Application of Gene Expression Analysis for Sediment Toxicity Stressor Identification

by
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EXECUTIVE SUMMARY

Sediment toxicity tests are frequently used to assess sediment quality along the west coast of North America. However, the use of sediment toxicity information to assist in the development of management actions (e.g., source control, sediment remediation) is limited by the difficulty of determining which contaminants are responsible for the toxic effects. An investigative process known as a Toxicity Identification Evaluation (TIE) is one approach commonly used to determine the toxicants present in environmental samples. However, the TIE process can be expensive, time consuming, and may yield a nonspecific result due to low toxicity or a lack of analytical methods. These constraints limit the application of TIEs, resulting in uncertainty regarding the cause of sediment toxicity in coastal water bodies such as San Francisco Bay.

This report describes the results of a project to investigate the feasibility of a new sediment TIE approach, known as a molecular TIE. This approach uses changes in gene expression of the toxicity test organism, rather than chemical manipulation, as a method for identifying the cause of sediment toxicity. The research utilized a newly-developed gene microarray for the estuarine amphipod *Eohaustorius estuarius,* a widely used sediment toxicity test organism. The project was designed to accomplish three objectives:

- **Determine gene expression in amphipods exposed to diverse contaminant types.**
- **Develop tools for toxicant identification.**
- **Evaluate toxicant identification ability**.

Samples of *E. estuarius* from a variety of sediment and water toxicity tests were analyzed using the microarray. A training data set consisting of a total of 16 different chemical treatments, each with matching controls, was analyzed. The treatments represented a diversity of contaminants of concern, such as trace metals, chlorinated pesticides, petroleum hydrocarbons, and current use pesticides. The RNA from each replicate was extracted and analyzed using the microarray. The gene expression results for the exposed samples were compared to matching controls in order to calculate differential gene expression (i.e., ratio of expression in test sample relative to control).

Two preliminary approaches for classifying unknown samples were developed using the training data: cluster analysis and class prediction models based on random forest analysis. The effectiveness of the classification methods was tested by analyzing independent evaluation samples having different chemical characteristics.

Each training sample analyzed showed evidence of differential gene expression. Both up-regulation and down-regulation of genes were evident in all samples. A total of 3182 microarray probes with statistically significant differential gene expression (i.e., candidate genes) were identified in the training samples. Between 12% and 54% of the candidate genes in a treatment group were uniquely expressed (i.e., had significant differential expression in only one chemical treatment group). The magnitude of differential expression for individual gene probes also varied widely among the samples, with extremes ranging from 40-fold down-regulation for fipronil to 100-fold up-regulation for DDT.

Cluster analysis identified seven clusters of the 16 training sample types, indicating that characteristic patterns of gene expression were present among the samples. Three clusters were

composed of single chemicals (copper 750, DDT, and fipronil), suggesting that these chemicals produced very distinctive patterns of expression. The remaining clusters each contained 2-4 chemical treatments of variable composition. For example, one cluster was comprised of samples exposed to pyrene, chlordane, and cadmium. Samples representing different exposure concentrations of the same chemical usually grouped in the same cluster, suggesting consistency of gene expression patterns within chemical type.

Three independent evaluation samples (not part of the training data set) were used to test the ability of the candidate molecular TIE methods to identify the cause of toxicity. One sample (T1) was from a spiked sediment exposure to cyfluthrin, a pyrethroid pesticide. A second sample (T2) was from a field site in southern California where TIEs had determined the cause of toxicity to be pyrethroid pesticides. The third evaluation sample (T3) was from RMP monitoring in San Francisco Bay and the cause of toxicity was not known. Both of the field sediment samples were contaminated by a complex mixture of trace metals and trace organics.

Cluster analysis yielded limited success in classifying the evaluation samples. Sample T1 (cyfluthrin exposure) was grouped into the first cluster, which also contained samples exposed to bifenthrin, ammonia, and pyrene. It was expected that sample TI would cluster with the other cyfluthrin samples, which were grouped nearby in a separate cluster. However, bifenthrin is a pyrethroid pesticide that is chemically similar to cyfluthrin and has a similar mode of toxic action. Sample T2 (pyrethroid contaminated) was grouped into a cluster with two other chemicals: chlordane and pyrene. Neither of these chemicals has a chemical similarity to pyrethroids, which were identified by TIE as the cause of toxicity at this site.

Results for the third evaluation sample (T3) were similar to those for T1; this samples was grouped into the same cluster associated with bifenthrin, ammonia, and pyrene. There was no a priori expectation regarding the cause of toxicity in this sample. However, both PAHs (such as pyrene) and ammonia have been suggested as contributing to sediment toxicity in portions of San Francisco Bay.

More accurate classification results were obtained using a class prediction model developed using random forest analysis. This analysis developed a prediction model based on three stressor classes: pyrethroid pesticide, trace organic (other than pyrethroids), and other (e.g., metals, ammonia). Class predictions using the evaluation samples gave results consistent with expectations. Replicate samples of T1 and T2 were correctly classified into the Pyrethroid category 100% of the time when average response values were used. Variable results were obtained for sample T3 using random forest, with classification into both the Pyrethroid and Trace Organic categories.

This project has achieved several important milestones in the effort to develop a molecular TIE method for sediment toxicity. First, a gene microarray for *E. estuarius* was successfully developed based on the first ever sequencing of RNA from this amphipod. Second, this microarray was used to identify a subset of candidate genes having differential expression in response to 11 different types of chemical exposure. Third, many of the chemical treatments were shown to produce distinctive patterns of differential expression, confirming a key assumption of the approach. Finally, we developed and applied multiple approaches for

evaluating unknown samples. Some of these approaches were shown to correctly identify the cause of toxicity in independent evaluation samples, providing a demonstration that a molecular TIE approach is feasible and has the potential to provide an effective and powerful tool for sediment TIEs.

Several data gaps and areas of uncertainty need to be addressed before this molecular TIE approach can be used with confidence in monitoring programs. A primary data gap is the lack of gene expression information for many contaminants of concern. Contaminant groups particularly underrepresented in the current training set include PAHs, trace metals, and PCBs. It is possible that greater discrimination between the evaluation sample from San Francisco Bay (T3) and the other evaluation samples would have been obtained had the training data set included these additional constituents.

Standardization of sample preparation and analysis methods is needed to develop a reliable tool for use in multiple laboratories. There are few standard protocols for sample preservation, extraction, analysis, and quality assurance, especially for marine invertebrates. Consequently, data for the same sample analyzed by different laboratories may vary as a result of method variations, possibly leading to incorrect conclusions regarding the gene expression patterns obtained. Interlaboratory studies are needed to investigate these issues and develop methods that yield comparable results among laboratories. This issue is of importance to all TIE methods, as the methods must be accurate and reliable in order for end users to have confidence in their use for guiding environmental management actions that may have high cost.

Finally, further development and evaluation of classification models based on gene expression is needed. This study investigated only two types of statistical approaches for stressor identification: clustering and random forest analysis. Alternative data analysis methods should be investigated to help in determining the most accurate approach for TIE applications. A molecular TIE method, like other stressor identification methods, must be accurate and reliable in order for the results to be accepted by management agencies and used to guide potentially costly clean up and control actions.

The research conducted in this study has laid the foundation for development of a new and potentially effective TIE approach with wide application. While further development and validation is needed, this project has demonstrated that rapid progress can be made in these areas through collaboration and partnership.

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INTRODUCTION

Sediments in marine ecosystems accumulate numerous contaminants from point sources (e.g., municipal and industrial effluents) and non point sources (e.g., urban and agricultural runoff) and may become toxic to sediment dwelling organisms. The assessment of sediment quality is required by many monitoring and regulatory programs. Sediment toxicity tests with sediment dwelling organisms, such as the marine amphipod *Eohaustorius estuarius*, are frequently used to assess sediment quality along the west coast of North America. However, the use of sediment toxicity information to assist in the development of management actions (e.g., source control, sediment remediation) is limited by the difficulty of determining which contaminants are responsible for the toxic effects. This difficulty is due to the presence of complex mixtures of contaminants at most sites, incomplete chemical characterization of the sediments, and limitations in the ability of chemical measurements to reliably determine the bioavailable fraction of toxicants.

The Toxicity Identification Evaluation (TIE) process is used to determine the causal agents in environmental samples. This process uses a variety of chemical/physical separation methods and treatments to remove one or more toxicant classes, coupled with toxicity testing following each manipulation. A variety of sediment TIE treatments have been developed for use with either whole sediment or pore water (USEPA, 2007; Holmes et al., 2008). These methods are most successful in differentiating broad classes of toxicants in sediments where a high level of acute toxicity is present. The use of sediment TIEs in assessment programs is frequently hampered by their high cost, sample volume, and time requirements (due to the many treatments that must be evaluated), and by their limited effectiveness in differentiating among specific types of contaminants. The limitations to the effectiveness of existing sediment TIE methods are related to a variety of factors, including: 1) most toxicity is at a low to moderate level where the results are difficult to interpret due to variability in test response; 2) complex mixtures of contaminants are present that are difficult to separate from one another for evaluation; and 3) lack of chemical-specific identification methods for many types of trace organic contaminants. These factors are likely to become even more limiting with regards to evaluating the significance of contamination by new compounds of emerging concern, for which TIE methods are unavailable.

Multiple research institutions are investigating a new TIE approach that has the potential to reduce the limitations identified above. This method, known as a molecular TIE, is based on measuring changes in gene expression of the test organism (Poynton et al. 2008a, Poynton and Vulpe 2008). Recent research has demonstrated that aquatic organisms produce distinctive patterns of gene expression in response to contaminant stress, and that these patterns can be used in a diagnostic manner to investigate the cause of toxicity (Garcia-Reyero et al. 2009, Poynton et al. 2008b,c). The molecular TIE approach has the potential to greatly enhance the success and applicability of sediment TIEs, yet the method must first be developed and evaluated. A key assumption of the approach must be confirmed: that there is a predictive and chemical specific relationship between contaminant- induced responses at the molecular level (e.g., gene expression) and toxicity (e.g., mortality) that can be used to identify the stressor(s). Most molecular TIE development research has focused on water column species such as the cladoceran *Daphnia magna*, and have limited applicability for use with sediments.

Prior grant support from Environment Canada has resulted in significant progress in the development of a molecular TIE method for use with sediment toxicity testing. Recent accomplishments include *de novo* sequencing and gene microarray development for the estuarine amphipod *Eohaustorius estuarius* (Bay et al. 2010a). The custom microarray resulting from this effort contains 8,610 probes for unique gene sequences. This array is commercially available and can be used with equipment and methods widely available in most genomics laboratories.

Several additional development steps are needed before the *E. estuarius* molecular TIE tool is ready for application in monitoring and assessment programs; these include a demonstration of the approach's ability to identify contaminants causing toxicity in test samples, development of molecular and statistical analytical methods, and evaluation of interlaboratory data comparability.

Funding was provided by the San Francisco Bay Regional Monitoring Program (RMP) and Environment Canada's Marine Protection Programs Section to investigate the feasibility of gene expression analysis as a method for identifying the cause of sediment toxicity in San Francisco Estuary and other coastal water bodies. The project was designed to accomplish the following objectives:

1) **Determine gene expression in amphipods exposed to diverse contaminant types.** Samples of amphipods exposed to selected contaminants of concern for sediment quality assessment will be analyzed using the microarray to determine changes in gene expression relative to controls (differential gene expression).
2) **Develop tools for toxicant identification.** Statistical analysis will be used determine whether distinctive gene expression patterns are produced by exposure to each contaminant type. These data will be used to develop preliminary models for toxicant class prediction.
3) **Evaluate toxicant identification ability**. Gene expression results for an independent set of evaluation samples will be analyzed to determine if the toxicant class prediction models can correctly identify the cause of toxicity in blind samples.

This report describes the results of the project.

METHODS

Study Design

Four tasks were conducted to accomplish the project objectives:

1) **Analysis of training samples.** Samples of *E. estuarius* from a variety of sediment and water toxicity tests were analyzed using the microarray. Replicate samples from each experiment were analyzed. A total of 16 different chemical treatments, each with matching controls, were analyzed. The treatments represented a diversity of contaminants of concern, such as trace metals, chlorinated pesticides, petroleum hydrocarbons, and current use pesticides. The RNA from each replicate was extracted and analyzed using the microarray.
2) **Identification of candidate genes.** The gene expression results for the exposed samples were compared to matching controls in order to calculate differential gene expression (i.e., ratio of expression in test sample relative to control). Data for replicate samples and duplicate microarray probes were combined to generate a subset of robust mean differential expression values for use in subsequent analyses for toxicant identification.
3) **Development of toxicity identification approaches.** Numerous statistical methods are available for making class predictions (e.g., toxicant type identification) from gene expression data. Two candidate molecular TIE analysis methods, representing different statistical approaches, were used in this study: cluster analysis and tree-structured classifiers based on identically distributed random vectors (random forest). Cluster analysis was used to determine whether the treatments had distinctive gene expression patterns and grouped into clusters related by chemical class or exposure level. Random forests were used to develop a prediction model for three stressor classes: pyrethroid pesticide, trace organic (other than pyrethroids), and other (e.g., metals, ammonia).
4) **Analysis of evaluation samples**. Three independent evaluation samples (not part of the training data set) were used to test the ability of the candidate molecular TIE methods to identify the cause of toxicity. One sample was from a spiked chemical exposure, one from a field study were the cause of toxicity was determined using conventional TIE methods, and the final sample was from San Francisco Bay where the cause of toxicity was unknown. The identity and probable cause of toxicity for each sample was not known to the analyst.

Toxicant Exposures

E. estuarius were collected from Yaquina Bay (Oregon) and acclimated under laboratory conditions. Amphipods were exposed to various treatments in sediment or seawater for four to ten days according to standard procedures (USEPA 1994). Tests were 10-d sediment exposures or 4-d to 10-d water exposures conducted under controlled laboratory conditions. Tests were conducted at 15 C, a salinity of 20 g/kg, and under constant illumination (sediment exposure) or constant darkness (water exposure). Multiple replicate test chambers, consisting of 20 (sediment) or 10 (water) amphipods were exposed for each treatment.

Samples for training (e.g., candidate gene identification or molecular TIE approach development) or evaluation were obtained from experiments conducted by laboratories in California (USA) or Vancouver (British Columbia, Canada). The training samples represented exposure to a diverse array of contaminants spiked into sediment or water, and included metals,

chlorinated pesticides, pyrethroid pesticides, and ammonia (Table 1). Most treatments represented low concentrations that were near the threshold of mortality for *E. estuarius*. Samples from exposures producing a low level of mortality were intentionally selected in order to increase the likelihood of detecting contaminant-specific gene expression patterns that were not obscured by potentially nonspecific responses indicative of incipient death. The relationship of the training sample dose to the mortality response is shown for most of the training samples in Appendix G.

Three sets of evaluation samples were obtained from separate experiments not used for training data set development. One sample set was from a spiked sediment test using the pyrethroid pesticide cyfluthrin. Two additional evaluation sample sets consisted of amphipods exposed to toxic sediments collected from San Francisco Bay (station BA41) or Ballona Creek Estuary in southern California (station BCE2).

Samples for microarray analysis consisted of a composite of 3-5 surviving amphipods from a single replicate that were placed in preservative (RNAlater) and stored frozen (-80 C) until extraction (Appendix A). An equal number of control samples (e.g., laboratory seawater or uncontaminated sediment) from each experiment were also saved for use in determining differential gene expression.

Microarray

Development of the *E. estuarius* gene microarray was based on a set of 8610 unique RNA sequences (contigs) obtained in previous research (Bay et. al. 2010). Sequencing was done at the Vincent J. Coates sequencing facility at UC Berkeley on an Illumina Solexa Genome Analyzer. *De novo* assembly and annotation of the contigs was also conducted at UC Berkeley. Annotation of the contigs was conducted using a web-based protein Basic Local Alignment Search Tool (BLAST), blast2go (blast2go.org). BLAST converts nucleotide sequences into their corresponding protein sequences and compares the data to a large database. It then finds similar regions in the proteins and assigns quality scores and likely functions. The unique contigs were blasted against the non-redundant protein database. Annotation results are included in Appendix B.

The *E. estuarius* gene microarray was designed using Agilent eArray (https://earray.chem.agilent.com/earray/). The array design consists of a 15,000-probe microarray composed of 60-nucleotide long probes with a 5-nucleotide long linker. The on-line eArray program uses an algorithm to pick probes that are most likely to hybridize best. It also includes printing and hybridization quality controls within the array. The array design included all annotated and non-annotated contigs from the previous sequencing effort. Two discrete probes were selected for each annotated contig and non-annotated contigs greater than 415 bp. One probe was selected for each non-annotated contig less than 415 bp. Probes for the set of annotated contigs were chosen within the sequenced region that aligned with the protein sequence of its top BLAST result. A total of 14,723 amphipod gene probes are contained in the microarray.

Table 1. Characteristics of the samples in the training and evaluation data sets. The Experiment ID identifies each experiment batch used to obtain treatment and matching control samples. N = number of replicate samples for each treatment. Concentration indicates the nominal concentration of the chemical used in the exposure.

Experiment ID	Date	Treatment	Concentration	Matrix	N	Survival (%)	Exposure Duration (d)
			Training Samples				
ECV0711	8/26/2010	Control	Control	Water	3	100	4
ECV0711	8/26/2010	Cd	10000 ug/l	Water	3	83	4
EE114	10/16/2009	Control	Control	Water	2	90	10
EE114	10/16/2009	DDE	4 ug/L	Water	1	90	10
EE121	12/14/2009	Control	Control	Water	2	100	10
EE121	12/14/2009	Ammonia	100000 ug/L	Water	2	75	10
EE123	1/29/2010	Control	Control	Sediment	2	95	10
EE123	1/29/2010	DDT	2400 ug/kg	Sediment	2	58	10
EE127	3/29/2010	Control	Control	Sediment	3	93	10
EE127	3/29/2010	Cyfluthrin	0.8 ug/kg	Sediment	3	88	10
EE127	3/29/2010	Cyfluthrin	1.6 ug/kg	Sediment	3	60	10
EE136	7/2/2010	Control	Control	Sediment	2	88	10
EE136	7/2/2010	Fipronil	10 ug/kg	Sediment	2	82	10
EE138	11/15/2010	Control	Control	Water	3	96	10
EE138	11/15/2010	Copper	250 ug/L	Water	3	100	10
EE138	11/15/2010	Copper	750 ug/L	Water	3	98	10
EE139	12/20/2010	Control	Control	Water	3	93	10
EE139	12/14/2010	Bifenthrin	0.01 ug/L	Water	2	80	10
EE139	12/20/2010	Bifenthrin	0.03 ug/L	Water	2	55	7
EE139	12/17/2010	Cypermethrin	0.01 ug/L	Water	3	87	10
EE139	12/20/2010	Cypermethrin	0.03 ug/L	Water	3	100	4
GCM1	10/11/2010	Control	Control	Water	2	71	4
GCM1	10/11/2010	Chlordane	100 ug/L	Water	2	58	4
GCM1	10/11/2010	Pyrene	10 ug/L	Water	2	38	4
GCM2	10/18/2010	Control	Control	Sediment	2	90	10
GCM2	10/18/2010	Pyrene	25000 ug/kg	Sediment	2	89	10
			Evaluation Samples				
EE113	10/12/2009	Control	Control	Sediment	3	100	10
EE113	10/12/2009	Field Sample	BCE2	Sediment	3	40	10
EE116	11/9/2009	Control	Control	Sediment	2	100	10
EE116	11/9/2009	Cyfluthrin	1 ug/kg	Sediment	2	78	10
RMP100209	10/2/2009	Control	Control	Sediment	3	93	10
RMP100209	10/2/2009	Field Sample	BA41	Sediment	3	58	10

Sample Analysis.

All amphipods (generally 5 individuals) contained in a preserved sample were combined and extracted for gene expression analysis. Total ribonucleic acid (RNA) was extracted using the Qiagen RNeasy method (Appendix C) and converted into complementary deoxyribonucleic acid (cDNA). Subsequently, the cDNA was amplified into complementary ribonucleic acid (cRNA; Poynton et al. 2008a,b). Agilent's Quick Amp, one color labeling kit was used to fluorescently label samples (Agilent, CA, USA). Each amplified treatment or control sample was labelled with cyanine 3 and hybridized to the microarray. Hybridized slides were analyzed using a GenePix® 4000B microarray scanner. Scans were processed using GenePix Pro software.

Data Analysis

Agilent one color microarray data sets were grouped as "Experiment vs. Control" pairs. The statistical methods used to preprocess the data and identify differentially expressed genes are described in Loguinov et al. (2004). All positive values were log (base-2)-transformed. Relative intensity values (in the scatter plots of log2 transformed ratios vs. average spot intensity) were corrected for non-linear trends with loess global normalization methods (Yang et al. 2002). A variance estimation algorithm was used to take heteroscedasticity into consideration. Each gene in a given Experiment vs. Control pair was characterized by a normalized log-transformed ratio and a corresponding q-value. A multiple slide method was used to detect candidate genes. The technique treated the differential gene expression outcomes as Bernoulli trials. Then a modified Fisher's method of meta-analysis was applied to combine q-values and the resulting p-values were adjusted with a Bonferroni correction. This method does not require the use of between-array normalization because it does not apply any scale estimator. Following these steps, a list of candidate genes was identified for each treatment that had potential value for stressor identification. A gene was included in this list if it was detected as differentially expressed in at least one treatment.

Cluster analysis was conducted using *hopach* and *cluster* R packages (www.Bioconductor.org): HOPACH, PAM, DIANA and HCLUST. We consider HOPACH clustering results as more reliable because the algorithm uses non-parametric bootstrap to evaluate stability of clustering results (Van der Laan and Pollard 2003). The input matrix for cluster analysis consisted of the robust mean differential expression ratio (log2 transformed) of all replicate pairs for each treatment (i.e., treatment vs. control). Values for candidate genes represented by duplicate probes (from same contig) were combined (average) to eliminate redundancy in the data set. The final analysis data set contained 2585 genes (Appendix D). Two sets of cluster analyses were conducted: one using only the training data and one using the training data combined with the evaluation samples.

Random forest analysis was conducted using package *varSelRF* from Bioconductor.org (Díaz-Uriarte and Alvarez de Andrés, 2006). The input data for random forest was a subset of the nonredundant data set used for cluster analysis. The cluster analysis gene list was filtered to exclude genes that showed significant differential expression among different groups of control samples. The intent of this step was to develop a robust list of candidate genes having greater specificity for contaminant-related responses. Also, empty entries (NAs) are not allowed in the RF algorithm. As a result, all genes (i.e., rows in the input matrix) with one or more NAs through their replicates were removed. The final input data set consisted of 1899 candidate

genes (Appendix E). Values for each replicate of the training samples were used to build the classifier.

The analysis was conducted in two steps. First a series of trees were developed based on grouping the training data set into three prediction classes: Pyrethroids, Trace Organics, and Other. The Pyrethroid class consisted of six sample sets representing exposure to three different pyrethroid pesticides (two exposure levels/pesticide): cyfluthrin, cypermethrin, and bifenthrin. The Trace Organics class consisted of six sample sets of nonpolar toxicants other than pyrethroids: DDT, DDE, pyrene (two exposure levels), chlordane, and fipronil. The Other class consisted of four sample sets: copper (two exposure levels), cadmium, and ammonia. A set of 3000 random trees was generated and saved for subsequent use with the evaluation samples.

The second step of random forest analysis consisted of using the previously developed trees together with the evaluation samples to predict the likely class for each sample replicate. Each of the three evaluation sample sets was analyzed separately. Two versions of the evaluation sample data were analyzed; 1) all individual replicates and 2) the average of each replicate from the chemical or field sediment exposures versus all controls.

RESULTS

Sample Results and Variability

Successful hybridization of the RNA extracts with the microarray was obtained for all samples. Each sample analyzed showed evidence of differential gene expression relative to the control. Both up-regulation and down-regulation of genes were evident in most samples (Figure 1). Hundreds of probes on each microarray indicated significant differential expression (i.e., greater than statistical tolerance interval), with some probes indicating more than a 10-fold change in RNA content in the exposed sample relative to the control.

Visual inspection of differential expression vs. intensity plots indicated different patterns of results for different exposure groups, such as shown in Figure 1 for ammonia and copper. These results provide a visual indication that distinct patterns of gene expression are produced by different types of chemical exposure.

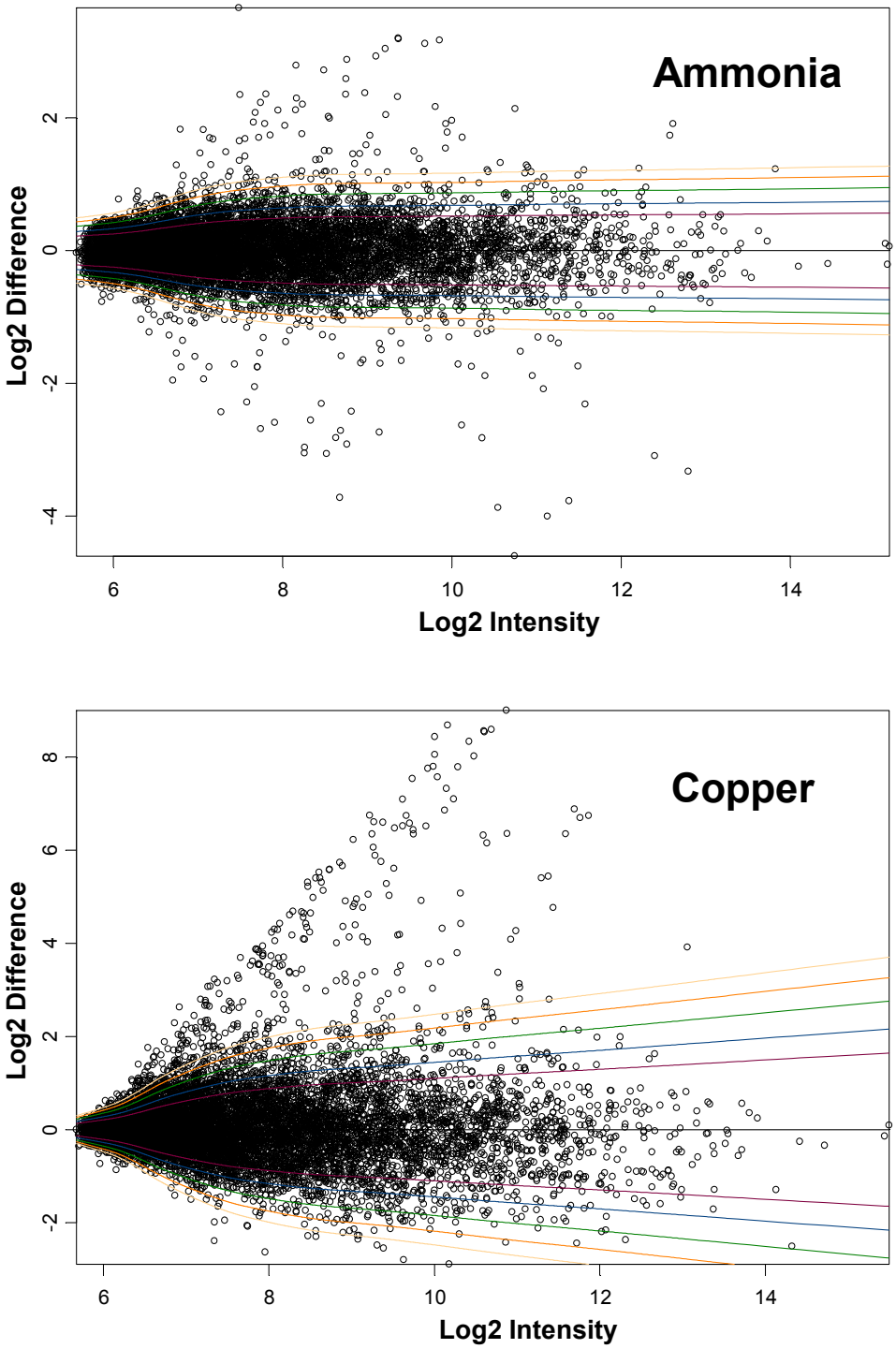

Figure 1. Differential gene expression versus spot intensity plots of microarray results. Results (log_2 exposed - log_2 control) are shown for *E. estuarius* exposed to ammonia (top) and copper (bottom) in water. Colored lines represent the 95%, 99%, 99.8%, 99.98%, and 99.998% simultaneous tolerance intervals relative to the zero line.

Two to three replicates of each test or control sample were analyzed. Differential expression analysis and candidate gene selection was conducted on each pairwise combination of the test sample and corresponding control replicates. For example, three replicate exposed and three replicate control samples were analyzed for the cyfluthrin exposure, resulting in a total of nine comparisons (Figure 2). Substantial variation in differential expression values for some probes was present among the individual replicate comparisons, depending upon the specific replicates compared. In some cases the results varied from substantial up-regulation to substantial down-regulation for the same probe (e.g., contig_1178 in Figure 2). Such variation is not uncommon with gene expression data and may reflect variation in organism response or uncontrolled variables in sample preparation or analysis.

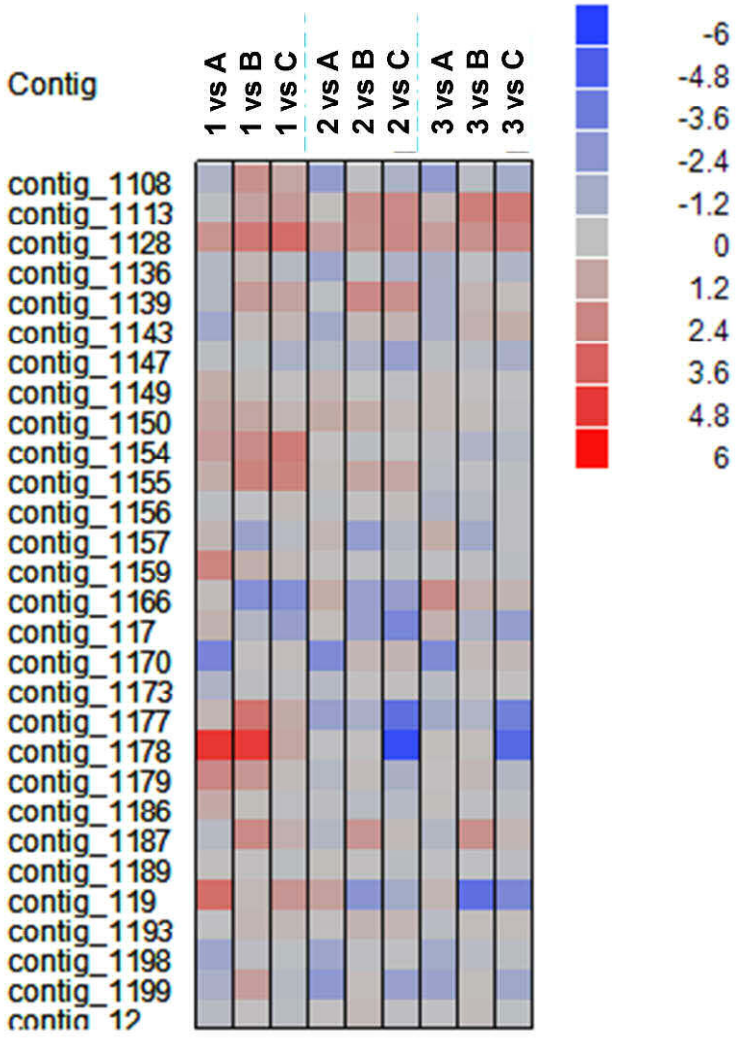

Figure 2. Differential gene expression (log_2 ratio) for replicate samples of *E. estuarius* exposed to cyfluthrin in sediment. Each column represents a pairwise comparison between one of three replicate exposed samples (1, 2, or 3) and a control (A,B, or C). Each row corresponds to a single microarray probe, identified by its contig number.

Candidate Gene Selection

Analysis of the normalized log2 ratio data identified 3182 microarray probes with statistically significant differential gene expression relative to controls (i.e., candidate genes). The number of candidate genes identified for a given treatment group was variable (Figure 3), ranging from 102 for pyrene (sediment exposure) to 770 for copper (250 ug/l water exposure). There were no candidate genes that were common to all 16 chemical treatment groups. Two alternate microarray probes were designed from many of the contigs. Thus, the number of candidate genes from this analysis corresponds to a smaller number of unique *E. estuarius* genes.

Between 12% and 54% of the differentially expressed genes in a treatment group were uniquely expressed (i.e., had significant differential expression in only one chemical treatment group), as shown in Figure 3. Both up-regulated and down-regulated genes were present among the list of unique candidate genes for each sample type (Table 2). There was no obvious relationship between the numbers of differentially expressed genes and amphipod response (% survival) For example, samples with the lowest number of uniquely expressed genes (<50) had survival values ranging from 58-100%.

The greatest numbers of uniquely expressed genes were produced by exposure to cadmium (257) and copper (250 ug/l; 219). There was no consistent pattern in terms of the number of up-regulated vs. number of down-regulated genes. Approximately one third of the samples had a similar number of up- and down-regulated genes (e.g., chlordane), while approximately equal numbers of the remaining samples had 2-3 times more up- or down-regulated genes. The magnitude of differential expression for individual gene probes also varied widely among the samples, with extremes ranging from 40-fold down-regulation for fipronil to 100-fold up-regulation for DDT (Table 2).

Table 2 Number of genes uniquely expressed by each chemical. Minimum and maximum expression values represent the ratio of intensity in the chemical exposure group divided by control intensity.

		Down-Regulated		Up-Regulated	
Treatment	Total Unique	N	Minimum Expression	N	Maximum Expression
Cyfluthrin (1.6 ug/kg)	130	36	0.165	94	16.000
Cyfluthrin (0.8 ug/kg)	86	44	0.233	42	4.287
Cypermethrin (0.03 ug/L)	184	144	0.077	40	2.828
Cypermethrin (0.01 ug/L)	112	41	0.058	71	2.462
Bifenthrin (0.03 ug/L)	79	27	0.330	52	48.503
Bifenthrin (0.01 ug/L)	110	62	0.047	48	3.031
Chlordane (100 ug/L)	47	27	0.058	20	6.964
DDE (4 ug/L)	70	46	0.027	24	3.732
DDT (2400 ug/kg)	107	44	0.095	63	111.430
Fipronil (10 ug/kg)	47	18	0.024	29	13.929
Pyrene (10 ug/L)	56	27	0.072	29	3.031
Pyrene (25000 ug/kg)	21	11	0.287	10	4.000
Ammonia (100000 ug/L)	40	22	0.165	18	8.000
Cadmium (10000 ug/L)	257	68	0.144	189	19.698
Copper (250 ug/L)	219	171	0.144	48	5.278
Copper (750 ug/L)	129	37	0.029	92	5.278

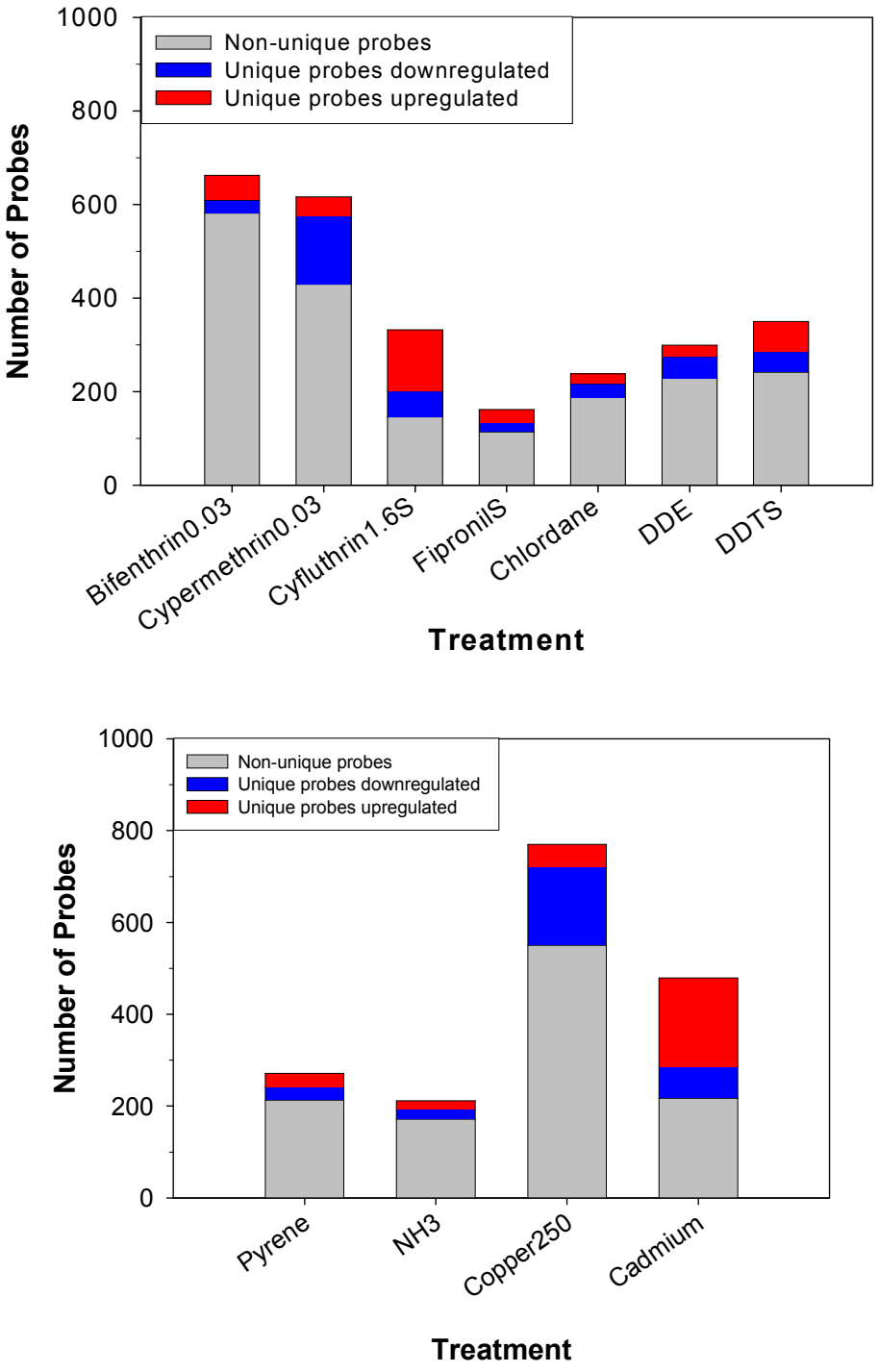

Figure 3. Summary of candidate gene selection results for selected treatments. Top plot shows results for pesticides; bottom plot shows results for other toxicant types.

Differential gene expression results differed substantially between different exposure concentrations of the same chemical. The nature of the difference was not consistent among chemicals. For example, there were only about 10% candidate genes in common between two dose levels of bifenthrin, with the higher dose containing more than twice as many candidate genes as the lower dose (Figure 4). Comparison of doses for copper produced a different result. The lower copper dose contained more than twice as many candidate genes as the higher dose, with about 4% of the genes in common.

Ordered gene expression plots show the dose comparison results in greater detail (Figure 5). While the overlap in candidate genes is small, the direction of the differential expression for the common genes (either up or down) is similar for both copper and bifenthrin. Genes showing consistent patterns of differential expression are likely the most reliable candidates for use in developing a tool for use in TIE applications.

These results indicate that the exposure concentration is an important factor influencing gene expression, as expected. Thus, using gene expression to identify the toxicant in complex environmental samples is likely to require a multivariate statistical approach based on a large training data set that includes multiple exposure levels.

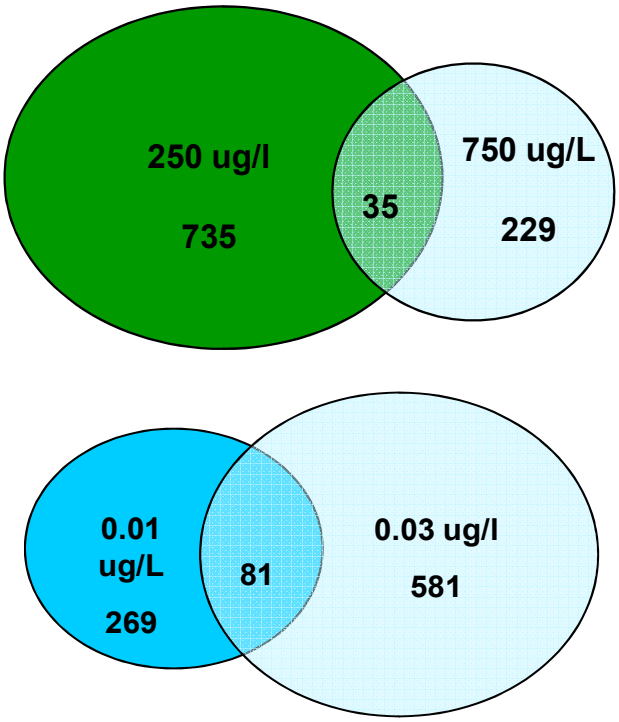

Figure 4. Summary of candidate genes in common between exposure concentrations. Top diagram shows numbers of unique and common gene probes for copper; bottom figure shows results for bifenthrin.

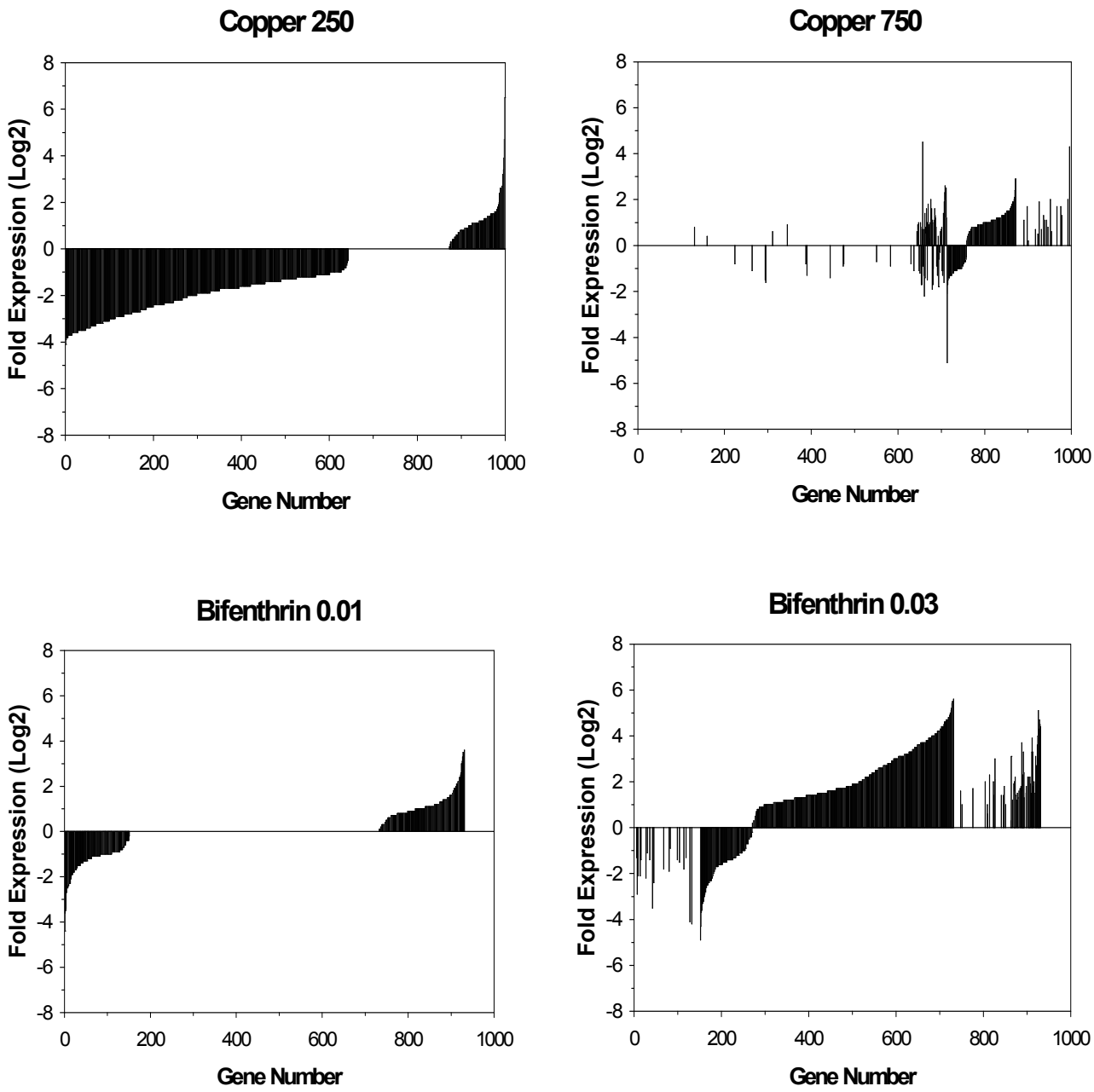

Figure 5. Ordered expression plots for different copper and bifenthrin concentrations. Genes are plotted on the x-axis in the same order for each test concentration of the same chemical. An expression value of zero indicates no significant differential expression for that gene probe.

Evaluation Sample Analysis

The differential gene expression for three sets of evaluation samples was determined using the same methods applied to the training samples. These samples represented three distinct exposure conditions (Table 3). Sample T1 consisted of amphipods exposed to reference site sediment spiked with cyfluthrin (pyrethroid pesticide) in a different experiment from the one used to provide the training samples. This exposure concentration was just below the lowest observed effect level determined in other experiments and did not produce significant mortality.

The other two evaluation samples contained a complex mixture of multiple trace organic and metals contaminants, including PAHs, PCBs, DDTs, chlordane, copper, and mercury. Sample T2 was a field sample collected from the Ballona Creek Estuary (station BCE2). This sample was highly toxic (60% mortality) and TIEs conducted on sediments from this site consistently indicated toxic levels of multiple pyrethroid pesticides, with most of the toxicity attributed to cyfluthrin and bifenthrin (Bay et al. 2010b). This sample was also contaminated with legacy pesticides and trace metals.

Sample T3 was a field sample from the 2009 RMP program (station BA41). This sample was also highly toxic (42% mortality), but the cause of toxicity is not known. Sample T3 had lower concentrations of most contaminants relative to T2, but contained much higher concentrations of PAHs (Table 3).

Table 3. Chemical characteristics of sediment evaluation samples. Sample T1 was not analyzed for PAHs or trace metals.

Parameter	Units	Sample		
		T1 (cyfluthrin spike)	T2 (BCE2)	T3 (BA41)
Chlordanes	µg/kg	0.3	27.6	0.2
DDTs	µg/kg	47	16	4
PAHs	µg/kg	NA	560[1]	2803
PCBs	µg/kg	15	20	14
Pyrethroids	µg/kg	1.0[2]	77.2	0.2
Copper	mg/kg	11.5	56.8[1]	27.2
Lead	mg/kg	12.6	74.2[1]	17.4
Mercury	mg/kg	0.11	0.12[1]	0.16
Zinc	mg/kg	50	278[1]	77

[1] Typical value for station, evaluation sample not analyzed for constituent.
[2] Nominal concentration.

The number of differentially expressed genes varied among the evaluation samples (Table 4). Nearly twice as many candidate genes (having significant differential expression) were identified for samples T1 and T2 compared to T3. Over one-third of the candidate genes for each evaluation sample were uniquely expressed (i.e., not differentially expressed in any of the training samples). The relative number of up- and down-regulated genes also varied among samples, with T1 and T3 having a greater proportion of down-regulated unique genes relative to T2.

Table 4. Summary of candidate genes identified for evaluation samples. The total number of candidate genes and numbers of uniquely expressed genes are shown. Minimum and maximum expression values represent the ratio of intensity in the chemical exposure group divided by control intensity.

		Unique Down-Regulated[1]		Unique Up-Regulated[1]	
Sample	Total Genes[1]	N	Minimum Expression	N	Maximum Expression
T1 (cyfluthrin)	467	103	0.088	51	26.0
T2 (Ballona Creek Estuary)	463	61	0.019	56	6.5
T3 (San Francisco Bay)	280	62	0.354	36	4.6

[1] Note that the number of candidate genes for the evaluation samples is based on the nonredundant results data set (results for duplicate probes combined), and thus are not directly comparable to results for the training samples shown in Table 2.

Many of the specific candidate genes identified in the evaluation samples matched those identified in each of the training samples (i.e., had same identity and direction of change). The degree of match varied considerably among the training samples, varying from 1% for DDE or fipronil to over 20% for bifenthrin (Figure 6). The greatest correspondence was present for sample T2 and bifenthrin, where 25% of the evaluation sample candidate genes were also present in the 0.03 ug/l bifenthrin exposure. The bifenthrin 0.03 training sample also showed relatively high matches with the other two evaluation samples.

An unexpected result was the low degree of match between sample T1 and the cyfluthrin training samples (2%). A higher level of match was anticipated for this comparison since T1 was a spiked sediment exposure with cyfluthrin. However, sample T1 candidate genes showed relatively high matches for the other two pyrethoids in the training data set (cypermetrin and bifenthrin).

Several of the training samples showed substantial variation in degree of match relative to each evaluation sample. For example, the cypermethrin 0.03 sample had the highest match with T2 (14 %) and a much lower match with T3 (4%). While % match analysis is relatively simple method of comparing the results, it illustrates two key characteristics of the data. First, differences in gene expression patterns among the evaluation samples are evident (e.g., cypermethrin). Second, differences among the evaluation samples are not absolute (e.g., some match with all training samples, concentration-dependent patterns within a chemical type), which suggests that careful consideration of statistical data analysis methods is needed.

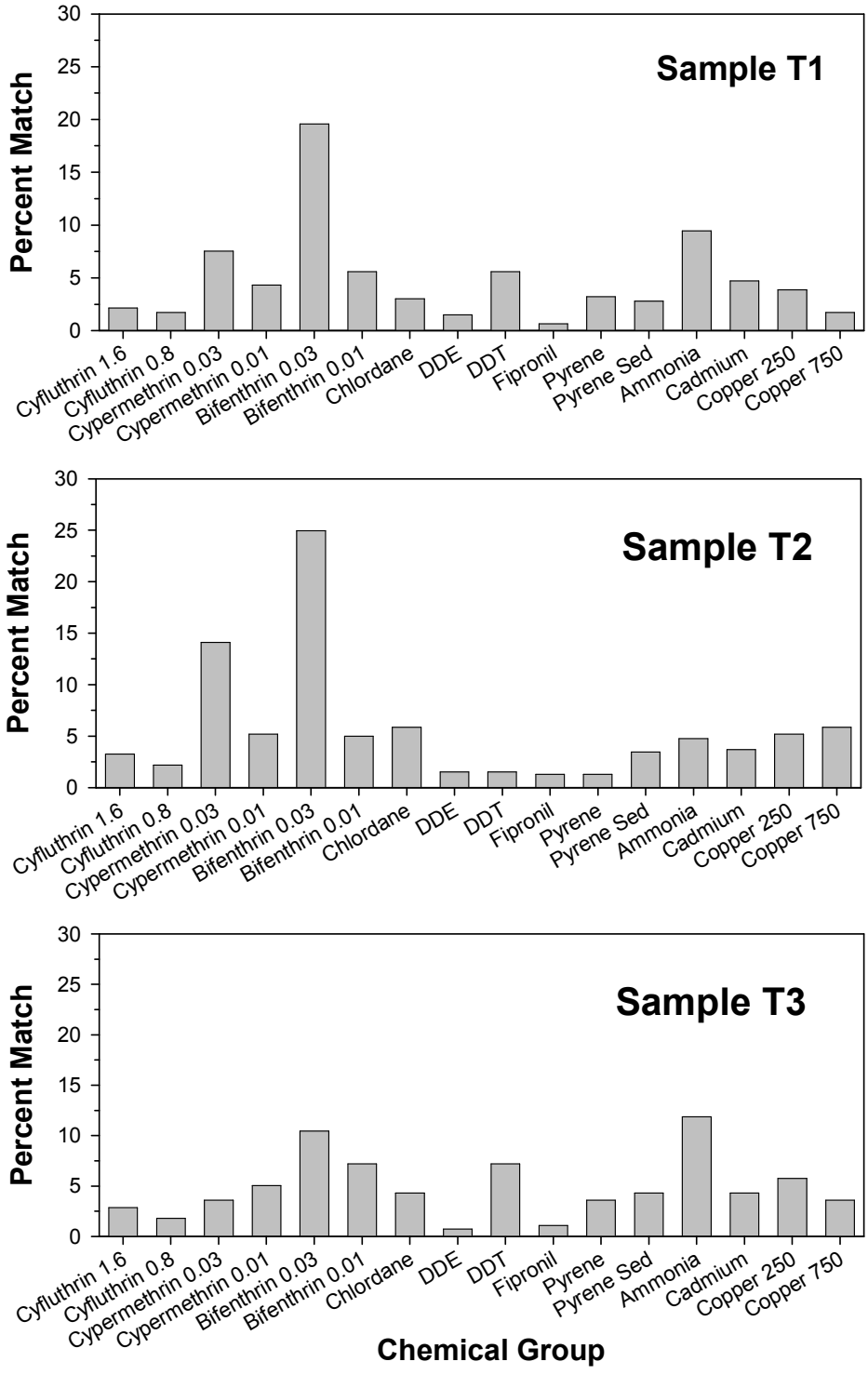

Figure 6. Percentage match of evaluation sample candidate genes with training samples. Percent match is based on the number of common candidate genes relative to the total number of candidate genes for the evaluation sample.

Cluster Analysis

Chemical-specific patterns.

Cluster analysis using the HOPACH method was conducted using the nonredundant set of all 2585 differentially expressed genes. The 16 chemical treatments in the training data set were grouped into seven clusters (Figure 7). Three clusters were composed of single chemicals (copper 750, DDT, and fipronil), suggesting that these chemical produced very distinctive patterns of expression. The remaining clusters each contained 2-4 chemical treatments.

With the exception of copper, treatments consisting of different doses of the same compound clustered together (e.g., cyfluthrin 1.6 and cyfluthrin 0.8), suggesting greater similarity of gene expression patterns within chemical type. Clusters 1, 2, and 7 were composed of dissimilar types of compounds expected to have different modes of toxic action. For example, cluster 1 contained the pyrethroid pesticide bifenthrin, a neurotoxin, and ammonia, a strong irritant to membranes and potential disruptor of metabolism.

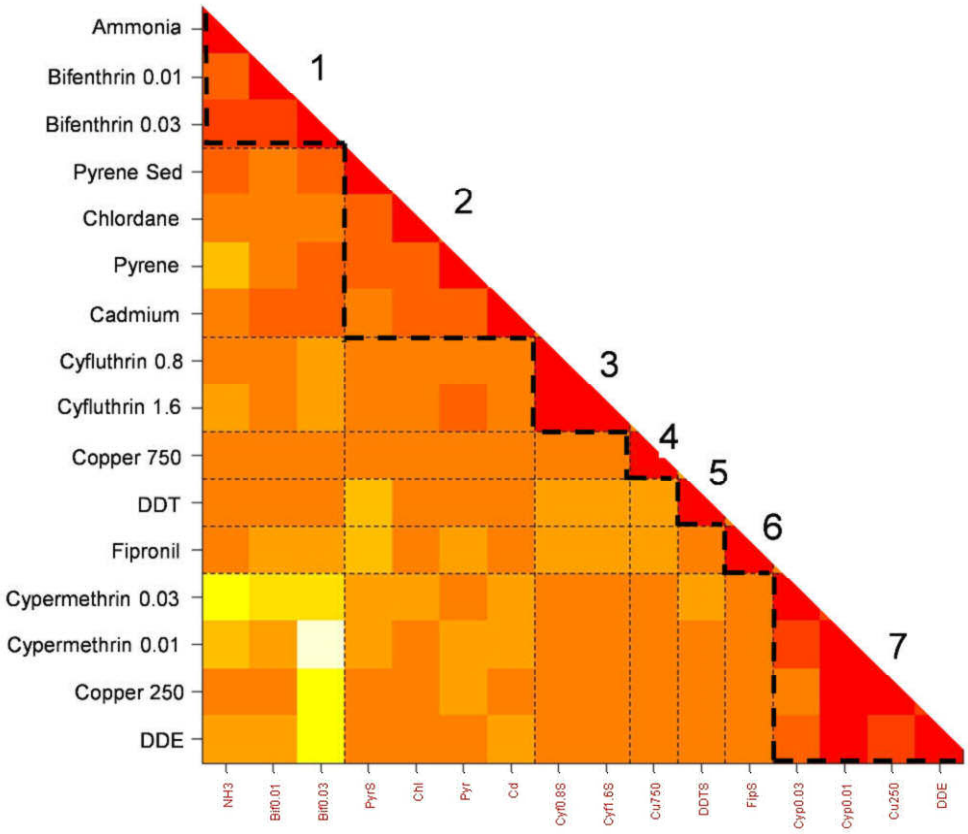

Figure 7. Ordered distance matrix of HOPACH cluster analysis results. Intensity of shading indicates chemical types with greater similarity to each other, with cosine-angle distance as metric. Red color means "small dissimilarity" and white - "large dissimilarity" corresponding to positive and negative correlation, respectively, and yellow to "no correlation" Dashed lines indicate seven stable cluster groups.

Evaluation sample classification

A separate HOPACH cluster analysis was used to classify each of the three evaluation samples: T1, T2, and T3. Sample T1 (cyfluthrin exposure) was grouped into the first cluster of the ordered distance matrix (Figure 8). A diverse set of other chemicals were also in this cluster: bifenthrin, ammonia, and pyrene. It was expected that sample TI would cluster with the other cyfluthrin samples, which were grouped nearby in a separate cluster. However, bifenthrin is a pyrethroid pesticide that is chemically similar to cyfluthrin and has a similar mode of toxic action.

Sample T2 (Ballona Creek Estuary) was grouped into a cluster with two other chemicals: chlordane and pyrene (Figure 9). Neither of these chemicals has a chemical similarity to pyrethroids, which were identified by TIE as the cause of toxicity at Ballona Creek Estuary.

Sample T3 (San Francisco Bay) was grouped into the same cluster associated with sample T1, which contained bifenthrin, ammonia, and pyrene (Figure 10). There was no a priori expectation regarding the cause of toxicity in this sample. However, both PAHs (such as pyrene) and ammonia have been suggested as contributing to sediment toxicity in portions of San Francisco Bay.

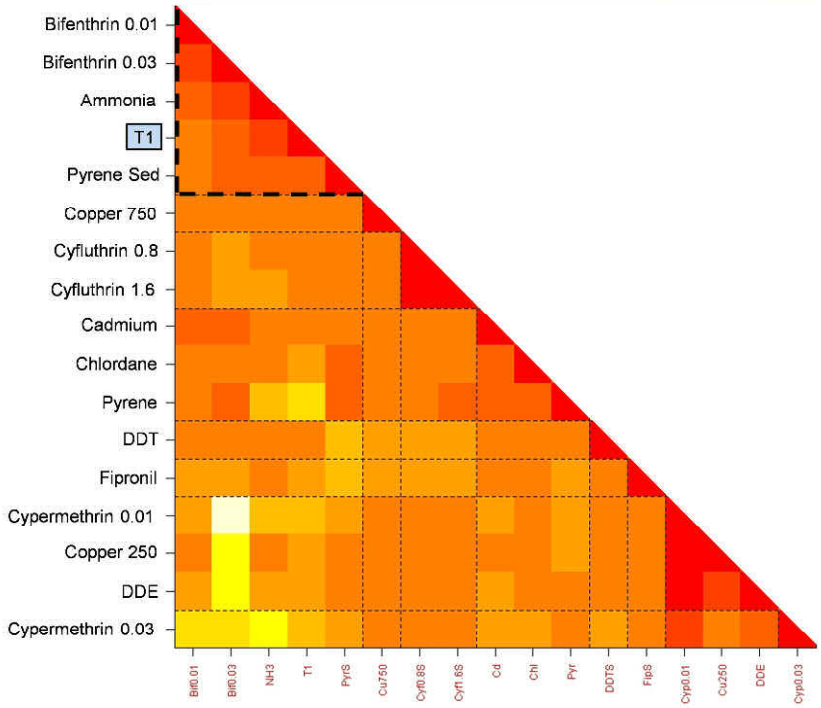

Figure 8. HOPACH cluster analysis results for evaluation sample T1. Heavy dashed line indicates cluster group containing the evaluation sample.

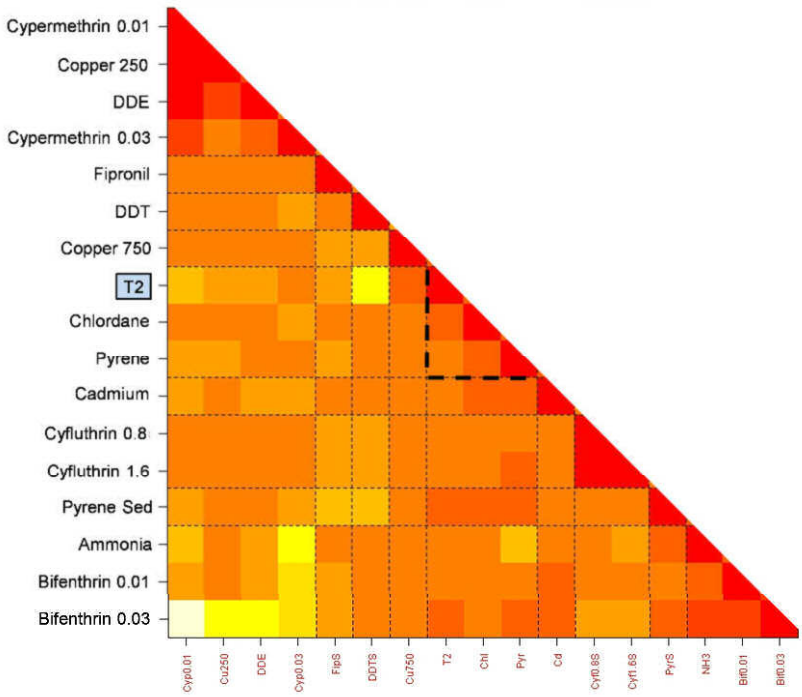

Figure 9. HOPACH cluster analysis results for evaluation sample T2. Heavy dashed line indicates cluster group containing the evaluation sample.

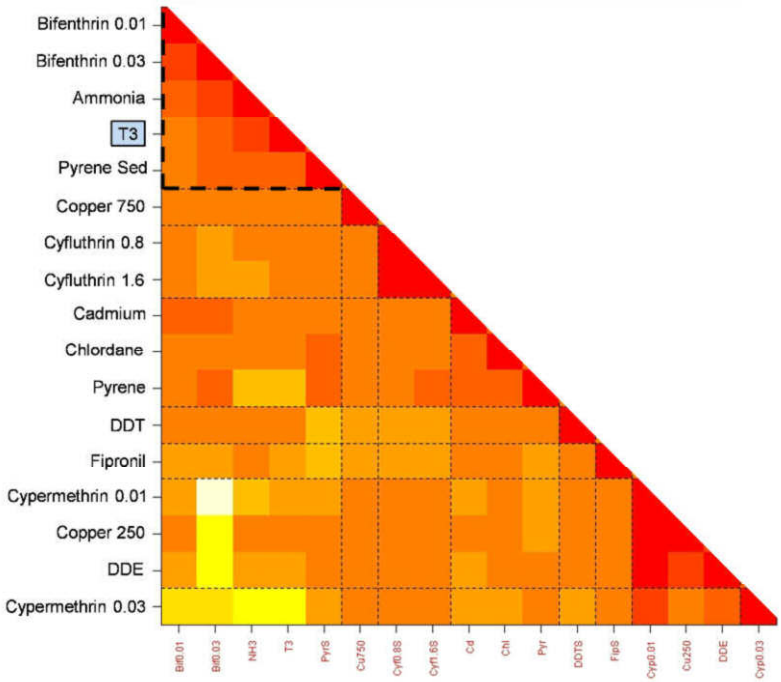

Figure 10. HOPACH cluster analysis results for evaluation sample T3. Heavy dashed line indicates cluster group containing the evaluation sample.

Random Forest Analysis

The input data set for random forest analysis consisted of 1899 genes. The analysis was based on 3000 trees with 3 prediction classes: Pyrethroids, Trace Organics, and Other. A set of 76 genes was selected by the algorithm as important predictors for classification (Appendix F).

Cross validation analysis of the training data indicated that a highly accurate 3 class prediction model was developed by random forest. Most of the training data set replicates were correctly classified into their respective classes (Table 5). An overall out of bag (OOB) error rate of 5% was obtained. The OOB error rate describes the classification error on a random subset of the training data sample replicates that was withheld from use in generating each of the classification trees.

Table 5. Random forest class prediction results for training data replicates.

	Number of Replicates in Predicted Class			
True Class	**Other**	**Trace Organic**	**Pyrethroid**	**OOB Class Error**
Other	23	1	1	0.080
Trace Organic	1	21	2	0.125
Pyrethroid	0	0	48	0.000

Most of these predictor genes identified by random forest did not correspond to protein sequences contained in public databases and thus could not be identified or characterized in terms of biological function (annotated). Tentative identifications were available for 33 of the predictor genes (Table 6). The genome of *E. estuarius* or similar taxa has not been sequenced, so annotation of the candidate genes is dependent upon locating similar RNA or protein sequences that have been reported by other researchers.

Gene Ontology (GO) terms were available for most of the annotated genes. These terms identify functions and characteristics of the genes that have been described for other species. A wide variety of biological processes were associated with these genes. Various types of metabolic or biosynthetic processes were among the most prevalent biological processes identified for the genes. The annotated predictor genes did not include gene types commonly associated with response to contaminant exposure, such as metallothionein, heat shock protein, glutathione S-transferase, or cytochrome P450.

Table 6. Descriptions of annotated random forest predictor genes. GO Terms: F= Molecular function; P= Biological process; C= Cellular components.

Contig ID	Sequence Description	GO Terms
contig_1655	Myosin light chain smooth muscle	F:protein kinase activity; ATP binding; nucleotide binding; protein serine/threonine kinase activity. P:protein amino acid phosphorylation; auxin biosynthetic process
contig_1527	Sorting nexin	F:lipid binding; protein binding. P:cell communication
contig_5334	Ypf01_plaf7 uncharacterized protein	F:molecular_function; chromatin binding. P:symbiosis, encompassing mutualism through parasitism; chromatin assembly or disassembly
contig_481	Ribosomal protein l22	F:carbohydrate binding; structural molecule activity. P:translation; cell differentiation; multicellular organismal development.
contig_770	Amyrel	F:binding; hydrolase activity. P:carbohydrate metabolic process
contig_1428	Phosphoenolpyruvate carboxykinase	F:binding; kinase activity, catalytic activity; nucleotide binding. P:lipid metabolic process; biosynthetic process; carbohydrate metabolic process; generation of precursor metabolites and energy
contig_4500	Necdin-like 2	P:regulation of biological process
contig_601	Lim domain-binding	F:binding; cytoskeletal protein binding. P:regulation of biological process
contig_7599	Lantibiotic synthetase component c-like 2	F:lipid binding; nucleotide binding. P:regulation of biological process; signal transduction; response to endogenous stimulus; transcription
contig_2195	Kelch repeat type 1-containing protein	F:protein binding
contig_2352	Serine protease	F:peptidase activity. P:protein metabolic process; catabolic process
contig_2623	Iq domain-containing protein g	-
contig_5779	Hypothetical protein [Branchiostoma floridae]	-
contig_3886	Growth hormone-inducible transmembrane protein	C:integral to membrane; membrane
contig_588	Guanine nucleotide-binding protein beta 3	F:signal transducer activity; transferase activity. P:reproduction; behavior; regulation of biological process; embryonic development; signal transduction; anatomical structure morphogenesis; cytoskelet
contig_1880	Extensin-like protein	F:oxidoreductase activity; 2-alkenal reductase activity; protein binding. P:plant-type cell wall organization; oxidation reduction; cellular cell wall organization
contig_312	Universal minicircle sequence binding protein	F:nucleic acid binding; hydrolase activity. P:regulation of biological process; transcription; cell proliferation
contig_1757	Dusky- isoform a	-
contig_65	Tubulin beta-2c chain	F:structural molecule activity; nucleotide binding; hydrolase activity. P:multicellular organismal development; cellular component organization; biological_process
contig_6439	ABC subfamily	F:hydrolase activity; nucleotide binding. P:biosynthetic process; transport
contig_2318	Aspartate ammonia lyase	F:catalytic activity. P:biological process; metabolic process; generation of precursor metabolites and energy; catabolic process
contig_3709	Trypsin 4	F:peptidase activity. P:protein metabolic process; catabolic process
contig_5350	Troponin c	F:calcium ion binding
contig_833	Alpha-amylase	F:hydrolase activity; binding. P:carbohydrate metabolic process
contig_2614	Cg3305-pa isoform 2	C:membrane
contig_1580	Cuticular protein 49aa	F:structural constituent of chitin-based cuticle; structural constituent of cuticle
contig_3791	Copia protein	-
contig_379	Cop9 signalosome complex subunit 5	F:binding; protein binding; peptidase activity. P:protein modification process; regulation of biological process; cell cycle; transport; biosynthetic process; lipid metabolic process
contig_6821	Collagen alpha-1 chain	F:chitin binding. P:chitin metabolic process
contig_1987	Isoform q	F:calcium ion binding; vinculin binding; myostimulatory hormone. P:actin polymerization or depolymerization; oogenesis; epithelial fluid transport actin filament organization
contig_1567	Calcified cuticle protein	F:structural constituent of cuticle
contig_2879	Tho complex	F:nucleic acid binding; nucleotide binding
contig_6784	Sulfate transporter	F:transporter activity. P:transport

Class predictions using the replicate evaluation data showed high consistency with expected results (Figure 11). Toxicity in 75% of the replicates for T2 and T2 was predicted to similar to pyrethroids, which was consistent with expectations. Gene expression in the other replicates was predicted to be similar to trace organic toxicants (Trace Organic class). Results for sample T3 were similar to the other evaluation samples, with two-thirds of the replicates predicted to have toxicity similar to pyrethroids. No replicates were predicted to have toxicity similar to other compounds (i.e., non organics).

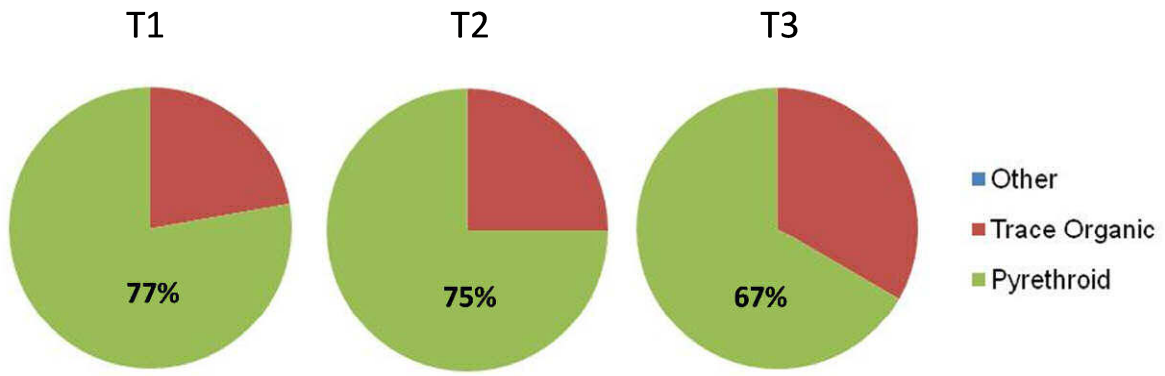

Figure 11. Random forest class prediction results for evaluation sample replicates. Percentage of replicates classified in the Pyrethroid group is indicated. N=9 for T1 and T3; n = 4 for T2.

Class predictions were also made using random forest applied to averages of the evaluation samples. This approach was used to determine if the use of averages would result in more consistent predictions among replicates. The results were very consistent for samples T1 and T2; all replicates for these two samples were correctly classified into the Pyrethroid class (Table 7).

Table 7. Random forest class prediction results for evaluation sample averages.

	Number of Samples in Predicted Class			
Sample	Other	Trace Organic	Pyrethroid	Accuracy
T1	0	0	3	100%
T2	0	0	2	100%
T3	0	1	2	NA

DISCUSSION

This project has achieved several important milestones in the effort to develop a molecular TIE method for sediment toxicity. First, a gene microarray for *E. estuarius* was successfully developed based on the first ever sequencing of RNA from this amphipod. Second, this microarray was used to analyze a training set of samples and identify a subset of candidate genes having differential expression in response to 11 different types of chemical exposure. Third, many of the chemical treatments were shown to produce distinctive patterns of differential expression, confirming a key assumption of the approach. Finally, we developed and applied multiple approaches for evaluating unknown samples. Some of these approaches were shown to correctly identify the class of compounds causing toxicity in independent evaluation samples, providing a demonstration that a molecular TIE approach is feasible and has the potential to provide an effective and powerful tool for sediment TIEs. Results for the evaluation sample from Ballona Creek Estuary are especially encouraging, as the cause of toxicity (pyrethroids) was correctly identified in spite of the presence of elevated concentrations of many other contaminants.

This is also the first study to demonstrate that a molecular TIE approach based on a routine sediment toxicity test method (10-day amphipod survival test) may be feasible. Developing a molecular TIE approach that is compatible with existing test methods offers a powerful advantage: animal samples from such tests can be saved and analyzed for TIE without having to incur additional costs or time delays in conducting additional tests (required for conventional TIEs).

The results described in this report are encouraging with respect to the prospects for developing a more complete molecular TIE approach in the near future. Significant differential gene expression was identified in approximately 20% of the gene probes on the microarray. Furthermore, 50% of these genes showed unique expression for single chemicals. If these results are shown to be consistent in future studies, then there is good potential for the molecular TIE approach to be effective for the many types of contaminants of interest to US and Canadian environmental management agencies.

This study has also helped to identify data gaps and areas of uncertainty that need to be addressed before this molecular TIE approach can be used with confidence. A primary data gap is the lack of gene expression information for many contaminants of concern. This study analyzed 11 different contaminants, using a single exposure dose in most cases. However, other contaminants need to be investigated to develop a classification model with greater resolution that the three class model used in the present study. Contaminant groups particularly underrepresented in the current training set include PAHs, trace metals other than Cu or Cd, and PCBs. It is possible that greater discrimination between the evaluation sample from San Francisco Bay (T3) and the other evaluation samples would have been obtained had the training data set included these additional constituents.

There is also high uncertainty regarding the influence of exposure dose and exposure duration on the ability to identify chemical-specific gene expression patterns. Other studies have shown that

gene expression in aquatic animals exposed to contaminants varies with respect to exposure duration, and our results for copper and pyrethroids illustrate that dose can have a strong effect on gene expression. Training samples were obtained from experiments ranging in duration from 4-10 days. In addition, the level of effect measured in the training data varied from highly toxic (e.g.,. >40% mortality) to nontoxic (0% mortality). Duration and dose varied as a consequence of using samples from other monitoring and research programs for training and evaluation. The limited control of exposure conditions in this project and represent potentially significant confounding factors. Collaborative research is underway at Hollings Marine Laboratory (Charleston, South Carolina) to investigate dose and duration effects on *E. estuarius* gene expression. Analysis of these forthcoming data should help to determine key test design parameters for future research. It is possible that some of the training data set samples analyzed in the present study will need to be repeated with samples obtained under different exposure conditions.

The training data set was also limited in that contaminant mixtures were not represented. Most environmental contamination is composed of complex mixtures of multiple types of contaminants, such as metals, petroleum hydrocarbons, and pesticides. The training data set used in the present study was intentionally composed entirely of single contaminant exposures in order to simplify the experimental design and data analyses. Additional research is needed to determine how the accuracy of a molecular TIE approach is influenced by contaminant mixtures.

Standardization and optimization of sample preparation and analysis methods is also needed to develop a reliable tool for use in multiple laboratories. There are few standard protocols for sample preservation, extraction, and analysis, and quality assurance. Consequently, data for the same sample analyzed by different laboratories may vary as a result of method variations, possibly leading to incorrect conclusions regarding the gene expression patterns obtained. Interlaboratory studies are needed to investigate these issues and develop methods that yield comparable results among laboratories. An interlaboratory study using the *E. estuarius* microarray is under development and is expected to result in greater standardization of methods and data interpretation.

Finally, further development and evaluation of classification models based on gene expression is needed. This study investigated only two types of statistical approaches for stressor identification: clustering and random forest analysis. These two approaches varied in effectiveness, with random forest yielding the most accurate results. Cluster analysis was shown to produce variable results depending on the composition of the input data set and this method does not appear to be amenable to use for stressor identification. Other statistical methods for classifier development, such as discriminant analysis and centroid analysis, have not yet been evaluated. Such alternative data analysis methods should be investigated to help in determining the most effective approach for TIE applications. In addition, a limited number of evaluation samples were used in the present study, and only two of these samples could be used to determine accuracy of the molecular TIE approach. Analysis of additional evaluation samples (where the cause of toxicity is known) that represent different causes of toxicity, mixtures, and variation in magnitude of effect is needed in order to provide a more complete assessment of classification method accuracy.

The research conducted in this study has laid the foundation for development of a new and potentially effective TIE approach with wide application. While further development and validation is needed, this project has demonstrated that rapid progress can be made in these areas through collaboration and partnership. Although extraction of RNA, microarray analysis and data interpretation require expertise and equipment not normally present in a typical aquatic toxicology laboratory, this is not expected to prevent implementation of this method. There should be sufficient capability to widely apply a molecular TIE method in the near future, once the method development and validation have progressed. The needed expertise and equipment are already accessible at most universities and many government laboratories, and the rapid pace of technology development ensures that these methods will be even more reliable and less expensive in the near future.

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APPENDIX A: SAMPLE PREPARATION METHOD

Eohaustorius estuarius Preparation for Microarray Analysis SOP
November 16, 2010

Purpose: To prepare the amphipod, *Eohaustorius estuarius,* for microarray analysis.

Equipment/Supplies:
500µm sieve
Teflon coated forceps
RNA*later*®
1.5ml vials (labeled and filled with 1.0ml of RNA*later*)
Methanol
Deionized water
Wash bottles
Ice cooler
Dry ice

Overview: A gene microarray for *Eohaustorius estuarius* has been development. This microarray will be used to analyze gene expression in amphipods exposed to various whole sediment and water samples for a wide range of potential contaminants and concentrations. This document will explain the process of collecting and preparing the amphipod samples at the end of an exposure.

Exposure: Ideally for the *E. estuarius* microarray development, each test should consist of 5 replicates per treatment. However, depending upon the goals of a given study, the number of replicates may vary.

Test Breakdown: When breaking down a whole sediment test a 500µm sieve should be used to remove the amphipods from the sediment. Amphipods should be counted for survival directly on the sieve and retrieved with a pair of Teflon coated forceps or nitex screen. For water only exposures, amphipods should be counted for survival and directly removed from the test container with a pair of Teflon coated forceps. Only surviving amphipods should be selected for the microarray analysis. Each amphipod selected for the microarray should be crushed with forceps and added to a 1.5ml centrifuge vial pre-filled with 1.0ml of RNA*later* for tissue stabilization. No more than five amphipods should be added to each centrifuge vial. Forceps should be cleaned with methanol and deionized water rinses between treatments.

Storage: Allow for the amphipods to remain in the RNA*later* at room temperature for 1 hour and then store at 4°C overnight to allow for the stabilization reagent to penetrate the tissues. After the overnight incubation period, remove the RNA*later*® from the 1.5 mL vial with a glass pipet. The amphipod samples should then be frozen at -80°C. The storage time is indefinite under these conditions.

Shipment: The amphipod samples should be shipped frozen on dry ice.

APPENDIX B: MICROARRAY PROBE ANNOTATION RESULTS

Results for annotation can be found in the following file: AppendixB_Annotation.xlsx.

APPENDIX C: RNA EXTRACTION METHOD

RNA Extraction using Qiagen RNeasy Mini Kit with DNase Set
Method adapted by UC Berkeley

RLT buffer: Add β-Mercaptoethanol to buffer RLT before use. (10 µL β-Mercaptoethanol per 1 mL buffer RLT)

DNase I stock solution: Dissolve the lyophilized DNase I in 550 µL of the RNase free water by using needle and syringe, and mix by inverting. Then divide it into single-use aliquots (about 55 µL), and store at -20 ^{o}C for up to 9 months.

1. Collect 3-5 arthropods and add 600 µL buffer RLT.
2. Homogenize with tissue lyser. (usually 20~40 seconds)
3. Centrifuge the lysate for 3 min at full speed and carefully transfer the supernatant to a new microcentrifuge tube by pipetting.
4. Add 600 µL of 70% EtOH and mix immediately by pipetting.
5. Transfer up to 700 µL to an RNeasy spin column placed in a 2 mL collection tube. If the sample volume exceeds 700 µL, centrifuge successive aliquots in the same RNeasy spin column (Repeat step 5 and 6).
6. Centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm) and discard the flow-through.
7. Add 350 µL buffer RW1 to the RNeasy spin column and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm). Discard the flow-through
8. Add 10 µL DNase I stock solution to 70 µL buffer RDD and mix by gently inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.
9. Add the DNase I incubation mix (80 µL) directly to the RNeasy spin column membrane, and place on the benchtop (room temperature) for 15 min.
10. Add 350 µL buffer RW1 to the RNeasy spin column and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm). Discard the flow-through
11. Add 500 µL of buffer RPE to the RNeasy spin column and centrifuge for 15 s at ≥8,000 x g (≥10,000 rpm) and discard the flow-through.
12. Add 500 µL of buffer RPE to the RNeasy spin column and centrifuge for 2 min at ≥8,000 x g (≥10,000 rpm) and discard the flow-through.
13. Place the RNeasy spin column in a new 1.5 mL collection tube and add 30-50 µL RNase-free water directly to the spin column membrane. Centrifuge for 1 min at ≥8,000 x g (≥10,000 rpm).

APPENDIX D: LIST OF CANDIDATE GENES

Data contained in file: AppendixD_CandidateGenes.xlsx

Contents include microarray probe name for all nonredundant candidate genes, annotation information, and mean differential expression (log_2) for each training sample or evaluation sample. Data were used for cluster analysis.

APPENDIX E: INPUT DATA FOR RANDOM FOREST ANALYSIS

Data contained in file: AppendixE_RF_InputData.xlsx

Contents include microarray probe name for 1899 candidate genes and differential expression (log_2) for individual replicates of each training sample or evaluation sample.

APPENDIX F: RANDOM FOREST PREDICTOR GENES

Table F-1. Legend for Table F-2.

Table Heading	Sample Name
Cyf16	Cyfluthrin (1.6 ug/kg)
Cyf08	Cyfluthrin (0.8 ug/kg)
Cyp003	Cypermethrin (0.03 ug/L)
Cyp001	Cypermethrin (0.01 ug/L)
Bif003	Bifenthrin (0.03 ug/L)
Bif001	Bifenthrin (0.01 ug/L)
Chl	Chlordane (100 ug/L)
DDE	DDE (4 ug/L)
DDT	DDT (2400 ug/kg)
Fip	Fipronil (10 ug/kg)
Pyr	Pyrene (10 ug/L)
PyrS	Pyrene (25000 ug/kg)
NH3	Ammonia (100000 ug/L)
Cd	Cadmium (10000 ug/L)
Cu250	Copper (250 ug/L)
Cu750	Copper (750 ug/L)
T1	T1 (cyfluthrin)
T2	T2 (Ballona Creek Estuary)
T3	T3 (San Francisco Bay)

Table F-2. Predictor gene set for random forest classification. Mean differential expression (log_2) for each sample type is shown to illustrate relationships. Analysis conducted with replicate values for each sample type.

Contig	Cyf16	Cyf08	Cyp003	Cyp001	Bif003	Bif001	Chl	DDE	DDT	Fip	Pyr	PyrS	NH3	Cd	Cu250	Cu750	T1	T2	T3
contig_4717									1.5									-1.0	-1.1
contig_481											1.4								0.7
contig_770				0.9		-0.9		0.3									1.8		
contig_2267																	-1.0		
contig_1428			-1.3	-0.8		1.0												0.8	
contig_4629			-0.9				1.0										-1.0		
contig_1880	-2.2		-1.2					-1.9											0.7
contig_65			-1.4	0.8									-1.4			1.0		1.4	1.0
contig_3709		-2.8	-0.7											1.0			0.7		
contig_3791						1.0													-0.8
contig_1567							1.3					0.2						1.1	
contig_8075				-1.3		1.6								-2.0			1.6	0.6	
contig_1655				0.5		-0.4										-0.7			
contig_5069	-1.4																		
contig_2540																1.2			
contig_2858	-0.4	-0.8																	
contig_8617	-2									2.0									
contig_1527							1.3			1.1	1.4					1.1			
contig_5334					-1.5									0.3					
contig_4260														1.6					
contig_5907		0.1														0.9			
contig_3929														-1.0					
contig_7995			-1.4			0.9								1.9					
contig_2101											1.5								
contig_6229																			
contig_7495		-0.8												1.4					
contig_715		1.1																	
contig_3241																			
contig_6557																			
contig_6016			-1.5																
contig_4931														1.4					
contig_7224																			
contig_14				1.5	-1.4					1.4									
contig_2250																			
contig_2648														1.3					
contig_5687														1.3					
contig_8564																0.8			
contig_3263														0.4					
contig_1756											-1.3								

Contig	Cyf16	Cyf08	Cyp003	Cyp001	Bif003	Bif001	Chl	DDE	DDT	Fip	Pyr	PyrS	NH3	Cd	Cu250	Cu750	T1	T2	T3
contig_4698		-1.2												1.7		1.0			
contig_4500																			
contig_5494																			
contig_3501																1.0			
contig_601																1.2			
contig_7599									-1.3										
contig_2195	-2.1	-1.7		1.2					1.1							-0.9			
contig_8302																			
contig_2352														-1.0					
contig_3231		-1.5																	
contig_2623																			
contig_5779			-1.0																
contig_6919		-1.2																	
contig_6617								-1.3					1.1						
contig_3886																			
contig_1681																			
contig_588	2.4	2.0																	
contig_5583													-1.3						
contig_312	2.1																		
contig_1757																			
contig_8130				1.2	-1.5														
contig_5421	-3.1	-1.8			-1.0				1.1		-1.7								
contig_2320				0.7			1.0		0.8	2.0	-1.1				1.6				
contig_6439	1.8																		
contig_2318																			
contig_5350																			
contig_833																			
contig_2614																			
contig_4146														1.4					
contig_1580			-0.8			0.4	1.0	0.4			1.3			-1.0	1.0	1.8			
contig_379																0.8			
contig_6821																			
contig_1987		1.4														0.9			
contig_2879	1.5																		
contig_2209																			
contig_8012																1.0			
contig_6784														-1.0		0.9			

APPENDIX G: DOSE RESPONSE PLOTS

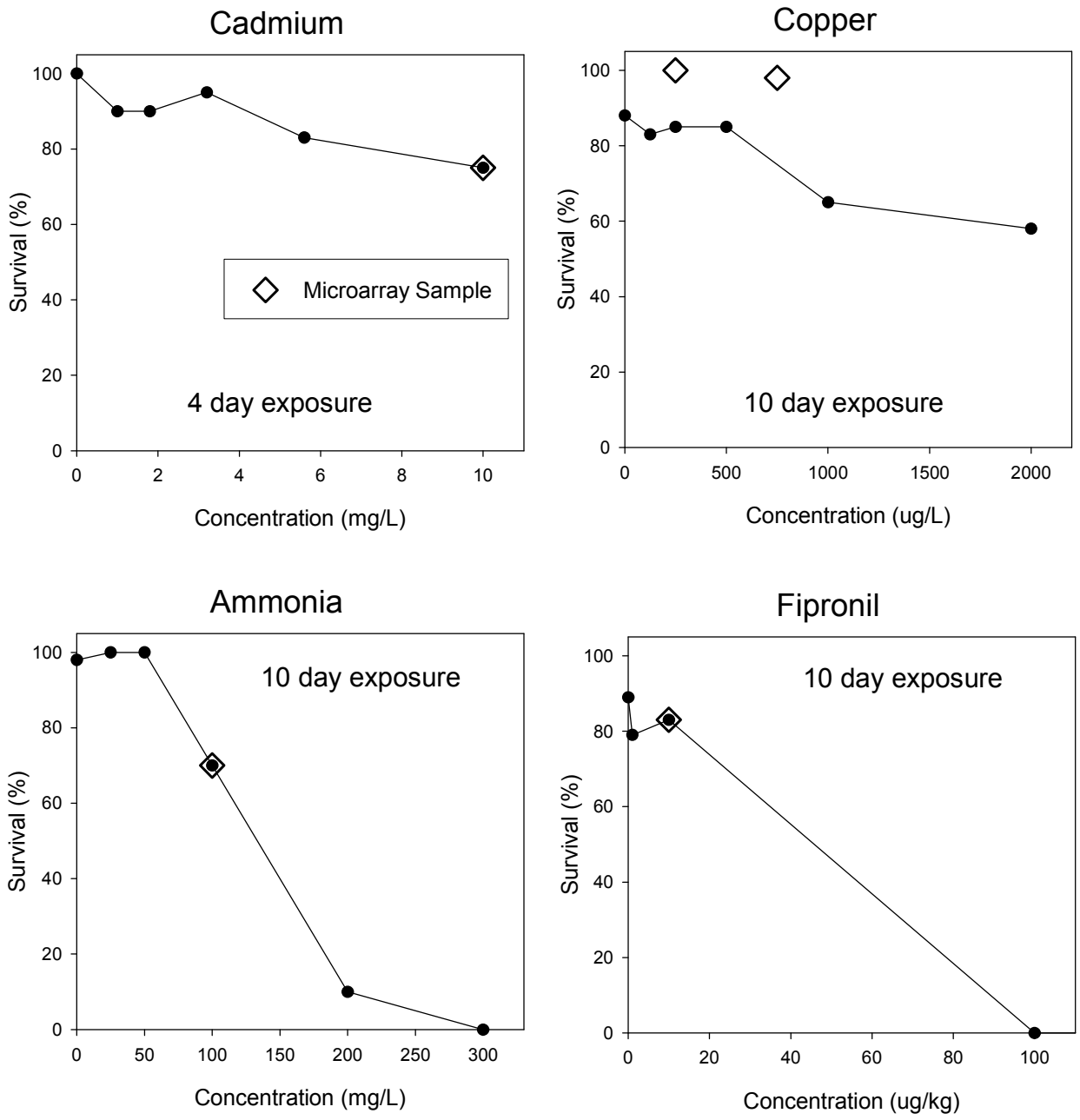

Table G-1. Amphipod survival dose response plots for cadmium, copper, ammonia, and fipronil training samples. Diamond symbol indicates sample used in microarray. Note that microarray samples for copper were obtained from a different experiment than the one used to generate the dose response plot.

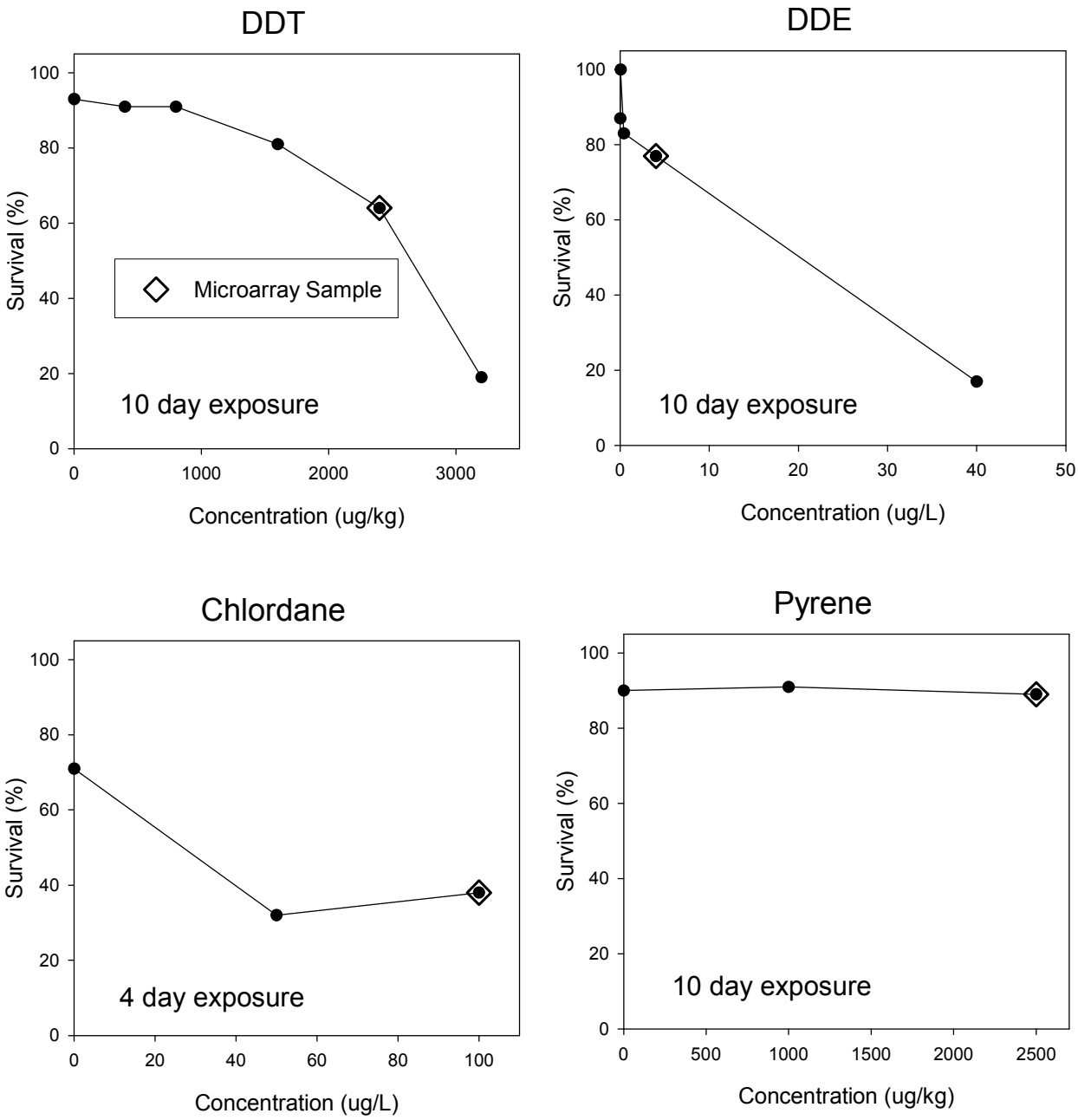

Table G-2. Amphipod survival dose response plots for DDT, DDE, chlordane, and pyrene training samples. Diamond symbol indicates sample used in microarray.

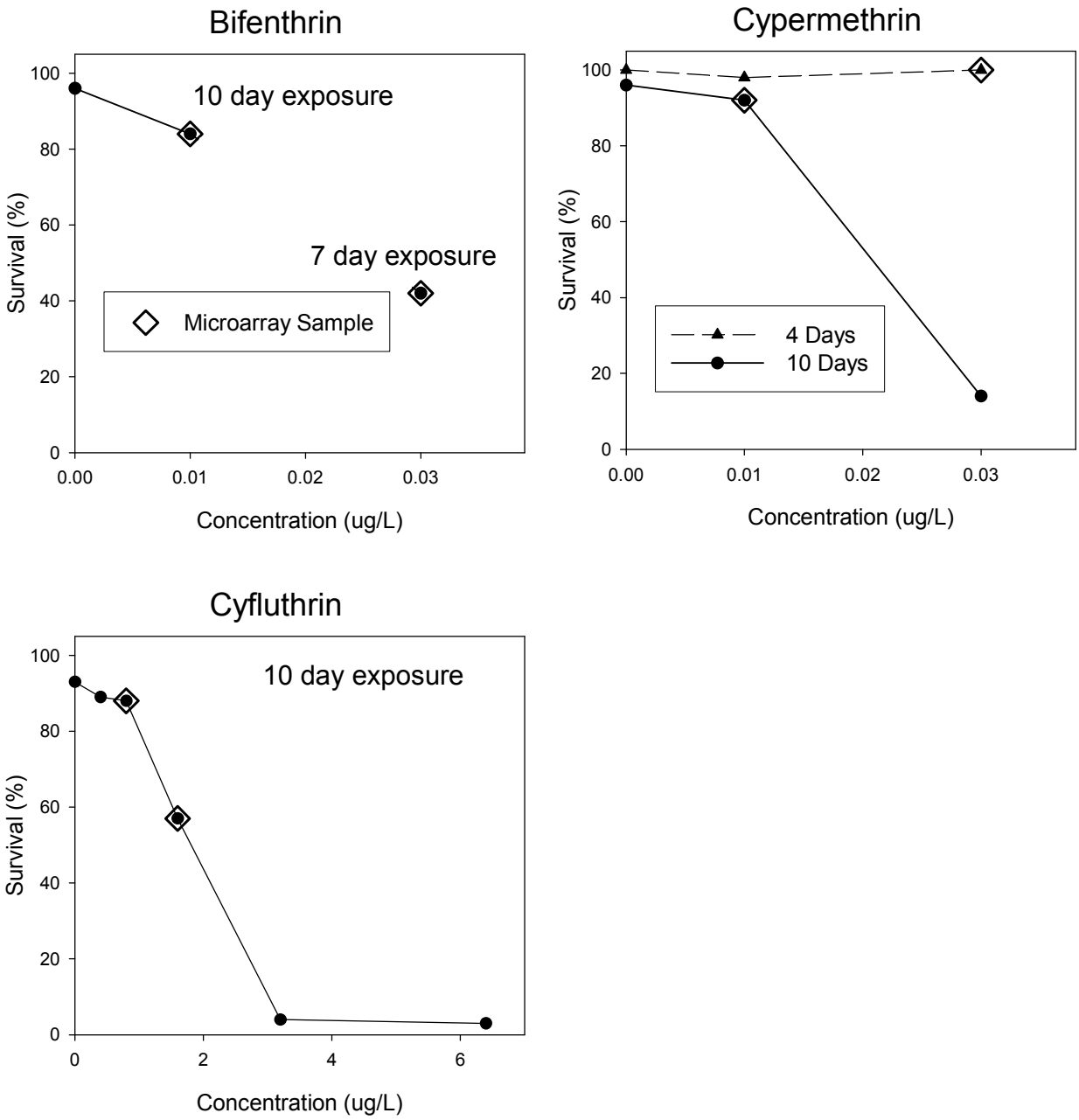

Table G-3. Amphipod survival dose response plots for pyrethroid pesticide training samples. Diamond symbol indicates sample used in microarray. Note that training samples for bifenthrin and cypermethrin were obtained from various exposure durations in order to represent doses producing a low-moderate effects.