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Gene expression patterns in rainbow trout, *Oncorhynchus mykiss*, exposed to a suite of model toxicants

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Abstract

The increased availability and use of DNA microarrays has allowed the characterization of gene expression patterns associated with exposure to different toxicants. An important question is whether toxicant induced changes in gene expression in fish are sufficiently diverse to allow for identification of specific modes of action and/or specific contaminants. In theory, each class of toxicant may generate a gene expression profile unique to its mode of toxic action. In this study, isogenic (cloned) rainbow trout *Oncorhynchus mykiss* were exposed to sublethal levels of a series of model toxicants with varying modes of action, including ethynylestradiol (xeno-estrogen), 2,2,4,4'-tetrabromodiphenyl ether (BDE-47, thyroid active), diquat (oxidant stressor), chromium VI, and benzo[a]pyrene (BaP) for a period of 1–3 weeks. An additional experiment measured trenbolone (anabolic steroid; model androgen) induced gene expression changes in sexually mature female trout. Following exposure, fish were euthanized, livers removed and RNA extracted. Fluorescently labeled cDNA were generated and hybridized against a commercially available Atlantic Salmon/Trout array (GRASP project, University of Victoria) spotted with 16,000 cDNA's. The slides were scanned to measure abundance of a given transcript in each sample relative to controls. Data were analyzed via Genespring (Silicon Genetics) to identify a list of up- and downregulated genes, as well as to determine gene clustering patterns that can be used as "expression signatures". The results indicate each toxicant exposure caused between 64 and 222 genes to be significantly altered in expression. Most genes exhibiting altered expression responded to only one of the toxicants and relatively few were co-expressed in multiple treatments. For example, BaP and Diquat, both of which exert toxicity via oxidative stress, upregulated 28 of the same genes, of over 100 genes altered by either treatment. Other genes associated with steroidogenesis, p450 and estrogen responsive genes appear to be useful for selectively identifying toxicant mode of action in fish, suggesting a link between gene expression profile and mode of toxicity. Our array results showed good agreement with quantitative real time polymerase chain reaction (qRT PCR), which demonstrates that the arrays are an accurate measure of gene expression. The specificity of the gene expression profile in response to a model toxicant, the link between genes with altered expression and mode of toxic action, and the consistency between array and qRT PCR results all suggest that cDNA microarrays have the potential to screen environmental contaminants for biomarkers and mode of toxic action.

Keywords

Rainbow trout; *Oncorhynchus mykiss*; Xeno-estrogen; Gene expression; Microarrays; Genomics

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1. Introduction

The last decade has seen rapid advances in genomic analysis. Among the developments are microarray technologies, typically cDNA or DNA oligos spotted onto glass slides or nylon membranes (Waters and Fostel, 2004). Microarrays allow for the simultaneous measurement of 1000's of expressed genes, potentially allowing the monitoring of the entire transcriptome of an organism (Schena et al., 1996; Bartosiewicz et al., 2000). Their use has revolutionized oncology and pharmacology (vanDelft et al., 2004) and a similar impact is beginning to occur in toxicology (Hamadeh et al., 2001; Aardema and MacGregor, 2002).

An important advantage of genomic analysis with regard to toxicological investigations is that gene expression changes are likely to be an initial response compared to more traditional toxicological endpoints. This would allow for increased sensitivity, earlier detection and measurement of toxicant effects at more environmentally relevant concentrations (Aardema and MacGregor, 2002; Waters and Fostel, 2004). Recent studies in rodents also suggest genomic analysis may offer improved analysis of the effects of complex mixtures (Hamadeh et al., 2001; Amin et al., 2002; Aardema and MacGregor, 2002).

The application of genomic analysis in toxicology offers the potential for improved assessment of toxicant mode of action. The response to contaminant exposure may involve a cascade of gene interactions, rather than a change in a single gene or a few genes (Aardema and MacGregor, 2002). Metabolic pathways are often controlled by master genes or "nodes" and changes in these master genes could have pleiotrophic outcomes which could be monitored by genomic approaches (Neumann and Galvez, 2002). Recent work suggests that diverse toxicants produce a distinctive gene expression signature (Bartosiewicz et al., 2001; Amin et al., 2002; Hamadeh et al., 2002b). For instance, genotoxic and nongenotoxic carcinogens produce distinctly different patterns of gene expression (vanDelft et al., 2004). Because expression profiles are more closely linked to the toxic mechanism of action as opposed to chemical structure, modes of toxic action for unknown compounds can be discovered (Bartosiewicz et al., 2001; Hamadeh et al., 2001). For example, distinct patterns of gene expression were obtained from mice exposed to two different classes of hepatotoxins: peroxisome proliferators and phenobaritol-like enzyme inducers (Hamadeh et al., 2002b). Hepatotoxins of unknown function were classified as to mode of toxic action according to expression fingerprints (Hamadeh et al., 2002a). This experimental approach has applications in ecotoxicology where increasingly, emphasis is being placed on understanding the mechanism of toxic action of environmental contaminants (Snape et al., 2004).

Despite the potential of gene expression profiling in ecotoxicology, relatively few studies have utilized this technique in fish (Koskinen et al., 2004). Some preliminary work using arrays to identify contaminant exposure in field collected fish and in fish exposed to effluents has been done (Williams et al., 2003; Denslow et al., 2004). Estrogenic compounds have been shown to cause measurable increases in genes involved in female gametogenesis (Larkin et al., 2002, 2003a), but this work has not been extended to other classes of toxicants. If gene expression profiling is to have the same impact on environmental assessments as it has in human health, more exhaustive "proof of concept" work must be done to demonstrate that transcriptomic responses in lower vertebrates, such as fish, are sufficiently diverse to distinguish between classes of toxicants. Importantly, a stronger linkage between a specific mode of toxic action and gene expression profile needs to be demonstrated.

A primary aim of this study is to demonstrate that structurally diverse contaminants exhibiting a variety of toxic modes of action will generate unique patterns of gene expression. We exposed rainbow trout (*Oncorhynchus mykiss*) to one of six different model toxicants: ethinylestradiol (EE2; a potent synthetic estrogen), trenbolone (Trb; a potent synthetic androgen), 2,2,4,4'-

tetrabromodiphenyl ether (BDE-47; a flame retardant suspected of having thyroid disrupting properties), benzo[*a*]pyrene (BaP, a carcinogen and genotoxicant), Diquat (Diquat, an aquatic herbicide and potent oxidative stressor), and chromium VI (Cr, a metal and oxidative stressor). Following separate, short-term exposures to each toxicant, the liver was collected and RNA extracted for gene expression profiling using a high density cDNA microarray and subsequent qRT PCR validation of array results.

2. Materials and methods

2.1. Fish

All fish were maintained according to the guidelines established by the Institutional Animal Care and Use Committee (IACUC) of Battelle. All experiments except for the Trb exposures used male isogenic rainbow trout of the OSU × Swanson cross (Young et al., 1996). These fish were transferred to the Battelle Marine Research Operations Sequim, WA laboratory at 530 degree days in age. Throughout the study, all fish were maintained in single pass flow through tanks under natural photoperiod conditions and fed Bio-Oregon® soft moist pellets of various sizes based on fish size. At the time of the exposures, the isogenic trout ranged in age from 1800 to 2460 degree days (5–7 months) and averaged 0.01–0.03 kg in weight.

For the Trb exposures, sexually mature female trout (0.71–0.95 kg) were obtained from a local hatchery (Nisqually Trout Farm, Lacey WA, USA) and acclimated for a minimum of 2 weeks prior to exposure. These trout were initially group housed in 1400 l circular tanks and then individually housed in 370 l circular tanks. Other conditions were as described above. Throughout the study, various water quality parameters were routinely measured in holding and treatment tanks and averaged 12 °C, >9 mg/l dissolved oxygen, pH 7.9, total alkalinity 200 mg/l (as CaCO₃), ammonia <0.05 mg/l, and nitrate–nitrite <0.01 mg/l.

2.2. Chemicals

The study contaminants were >99% purity and were obtained from the following sources: EE2, Trb, BaP, and Cr-VI were obtained from Sigma (Colombia MO); BDE-47 and Diquat were obtained from Chem Service (West Chester, PA). All other chemicals used were of reagent grade.

2.3. Exposures

Different routes of exposure were selected based on past experience and perceived environmental relevance and/or experimental necessity. For the EE2, Trb and BaP exposures, a minimum of three fish were exposed for 7 days to each contaminant using a flow-through exposure system. Nominal exposure levels of 50 ng/l EE2, 1 µg/l Trb and 1 µg/l BaP was used. For each exposure, a concentrated stock solution was prepared in methanol and slowly added to the exposure tanks using a peristaltic pump at a flow rate of 0.10 ml/min (equals 0.0005% methanol in tanks). Control tanks had only methanol added (solvent control). The exposure tanks were allowed to equilibrate with each contaminant dosing system for 2–3 days prior to the addition of the trout. The EE2 and Trb concentrations were monitored before and after the exposure by GC–MS using analytical methods previously described in Schultz et al. (2001). The mean of the measured exposure levels is shown in Table 1.

For BDE-47, an oral exposure route was used. Five individually housed trout were fed for 21 days live adult *Artemia* sp. (purchased from Northeast Brine Shrimp, Oak Hill, FL), which were used to bioencapsulate BDE-47 prior to feeding. For this procedure, adult *Artemia* (approximately 100–150) were placed in a 0.04 l flat bottomed, Pyrex tube previously coated with 660 µg of BDE-47 and filled with sterile filtered, Sequim Bay seawater. The adult *Artemia* were incubated in the tube overnight with light aeration. The following morning

(approximately 18–24 h incubation), the live *Artemia* were removed using a fine mesh net, rinsed with filtered seawater and then immediately fed to the trout. Additional details on the bioencapsulation of BDE-47 can be found in Muirhead et al. (2006). Subsequent to this feeding, trout were fed the normal pelleted diet twice each day. A separate group of control trout were maintained and fed live adult *Artemia* which were not exposed to BDE-47. These latter trout were used as appropriate vehicle controls in subsequent microarray hybridization experiments. The BDE-47 content in the *Artemia* and also the plasma, liver and carcass of the exposed trout were measured using GC-ECD as described in Muirhead et al. (2006). A summary of these values are shown in Table 1. For the Cr-VI and Diq exposures, five trout were administered an interperitoneal (i.p.) injection of (25 and 500 µg/kg, respectively) each contaminant dissolved in 0.9% (w/v) NaCl. A separate group of three trout were administered an i.p. injection of only 0.9% NaCl dosing vehicle. After 24 h, the fish were euthanized as described below.

2.4. Sampling and RNA extractions

Fish were euthanized with a lethal overdose of MS 222 (250 mg/l). Blood was collected from the caudal vein and the plasma obtained by centrifugation ($3000 \times g$ for 5 min) and stored at -80°C for chemical analysis. The liver was immediately removed and subsectioned. One subsection was frozen for chemical analysis and the remaining pieces placed in RNeasy lysis buffer (Qiagen) and stored following the manufacturer's protocols. RNA was extracted using a standard TRIzol procedure (Invitrogen) and purified either via a mRNA cleanup kit (Qiagen) (BDE and BaP) or via the TURBO DNAfree kit (Ambion) (EE2, Trb, Cr, Diq). Total RNA was quantified via fluorometry using ribogreen reagent (Molecular Probes) and RNA quality was verified via gel electrophoresis. After processing, SUPRNasin (RNase inhibitor, Ambion) was added to help maintain sample integrity and RNA was stored at -80°C .

2.5. Microarray methods

Salmonid cDNA microarrays were obtained from the GRASP consortium (Dr. Ben Koop, University of Victoria, Canada). These arrays have 16,000 cDNA and EST's from either Atlantic Salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*). The array has 6998 unknown EST's; the remainder of the spots have a putative identity available on the GRASP website <http://web.uvic.ca/cbr/grasp/>. The methods for obtaining the cDNAs for the array, developing the arrays and validating the arrays themselves are described in detail in Rise et al. (2004a). Sequence homology between the two species is sufficiently high, allowing for the cross species use of the array (Rise et al., 2004a). Array hybridizations were performed in a 3×3 replicate design; with three animal replicates and three technical replicates. RNA was transcribed into cDNA and indirectly labeled via an aminoallyl technique (Invitrogen's Superscript cDNA Indirect Labelling kit). Control cDNA was labeled with Cy3 (Amersham), and exposed cDNA was labeled with Cy5. A split control experiment (where control RNA is put into two separate tubes, labeled with Cy3 or Cy5, then recombined for array hybridization) was also performed to examine genes selected as significant due to differences in dye incorporation (Draghici, 2003). Exposed and control samples were paired according to cDNA yield and label incorporation, combined, and reduced in volume to 32 µl in a vacuum concentrator. Samples were mixed with 20 µg tRNA and 20 µg Herring Sperm DNA to prevent non specific hybridization, then mixed with 35 µl of modified "Genisphere" hybridization buffer (50% formamide, 40% 20× SSC, 9% Denhardt's solution, 1% SDS). This mixture was then applied to the arrays and allowed to hybridize overnight (16 h) at 45°C . After hybridization, arrays were washed in SSC/SDS buffers with descending stringency to remove any unhybridized or weakly (nonspecifically) hybridized cDNA's. Arrays were scanned using a Perkin-Elmer ScanArray Express, with laser power and PMT gain varied to equalize fluorescence intensity between channels and to prevent over saturation of signal intensity.

2.6. Microarray data analysis

Data were extracted using ScanArray Express software (Perkin-Elmer). The median fluorescence intensity with background subtracted was imported into a MIAME compliant database (Brazma et al., 2001). Genespring (Silicon Genetics) microarray analysis software was used for further analysis. Data were LOWESS normalized (Draghici, 2003), and spots that did not meet minimum signal intensity were removed. The resultant signal information was analyzed using one way ANOVA ($p = 0.05$) with a Benjamani–Hochberg correction for multiple comparisons (GeneSpring). A list of differentially expressed genes was prepared comprised of those genes that demonstrated a statistically significant change in expression for each toxicant treatment (Draghici, 2003). The identity of genes with altered expression was verified by submitting the sequences to BLAST (NCBI) (Altschul et al., 1990). Gene ontology (GO) terms were taken from the information available on the GRASP website <http://web.uvic.ca/cbr/grasp/>. These terms were assigned by members of the GRASP consortium as described on the website (von Schalburg et al., 2005a).

Gene expression data were further analyzed via Hierarchical Cluster Analysis. Gene trees were created to group similar genes and allow for better visualization of the data (Butte, 2002). Genes that were found to be significantly different in expression in at least one treatment were used to create a gene tree in GeneSpring. Different treatments were clustered using Genespring's Condition Tree function. Trees were created using a Pearson's correlation (Claverie, 1999) as a similarity measure, and branches that were more than 95% similar were merged.

2.7. Quantitative real time PCR

RNA for quantitative real time PCR (qRT PCR) was collected, purified, quantified and stored as described in Section 2.4. All qRT PCR analyses were performed using Applied Biosystems 7300 Real time system and the one step RT PCR master mix reagents (Applied Biosystems). Standards for each of the specific genes to be validated via qRT PCR were made either from the cDNA clones used to print the array (a kind gift from Dr. Ben Koop, University of Victoria) or from previously isolated rainbow trout genes (J.A. Small, unpublished data). Plasmids of genes to be used as standards were transcribed in vitro (Riboprobe system, Promega) and quantified via fluorometry (Ribogreen quantitation kit, Molecular Probes). Primers used for qRT PCR are given in Table 2. Transcription levels in treated and untreated fish were compared to a dilution series of the above standards. All measurements (samples and standards) were made in triplicate, and measurements were taken from three replicates for each treatment. All samples and standards were compared to a no reverse transcriptase control (to eliminate the possibility that signal resulted from DNA contamination), and each plate contained no template controls to serve as blanks. Data were normalized to expression levels of beta actin. Significance was determined via a Student's t -test ($p < 0.05$).

3. Results

3.1. Exposure results

A summary of the measured and nominal exposure levels are shown in Table 1. Selected internal dose metrics are also shown for specific contaminants in Table 1. There were no unscheduled mortalities during any of the exposures. At time of sampling, gross necropsy did not indicate any signs of necrosis or overt pathology in the livers of control or exposed fish. For fish receiving an i.p. injection, there were mild signs of inflammation, but this was localized to the site of injection.

3.2. Microarray results

Results obtained from the microarray analysis appear to accurately reflect the transcriptomic changes. In our split control experiment, no genes were found to be significantly altered in expression (data not shown). However, in each of the chemical exposures there were a subset of genes, between 64 (Trb exposure) and 222 (BDE exposure), that were significantly (ANOVA, $p < 0.05$) altered in expression. Complete lists of the identities of these genes, their fold change and associated confidence, and their closest homolog in a BLAST are available as Supplementary material. To summarize the data, histograms of the contaminant induced changes in gene expression are presented in Fig. 1, along with identities for a few chosen genes. Plots of changes in gene function (determined using the associated GO terms) are also presented in Fig. 2–Fig. 7. Typically 25–50% of the genes with altered expression had unknown function. The GO category with an identifiable function exhibiting the greatest number of genes are those involved in transport. Additional broad generalizations are difficult to discern and a contaminant by contaminant discussion of the results is presented below.

Genes with altered expression in response to the EE2 exposure are shown in Fig. 1A (reprinted from Skillman et al., 2006), with gene annotation listed in Table 1 (supplementary material). In total, 189 genes had altered expression: 48 genes were upregulated, 141 were downregulated. Among the most significantly upregulated genes were vitellogenin (gene a, 10.4-fold increase over control, $p < 0.001$), the vitelline envelope protein (gene b, 7.4-fold change, $p < 0.001$) and an apolipoprotein (gene c, 3-fold change, $p < 0.001$). A different apolipoprotein (gene number d), a fatty acid protein (gene number f) and pentraxin (gene number e) were among the most downregulated genes: fold changes were -4.7 , -6.9 and -5.7 , respectively, with p values of less than 0.001 in each case. Fig. 2 shows the molecular functions of the genes with EE2 altered expression and the biological process in which the genes are involved. In addition to transport, other groups of genes upregulated by EE2 include those with nucleic acid binding, kinase activity, isomerase activity and hydrolase activity. Heparin binding activity was downregulated. When the biological processes in which the genes with altered expression are examined, protein folding and nucleotide metabolism are upregulated, while inflammatory response, immune response, hyaluronan metabolism, and glycolysis are downregulated (Fig. 2).

Compared to EE2, fewer genes were altered in response to Trb exposure although many more genes were upregulated than downregulated (Fig. 1B, Table 2; see also supplementary material). An especially high percentage of genes have of unknown function making it difficult at present to discern clear trends or patterns. Because there are so few genes downregulated following exposure to Trb, they are not plotted as was done for EE2 (Fig. 3.). Among the genes with an identified function are NADH dehydrogenase (gene g, 31.7-fold change, $p = 0.0133$), alkaline phosphatase (gene h, 11.2-fold change, $p = 0.0006$) and an apolipoprotein (gene i, 3.8-fold change, $p = 0.009$). The downregulated genes include glyceraldehydes 3-phosphatase, a plasma regulatory protein, and a ribosomal protein (genes labeled j, k and l, respectively, downregulated roughly 2-fold, $p < 0.05$).

Genes with altered expression after the BDE-47 exposure are shown in Fig. 1C and Table 2 (supplementary material). Overall, more genes were downregulated than upregulated. Again, many of the genes with increased expression levels have unknown function, however genes associated with apoptosis and cell signaling were upregulated: Caspase 8, upregulated 3.3-fold ($p < 0.005$; labeled m on graph) and a regulator of G protein signaling is upregulated 4-fold ($p < 0.005$; labeled n on graph), Fig. 1C. Interesting downregulated genes were serotransferrin II and apolipoprotein B (genes labeled o and p, both downregulated 4-fold, $p < 0.05$ in all examples). Besides the unknown and transport GO categories, other discernible trends include the downregulation of genes with lipid binding function and the upregulation of genes with

hydrolase function. In addition, genes involved in protein biosynthesis are downregulated, as are those that protect against apoptosis (Fig. 4).

Genes with altered response following BaP exposure are shown in Fig. 1D, and an annotated gene list is provided in Table 4 (see supplementary material). An acyl carrier protein is strongly upregulated (gene q, 54 fold, $p < 0.01$). Among the other genes upregulated in response to BaP are HSP 90, cytochrome p450 1a3, and glutathione *S* transferase Mu 6, which were altered (genes r, s and t), 7.5-fold, $p = 0.016$; 7.1-fold, $p < 0.001$ and 3.6-fold, $p < 0.001$, respectively. Alpha and betaglobin are among the downregulated genes (u and v, 3-fold, $p < 0.05$), as are some of the apolipoproteins (downregulated 2–3-fold, $p < 0.05$). In addition to those genes involved with transport processes or unknown function, a relatively large proportion (>20%) of genes associated with oxygen binding were downregulated. One monooxygenase was upregulated (Cyp1a3, 7.1-fold), and two were downregulated (Cyp 2K1, and an unidentified Cyp isoform, both downregulated less than 2-fold). Among the biological processes associated with genes that were upregulated include DNA damage response and proteolysis and peptidolysis (Fig. 5).

Exposure to Cr caused the greatest number of transcriptomic changes, as shown in Fig. 1 E and Table 5 (supplementary material). It also caused the greatest difference between up- and downregulated genes: 210 of the 218 genes with altered expression are upregulated. Among these are genes involved in growth, protein synthesis, protein binding, oxidoreductase activity, nucleic acid binding, copper ion binding, mitochondrial electron transport and metabolism (Fig. 6). For instance, a ribosomal protein is upregulated 11.5-fold (gene w, $p = 0.03$) and a C1 inhibitor is upregulated 7-fold (gene x, $p < 0.001$). Most downregulated genes have unknown function. As a result, their functions and biological processes are not plotted in Fig. 6.

Diq also caused large changes in gene expression. As shown in Fig. 1 F and in Table 6 (see supplementary material), more genes are upregulated more than 10-fold than in response to any other treatment. The highly upregulated genes include methylenetetrahydrofolate dehydrogenase, acyl carrier protein, and phosphoinositide-3-kinase (genes number y, x, aa, respectively), all upregulated more than 20-fold ($p < 0.005$ for all). Like Cr, many more genes were upregulated than downregulated. Among the downregulated genes were glyceraldehyde 3-phosphatase (gene bb) and a heparin binding factor (gene cc). The GO classifications of the genes altered by Diq are shown in Fig. 7. Of the genes with known function, oxygen binding and oxidoreductase activity genes were downregulated following exposure to Diq, and nucleic acid binding genes are upregulated. Also, genes involved in glycolysis are downregulated.

3.3. qRT PCR results

A subset of genes with significantly altered expression was chosen for qRT PCR analysis. The data, normalized for beta actin expression unless noted, are presented in Table 3. Statistical significance of the difference from control was determined using the *t*-test assuming equal variance ($p = 0.05$). In all cases, expression levels presented are several orders of magnitude different from negative PCR controls ($p < 0.001$ for both the no template and no reverse transcriptase controls). Also, expression levels of the genes selected do not differ significantly among controls ($p > 0.05$). In general, agreement between qRT PCR data and array data is good, though the magnitude of difference between controls and exposed is typically greater in qRT PCR measurements than in array data (Table 3). Some notable exceptions to this trend include the estrogen receptor, which was not found to be altered by the array but upregulated 33-fold ($p < 0.01$) when measured via qPCR, and GADPH, toxin-1 and Apolipoprotein cII, which were not significantly altered in every treatment (shown in Table 3). This result is typical as qRT PCR is a much more sensitive assay for mRNA quantification than microarray based measurements.

A comparison of the genes listed in the supplementary Tables 1–6 show that there are some genes that are altered in expression in response to multiple contaminant exposures. However, as shown in Table 4, the majority of genes are expressed uniquely in response to only one contaminant. For instance, EE2 and Trb have 10 genes that are expressed in response to both, EE2 and BDE have 72, EE2 and BaP have 14, and so on, but the greatest fraction, 104 genes, are expressed solely in response to EE2. Furthermore, the greatest degree of overlap is seen between compounds with similar modes of toxic action. For instance Cr and Diq, both oxidative stressors, have 52 genes in common, Diq and BaP (which has been shown to induce some oxidative stress) have 27. However, each of these compounds still generate unique profiles. In contrast, Trb and Diq have only 13 genes in common. In addition to different individual genes being altered by each contaminant, the functional classification of these genes, as determined by the associated GO terms, is unique to each contaminant exposure, as shown in Fig. 2–Fig. 7.

3.4. Hierarchical clustering results

Hierarchical clustering results further demonstrate the unique profiles generated by each compound via hierarchical clustering algorithms. Fig. 8 shows a gene tree generated in Genespring (Silicon Genetics). Genes are clustered (vertically) according to the similarity in their expression across exposure to different model toxicants, and replicate exposures are clustered horizontally according to the similarity in the gene response they elicit. Genes with similar function are clustered together (Eisen et al., 1998) and model toxicants that cause similar responses in gene expression are also clustered together. The horizontal clustering places contaminants that cause similar changes in gene expression adjacently. For instance, the two oxidative stressors, Cr and Diq, are more closely aligned together than they were to the other study contaminants.

4. Discussion

The overall goal of this work was to allow for characterization of gene expression profiles in trout liver that are directly related to the toxicant exposure. We used a male isogenic strain of rainbow trout for most exposures (Trb being the exception) to allow characterization of expression profiles in fish possessing a uniform genome and exposed to contaminants under consistent environmental conditions. Since Trb is an androgen, adult female trout were used for both the exposures and controls to attempt to maximize changes in gene expression. However, we would hypothesize that many of the changes in gene expression would transcend the differences in gender and age and would be specific to the contaminant response. The exposure levels were chosen to avoid overt signs of toxicity and thus minimize the potentially confounding influences of necrosis and inflammation. Where possible, the exposure duration for the water and oral exposures was selected to allow for the assessment of gene expression under near steady state conditions, and at doses thought to approach environmentally relevant levels.

The design of this experiment and the analysis of the resulting data aim to reduce experimental “noise” and yet maximize the experimental “signal”. Although cloned fish were used to reduce to inter-individual variability, this component of the variation may still be greater than array-to-array variability. The genes presented as significantly altered were chosen on the basis of statistical significance rather than an arbitrary fold change. A drawback of selecting genes based on fold change is that this method may select for large changes in genes that fluctuate irregardless of toxicant exposure above smaller changes in more tightly regulated genes which may be more biologically significant (Draghici, 2003). The *z* score was not used because it will always choose a set fraction of genes as being altered in response regardless of the number that actually changes in response to a given chemical treatment (Draghici, 2003).

The qRT PCR data and array data are in general agreement as to the direction of gene change, although the data often do not agree as to scale. Other studies with the GRASP arrays have also found agreement between the array data and PCR validation (Rise et al., 2004b; von Schalburg et al., 2005b). The difference in scale may be due in part to the semi-quantitative nature of arrays, which were found to be similar to northern blots in previous studies (Bartosiewicz et al., 2000). Also, there is some indication that spotted glass arrays such as the GRASP arrays are more prone to saturation of the response as compared to membrane-type arrays (Lopez et al., 2004). This would explain the large differences in scale for some cDNA sequences such as vitellogenin, which can be induced over a 1000-fold by xeno-estrogen treatments. Typically, the qRT PCR results are most likely to agree with the array results if there is a high degree of confidence (i.e. low p value). Our results with expression of vitellogenin best support this argument. The estrogen receptor results differed sharply between the array and qRT PCR. The reason for this difference may be that the two forms were measuring two different isoforms of the trout estrogen receptor. The sequence on the array aligns most closely to *Oncorhynchus mykiss* GRE gene encoding estrogen receptor (Z16149 accession number, 6e–77e value). The sequence used for qRT PCR aligns most closely to estrogen receptor (*Oncorhynchus mykiss*) (CAB45140 accession number, 4e–75e value). The two may be different isoforms of the receptor, which have been shown to have different levels of expression following exposure to estrogenic compounds in fishes (Pakdel et al., 2000; Thomas, 2000; Sabo-Atwood et al., 2004). The same gene has two isoforms in trout and their transcription depends on which promoter is used. One gene is basally transcribed and the other is induced by estrogen and presumably by estrogenic compounds (Pakdel et al., 2000). Although the alpha and gamma isoforms were induced in female Largemouth Bass at the onset of reproduction, the expression levels of the beta receptor were not shown to change during the reproductive cycle (Sabo-Atwood et al., 2004). The degree to which different isoforms of the estrogen receptor are induced also varies depending on tissue (Thomas, 2000).

qRT PCR data is typically normalized to a housekeeping gene, such as beta-actin, to ensure that RNA samples are correctly diluted and quantified (Rees et al., 2003). However, several of the study contaminants caused changes in the expression of actin when measured via qRT PCR. In these cases, the qRT PCR data are presented without actin normalization to avoid confounding the data.

The contaminants used in this study with the best established toxic mode of action are EE2, Trb and Diq. Both EE2 and Trb are synthetic steroids and established to be strong estrogen and androgen agonists, respectively, in fish (Schultz et al., 2001; Skillman et al., 2006; Ankley et al., 2003; Wilson et al., 2004). Diq is a bipyrillium aquatic herbicide that is selectively accumulated in the fish liver where it readily undergoes redox cycling causing formation of superoxide (Schultz et al., 1992, 1995). The latter when formed in excess, can overwhelm cellular defenses and lead to oxidative stress. As for the other test compounds, BDE-71, BaP and Cr-VI, some inference about mode of action can be made (e.g. thyroid active, oxidative stress) but it is also likely that multiple or mixed modes of action may be occurring. As might be expected, correlation of the gene expression signatures with the established mechanism of toxic action varies among the study contaminants. For instance, the most commonly used biomarker genes for exposure to estrogenic compounds, vitellogenin and the vitelline envelope protein (Arukwe et al., 2001) were upregulated in response to EE2. Cathepsin D, which processes vitellogenin (Patino and Sullivan, 2002) was also upregulated. Cytochrome p450 1A, a commonly used biomarker for exposure to BaP (Stegeman et al., 1988), was significantly induced in fish exposed to BaP. Previous in vitro studies using mammalian cell cultures have shown that many genes are potentially involved in the oxidative stress response, and that different oxidative stressors generate unique gene expression profiles (Weigel et al., 2002; Thorpe et al., 2004). We also found a high number of genes with altered expression following exposure to Diq and Cr-VI (Fig. 6 and Fig. 7), and that the expression signatures generated by

these two oxidative stressors are different. Other studies have noted that many ribosomal genes and heat shock proteins were altered in response to oxidative stressors, which is consistent with our findings (Thorpe et al., 2004; Afonso et al., 2003). Recent studies using human cell lines found that the signal transduction genes map kinase-kinase and phosphoinositide-3-kinase were downregulated following exposure to H₂O₂, 4-hydroxynoneal and *tert*-butylhydroperoxide (Weigel et al., 2002). Phosphoinositide-3-kinase was upregulated in response to BaP and both genes were upregulated in response to Diq in this study.

For Trb and BDE-47, obvious links between genes expression changes and mode of action were less apparent. This is due in part to the large number of genes with unknown function altered in response to each contaminant, and perhaps to the fact that the hepatic gene expression patterns associated with these toxic modes of action is not as well understood as the response to estrogenic compounds and oxidative stressors. Interestingly, alterations in UDPGT or in deiodinases following BDE exposure were not apparent, as has been suggested in other studies using embryonic rats and other BDE congeners (BDE 71,79, 83) (Zhou et al., 2001, 2002). The liver may also have not been the best tissue to select for array analysis. Different responses may have been observed if a different tissue had been chosen such as the brain or the gonad, as the transcriptomic response has been shown to vary with tissue sampled (Volz et al., 2005).

When the GO terms are examined to determine the function of genes with altered patterns of expression, the most striking finding is the high proportion of altered genes with uncharacterized function. This is in part a consequence of the trout genome being less characterized in comparison to other fishes such as the zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), although a fully sequenced genome is not necessarily a prerequisite for understanding biological function. For example in humans, much of the sequenced genome codes for genes with unknown function (Collins et al., 2003). Another interesting result from the GO analysis is the high number of genes involved in transport. This may be a consequence of broad generalizations sometimes used in assigning GO terms. In order to meaningfully reduce the complexity of the data, GO terms were selected at the broadest category of differentiation. In this case, “transport” likely represents many types of cellular transport processes and as a result, may compile genes with dissimilar cellular function. An additional limitation of organizing genes via their GO functions is when the functional group mono-oxygenases are examined following exposure to BaP, there is one upregulated and two downregulated. Since the one upregulated gene (Cyp 1a) is upregulated roughly seven-fold and the two downregulated genes are downregulated less than two-fold, the 1:2 representation may be misleading. However, despite these caveats, the GO terms may be helpful in interpreting the array data. For instance, it could be speculated that the increase in protein folding genes in the liver has utility in packaging vitellogenin for transport to the ovary and ultimately the oocyte. The downregulation of immune function genes following EE2 exposure suggest that there may be increased susceptibility of organisms chronically exposed to EE2 to pathogens. Furthermore, the increase in apoptosis and the decrease in antiapoptosis genes (only noted following exposure to BDE) suggest that increased programmed cell death may occur following exposure to BDE. It could also be hypothesized that the downregulation of oxygen binding molecules, oxidoreductase activity, and the increase in proteolysis and petidolysis, protein biosynthesis following exposure to Diq, Cr-VI and BaP may be characteristic of an oxidative stress response.

In general, our results are comparable to the limited number of studies measuring gene expression in laboratory exposed or field collected fish. Previous studies focusing on the effects of estrogens have observed that many genes including vitellogenin and the vitelline envelope proteins were upregulated while others, notably transferrin was downregulated (Larkin et al., 2002, 2003a,b). Our studies reveal similar changes in expression of these genes and others

following exposure to EE2, though vitellogenin and the vitelline envelope protein were not upregulated to the same degree in our work. European flounder (*Platichthys flesus*) collected from rivers known to be heavily polluted with PAHs among other industrial contaminants were examined for toxic stress response. Few genes were found to be significantly different due to inter-individual variation, but Cyp 1A was upregulated, while elongation factors and complement component C3 were among the downregulated genes (Williams et al., 2003). These genes were altered following exposure to BaP (cyp 1A), EE2 (elongation factors and complement component C3) in this study. Suppressive subtractive hybridization was used to identify genes expressed by male largemouth bass exposed to dihydrotestosterone and 11-ketotestosterone in another study (Blum et al., 2004). These genes were then used to construct a cDNA macroarray. Like our results with Trb, the authors found relatively few genes with altered expression. Few of the same genes were altered in both studies, possibly because we were studying sexually mature females in the Trb exposures as opposed to immature males. Differential display was used to identify genes expressed in fish exposed to trivalent chromium (Maples and Bain, 2004). Eukaryotic initiation factors were found to be upregulated in both that study and the present study using Cr-VI (Fig. 6). In a similar study with Cr-VI, serine proteinase inhibitors, a carboxypeptidase, and elongation factors were among the genes with altered expression following Cr-VI (Chapman et al., 2004). These results are consistent with the findings in the present study (Fig. 6).

Our results demonstrate that the overall patterns of gene expression are unique to each model toxicant, as shown in Fig. 8. Furthermore, as shown in Fig. 2–Fig. 7, the functions of genes with altered expression also has a unique pattern for each of the contaminants tested. While each toxicant signal is unique, the degree of overlap correlates with function. For instance, the Diq gene expression profile are more closely aligned to those expressed in response to other oxidative stressors, and the endocrine active compounds have more similar expression patterns to each other than to the other contaminant classes. Hierarchical clustering organizes gene expression data such that genes with similar patterns of expression are grouped together (Eisen et al., 1998; Butte, 2002). Our clustering results (given in Fig. 8) show that unique expression profiles are generated for each compound, that these profiles are non-random, and that compounds with similar function are more tightly grouped together than compounds with disparate function. Although EE2 and Trb are both considered endocrine disruptors, their modes of action are antagonistic (Trb is an androgen and EE2 is an estrogen), and as a consequence, their gene expression profiles do not resemble each other. This finding that expression of genes with similar function can be grouped together into clusters likely arises because genes with similar function are often co-expressed (Eisen et al., 1998; Heyer et al., 1999; Wu, 2001). Gene expression data from animals treated with the same compound group together when subjected to principle component analysis, and transcriptomic responses from animals treated with similar compounds also cluster (Amin et al., 2002). Other studies in trout have also found that altered patterns of gene expression generated from array results can be displayed in hierarchical clusters (Koskinen et al., 2004). This previous study also examined alterations in gene expression in rainbow trout exposed to different model toxicants at three different doses. They also found that their expression profiles clustered according to the contaminant at low and medium doses. Surprisingly, the transcriptomic patterns did not form exposure related clusters as well at high doses, which the authors suggested may arise from non-specific stress responses (Koskinen et al., 2004).

In conclusion, we exposed rainbow trout to six different model toxicants and compared the resultant gene expression patterns using cDNA microarrays. Our work demonstrates that each compound generates a unique gene expression signature, and that these patterns can be verified via qRT PCR. While this initial study demonstrates the specificity of gene expression profiles to individual chemical contaminants, it does not address how these patterns vary with contaminant dose or duration of exposure. We also frequently encountered a current limitation

of cDNA microarray based gene expression analysis in that many of the genes with altered expression have no known or documented function. This situation is likely to improve in the future, due to the rapid expansion of genomic studies in fish and trout specifically. Overall, this study bolsters the promise of the application of toxicogenomics towards ecotoxicology. The gene expression signatures generated by a compound are unique, even when compared to compounds with similar modes of toxic action. Consequently, arrays may be a means to identify highly specific biomarkers for each toxicant, or at least each class of toxicant. Since many cellular functions are conserved across taxa (Ballatori and Villalobos, 2002), findings of studies using microarray technology may have implications for species other than the organism tested. Furthermore, because some genes with altered expression could be correlated to mode of toxic action, microarray data could be used to determine the potential impact of novel compounds and environmental toxicants (Miracle and Ankley, 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.aquatox.2006.01.007.

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Fig. 1.

Genes with altered response following contaminant exposure Mean fold change is plotted on the y-axis, gene numbers are plotted on the x-axis. Identities of each gene, Gen Bank accession number, and confidence are provided in Table 1– Table 6 in supplementary material. Genes were considered to be altered if they were significantly different than 1 in the given chemical treatment (ANOVA with Benjamini–Hochberg multiple test correction, $p < 0.05$). Panel A shows exposure to EE2, panel B shows exposure to Trb, panel C shows exposure to BDE, panel D shows exposure to BaP, panel E shows exposure to Cr, panel F shows exposure to Diq. The labeled genes are (a) vitellogenin, (b) vitelline envelope protein, (c) apolipoprotein A-IV3, (d) apolipoprotein B, (e) pentraxin, (f) liver-basic fatty acid binding protein, (g) NADH

dehydrogenase, (h) Alpl-prov protein, (i) apolipoportein, (j) glyceraldehyde 3-phosphate dehydrogenase, (k) complement regulatory plasma protein SB1, (l) 60S ribosomal protein L38, (m) Caspase 8, (n) regulator of G-protein signaling 5, (o) serotransferrin II, (p) apolipoprotein B, (q) acyl carrier protein, (r) HSP 90, (s) cytochrome p450 1a3, (t) glutathione *S* transferase, (u) alphaglobin, (v) betaglobin, (w) ribosomal protein S3, (x) C1 inhibitor, (y) include methelynetetrahydrofolate dehyrdogenase, (z) acyl carrier protein, (aa) phosphoinositide-3-kinase, (bb) glyceraldehyde 3-phosphatase and (cc) heparin binding factor.

Fig. 2.

The function of genes with altered expression following exposure to EE2. The number of genes in each category (given as a percentage of the total up- or downregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 31.25% of molecular function or 34.10% of the biological process of the upregulated genes, and 40.14 and 45.45% of the molecular function and biological process of downregulated genes, are not plotted.

Fig. 3.

The function of genes with altered expression following exposure to Trb. Only upregulated genes are plotted because there are too few downregulated genes to infer their function. The number of genes in each category (given as a percentage of the total upregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 45.76% of molecular function or 47.45% of the biological process of the upregulated genes.

Fig. 4.

The function of genes with altered expression following exposure to BDE. Upregulated genes are plotted as positive numbers in black, while downregulated genes are plotted as negative numbers in grey. The number of genes in each category (given as a percentage of the total up- or downregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 57.38% of both molecular function and biological process of the upregulated genes, and 44.18% of both molecular function and biological process of downregulated genes, are not plotted.

Fig. 5.

The function of genes with altered expression following exposure to BaP. Upregulated genes are plotted as positive numbers in black, while downregulated genes are plotted as negative numbers in grey. The number of genes in each category (given as a percentage of the total up- or downregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 47.27% of molecular function or 41.81% of the biological process of the upregulated genes, and 55 and 60% of the molecular function and biological process of downregulated genes, are not plotted.

Fig. 6.

The function of genes with altered expression following exposure to Cr. Only upregulated genes are plotted because there are too few downregulated genes to infer their function. The number of genes in each category (given as a percentage of the total upregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 43.33% of molecular function or 47.14% of the biological process of the upregulated genes are also not plotted.

Fig. 7.

The function of genes with altered expression following exposure to Diq. Upregulated genes are plotted as positive numbers in black, while downregulated genes are plotted as negative numbers in grey. The number of genes in each category (given as a percentage of the total up- or downregulated genes) are plotted on the y-axis. Molecular function and biological processes are plotted on the x-axis. For clarity, functional categories with less than 2% contribution are not plotted. Unknown genes, which comprise 45.5% of both molecular function and the biological process of the upregulated genes, and 39.4% and 45.45% of the molecular function and biological process of downregulated genes, are not plotted.

Fig. 8.

Gene tree generated via clustering algorithms showing relationships between expressed genes and different classes of contaminants as determined by Pearson's correlation. Only those genes that were found to be significantly different from control in at least one treatment. Gene tree is colored as a gradient with respect to expression level, with red denoting five-fold induction, yellow denoting no change (fold change of 1), and green denoting five-fold reduction in expression levels. Treatments are identified below gene expression profiles with the following abbreviations: BDE= brominated diphenylether-47, BaP = benzo[α]pyrene, Cr = chromium, Diq = Diquat, EE2 = ethinylestradiol, Trb = trenbolone. For clarity, individual genes are not labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Chemical exposures and resultant tissue concentrations

Contaminant	Exposure method	Nominal level	Measured level	Tissue concentration ^a
Ethinyl estradiol	Dissolved (methanol carrier)	50 ng/l	37 µg/l	n.m.
Trenbolone	Dissolved (methanol carrier)	1 µg/l	0.939 µg/l	13.6 ± 1.7 µg/g (L)
Brominated diphenyl ether-47	Artemia food	n.m.	500 µg/kg	8.7 ± 2.0 µg/ml (P), 8.8 ± 6.1 µg/g (L)
Benzo[α]pyrene	Dissolved	1 µg/l	n.m.	n.m.
Chromium(VI)	Intraperitoneal injection	25 µg/kg	n.m.	n.m.
Diquat	Intraperitoneal injection	500 µg/kg	n.m.	n.m.

^aL = liver, P = plasma.

Table 2

Primers used for q RT PCR

Gene	Forward	Reverse	Fluorescently labeled
Androgen receptor	AGCGCCAACTGGTCGAA	CACATGCAGATTCCGAAAACC	TGGTCAAGTGGGCCAAAGGCATG
Apolipoprotein A2	ACCTGAGCCATGTACTCCATCAT	CCCATGCAGGCTGATGCT	CTACCTTCACATGCTCCAGCTGAGA
Apolipoprotein CII	GCAGGATGCCAGCGTAAAGTC	AGGCCACGAATGTCCTCAGT	TCGCTGCCACGGTGGTGT
Beta actin	ACGGCCAGAGGCGTACAG	TTCAACCCTGCCATGT	ACAACACGGCCTGGATGGCCA
Cytochrome p4501a3	CCCCTTCCGCCATATTGTC	CGGCCGAAGCACATTCC	TATCGGTGGCCAACGTCATCTG
Delta-6-dehydrogenase	GGGAAGTCCATGTTTCTCACACA	CATGCCCCGTCATAACTACCA	AGCACGGACCAGAGGAGCCACCA
Estrogen receptor	GCAGGACCAAACCTCCGTAGTG	TGGCCAACGCGAGGTA	TACCCAGAGGCCAAAGTCGCTGCAGA
GADPH	TGACCGTCCGTCTGGAGAA	TCGGCAGCAGCCTTAACAA	CCTGCCAGCTATGATGCCATCAAGAAG
Haptoglobin	CACGGCACAGGACTTATCGA	CAGTCCAGGACCCCAAAGAC	ACAGGATCCCTGCAGCATACACTCT
Polyubiquitin	GGCCATCTTCCAGCTGCTT	GCCAAGATCCAGGACAAGGA	AGCCTCTGCTGGTCTGGCGGG
Procathpsin B	CTTTCCCCAGCCCAGGAT	ACCAGCATGTGACTGGACAGAT	TTGATGGCATGACCCCCCAG
Toxin-1	GCATTGCAGCGGTTCTGAT	GGCTGTGCTAGGCTGGAGTT	CAGCACTTCCAGCCTGTTCGAGAGC
Vitellogenin	CTTGTAACCCTGAGATC	GCAGCTGGGACGAAAGG	TTGAGTACAGTGGTGTGTGGCCCAAAGA
Vitelline envelope	GCCGGTTCCTCCTCCAAAT	TCCGCTGCCCAGTCTGA	CTGATATAGCTCCTGGGCCCCCTCATAGTTG

All primers are listed from 5' to 3'. Fluorescently labeled primers have a 6-FAM fluor on the 5' end and a TAMRA fluor on the 3' end.

Table 3

Comparison between array data and qPCR data

Gene	Contaminant	Array		qPCR	
		Fold change	p-Value	Fold change	p-Value
Cytochrome p450 1a3	BaP	7.1	<10 ⁻⁴	2.5	<0.05
	EE2	-1.81	0.02	-3.68	<0.01
Estrogen receptor	EE2	1.11	0.223	33	<0.01
Vitellogenin	EE2	10.42	<10 ⁻³	65000	<10 ⁻³
Vitellogenin envelope	EE2	7.22	<10 ⁻³	36500	<10 ⁻⁶
GADPH	EE2	-3	<0.01	-2.5	0.06
	BaP	-1.75	0.08	-5.6	0.0015
	Diq	-2.78	0.04	1.3	0.19
	Trb	-1.5	0.08	-3.79	0.18
Polyubiquitin	BaP	1.95	<0.0001	5.35 ^a	<0.001 ^a
	Diq	1.96	<0.0001	10	0.005
	Cr (VI)	1.292	0.211	2.5	0.007
Androgen receptor	EE2			2.73	<0.005
	Trb	-2.6	<0.01	2.17	0.15
Apolipoprotein CIII	EE2	2.45	0.02	-1.5	0.16
	Cr	-2.2	0.06	10.4	0.13
	BaP	2.8	<10 ⁻⁴	18	0.03
Delta-6-desaturase	EE2	1.57	0.05	2.5	<10 ⁻⁵
	Trb	-1.78	0.06	36	0.05
Haptoglobin	BaP	2.34	<0.02	-8	0.002
	Trb	2	<0.001	2	0.13
Procathepsin B	Diq	-2.15	0.02	-2	0.05
Toxin-1	EE2	2.30	<0.01	1.04	0.42
	Cr	1.74	0.08	7	0.03
	Trb			3	0.15

For each cDNA tested, the gene name and chemical treatment are given. Mean fold change was determined by comparing expression levels in treated fish to that in appropriate controls, as described in the text. *p*-Values were determined via a Student's *t*-test assuming equal variance, compared to a fold change of one for the array data. For the qPCR data, the *p*-values were determined via a Student's *t*-test assuming equal variance, and compare the expression levels in exposed versus control fish. qPCR data are normalized to actin expression levels, except as noted, as described in the text.

^aData that are not actin normalized.

Table 4

Specificity of the gene expression profiles

	BaP (94 total)	BDE (223 total)	Cr-VI (218 total)	Diq (146 total)	EE2 (190 total)	Trb (65 total)
BaP (94 total)	44 unique	29	1	27	14	14
BDE (223 total)	29	148 unique	7	24	72	15
Cr-VI (218 total)	1	7	137 unique	52	2	8
Diq (146 total)	27	24	52	33 unique	13	14
EE2 (190 total)	14	72	2	13	104 unique	10
Trb (65 total)	14	15	10	13	10	38 unique

In each grid, the number of genes with significantly altered expression for the corresponding chemical treatment are given.