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Linkage of Genomic Biomarkers to Whole Organism End Points in a Toxicity Identification Evaluation (TIE)

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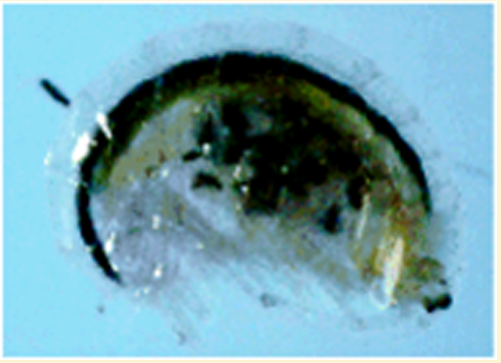
Supporting Information

ABSTRACT: Aquatic organisms are exposed to many toxic chemicals and interpreting the cause and effect relationships between occurrence and impairment is difficult. Toxicity Identification Evaluation (TIE) provides a systematic approach for identifying responsible toxicants. TIE relies on relatively uninformative and potentially insensitive toxicological end points. Gene expression analysis may provide needed sensitivity and specificity aiding in the identification of primary toxicants. The current work aims to determine the added benefit of integrating gene expression end points into the TIE process. A cDNA library and a custom microarray were constructed for the marine amphipod *Ampelisca abdita*. Phase 1 TIEs were conducted using 10% and 40% dilutions of acutely toxic sediment. Gene expression was monitored in survivors and controls. An expression-based classifier was developed and evaluated against control organisms, organisms exposed to low or medium toxicity diluted sediment, and chemically selective manipulations of highly toxic sediment. The expression-based classifier correctly identified organisms exposed to toxic sediment even when little mortality was observed, suggesting enhanced sensitivity of the TIE process. The ability of the expression-based end point to correctly identify toxic sediment was lost concomitantly with acute toxicity when organic contaminants were removed. Taken together, this suggests that gene expression enhances the performance of the TIE process.



INTRODUCTION

Aquatic environments near urban and industrial areas are often contaminated with mixtures of chemicals, which may be capable of exerting toxic effects on aquatic species. Interpreting the relationship between exposure to this mixture of chemicals and observed ecotoxicological impairment is often difficult. Toxicity Identification Evaluation (TIE) is an approach to systematically identify causes of toxicity by coupling biological responses with physicochemical manipulations of environmental samples and chemical analytical methods.[1,2] This approach has been used effectively in a variety of environmental media, including fresh[3] and marine waters,[4] wastewater effluent,[3] pore (interstitial waters)[5] and whole sediment.[6] Moreover, TIEs have also been integrated into a regulatory context as part of permitting under the National Pollutant Discharge Elimination System (NPDES)[7] and implementation of total maximum daily load (TMDL) process as required by the Clean Water Act.[8]

The TIE process consists of three phases: characterization (Phase 1), identification (Phase 2), and confirmation (Phase 3). Phase 1 manipulations are intended to generally characterize toxicants into broad categories (e.g., nonionic organic chemicals, metals, ammonia) which are used to direct phase 2 analyses.[2,3] Phase 2 builds on phase 1 data and employs more refined, targeted manipulations in combination with analytical chemistry to identify the specific toxicant(s) putatively responsible for the observed toxicological response (e.g., the pesticide DDT, copper).[2,9] Finally, the aim of phase 3 is then to confirm, using independent methods, that the toxicant(s) identified in phases 1 and 2 are indeed responsible for the toxicity of the whole sample.[2,10]

There have been significant improvements in the specificity of the manipulations[11,12] and analytical chemical methods used in the TIE process; however, there has not been corresponding progress in the diversity of bioassays employed. The TIE process emphasizes the use of whole organisms and typically focuses on growth, reproduction and mortality in order to link the toxicological response to observed ecotoxicological impair-

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ment. Although these end points have been shown to provide meaningful linkages between laboratory results and observed ecological effects,[13,14] they may be somewhat uninformative in terms of identifying causative agents, as they can be affected by a diverse set of toxicants. In contrast to TIE, Effects Directed Analysis (EDA) tends to use cellular end points aimed at targeting toxicologically important modes of actions (MOA) such as mutagenicity, and teratogenesis [15] or groups of toxicants such as endocrine disruptors.[16] Although these end points are more informative, they are more difficult to relate to ecotoxicological effects due to the use of cultured cells, which do not account for organ and organismal biotransformation of toxicants, and from technical artifacts resulting from manipulations of the environmental samples which may alter the bioavailability or toxicity of toxicants in the resulting sample.[1] With genomic technologies it is possible to measure cellular responses within the same organism that whole organism end points are measured. By focusing within the same organism, alternative toxicity end points may increase the sensitivity and discriminative ability of the TIE process without sacrificing ecological relevance.

Genomic technologies are able to identify genes or proteins that respond to chemical exposures. Commercially available platforms have the ability to monitor the expression of thousands of genes simultaneously, which has led to the hope that these end points may be used to establish transcriptional "fingerprints" of particular classes of toxicants or individual contaminants. Reproducible responses of genomic end points to exposures to a number of chemical toxicants in aquatic organisms has previously been demonstrated.[17−20] There have been several examples where genomic tools have been shown to be helpful in identifying responsible chemical stressors in complex environmental exposures.[21,22] Expression of genes or proteins are thought to be associated with particular modes of action, which can be useful in relating genomic end points to impacted traditional ecotoxicological end points, thereby providing additional evidence of ecotoxicological relevance and specificity to the TIE process.[23] Additionally, by incorporating genomic end points early in the TIE process, provided data may be used to direct subsequent phases increasing throughput and efficiency

The current project aims to link genomic biomarkers to traditional toxicity responses through the whole sediment TIE process.[2] The use of physical-chemical manipulations of environmental samples as part of the TIE and its reliance on whole organism end points when coupled with the sensitivity and discriminative ability of genomic end points is hypothesized to offer complementary information to further build a weight of evidence for the identification of unknown toxicants. For the current effort, the marine amphipod *Ampelisca abdita* was selected as the toxicity testing organism. Although used extensively in sediment testing and regulation, DNA sequence data for this organism has not been previously reported, and microarrays were not available for its study. Here, we provide a partial transcriptome sequence for *A. abdita*, which was subsequently used to develop a functional microarray. We then compared results from a side-by-side TIE conducted on a contaminated marine sediment using traditional toxicity testing of *A. abdita* and our now publicly available microarray.

MATERIALS AND METHODS

Sediment Collection. Elizabeth River (ER) sediments were collected from the Eppinger and Russell creosote site (36° 7.7′ N and 76° 3.0′ W). Surface sediment samples (∼2 cm) were collected using a Van Veen sampler in approximately four meters of water on 14 May 2003. Following collection and refrigerated shipment to the laboratory, large components of the sediment including rocks and sticks were removed. Reference sediments from Long Island Sound (LIS) were collected April 2005 at 41° 7.95′ N 72° 52.7′ W in ∼24 m of water using a Smith MacIntyre grab (0.1 m^2). Prior to use, LIS sediment was press-sieved through a 2 mm stainless steel sieve. Dilutions of the ER River sediment were prepared using LIS sediment. To prepare a dilution, wet LIS and ER sediments were combined by mass in the required ratio in a large glass jar (e.g., 10%, 40% ER), mixed manually, and then mixed mechanically for several minutes. All sediments were stored at 4 °C in the dark in sealed glass jars until used in the studies.

Preparation of Model Toxicant-amended Sediments. Test samples of model toxicants suspected or known to occur in Elizabeth River sediment included the polycyclic aromatic hydrocarbons (PAHs) phenanthrene and benzo[a]pyrene(B-[a]P), the pesticides DDT and dieldrin, the antimicrobial agent triclosan (Sigma-Aldrich, St. Louis, MO, U.S.), and a degraded fuel oil mixture consisting of *n*-heptadecane, *n*-octadecane, pristane and phytane (ULTRA Scientific, North Kingtown, RI, U.S.) Three higher order mixtures were also prepared: (1) phenanthrene and benzo[a]pyrene; (2) a mixture of three toxic divalent transition metals, cadmium ($CdCl_2$), nickel ($NiCl_2{\cdot}6H_2O$) and zinc ($ZnSO_4{\cdot}7H_2O$), and (3) phenanthrene, benzo[a]pyrene, DDT, dieldrin, the degraded fuel oil mixture, triclosan and the three metals (i.e., "grand mixture"; Supporting Information, SI, Table 1). Organic model toxicants were amended into LIS reference sediment at a concentration equivalent to 10% of the known or estimated water-only LC_{50} value. If an LC_{50} was not available in the scientific literature, then narcosis theory was used to estimate an LC_{50} in water.[24−26] Further, using the known or estimated 10% LC_{50} value, equilibrium partitioning was used to calculate the sediment LC_{50} values (μg/g organic carbon (OC)).[27] For metals, the acid volatile sulfide (AVS) content of the LIS reference sediment was used to prepare a nonacutely toxic sediment concentration 0.1 metal:AVS ratio (i.e., at a metal:AVS ratio greater than 1.0 the sediment would be acutely toxic).[28,29] The three mixtures were also prepared such that the total toxicity would be equivalent to 10% of the LC_{50}s of the chemicals present or a metal:AVS ratio of 0.1.

For sediment amendments, organic chemicals were prepared in acetone and plated onto the wall of a glass jar, a small amount of muffled fine grained sand was added, the solvent was allowed to evaporate, and wet LIS reference sediment was added. The sediments were then homogenized vigorously in the jars manually for several minutes. For metals, metal salts were added to the glass jars and allowed to dissolve into a small volume of deionized water with fine grained beach sand, the wet LIS reference sediment was then added, and the jar contents homogenized vigorously manually for several minutes. Prepared sediments were stored at 4 °C in the dark in sealed glass jars for approximately six weeks until use in these studies. Prior to use, all sediments were thoroughly homogenized.

Elizabeth River Sediment Exposures. *A. abdita* were field collected from the John H. Chafee National Wildlife Refuge at Pettaquamscutt Cove (Narrow River), Narragansett, Rhode Island, U.S. (N 41826.920, W 71827.510). This area is an estuarine wildlife refuge of over 300 acres of saltmarsh and surrounding forest habitat (salinity range 20−30 ppth), with no

noted sources of contaminants. Juvenile organisms that passed through a 1.0 mm screen, but were retained on a 0.7 mm screen were selected for the experiments. During holding and acclimation, amphipods were fed cultured algae (*Tetraselmis suecica*), and held in LIS reference sediment at 20 ± 3 °C for at least 24 h prior to testing. Each replicate consisted of 20 g of wet sediment and 60 mL of overlying reconstituted seawater.[2] Sediment-water replicates were allowed to equilibrate for 24 h prior to adding organisms. Ten amphipods were added to each replicate. All tests were static, aerated exposures using 30 parts per thousand reconstituted seawater. Tests were conducted for seven days because it was determined that this duration was sufficient to determine if the TIE manipulations were effective.

Two initial toxicity tests were performed to determine the concentration of ER sediment that caused 30−40% mortality in organisms. Range finding experiments were conducted at high (100%, 75%, 50% and 25%) and low (50%, 25%, 12.5%, 6.25%, 3.13% and 1.56%) percentages of ER. On the basis of range finding toxicity tests, the initial TIE was performed on a mixture of 10% ER: 90% LIS sediment. TIE manipulations for both ER and LIS controls included additions of: (1) coconut charcoal (CC) to bind and thereby reduce the bioavailability of organic toxicants, (2) cation exchange resin (CE) to bind metal toxicants, and (3) zeolite (Z) to reduce ammonia-related toxicity.[2] A minimum of six replicates for each manipulation and operational control were used. A second TIE was performed as described above, except a 40% ER: 60% LIS sediment mixture was used.

Upon test termination, the amphipods were sieved through a 0.5 mm screen and enumerated. Missing organisms were scored as dead. For the high and low range finding toxicity tests, 10% ER TIE and 40% ER TIE and corresponding manipulations, live organisms from each replicate were divided into groups of four or less, placed in a microcentrifuge tube, immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. In order to minimize the effect of individual variability and to obtain a more stable and representative gene expression profile for the model toxicants, all surviving organisms from model toxicant exposures within a replicate (exposure jar) were pooled in a single tube, otherwise they were handled as described above.

Microarray Development. In order to generate gene expression data, a microarray was developed for *A. abdita*. Organisms used for gene expression libraries were from two naturally occurring populations, Narrow River, Narragansett, RI and Jamaica Bay, NY, USA and included multiple age classes, as well as organisms exposed to model chemicals (SI Table 2). Total RNA from all samples was isolated using TriReagent (MRC Research, Cincinnati, OH) according to the manufacturer's protocol and genomic contamination was removed (DNA-free, Life Technologies, Grand Island, NY). RNA purity and quality were assessed using 260:280 ratios and glyoxal gel electrophoresis. Following isolation, equal mass amounts of each RNA sample was pooled (see SI Table 2 for samples), precipitated and resuspended in water. Normalized cDNA libraries were constructed using The Trimmer Direct cDNA Normalization kit and protocol (Evrogen, Moscow, Russia). A detailed description of the normalized libraray construction can be found in the Supporting Information.

Finished sequence data was submitted to Agilent Technologies (Santa Clara, CA) using their e-array web application (https://earray.chem.agilent.com/earray/) for probe design and printing using their 8 × 15k custom microarray (catalog number G2509F) technology. There are 7351 noncontrol probesets per array, consisting of 2 (n = 7331 probesets) or 22 (n = 20) identical 60-mer probes per probeset.

Gene Expression Analysis and Hybridizations. RNA was isolated and genomic DNA contamination was removed as described above. Though surviving organisms from each exposure vessel were split into multiple tubes (with the exception of model chemical exposures), each containing 1−4 organisms, only a single tube from each exposure vessel was used for microarray analysis. The remaining tubes from a single replicate exposure were saved and were used only if technical issues arose in labeling or hybridization. Total RNA (90 ng) was labeled with cyanine 3-labeled CTP using the One-color Low Input Quick Amp Labeling kit (Agilent, Santa Clara, CA) according to the manufacturer's protocol (v 6.5: part number G4140−90040). Labeled RNAs were purified using RNeasy mini spin columns (Qiagen) and the yield and specific activity were determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). If cRNA yield was below 0.825 μg or the specific activity was below 6 pmol per μg cRNA, then an alternative replicate sample from the same exposure vessel was labeled. If none existed, a new labeling reaction with the original sample was conducted.

Sample placement on the slides was randomized. Due to the large number of samples (168), hybridizations were carried out over two consecutive days. Hybridizations were done according to the manufacturer's protocol (v 6.5). Following hybridizations and washing, the arrays were immersed in drying and stabilization solution (Agilent), and covered with ozone barrier slide covers and immediately placed in the Agilent G2565BA scanner and scanned. If samples failed QA/QC following hybridization and scanning, then samples were hybridized again; however, sample placement on the arrays and within the array set was blocked rather than randomized so that samples from the same treatment group were not run together.

Microarray Analysis. General data processing was performed using Perl. Sequence comparisons were performed using BLAST [30] version 2.2.14 (ftp://ftp.ncbi.nlm.nih.gov/blast). Statistical analysis was performed using R[31] version 2.12.1. In addition to using functions in the standard R base packages, L1 regularized logistic regression[32] was conducted using package glmnet (version 1.7.1), and random forest classification[33] was conducted using package randomForest (version 4.6.2). Refer to Supporting Information for specifics regarding microarray analysis

The entire microarray data set is available through the National Center for Biotechnology Information Gene Expression Omnibus (accession: GSE41463).

Experimental Design. Samples were divided into two groups. The first group, which was used for developing the classifier, consisted of all whole (unmanipulated) ER samples displaying greater than 20% mortality and all whole LIS controls. The mortality cutoff of 20% was selected to ensure that organisms included in the "exposed" group of the training set, were exposed to sufficient levels of contaminants to ensure expression responses. The second group, which consisted of all whole ER samples that displayed less than 20% mortality, ER samples manipulated during the TIE process, TIE manipulated LIS controls and model chemical exposures, were grouped into what will henceforth be referred to as the "evaluation set."

For classifier tuning and evaluation, LIS samples were treated as controls, and surviving samples from ER dilutions showing more than 20% lethality were treated as cases. 7-fold stratified

cross-validation repeated with 72 randomly varied splits into folds,[34] generating 504 distinct training set/test set splits, was used to estimate classifier generalization error. Test-set accuracy was expressed as the empirical area under the operating characteristic curve (eAUC).[35] For each successive "training set", a two-sample *t* test (cases vs controls) was conducted for each probeset, and a *p*-value cutoff corresponding to a false discovery rate (FDR) of 0.01, was estimated by fitting the set of nominal *p*-values (one for each probeset) to a beta-uniform mixture model, representing a mixture of differentially expressed and nondifferentially expressed genes.[36] Probesets meeting the p-value cutoff from each training set were used to fit L1 regularized logistic regression (cv.glmnet parameters: family="binomial", nfolds=5, type.measure="deviance") and random forest classification (randomForest parameters: importance=F) models, which were then evaluated against the corresponding test set samples

Algorithm Accuracy and Comparison. LIS samples (n = 31) were treated as controls, and surviving samples from ER dilutions showing more than 20% lethality (n = 13) were treated as cases. In order to estimate generalization error, stratified cross-validation with randomly varied splits (resulting in 504 distinct training set−test set splits) was used to evaluate the accuracy of the classifier, which was expressed as the empirical area under the operating characteristic curve (eAUC). For regularized logistic regression-based analysis, eAUCs were between 0.75 and 1, with the vast majority of test sets (492/504) having an eAUC of 1, suggesting near perfect classification of the test set. Similarly, when random forest was used for classifier construction, eAUCs ranged between 0.75 and 1, with a slightly smaller majority of test sets (479/504) having an eAUC of 1. On the basis of this nominal difference in accuracy, as well as its tendency to select a much more parsimonious predictor set (data not shown), regularized logistic regression was selected for downstream analysis. Regularized logistic regression-based analysis chose two features (seq5064 and seq4524) as predictors in more than 500 out of 504 folds. Five additional features (seq1079, seq5751, seq1627, seq4331, and seq3030) were chosen in over half the folds.

A regularized logistic regression model was subsequently fit to the entire training data set (all 31 LIS control samples and 13 samples treated with diluted ER sediment showing more than 20% lethality). In order to reduce the number of probesets evaluated for the classifier, probesets were filtered using an FDR of 0.01 corresponding to a p value cutoff of 2.16×10^{-4}, resulting in retention of 129 probesets. Regularized logistic regression resulted in selection of 13 predictors, including the seven features selected in the majority of cross-validation iterations used to estimate generalization error (above), as well as 6 additional features (seq3269, seq4393, seq4466, seq580, seq5831, and seq5878). This model was used to predict the classes of samples in the evaluation set, which were not used to generate the classifier.

RESULTS AND DISCUSSION

Comparison of TIE and Genomic Classification Results. The predicted classifications for the evaluation set, as well as results from the mortality assay for the corresponding treatment group, are summarized in Table 1. The top block in this table shows results for the ER treatments. Little mortality was observed in any of the three ER dilutions, with only the 10% ER dilution displaying slightly increased mortality relative to the corresponding control (Fisher's exact test one-sided p-value ≤0.05). In comparison, the gene expression based classifier was able to correctly discriminate amphipods that were exposed to ER sediment (10% and 3.13% ER treatments) from those that were exposed to the corresponding LIS controls despite the absence of high levels of toxicity. Even in as little as 1.56% ER exposure, the gene expression based classifier identified 33% of the samples as belonging to ER. This suggests that in the current study, the expression-based classification was more sensitive than the traditional toxicological end point. Moreover, given that toxicant independent differences in soil type have previously been shown to affect gene expression,[37] the ability of the classifier to discriminate ER and LIS given the high dilution suggests that gene expression differences between ER and LIS were dependent on toxicants rather than differences in sediment type.

In order to evaluate the false positive rate, the classifier was tested using the LIS samples, which were run as controls during

Table 1. Comparison of Gene Expression Classifier and Toxicity End Points[a]

	gene expression			toxicity		
treatment	% identified as ER	control	ER	% mortality	live	dead
(1) ER samples <20% lethality						
ER 10%	83.3[b]	1	5	15[b]	51	9
ER 3.13%	66.7[b]	1	2	6.7	28	2
ER 1.56%	33.3	2	1	0.0	30	0
(2) manipulated LIS						
zeolite 40%	0	6	0	8.3	55	5
zeolite 10%	0	5	0	3.3	58	2
cation exchange 40%	0	5	0	11.1	54	6
cation exchange 10%	0	6	0	3.3	58	2
coconut charcoal 40%	0	6	0	3.3	58	2
coconut charcoal 10%	0	5	0	3.3	58	2
(3) manipulated ER (40% dilution)						
zeolite	100[b]	0	3	86.7[b]	8	52
cation exchange	100[b]	0	4	51.7[b]	29	31
coconut charcoal	16.7	5	1	6.7	56	4
(4) model chemical exposures						
triclosan	16.7%	5	1	1.7%	59	1
dieldran	0%	6	0	3.3%	58	2
DDT	0%	6	0	5.0%	57	3
phenanthrene	0%	6	0	3.3%	58	2
B[a]P	0%	6	0	1.7%	59	1
phenanthrene/B[a]P	0%	3	0	10%	45	5
metal mixture	0%	5	0	6.7%	56	4
grand mixture	0%	5	0	8.3%	55	5
degraded petroleum	0%	6	0	4.3%	67	3

[a]Gene expression classifiers were used to classify (1) surviving *A. abdita* exposed to a diluted Elizabeth River (ER) sediment, (2) LIS control sediment that have been manipulated as part of a TIE conducted on 10% or 40% ER, (3) ER sediment that has been manipulated as part of the ER process, (4) LIS sediment that has been amended with suspected toxicants. Numbers in Control or ER columns refer to the number of individuals that were classified as either control or ER. Live and Dead columns refer to the number of individuals in those respective groups. [b]Indicates statistical significance at $p \leq 0.05$ (One-sided Fisher's Exact Test).

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each of the TIEs (Table 1, second block). These controls were manipulated in an identical manner to the ER samples. No significant mortality was observed in any of these replicates (n = 33). The classifier correctly identified all of the LIS controls as controls (Table 1) with a very narrow range of probability scores among individuals within each group (Figure 1; SI Table 3), suggesting a low false positive rate for the microarray-based classification. That the classifier correctly identified all LIS samples as control, despite the TIE manipulations, further suggests that changes in gene expression observed following the TIE of ER sediment were not dependent on handling.

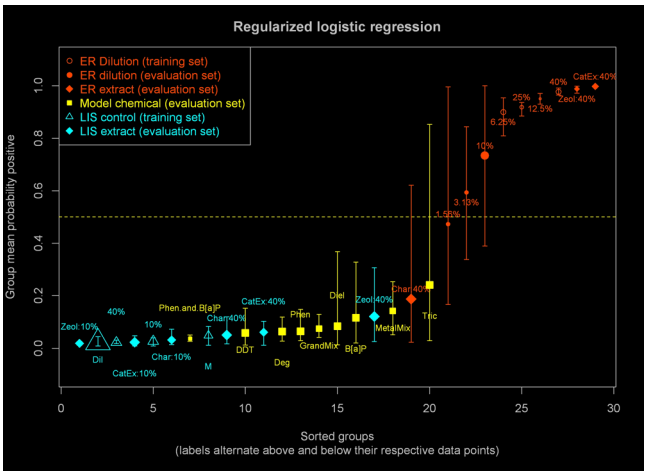

Figure 1. Group mean probability that test and training set samples were classified as ER using a gene expression-based regularized logistic regression model. Samples presented were either used in the design of the classifier (training) or were not (evaluation). Symbol size represents the number of samples in a particular set. Zeol, CatEx and Char refer to the zeolite fraction, cation exchange fraction and charcoal fractionation respectively. Phen and B[a]P, Deg, GrandMix, MetalMix, DDT, Diel, Tric refer to the amended LIS samples (SI Table 1) and M is the corresponding LIS control sample group. Dil refers to the LIS control group corresponding to ER dilution series. Percentages refer to the particular dilution of ER sediment. Error bars represent the range of probabilities within each group.

The performance of the classifier was evaluated relative to the mortality observed with the TIE manipulations of the 40% ER sediment in order to determine the relationship between gene expression and changes in mortality in the manipulated sediment. The ability of the classifier to correctly identify samples tracked fairly closely with toxicity (third block in Table 1). The zeolite manipulation did not reduce mortality and the classifier identified these samples as ER. A reduction in toxicity to control levels was observed in the CC manipulation and this treatment group was the only manipulated ER group to be classified predominately (5 of 6) as LIS by the gene expression classifier (Table 1). The concordance between the gene expression classification and the decreased mortality observed with the manipulations suggests that classifier performance is dependent on the primary toxic agent within the ER sediment rather than differences between ER and LIS sediments. The CC manipulation removes toxic nonionic organic compounds, which is consistent with previous work demonstrating high levels of PAH contamination in ER sediment.[38,39] Nevertheless, given the small number of rudimentary chemical manipulations of the phase 1 TIE, it remains possible that the microarray classifier may be tracking non- or weakly toxic substances in the ER sediment that cosegregate with the responsible toxicant in the manipulations. Functional annotation of the 13 genes comprising the classifier may provide a means to corroborate the effects of the manipulations on toxicity and the gene expression classification. In addition, the incorporation of more selective manipulations (Phase II TIE) would provide an even more reliable means to resolve potential toxicants.

CE manipulation resulted in a more modest reduction in observed mortality from 86.7% in the ER positive control to 51.7% after CE addition. However, the classifier identified all of the CE + ER treated samples as ER. This likely reflects the improved sensitivity of the gene expression classifier relative to the lethality assay, as suggested by the ER dilution results. Supporting this is the observation that the CE manipulation has been shown to reduce the toxicity of organic toxicants in addition to metals.[40] Thus, the partial sequestration of organic toxicants by CE, though significant enough to reduce mortality, may not have been effective enough to render the classifier incapable of identifying the manipulated sample as ER. As noted above, more selective fractionation, such as those used in the phase II TIE, may shed light on this.

In order to identify the toxicants responsible for ER classification by gene expression, the gene expression signature of the ER sediment was compared to that of LIS sediment amended with either single or mixtures of model toxicants (Bottom block in Table 1). The concentration of these toxicants was selected to be sublethal, with none of the toxicant treatments displaying significant lethality (one-sided Fisher's Exact Test p-value >0.05). The classifier consistently identified the amended sediments as LIS-like (98%; Figure 1), despite the presence of several toxicants known to occur at high levels in ER sediment, such as phenanthrene and B[a]P PAH mixtures.[38] Somewhat consistent with this is that the ER sediment is known to be contaminated with creosote as well as other PAHs,[41] which were not among the model toxicants, but still remain a possibility as the main driver of toxicity and would also be expected to be removed in the CC manipulation. Moreover, metabolites or breakdown products were not included among the model chemicals, which may have particular relevance here where many of the historical sources are no longer active and contaminants may have been present for several years. Of the amendments, only a single triclosan amended sample was classified as ER, although the result did not reach statistical significance (Fisher's exact test one sided p-value >0.05) for that treatment group as a whole. The single positive microarray result for a triclosan sample is difficult to interpret given the inconsistency of the classification among different triclosan-exposed samples, as it may represent an occasional false positive. The inability of the classifier to identify the chemical responsible for ER classification may also reflect the markedly low concentration of test toxicants in the exposures. Alternatively, it may suggest modified toxicity resulting from interactions of multiple toxicants in the ER sediment, as it is known to be contaminated with a number of known toxicants.[38] Again, more discriminative manipulations in a phase II TIE and comprehensive chemical analysis would aid in distinguishing between these possibilities.

Given the performance of microarray-based classification in cross-validation and classification of the evaluation set, our results show that gene expression-based biomarkers in *A. abdita* can provide clear separation or grouping of samples exposed to similar sediments with somewhat different chemical composition. Our data suggest this technology may augment the TIE

approach by identifying subtle responses more reliably and with greater specificity than a lethality-based assay. Further, following the gene expression classifier in both the whole and manipulated sediments may be useful in demonstrating that the toxicant identification did not result from technical artifact of the manipulations, thus aiding in the confirmation phase of the TIE process. A side-by-side approach that uses both gene expression based classifiers and traditional toxicological end points should provide a more powerful means of identifying toxicants than using either approach alone. The TIE process may be an ideal experimental construct for the integration of genomic technologies because it can achieve toxicological relevance of the gene expression response through the direct combination of gene expression with TIE manipulations and traditional toxicological end points. The addition of analytical chemistry confirmation of the occurrence of likely toxicants and the functional linkage of the genes differentially expressed to those toxicants and to mortality would provide a greatly strengthened weight of evidence for the prioritization of toxicants and subsequent remediation efforts.

■ ASSOCIATED CONTENT

Supporting Information

Model chemical ammendments to LIS sediment (Table S1); sample information (Table S2); supporting materials and methods describing the microarray development; and probability scores of classifications using either Random Forests or Generalized Linear Regression (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.B. developed the normalized transcriptional libraries, helped perform microarray experiments, and drafted parts of the manuscript. A.B. also helped organize the experimental workflow, helped plan the experimental design, and coordinated manuscript preparation. M.K. analyzed sequence and microarray data, developed the classifiers, and drafted the corresponding sections of the manuscript. D.B. and R.F. were involved in library construction and in conducting microarray experiments. K.T.H. and R.M.B. were involved in project coordination between the laboratories and establishing the study design. R.M.B. was also involved in preparing the model toxicants including their amendment into the reference sediment. K.T.H., M.M.P., L.M.P. and M.C.P. performed the whole sediment toxicity testing and TIEs. M.R. was involved in experimental study design, collection of sediments and organisms, and in manuscript editing.

Notes

The authors declare no competing financial interest.

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