*16-00392*

*Environmental Toxicology*



# AN IN SITU TOXICITY IDENTIFICATION AND EVALUATION WATER ANALYSIS SYSTEM: LABORATORY VALIDATION

AUGUST J. STEIGMEYER,† JIAMIN ZHANG,‡ JENNIFER M. DALEY,§ XIAOWEI ZHANG,*‡ and G. ALLEN BURTON, JR.†

†School of Natural Resources and Environment, University of Michigan, Ann Arbor, Michigan, USA

‡ School of the Environment, Nanjing University, Nanjing, Jiangsu, People's Republic of China

§ LimnoTech, Ann Arbor, Michigan, USA

*(Submitted 19 May 2016; Returned for Revision 8 August 2016; Accepted 23 November 2016)*

## Abstract

It is difficult to assess the toxicity of a single stressor and establish a strong stressor–causality link when multiple stressors coexist. Toxicity identification evaluation (TIE) methodology uses a series of chemical and physical manipulations to fractionate compounds within a matrix and systematically identify potential toxicants. The current US Environmental Protection Agency application of TIE can provide valuable information but often lacks ecological realism and is subject to laboratory-related artifacts. An in situ TIE device (iTIED) was designed to assess the sources of toxicity in aquatic ecosystems. For this laboratory validation, each unit was equipped with a sorbent resin chamber, an organism exposure chamber, a water collection container, and a peristaltic pump. Chemical analyses of water processed by each iTIED unit were compared with

both lethal and sublethal molecular responses of the organisms. The compound removal effectiveness of different sorbent resins was also compared. In addition to successfully fractionating diverse chemical mixtures, the iTIED demonstrated a potential for early detection of molecular biomarkers, which could identify chronic toxicity that may go unnoticed in traditional TIE assays. Utilizing this novel in situ system will reduce the uncertainty associated with laboratory-based simulations and aid management efforts in targeting compounds that pose the greatest threat.

**Keywords**: Toxicity identification evaluation (TIE), Endocrine-disrupting compounds, Mixture toxicology, Risk assessment, Aquatic toxicology

This article includes online-only Supplemental Data.

* Address correspondence to zhangxw@nju.edu.cn

Published online XXXX 2016 in Wiley Online Library (www.wileyonlinelibrary.com).

DOI: 10.1002/etc.3696

## INTRODUCTION

The causal link between a particular stressor and negative ecological effects is often difficult to ascertain when multiple confounding variables are present. In complex systems, simply demonstrating an incidence of organism stress response does not necessarily identify the cause. Toxicity identification and evaluation (TIE) is an experimental approach developed by the US Environmental Protection Agency (USEPA) to take a complicated matrix with established toxicity and partition the components to identify the compound(s) responsible [1]. A series of physical and chemical fractionation tests followed by a bioassay can support toxicity assessments of individual analytes. Such TIE experiments are intended to build a weight-of-evidence case against specific chemicals to better inform management decisions.

Although TIE has had some success, there are limitations, particularly those associated with laboratory sample manipulations [2]. Mixing test sediments with sorbent resins and chelating agents during phase I has been effective in removing specific classes of compounds prior to exposure tests [3,4]; however, the complexity of these matrices, and the artifacts created by sample manipulations, may reduce toxicity and impede causal linkages [3]. Furthermore, laboratory exposures of test organisms are constant and do not account for natural variables that may alter toxicity. Temporal variation in dissolved organic carbon, suspended solids, hardness, temperature, and pH can all affect the toxicity and bioavailability of metals and organics [3–7]. The choice of sampling times could also affect the composition of the sample for laboratory tests, exposing test organisms to only a snapshot of stream conditions [1,8]. Variations in these exposure conditions can influence toxicity compared with in situ conditions [9–11]. When one is addressing sites influenced by complex chemical mixtures, in situ evaluations provide a more accurate assessment.

Another limitation of current TIE approaches is a reliance on lethality endpoints, which could lead to false-negative results. Most TIE approaches use mortality to determine toxicity, ignoring chronic toxicity, bioaccumulation, and genomic disruption [1,12]. Comparing variations in gene expression and the presence of biomarkers in organisms exposed to various treatments could provide a more sensitive way to identify endocrine-disrupting compounds (EDCs) and other contaminants that lack acute toxicity but that may pose long-term threats [13]. Our previous studies demonstrated that a small number of genes in early responses could be used to predict adverse outcomes such as reproduction (fecundity) inhibition [14]. Further studies have demonstrated that changes to gene expression in *Daphnia magna,* the organism used in the present study, can be predictors of physical abnormalities and could be used to identify the

chemical class responsible with as little as 5 biomarker genes [14–16].

A novel in situ aquatic contaminant fractionation and exposure device was developed for the present study to address the limitations of laboratory-based TIE and the associated acute toxicity bioassays. The device was based on a similar approach that utilizes a 2-chamber resin-exposure system, developed by Burton and Nordstrom [17] for in situ toxicity identification and evaluation (iTIE) of sediment porewater. Using the 2-chamber concept, a new system was developed to support both sediment and open-water experiments conducted directly in the environment of focus.

The laboratory testing and design stage described in the present study determined the feasibility and effectiveness of the new system's core mechanisms. An assortment of commercially available sorptive resins, each designed to target a particular family of compounds, were tested in the iTIE device (iTIED) for selective removal capabilities. Furthermore, the present study used a molecular approach to the bioassay stage by comparing different exposures with early molecular indicators of chronic toxicity, which may offer a faster and more sensitive method for detecting sublethal effects of toxic trace compounds.

## METHODS

### *Overview*

The primary objectives for the laboratory validation of the iTIED system were to assess the mechanical functionality of the device, test the resins' ability to target specific compound types as the iTIED processes water samples, determine organism survival within the chamber, and compare gene expression in organisms from filtered and unfiltered bisphenol-A (BPA) treatments as a possible early indicator of endocrine disruption.

### *iTIED system design*

The dual-chamber spikes, for filtration and organism exposure, were constructed from acrylic, with rubber O-rings to seal the connections between pieces (Figure 1). 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 iTIED spikes using nylon one-eighth–inch hose-to-threaded male pipe adapters for one-fourth–inch (<**ZAQ;1**>inner diameter) tubing (McMaster-Carr). The interior outflow port in the organism chamber was covered with 0.25-mm nylon mesh.

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

Samples drawn from each iTIED chamber were pumped into 500-mL polyethylene bottles. The collection bottle caps contained both an inflow port (for treated water from the iTIED spike) and an outflow port. In the event 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 nonreturn air pump check valve.

*Resins*

Commercially available resins used were zeolite for ammonia; HLB (Sigma-Aldrich) and NDA-88 and NDA-150 (Nanjing University Environmental Protection) for organic compounds; TP-207 (Bayer) and Chelex (Solarbio) for metals; and activated carbon, which is commonly used for organics extraction but has an affinity for other types of compounds, including metals. Mainly composed of styrene and divinylbenzene, NDA-88 is modified by chloromethylation and

amine. With high specific surface area, NDA-88 can absorb carboxylic acids at the molecular level, and some phenols from biochemical metabolism. The NDA-150 resin also has high surface area and rich nano-adsorption pores. The skeleton of NDA-150 consists of polystyrene, which allows for the absorption of hydrophobic aromatic compounds and organic halogenated hydrocarbons.

*Calibration and blank run*

Before the system could effectively process chemically laced water, the optimum filtration speed through the chambers had to be established. A custom pump speed for each chamber was determined based on the resistance offered by each test resin.

The intake tubing for each iTIED chamber was submerged in Milli-Q water for the pump rate calibration test. 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. Some resins produced more resistance than others, so voltage was increased as needed to ensure similar flow rates for each treatment. Flow rate varied because of the inherent fluctuations in the pumps and air pockets in the resin chambers, so an acceptable flow rate range was established at 5 mL/min 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 acted as a negative control by targeting all types of compounds. Air was purged from interstitial resin spaces with a 2-h Milli-Q water soak. Immediately prior to adding the resin to the iTIED chamber, excess water and fine particles were drained off. Five grams of each resin were added to their respective chambers in triplicate. Two iTIED chambers contained no resin as a positive control. Each iTIED chamber contained glass wool above and below the resin to prevent movement and ensure tight surface

area and volume coverage. Contact with some resins can negatively impact test organisms [3], so a circular piece of cotton electrostatic vent filter (WEB) was placed at the top of the resin chamber to prevent movement of resin particles into the organism chamber.

Milli-Q water was pumped through the system for 2 h, after which 10-mL water samples were taken for analyses. These samples were analyzed for baseline concentrations to determine whether there was any leaching from the equipment. Other measurements included pH, temperature, dissolved oxygen, and flow rates.

*Resin effectiveness test I*

The resin effectiveness test was designed to assess the feasibility of compound fractionation within the iTIED and determine the adsorption abilities of several resin types. For a successful in situ TIE, the iTIED would have to remove or significantly reduce the concentration of target compounds as the source water passes through the resin chambers.

A 21-L spiked solution was used as the source water. The volume of solution was determined based on the combined flow rates of the 17 iTIED chambers and the length of the experiment (2 h). Cadmium (Alfa Aesar), cupric chloride (Nanjing Chemical Reagent), lead nitrate (Nanjing Chemical Reagent), zinc sulfate heptahydrate (Sigma-Aldrich), BPA (Aldrich), atrazine (AccuStandard), pyrene (AccuStandard), and ammonium chloride (Sigma) were added to 21 L of Milli-Q water in 2-mg/L concentrations. The iTIED processed this mixture for 2 h.

Resins utilized in the first test were zeolite, NDA-88, NDA-150, TP-207, and activated carbon. There were 3 replicate treatments for each resin, with 5 g of each sorbent in the respective chambers, and 2 replicates for the no-resin positive control chambers. Flow rate was recorded using the outflow from the check valves. Because of 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.

*Resin effectiveness test II*

A limited number of iTIED chambers were available, so a second resin test was conducted using 3 replicates each of activated carbon, Chelex (Solarbio), and NDA-150. The source water contained the same compounds as the first resin test, except for ammonia, in 2-mg/L concentrations. A 15-mL water sample was collected from each iTIED treatment replicate following 2 h of constant filtration for both resin tests. The samples were stored at 4 °C.

*Test organism*

*Daphnia magna* are common test organisms in TIE studies and have previously been used in experiments looking for chemical identification based on gene expression factors [1,15,16]. The BPA exposure tests were designed to test the feasibility of conducting bioassays within the iTIED and utilizing molecular methods for identifying sublethal toxicity in the source water. The primary goal of these tests was to demonstrate the iTIED's ability to prevent gene disruption in certain treatments through selective BPA removal, while allowing full exposure to the compound in other treatments. As there is still much uncertainty surrounding contaminant concentrations necessary to initiate clear biomarker responses, the present study first established a concentration–response curve. The concentrations were higher than those commonly found in natural systems, to ensure a clear genomic response in the positive control treatments. After demonstrating that selective removal in the iTIED can prevent gene disruptions, future studies could determine the device's detection limits for a variety of compounds prior to field applications.

The organisms were cultured in an established *D. magna* culture laboratory, fed daily

with green alga, and kept at 24 ± 0.5 °C with a 16:8-h light:dark cycle [14]. Organisms selected for the BPA exposure tests were 14-d to 16-d old. During the organism tests, nutrient solutions of $CaCl_2$, $MgSO_4$, $NaHCO_3$, and KCl were added to the spiked source water to replicate the *D. magna* culture water.

*BPA exposure test I: BPA concentration–response curve*

To establish a baseline molecular response curve before the iTIED organism tests, *D. magna* were exposed to 6 concentrations of BPA (0 mg/L, 0.3 mg/L, 1 mg/L, 3 mg/L, 10 mg/L, and 30 mg/L) with 3 replicates. After 12 h, 10 *D. magna* were taken from each treatment for RNA extraction. After an additional 12 h, organisms were again collected. Six *D. magna* genes were selected based on previous changes in their regulation in response to BPA exposure [14].

The selected genes were annotated manually by US National Center for Biotechnology Information (NCBI)/protein BLAST. Primers of target genes were designed by NCBI/Primer-BLAST software using gene messenger (m)RNA sequences. Two of these genes, DM06154 and DM07147 (Table 1), were selected for BPA exposure test II because they demonstrated fold increases in expression of approximately 1000 µg/L, which was lower than the BPA concentration planned for the subsequent iTIED application test.

*BPA exposure test II: iTIED fractionation and organism exposure*

Using the same protocol as the resin effectiveness tests, iTIED chambers were loaded with 3 replicates each of HLB and activated carbon. An additional 3 no-resin chambers acted as the control group. Ten 14-d- to 16-d-old *D. magna* were added to each iTIED organism chamber. Source water for the test contained 4 mg/L BPA. The iTIED processed the source water for 12 h, at which time the *D. magna* were collected for RNA extraction.

*RNA extraction and semiquantitative real-time polymerase chain reaction*

Samples of *D. magna* were collected after a 12-h exposure. Total RNA was isolated from the sample by use of the TRIzol Reagent (Ambion, Life Technologies) following the manufacture's protocol. Reverse transcription for each sample was performed using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in 96-well plates using QuantiTect SYBR Green PCR Master Mix (Qiagen). The amplification was performed on StepOne Plus (Life Technologies) with an initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The Ct values of the target genes were normalized by a housekeeping gene, β-actin, using the $^{\Delta\Delta}$Ct method. Fold change was calculated as $2^{-\Delta\Delta}$ Ct. Differences between control and exposure groups were evaluated by *t* test.

*Water sample analysis*

To detect dissolved metals, the water was filtered through a 0.45-μm water-based microfiltration membrane. A 500-μL sample of the filtered water was mixed with 500 μL of 0.1-M diluted nitric acid (analytical grade), and 20 μL of <**ZAQ;2**>115In standard solution (50 μg/L; ANPEL Scientific Instrument) as the internal standard. The inductively coupled plasma–mass spectrometry (ICP–MS) NexION 300X (PerkinElmer) was calibrated with a standard solution containing 1 μg/L <**ZAQ;3**>each of Be, Ce, Fe, In, Li, Mg, Pb, and U (PerkinElmer). Because of analysis restrictions, sample concentrations for each metal could not exceed 20 μg/L. The detection limit for each metal is shown in Table 2.

To measure the concentration of metal ions in the iTIED-filtered samples, ICP–MS with a NEX10N300X (PerkinElmer) was used. Water samples were analyzed for the presence of BPA, atrazine, and pyrene using high-performance liquid chromatography (Waters 2414 Refractive Index Detector).

*Data analysis*

An analysis of variance (ANOVA) test was used to analyze differences in concentration means between various treatments, with evidence against the null hypothesis defined as $p < 0.05$. 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 using R. **<ZAQ;4>**All data for the present study are available through the FigShare online storage system of this journal.

## RESULTS

*Calibration and blank run*

High-performance liquid chromatography analysis of the Milli-Q sample water collected by the iTIED during the calibration run yielded no peaks for the organic chemicals used in subsequent tests.

*Resins test: Flow rate*

Mean flow rates during the 2-h test did not differ between treatments ($p = 0.08$). Zeolite treatments had the slowest flows while TP-207 had the fastest. The pH varied between samples collected from different iTIE chambers. The average pH in NDA resin-treated water samples was lower compared with the no-resin control, whereas the other resin treatment samples had higher pH.

Flow rate differed significantly between some iTIED treatments during the second resin test ($p < 0.05$). There was no significant difference between the NDA-105 and carbon treatment flow rates ($p = 0.97$). However, rates between the Chelex and NDA-150 treatments did differ ($p < 0.05$). Flow rates also differed between the Chelex and carbon chambers ($p < 0.05$). The average water flow rate through the Chelex chamber was lower than the other 2 treatments.

*Resins test: Metals extraction*

Because only 17 iTIED chambers were available, the no-resin treatment in resins test I had only 2 replicates. 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, because it was untreated, similar to the iTIED control samples.

Following the 2-h resin effectiveness test, there was a difference in the concentration of metals between iTIED-treated samples (Figure 3). The resin present in the iTIED chamber significantly affected the concentration of metals ($p < 0.05$). The lowest metal concentrations were detected in water samples processed by the TP-207 chamber. The highest concentrations were observed in water passing through chambers without a resin (Figure 3), and there was a difference between the no-resin and TP-207 groups ($p < 0.05$). For example, the mean concentration of copper in TP-207 treatments (15.12 ± 6.34 µg/L) was 99.3% lower than in the samples processed by the no-resin iTIEs (2184.76 ± 101.56 µg/L). Zeolite treatments were also different from the no-resin groups ($p < 0.05$), with Cd, Cu, Pb, and Zn levels being 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 different from those in the TP-207 samples ($p = 0.51$).

The concentrations of metals in the NDA-88–treated water were not different from the no-resin group ($p = 0.99$). The 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 different ($p < 0.05$) in the NDA-150 treatment, with a 52.6% lower average concentration than the lead in no-resin treatments. The carbon treatments showed a difference in the concentrations of metals compared with the no-resin samples ($p < 0.05$).

*Resins test: Organic chemical extraction*

Water samples collected after the 2-h resins test showed variation in the concentration of organic chemicals based on the resin used in the iTIED chamber ($p < 0.05$). There was no difference in the concentrations of organic chemicals between the no-resin and TP-207 treatments samples (Figure 4). Concentrations were also not different between the zeolite and no-resin samples for all chemicals.

Carbon iTIED filtration resulted in different concentrations of atrazine ($p < 0.05$) and BPA ($p < 0.05$), compared with iTIED chambers with no resin, but the concentration of pyrene between these 2 groups did not differ ($p = 0.99$). The mean BPA concentration in carbon-treated water (0.13 ± 0.17 mg/L) was 98.2% lower than in the no-resin samples, while the mean atrazine concentration was 96.4% lower (0.086 ± 0.013 mg/L). When NDA-150 was compared with no-resin, atrazine and BPA levels were significantly different ($p < 0.05$), but pyrene concentrations (Figure 4) 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 chambers containing NDA-88 had significantly different levels for all 3 contaminants compared with no-resin. Atrazine, BPA, and pyrene concentrations were 81.6%, 75.8%, and 100% lower than in the unfiltered no-resin samples (Figure 4).

*Resins test: Ammonia extraction*

The concentration of ammonia in iTIED-processed water (Supplemental Data, Figure S1) differed based on the resin in the chamber ($p < 0.05$). The TP-207 and zeolite treatments were significantly different from the carbon and NDA treatments ($p < 0.05$). Ammonia concentration in TP-207 chambers' water samples was not different from the no-resin samples ($p = 0.06$). Zeolite sample concentrations were different from the no-resin ($p < 0.05$).

*BPA exposure test I: BPA concentration–response curve*

Expression of 6 genes was upregulated differently with concentration of BPA compared with samples in the control group at 12 h and 24 h, respectively (Supplemental Data, Figure S2). The mRNA of gene DM06154 increased from 0.1 μg/L to 3000 μg/L and then decreased at higher concentrations. Gene DM06154 was upregulated by 4.41-fold compared with control at 3000 μg/L. The BPA concentration-dependent mRNA expression of DM07147 showed hormesis at 0.1 μg/L and 1 μg/L, and the expression increased from 10 μg BPA/L to 30 000 μg BPA/L (Figure 5).

*BPA exposure test II: iTIED fractionation and organism exposure*

Two selected genes (Table 1) demonstrated differential gene expression in the HLB and no-resin treatment groups compared with the carbon treatment at 12 h (Figure 6). At 12 h, the BPA concentration in the no-resin and carbon-treated water was 4172 μg/L and 706 μg/L, respectively (Figure 6). The BPA concentration in HLB-treated water was below the detection limit (65 μg/L).

The transcriptional expression of the selected genes confirmed BPA exposure in different treatment groups. There was no significant difference in mRNA expression of DM06154 or DM07147 between HLB-treated and carbon-treated group after the 12-h exposure. However, the predicted gamma-gliadin–like protein coding gene DM06154 showed a mean of 5.51-fold increase in *Daphnia* from the no-resin treatment compared with the carbon treatment. The DM07147 gene was expressed 2.04-fold more in the no-resin treatment than in the carbon treatment. The fold changes of the 2 *D. magna* gene expressions in the iTIED chamber were consistent with that observed in the full concentration–response curves.

**DISCUSSION**

In a complex system in which multiple physical stressors and potential toxicants exist, it

can be difficult to find a causal link between observed ecological impacts and a specific compound [1,4]. The USEPA developed TIE protocols as a way to isolate variables in a field sample and build a weight-of-evidence case for the exact source of toxicity. Current application of TIE methods relies heavily on laboratory-based fractionation and exposure tests, which are subject to artifacts and variable biases [2,9,11]. Some studies have paired in situ bioassays with laboratory TIE to corroborate results in a natural setting [7,9,18]. While these pairings sometimes produce similar results, there are often drastic differences in survival rates between the 2 test groups, and the pattern is not consistent for all species or environments [11,18].

The goal of in situ TIE was to create the most realistic exposure test possible, accounting for natural stressors and temporal fluxes in toxicants, while reducing the influence of artifacts. The first deployment of an in situ TIE system demonstrated that phase I fractionation coupled with a bioassay was possible within streambed sediments [17]. As habitat risk assessments begin to focus on trace organic compounds and other contaminants of emerging concern, however, a more precise, adaptable, and reliable iTIE system is needed.

The novel iTIED tested in the present study was designed to work in a variety of aquatic ecosystems. With precise control mechanisms, the speed of each pump in the iTIED could be adjusted to accommodate the source of water (pore or overlaying) and ensure similar filtration rates across treatments, regardless of each resin's unique resistance. By isolating trace compounds in certain exposures, the iTIED could potentially identify toxicants that pose a long-term ecological risk, but would otherwise go unnoticed as acutely toxic compounds mask the effects of more subtle, sublethal compounds. Incorporating molecular biomarkers into the bioassays could aid in the identification of toxicants with the potential for endocrine disruption, intersex, and other forms of chronic toxicity. Because thousands of trace unregulated compounds

are being discovered in waterways, narrowing the source of toxicity to a particular group or compound will greatly aid habitat risk assessment studies and better inform management protocol.

Mechanically, the iTIED operated within the design parameters. Peristaltic pumps were able to draw the source water through the iTIED chambers and deposit the processed samples in collection containers, with overflow exiting through the one-way check valves. Adjusting the voltage delivered to each pump regulated the filtration rate through the iTIED chambers, making it possible to compensate for varying resistance between resins and achieve similar flow rates across all treatments. There was inherent mechanical variation between individual pumps, which required a larger than ideal range in flow rate. However, the circuit board design allows for easy pump replacement, so sturdier and more reliable pumps can be utilized in future studies. Conducting bioassays within the iTIED during future in situ deployments is also viable, as the *D. magna* in the organism chambers had 100% survival during the 24-h exposure test.

*Resin effectiveness*

Although concentrations of toxicants used for the present study were elevated above those observed at most contaminated sites to establish proof of concept, the significant reductions observed over the relatively short test period suggest that the iTIED filter chambers will be even more effective in situ [8,10]. Future studies can determine compound removal limitations of the iTIED, such as resin saturation points and chemical selectivity, as the present study has demonstrated the device's ability to conduct phase I fractionation.

The primary goal of phase I fractionation was achieved with the iTIED. Treatments using TP-207, for example, reduced the concentration of metals in their processed water compared with treatments not targeting metals (such as NDA-88 and the control). Likewise, iTIED

chambers containing NDA-88 and NDA-150, resins designed to target organic molecules, reduced the concentrations of atrazine and BPA, whereas other iTIED treatments were not different from the control. When one is trying to identify the source of toxicity in a stream environment, selective removal of compound types and a subsequent comparison of organism response could help narrow the focus to a particular group. This approach may also aid in identifying trace toxicants, which go unnoticed if a more dominant stressor masks their effects in traditional in situ cage bioassays. The in situ application of TIE can more accurately and thoroughly diagnose the stressors to target in remediation efforts or modifications of wastewater treatment protocols. There are, however, resin limitations that must be understood before the iTIED is applied to a habitat risk assessment study.

The commercially produced resins selected for the present study vary in their selectivity, which sometimes limited phase I fractionation within the iTIED. Although all metal concentrations were significantly reduced in the carbon treatments, the resin more successfully targeted copper and lead, which were each below 500 µg/L. Cadmium and zinc concentrations in that treatment were each greater than 1000 µg/L. Differences in resin affinity for organic compounds led to greater reductions of atrazine and BPA than pyrene in the carbon and NDA-150 chambers. These results suggest that the complete removal of all individual compounds in a particular category may not always be possible within the iTIED. If the compounds' concentrations are at least reduced below the toxicity threshold, however, a comparison with the control can still identify potential threats. More importantly, these differences in resin affinity could help identify a specific compound responsible for toxicity.

Several resins showed unique affinities for particular compounds. The NDA-150 treatments targeted lead for removal while leaving the other metals relatively untouched. Carbon

was able to remove nearly all traces of BPA and atrazine from the source water, but did not reduce pyrene levels as much, whereas NDA-88 was able to lower pyrene concentrations below detectable limits. These results suggest that phase II fractionation in situ may be possible. Phase II fractionation involves selective removal of specific compounds within a group linked to toxicity during phase I trials [1]. If, for example, a toxicant was linked to the metals group, an iTIED chamber with NDA-150 could target lead as a specific variable in 1 test, which could either implicate or eliminate lead as the likely cause of observed ecological effects. This advantage becomes more important in receiving waters for wastewater treatment discharge. With thousands of unregulated compounds in trace concentrations, it is not always clear what to test for in the water, and it is difficult to identify a particular threat. Using resins in the iTIED with an affinity for the compounds known to be present could, through a series of selective extractions, lead to the identification of an *unknown organic compound* as the toxicant. Water chemistry analyses could then be tailored to classify the unknown threat. This process can only work, however, if the metrics are in place to predict all threats to organism fitness.

*Molecular bioassays*

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 [14,19]. Targeted removal in organism exposures combined with genomic analysis could provide a more sensitive method for identifying toxicants that other screening tools might miss.

Observing differences in growth and reproduction is a common method for identifying molecular disruption [13], but this approach necessitates a longer experimental time, which

makes in situ and TIE approaches difficult. The 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 molecular biomarkers [16]. To integrate this approach into the iTIED, we tested selective removal of BPA, a compound known to cause variable gene expression in *D. magna* [8,14]. The purpose of these tests was to assess the feasibility of coupling molecular analyses with the iTIED, not to build a genetic response matrix for the test organism. For this reason, BPA was used at higher concentrations than are common in natural environments, to ensure a definitive, visible response in the control treatments for a mechanical assessment of the iTIED's capabilities, mitigating the impact of variables associated with limited knowledge of biomarker genes.

Our BPA concentration–response curves identified 6 *D. magna* genes that upregulate differently, based on the level of the contaminant. Two of these genes (DM06154 and DM07147) demonstrated fold changes in expression at approximately 1000 µg/L. Two resins (carbon and HLB) were capable of reducing the concentration of BPA below 1000 µg/L. During the iTIED organism test with BPA, the carbon and HLB treatments significantly reduced the concentration of BPA in the water, so the organisms in their respective chambers were exposed to levels below 1000 µg/L. The control chambers allowed all the BPA to pass through into the organism chamber, exposing those *D. magna* to over 4000 µg/L. The fold change in gene expression was significantly higher in the no-resin treatments than in either of the chambers that targeted BPA. The expression of these genes was altered only when the endocrine disruptor was present. The results suggest that it is possible to incorporate gene regulation into the bioassay portion of in situ TIE, increasing our ability to identify a range of contaminant types. The database of gene functions and responses to specific stressors is, however, limited for many indicator organisms,

so further study is needed to identify key biomarkers at ecologically realistic concentrations.

Previous studies have shown significant changes in *D. magna* gene expression after 24-h exposure to BPA concentrations as low as 0.3 µg/L [19], so in situ identification of EDCs during a brief iTIED deployment could be possible, but only with knowledge of the key biomarkers and predicted responses [14,16]. A complete chemical/molecular response database must be established for a test organism before this methodology can be effectively utilized in the field. For the present study, the iTIED has demonstrated an ability to potentially remove EDCs in some treatments, allowing for a molecular comparison with control treatments, which could identify compounds that pose the greatest risk for chronic toxicity. This early identification of EDCs can aid in preventative responses while allowing for a faster and less expensive identification of current causes of observed habitat impairment.

This laboratory validation of the iTIED tested the mechanical functionality of a prototype system for in situ identification of contaminants and other stressors in aquatic environments. The present study demonstrated general TIE phase I fractionation capabilities and showed promise for phase II fractionation as well as molecular approaches to aid in early and specific identification of contaminant threats. With the basic functionality of the prototype established, further tests can refine the device's components and identify limitations in field deployment. Understanding resin limitations, such as saturation points and affinity for specific compounds, will help build protocols for specific treatments, keeping them cost-effective by using the minimum amount of resin necessary and ensuring that the resin used has an affinity for all targeted compounds.

A field housing for the system, currently under development, will address flow challenges. The custom circuit board utilized in the present study, for example, adjusts the

voltage delivered to the pump to control pump speed. Slower speeds will be essential for porewater so the field iTIED will incorporate pumps operating at lower voltages. All electrical components will be sealed and deployed in a waterproof containment unit for in situ deployments in relatively shallow waters.

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, dissolved organic carbon, 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 multiple environmental stressors potentially masking the subtle effects of trace compounds, a new method of risk assessment is needed that more accurately characterizes exposures, which can then be better linked to effects. This preliminary laboratory test of the iTIED supports the in situ fractionation and exposure concept for ecological risk assessment.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3696.

*Acknowledgment*—We thank the Major Science and Technology Program for Water Pollution Control and Treatment (grants 2012ZX07506-003 and 2012ZX07101-007-01) and Jiangsu Province Funds for Distinguished Young Scientists (grant BK20130015). X. Zhang was supported by the Fundamental Research Funds for the Central Universities and the Collaborative Innovation Center for Regional Environmental Quality. We also thank M. Bradshaw and Research Technical Services at the University of Michigan for advice and assistance with the electrical components of the iTIED, as well as the LSA Scientific Instrument Shop at the

University of Michigan for help with constructing some iTIED mechanical components.

*Data Availability*—**<ZAQ;5>**All data have been uploaded to *Environmental Toxicology & Chemistry*'s FigShare online storage system. The data are also available on request. Please contact G. Allen Burton, Jr. (burtonal@umich.edu).

References

1. US 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, DC.
2. Ho KT, Gielazyn ML, Pelletier MC, Burgess RM, Cantwell MC, Perron MM, Serbst JR, Johnson RL. 2009. Do toxicity identification and evaluation laboratory-based methods reflect causes of field impairment? *Environ Sci Technol* 43:6857–6863.
3. Phillips BM, Anderson BS, Hunt JW, Huntley SA, Tjeerdema RS, Kapellas N, Worcester K. 2006. Solid-phase sediment toxicity identification evaluation in an agricultural stream. *Environ Toxicol Chem* 25:1671–1676.
4. Rotteveel SGP, Den Besten PJ. 2002. Differentiating metal from ammonia toxicity in toxicity identification evaluations. *Bull Environ Contam Toxicol* 69:576–585.
5. Burton GA Jr. 1999. Realistic assessments of ecotoxicity using traditional and novel approaches. *Aquat Ecosyst Health Manage* 2:1–8.
6. de Melo ED, Mounteer AH, de Souza Leão LH, Bahia RC, Campos IM. 2013. Toxicity identification evaluation of cosmetics industry wastewater. *J Hazard Mater* 15:329–334.
7. Farag AM, Harper DD, Skaar D. 2014. In situ laboratory toxicity of coalbed natural gas produced waters with elevated sodium bicarbonate. *Environ Toxicol Chem* 33:2086–2093.
8. Careghini A, Mastorgio AF, Saponaro S, Sezenna E. 2015. Bisphenol A, nonylphenols,

benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: A review. *Environ Sci Pollut Res* 22:5711–5741.

9. Anderson BS, Hunt JW, Phillips BM, Nicely PA, Tjeerdema RS, Martin M. 2004. A comparison of in situ and laboratory toxicity tests with the estuarine amphipod *Eohaustorius estuarius*. *Arch Environ Contam Toxicol* 46:52–60.
10. Bunch AR, Bernot MJ. 2011. Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. *Ecotoxicology* 20:97–109.
11. Hose GC, Murray BR, Park ML, Kelaher BP, Figueira WF. 2006. A meta-analysis comparing the toxicity of sediments in the laboratory and in situ. *Environ Toxicol Chem* 25:1148–1152.
12. US Environmental Protection Agency. 1991. Methods for aquatic toxicity identification evaluations: Phase I toxicity characterization procedures, 2nd ed. EPA/600/6-91/003. Office of Research and Development, Washington, DC.
13. Collard HRJ, Ji K., Lee S, Liu X, Kang S, Kho Y, Ahn B, Ryu J, Lee J, 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. *Ecotoxicol Environ Safe* 94:80–86.
14. Zhang X, Hecker M, Jones PD, Newsted J, Au D, Kong R, Wu RS, Giesy JP. 2008. Responses of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environ Sci Technol* 42:6762–6769.
15. Antczak P, Jo HJ, Woo S, Scanlan L, Poynton H, Loguinov A, Chan S, Falciani F, Vulpe C. 2013. Molecular toxicity identification evaluation (mTIE) approach predicts chemical exposure in *Daphnia magna*. *Environ Sci Technol* 47:11747–11756.

16. Kim J, Kim Y, Lee S, Kwak K, Chung WJ, Choi K. 2011. Determination of mRNA expression of DMRT93B, vitellogenin, and cuticle 12 in *Daphnia magna* and their biomarker potential for endocrine disruption. *Ecotoxicology* 20:1741–1748.
17. Burton GA Jr, Nordstrom JF. 2004. An in situ toxicity identification evaluation method, Part I: Laboratory validation. *Environ Toxicol Chem*12:2844–2850.
18. Phillips BM, Anderson BS, Hunt JW, Nicely PA, Kosaka RA, Tjeerdema RS, de Vlaming V, Richard N. 2004. In situ water and sediment toxicity in an agricultural watershed. *Environ Toxicol Chem* 23:435–442.
19. Ha MH, Choi J. 2009. Effects of environmental contaminants on hemoglobin gene expression in *Daphnia magna*: A potential biomarker for freshwater quality monitoring. *Arch Environ Contam Toxicol* 57:330–337.



Figure 1. The dual chamber acrylic in situ toxicity identification evaluation (iTIE) spike used for chemical fractionation and subsequent bioassay exposure.

Figure 2. Overview of the in situ toxicity identification evaluation device (iTIED) used for chemical fractionation and exposure tests.

Figure 3. Metal concentrations in water samples processed by various in situ toxicity identification evaluation device (iTIED) resin treatments. Asterisk denotes a significant difference from the control ($p < 0.05$). Values are mean ± standard deviation of 3 measurements. No resin = positive control.

Figure 4. Organic compound extraction by in situ toxicity identification evaluation device (iTIED) treatment. Asterisk denotes a significant difference from the control ($p < 0.05$). Values

are mean ± standard deviation of 3 measurements. No resin = positive control; BPA = bisphenol-A.

Figure 5. Bisphenol-A (BPA) concentration response curve for *Daphnia magna* genes (**A**) DM06154 and (**B**) DM07147.

Figure 6. Integration of in situ toxicity identification evaluation device (iTIED) and molecular TIE in the assessment of bisphenol-A (BPA): (**A**) BPA concentrations in the different (iTIED) treatments; (**B**) gene expression changes for 2 *Daphnia magna* genes in respective TIE chambers at 12 h. Asterisk denotes a significant difference from iTIE treatments ($p < 0.05$). No resin = positive control.

**<<ENOTE>> AQ1:** Please check: Correct definition of ID?

**<<ENOTE>> AQ2:** Please check: Text appears to be missing here.

**<<ENOTE>> AQ3:** Is addition of “each” OK here?

**<<ENOTE>> AQ4:** Please update: ET&C’s Figshare hub has not yet been activated. Is this available online in another format? Can this sentence be changed to say that data re in the Supplemental Data files?

**<<ENOTE>> AQ5:** Please update the Data Availability statement (ET&C’s Figshare hub is not yet active).



Table 1. Identification (ID) and primer sequences for the *Daphnia magna* genes used in the present study

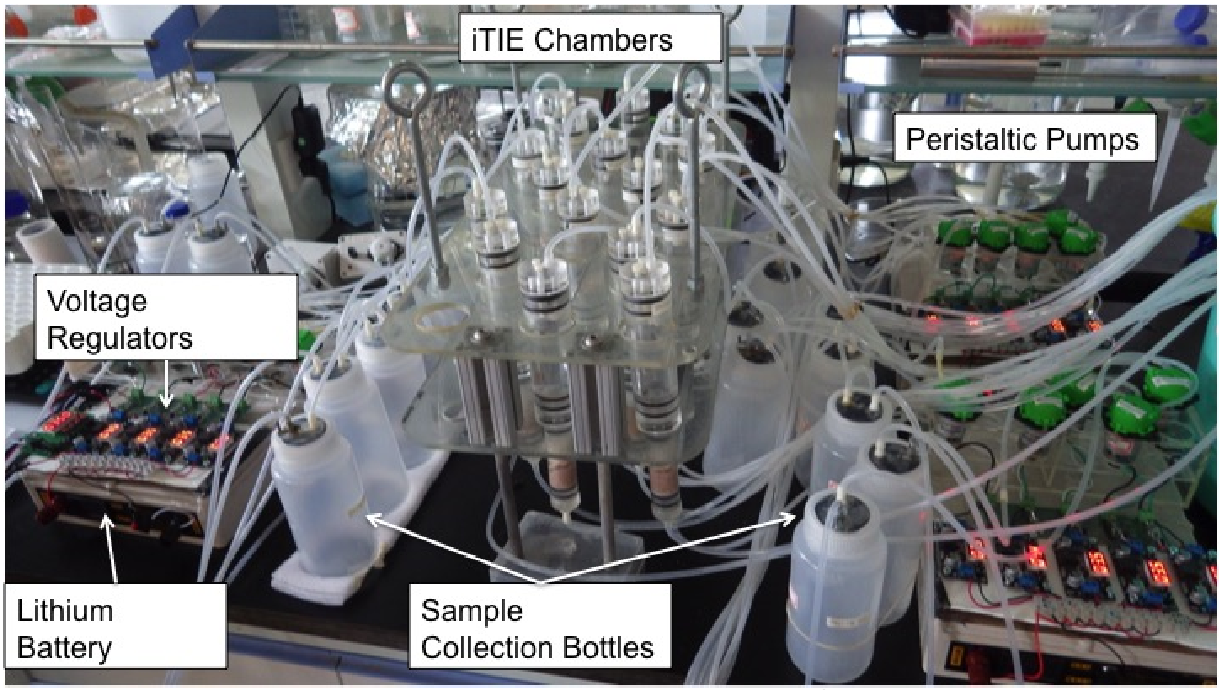
| Gene ID | Forward primer (5′→3′) | Reverse primer (5′→3′) | Gen |
|---|---|---|---|
| DM06154 | CAGCATATTCGATGGTCTTCAACTC | TATTAGTTTGTAACCGGTTCGTTGC | Predicted: Gam<br>*l* |
| DM07147 | CGGTACTAAACGAGATCGTTCAAAG | TTTTCTGTTTGTAGGCGAAGAACTC | Predicted: Hem<br>gamma-glut<br>(*Cer* |

Table 2. Detection limit of each metal

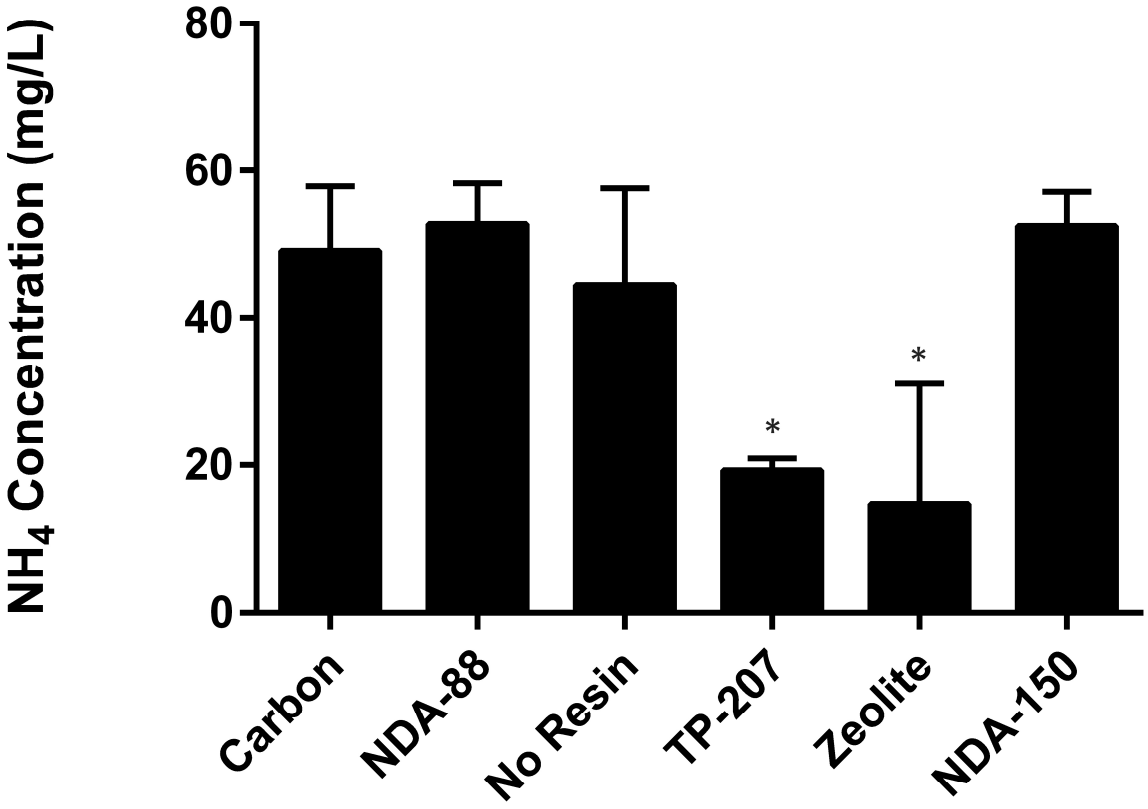
| Element | Detection limit (μg/L) |
|---|---|
| Cadmium | 0.00009[a] |
| Copper | 0.0002[b] |
| Lead | 0.00004[a] |
| Zinc | 0.0003[a] |

[a] In DRC mode in Class-100 Clean Room using Pt cones and quartz sample-introduction system.

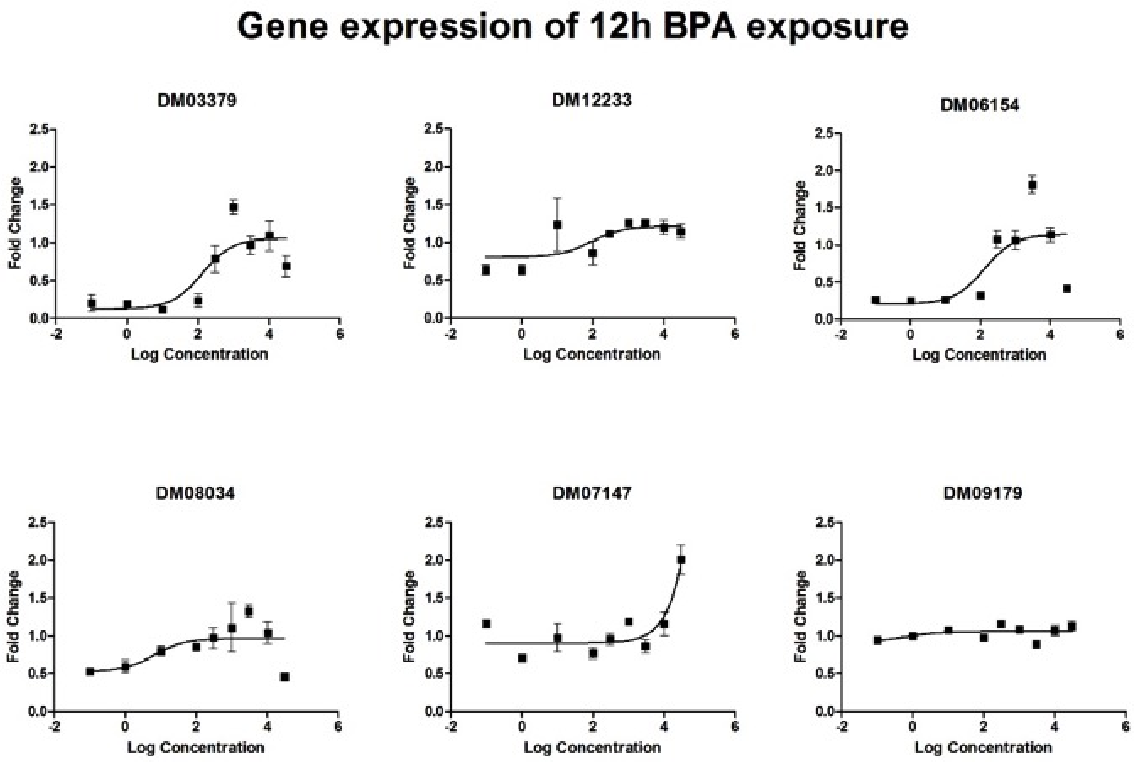
[b] In standard mode in Class-100 Clean Room using Pt cones and quartz sample-introduction system.




iTIE_System_Picture_Diagram .




Supplemental_Data%2C_Figure_S1 .




Supplemental_Data%2C_Figure_S2 .