *Environmental Toxicology and Chemistry*, *31*(12), 2674-2682.
33. Bunch, A. R., & Bernot, M. J. (2011). Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. *Ecotoxicology*, *20*(1), 97-109.
34. Hannas, B. R., Wang, Y. H., Thomson, S., Kwon, G., Li, H., & LeBlanc, G. A. (2011). Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (Daphnia magna). *Aquatic Toxicology*, *101*(2), 351-357.
35. Ho, K. T., & Burgess, R. M. 2013. What's causing toxicity in sediments? Results of 20 years of toxicity identification and evaluations. *Environmental Toxicology and Chemistry*, *32*(11), 2424-2432.
36. U.S. Environmental Protection Agency. 1991. Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures, Second Edition. EPA/600/6-91/003. Office of Research and Development, Washington, D.C.
37. Buloch, D., R. Lavado, and D. Schlenk. 2010. Ch. 26 – Bioassay Guided Fractionation (Toxicity Identification and Evaluation) for the Determination of Estrogenic Agents in Environmental Samples. In Ed. American Chemical Society. Meeting, & Halden, R. U. *Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations*. American Chemical Society.
38. Thomas, K. V., Hurst, M. R., Matthiessen, P., & Waldock, M. J. 2001. Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. *Environmental Toxicology and Chemistry*, *20*(10), 2165-2170.
39. Ho, K. T., Burgess, R. M., Pelletier, M. C., Serbst, J. R., Cook, H., Cantwell, M. G., ... & Petty, J. 2004. Use of powdered coconut charcoal as a toxicity identification and evaluation manipulation for organic toxicants in marine sediments. *Environmental toxicology and chemistry*, *23*(9), 2124-2131.
40. Weston, D. P., & Jackson, C. J. 2009. Use of engineered enzymes to identify organophosphate and pyrethroid-related toxicity in toxicity identification evaluations. *Environmental science & technology*, *43*(14), 5514-5520.
41. Villamar, C. A., Cañuta, T., Belmonte, M., & Vidal, G. 2012. Characterization of swine wastewater by toxicity identification evaluation methodology (TIE). *Water, Air, & Soil Pollution*, *223*(1), 363-369.
42. Ho, K. T., Gielazyn, M. L., Pelletier, M. C., Burgess, R. M., Cantwell, M. C., Perron, M. M., ... & Johnson, R. L. 2009. Do toxicity identification and evaluation laboratory-based methods reflect causes of field impairment?. *Environmental science & technology*, *43*(17), 6857-6863.

43. Phillips, B. M., Anderson, B. S., Hunt, J. W., Huntley, S. A., Tjeerdema, R. S., Kapellas, N., & Worcester, K. (2006). Solid‐phase sediment toxicity identification evaluation in an agricultural stream. *Environmental Toxicology and Chemistry*, *25*(6), 1671-1676.
44. Rotteveel, S. G. P., & Den Besten, P. J. 2002. Differentiating metal from ammonia toxicity in toxicity identification evaluations. *Bulletin of environmental contamination and toxicology*, *69*(4), 576-585.
45. de Melo, E. D., Mounteer, A. H., de Souza Leão, L. H., Bahia, R. C. B., & Campos, I. M. F. (2013). Toxicity identification evaluation of cosmetics industry wastewater. *Journal of hazardous materials*, *244*, 329-334.
46. Anderson, B. S., Hunt, J. W., Phillips, B. M., Nicely, P. A., Tjeerdema, R. S., & Martin, M. 2004. A comparison of in situ and laboratory toxicity tests with the estuarine amphipod Eohaustorius estuarius. *Archives of environmental contamination and toxicology*, *46*(1), 52-60.
47. Hose, G. C., Murray, B. R., Park, M. L., Kelaher, B. P., & Figueira, W. F. 2006. A meta‐analysis comparing the toxicity of sediments in the laboratory and in situ. *Environmental toxicology and chemistry*, *25*(4), 1148-1152.
48. Yi, X., Li, H., Ma, P., & You, J. 2015. Identifying the causes of sediment‐associated toxicity in urban waterways in South China: Incorporating bioavailabillity‐based measearuments into whole‐sediment toxicity identification evaluation. *Environmental Toxicology and Chemistry*.
49. Chen, F., Yao, Q., & Zhou, X. 2015. The Influence of Suspended Solids on the Combined Toxicity of Galaxolide and Lead to Daphnia magna. *Bulletin of environmental contamination and toxicology*, 1-7.
50. Zitova, A., Cross, M., Hernan, R., Davenport, J., & Papkovsky, D. B. 2009. Respirometric acute toxicity screening assay using Daphnia magna. *Chemistry and Ecology*, *25*(3), 217-227.
51. Lizotte, R. E., Shields, F. D., Murdock, J. N., & Knight, S. S. 2012. Responses of Hyalella azteca and phytoplankton to a simulated agricultural runoff event in a managed backwater wetland. *Chemosphere*, *87*(7), 684-691.
52. Chen, Y., Huang, J., Xing, L., Liu, H., Giesy, J. P., Yu, H., & Zhang, X. 2014. Effects of multigenerational exposures of D. magna to environmentally relevant concentrations of pentachlorophenol. *Environmental Science and Pollution Research*, *21*(1), 234-243.
53. Antczak, P., Jo, H. J., Woo, S., Scanlan, L., Poynton, H., Loguinov, A., ... & Vulpe, C. 2013. Molecular toxicity identification evaluation (mTIE) approach predicts chemical exposure in Daphnia magna. *Environmental science & technology*, *47*(20), 11747-11756.
54. Pollock, M. S., Dubé, M. G., & Schryer, R. 2010. Investigating the link between pulp mill effluent and endocrine disruption: attempts to explain the presence of intersex fish in the Wabigoon River, Ontario, Canada.*Environmental Toxicology and Chemistry*, *29*(4), 952-965.
55. Bahamonde, P. A., Munkittrick, K. R., & Martyniuk, C. J. 2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena?. *General and comparative endocrinology*, *192*, 25-35.
56. Haap, T., Triebskorn, R., & Köhler, H. R. 2008. Acute effects of diclofenac and DMSO to Daphnia magna: immobilisation and hsp70-induction. *Chemosphere*, *73*(3), 353-359.
57. Tay, K. S., Rahman, N. A., Abas, M., & Bin, R. (2011). Removal of selected endocrine disrupting chemicals and personal care products in surface waters and secondary wastewater by ozonation. *Water Environment Research*, *83*(8), 684-691.

IX. Appendix

Denver Data

Appendix 1. Organism survival counts – Denver Deployment (7-Sept-14)			
iTIE ID	Resin	H. azteca alive (of 10)	D. magna alive (of 10)
1A	Glass Wool	9	10
2A	Glass Wool	9	10
3A	Zeolite	8	8
4A	Zeolite	10	10
5A	Sep-Pak	10	8
6A	Sep-Pak	8	9
7A	Chelex	10	10
8A	Chelex	10	10
1B	Zeolite	10	10
2B	Zeolite	10	10
3B	Chelex	10	10
4B	Chelex	9	9
5B	HLB	10	10
6B	HLB	10	10
7B	Glass Wool	10	10
8B	Glass Wool	10	10
1C	Sep-Pak	10	10
2C	Sep-Pak	10	10
3C	Carbon	10	9
4C	Carbon	10	10
5C	HLB	10	10
6C	HLB	10	9
7C	Carbon	10	10
8C	Carbon	10	9

Schaumberg Data

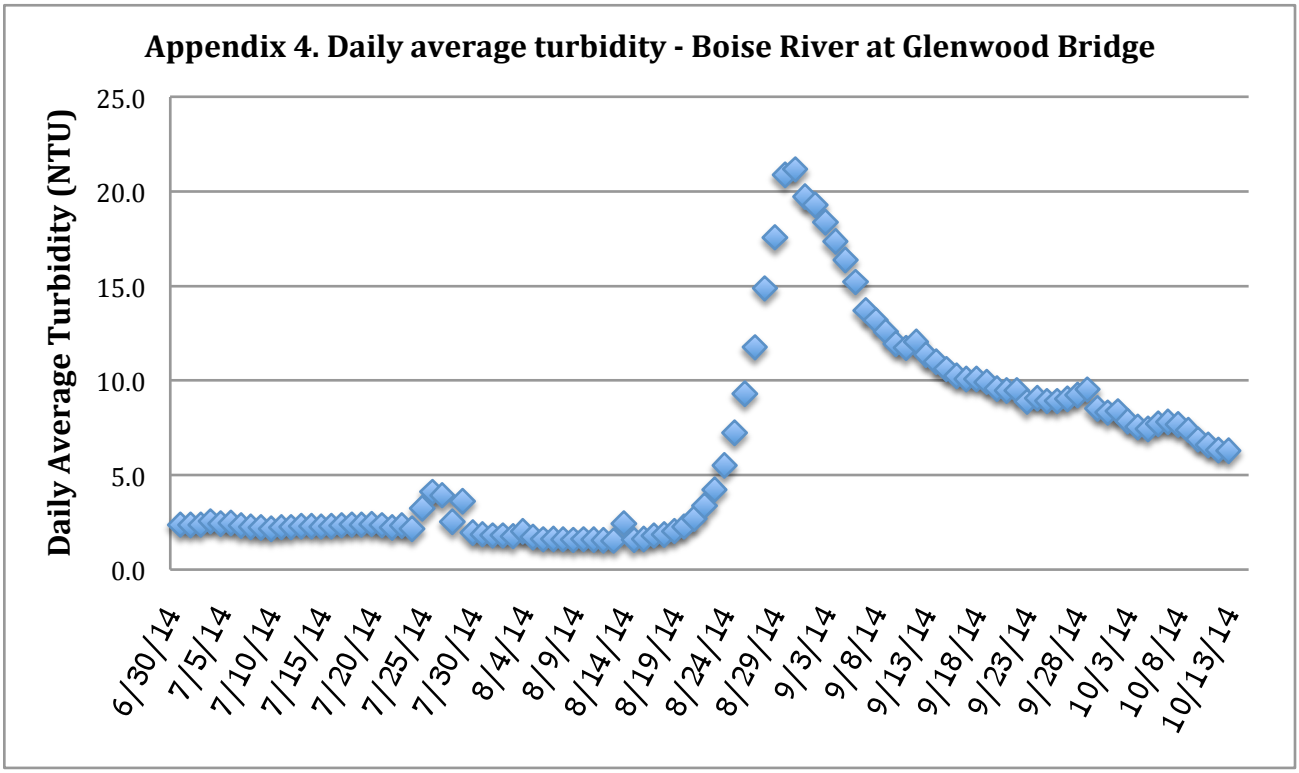
Appendix 2. Organism survival counts – Schaumberg Deployment (25-Sept-14)			
iTIE ID	Resin	H. azteca alive (of 10)	D. magna alive (of 10)
1A	Glass Wool	0	0
2A	Glass Wool	8	9
3A	Zeolite	9	10
4A	Zeolite	6	8
5A	Sep-Pak	3	0
6A	Sep-Pak	6	3
7A	Chelex	9	7
8A	Chelex	6	8
1B	Zeolite	7	4

2B	Zeolite	8	6
3B	Chelex	5	0
4B	Chelex	10	8
5B	HLB	9	8
6B	HLB	0	5
7B	Glass Wool	10	8
8B	Glass Wool	5	5
1C	Sep-Pak	7	8
2C	Sep-Pak	8	8
3C	Carbon	7	9
4C	Carbon	4	5
5C	HLB	8	7
6C	HLB	9	6
7C	Carbon	6	7
8C	Carbon	9	8

Boise Data

Appendix 3. Organism survival counts – Boise Deployment (25-Oct-14)

iTIE ID	Resin	H. azteca alive (of 10)	D. magna alive (of 10)
1A	Glass Wool	10	9
2A	Glass Wool	10	5
3A	Zeolite	10	9
4A	Zeolite	7	2
5A	Sep-Pak	10	1
6A	Sep-Pak	10	3
7A	Chelex	10	8
8A	Chelex	10	4
1B	Zeolite	8	6
2B	Zeolite	20	7
3B	Chelex	8	6
4B	Chelex	20	3
5B	HLB	7	6
6B	HLB	9	3
7B	Glass Wool	9	4
8B	Glass Wool	8	6
1C	Sep-Pak	10	9
2C	Sep-Pak	9	6
3C	Carbon	1	4
4C	Carbon	2	7
5C	HLB	7	5
6C	HLB	5	8
7C	Carbon	10	2
8C	Carbon	8	7



iTIE System Version III: China Lab Validation Data

Test 1: System Calibration

Appendix 5. pH of iTIE sample water during flow rate calibration and final system preparation

Treatment	pH
NDA-88 (1)	3.27
NDA-88 (2)	4.03
NDA-88 (3)	3.61
TP-207 (1)	10.72
TP-207 (2)	11.17
TP-207 (3)	11.65
NDA-150 (1)	5.52
NDA-150 (2)	8.12
NDA-150 (3)	7.41

Carbon (1)	10.07
Carbon (2)	9.89
Carbon (3)	9.72
Zeolite (1)	7.98
Zeolite (2)	7.07
Zeolite (3)	7.51
No Resin (1)	9.67
No Resin (2)	9.05

Appendix 6. HPLC analysis of iTIE processed water during calibration test.

Treatment	**area of BPA,ATR, PYR**
NDA-88 (1)	NO PEAK
NDA-88 (2)	NO PEAK
NDA-88 (3)	NO PEAK
TP-207 (1)	NO PEAK
TP-207 (2)	NO PEAK
TP-207 (3)	NO PEAK
NDA-150 (1)	NO PEAK
NDA-150 (2)	NO PEAK
NDA-150 (3)	NO PEAK
Carbon (1)	NO PEAK
Carbon (2)	NO PEAK
Carbon (3)	NO PEAK
Zeolite (1)	NO PEAK
Zeolite (2)	NO PEAK
Zeolite (3)	NO PEAK
No Resin (1)	NO PEAK
No Resin (2)	NO PEAK

Test 2: Resins Test – Part 1

Appendix 7. General test parameters for resin exposure to spiked water. Flow was rate measured twice during the 2h test, while pH was measured in the sample water at the end of the experiment.

	Flow Rate (ml/min)				
Treatment	**30 min**	**90 min**	**pH of sample water**	**Voltage Regulator Setting (V)**	**Notes**
NDA-88 (1)	10.2	10	3.82	3.5	
NDA-88 (2)	10.8	**	4.24	3.6	** Water draw stopped, intake tubing out of source water
NDA-88 (3)	7.4	7.9	4.03	3.5	
TP-207 (1)	8.6	8.6	9.91	3.5	
TP-207 (2)	11	11	10.11	3.9	
TP-207 (3)	9.1	8.4	10.29	3.7	
NDA-150 (1)	5.6	3.4	4.64	4	
NDA-150 (2)	9.1	8.3	5.65	3.8	
NDA-150 (3)	6.9	3.4	5.42	4	
Carbon (1)	7.2	8.2	7.93	3.4	
Carbon (2)	*	0	8.12	3.5	* Flow was occurring, but sample bottle not full yet
Carbon (3)	6.5	6	7.88	3.5	
Zeolite (1)	2.4	0.5	7.13	4.8	
Zeolite (2)	9.7	9.6	6.37	3.5	
Zeolite (3)	1.4	***	7.67	4	*** Water draw stopped, intake tubing out of source water
No Resin (1)	4.6	11.9	6.04	NA	No Resin treatments were on wall-powered peristaltic pumps. Flow control was possible, but there was no voltage reading
No Resin (2)	9.4	8	6.02	NA	
Spiked Water	NA	NA	6.05	NA	

Test 3: Organism exposure to BPA Spike (Juvenille Daphnia)

Appendix 8. General test parameters during 7-Day old *Daphnia magna* exposure to BPA-spiked water, filtered by iTIE

chambers.

	Flow Rate (ml/min)							
Treatment	**60 min**	**120 min**	**240 min**	**Voltage**	**pH**	**D.O. (12hr)**	**Temperature**	**Notes**
Carbon (1)	9.6	9.6	9	3.5	4.57	7.98	24.9	
Carbon (2)	*	5.6	1.6	3.7	6.68	8.05	24.3	
Carbon (3)	8.3	10.8	9.6	3.6	6.47			
NDA-88 (1)	*	*	7.1	8.9	7.69	8.2	24.4	* Pump having mechanical problems, had to keep voltage high for reasonable flow rate
NDA-88 (2)	15	9.8	7.7	3.4	7.27			
NDA-88 (3)	8	7	5.6	3.9	7.33	7.83	25.7	
NDA-150 (1)	*	8.6	9.1	4.5	7.64	8.22	23.5	
NDA-150 (2)	*	6.7	6.6	4	8.15	7.91	23.8	
NDA-150 (3)	*	*	8.4	4.3	7.86	7.57	24	
No Resin (1)	9.4	8.9	7.9	3.7	7.34	8.03	25.1	
No Resin (2)	11	6.8	10	3.4	7.5	8.24	24.4	
No Resin (3)		10.8	10	3.3	7.78	8.03	24.3	
Spiked Water	NA	NA	NA	NA	7.4	8.32		

* Sample bottle not full

D.O. measured in iTIE organism chamber

Appendix 9. *Daphnia magna* survival by treatment

	Daphnia Alive (of 16)	
Treatment	**12hr**	**24hr**
Carbon (1)	14	4
Carbon (2)	16	9

Carbon (3)	16	5
NDA-88 (1)	0	2
NDA-88 (2)	1	0
NDA-88 (3)	0	0
NDA-150 (1)	16	1
NDA-150 (2)	0	0
NDA-150 (3)	0	0
No Resin (1)	2	0
No Resin (2)	8	0
No Resin (3)	9	2

Test 4: Organism exposure to BPA Spike (Adult Daphnia)

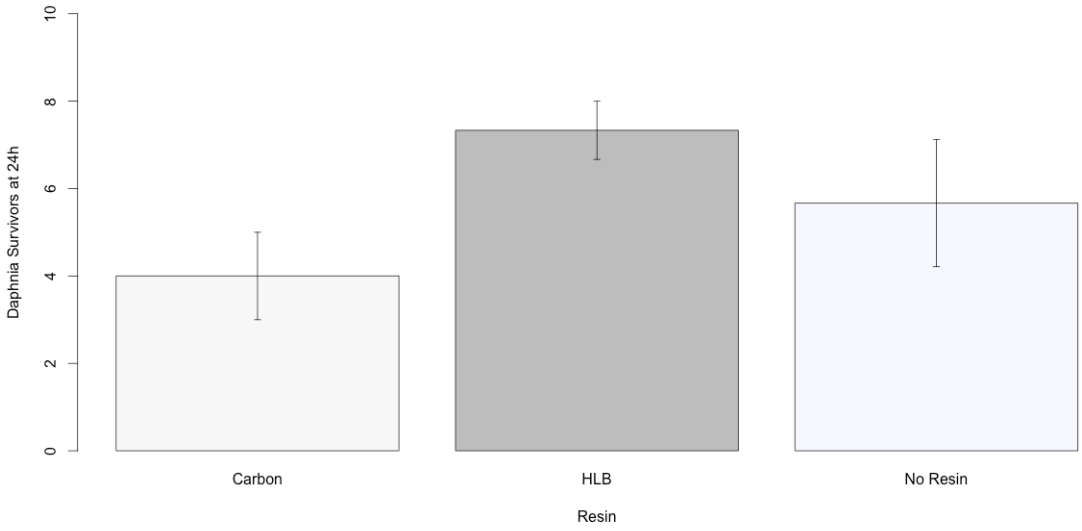
Appendix 10. General test parameters during 14-Day old *Daphnia magna* exposure to BPA-spiked water, filtered by iTIE chambers.

	Flow Rate (ml/min)					Dissolved Oxygen in Organism Chamber			
Treatment	**2h**	**5h**	**7hr**	**Voltage**	**pH**	**D.O. (12hr)**	**D.O. (24hr)**	**Temperature (°C)**	**Notes**
No Resin (1)	7.4	6.8	6.3	3.5	7.91	7.76	7.91	24.5	
No Resin (2)	11.2	11.9	12	3.6	7.97	7.35	7.97	24.1	
No Resin (3)	7.4	7	7.7	3.6	7.73	7.87	7.73	23.8	
Carbon (1)	10.8	6.6	7.4	3.4	8	8.02	8	24.1	
Carbon (2)	9.7	10	10.2	3.5	8.05	7.94	8.05	22.7	
Carbon (3)	7.3	8.2	5.8	3.5	8.13	7.54	8.13	23.7	
HLB (1)	10.6	9	10.1	4.0	7.99	8.22	7.99	23.1	

HLB (2)	*	7.6	5.8	3.5	7.91	8.9	7.91	24	* Pump had stopped, replaced at 2h and treatment restarted
HLB (3)	6.6	7	6.8	3.6	8.03	8.02	8.03	23.8	
Spiked Water	NA	NA	NA	NA	8.72		8.72		

Appendix 11. Organism survival during 24h BPA exposure test. At least 3 individuals were needed for mRNA extraction so this was the max number collected at 10h.

	10h			24h		
Treatment	Alive (of 10)	Dead	# Collected	Alive (of 10)	Dead	# Collected
No Resin (1)	9	1	3	5	1	3
No Resin (2)	10	0	3	5	2	5
No Resin (3)	4	6	2	0	2	1
Carbon (1)	6	4	2	4	0	3
Carbon (2)	6	4	2	4	0	0
Carbon (3)	8	2	3	4	1	2
HLB (1)	8	2	3	5	0	5
HLB (2)	10	0	3	5	2	5
HLB (3)	7	3	3	1	3	3



Appendix 12. Adult *Daphnia magna* survival during the BPA exposure test.

Test 5: Resins Test – Part II

Appendix 13. Flow Rate and pH during the second resin effectiveness test. pH was measured in the final water samples processed by the iTIE chambers.

	Flow Rate (ml/min)		
Treatment	**1h**	**2h**	**pH**
Carbon (1)	8.9	8.9	9.43
Carbon (2)	8.8	9.1	9.56
Carbon (3)	7.2	7	9.5
Chelex (1)	6.4	6	10.47
Chelex (2)	7	7.3	10.36
Chelex (3)	6.4	6	10.63
NDA-150 (1)	6.9	6.8	7.67
NDA-150 (2)	8.4	8.6	8.77
NDA-150 (3)	9.3	9.2	8.73