Comparative Transcriptomic Responses to Chronic Cadmium, Fluoranthene, and Atrazine Exposure in *Lumbricus rubellus*

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Transcriptional responses of a soil-dwelling organism (the earthworm *Lumbricus rubellus*) to three chemicals, cadmium (Cd), fluoranthene (FA), and atrazine (AZ), were measured following chronic exposure, with the aim of identifying the nature of any shared transcriptional response. Principal component analysis indicated full or partial separation of control and exposed samples for each compound but not for the composite set of all control and exposed samples. Partial least-squares discriminant analysis allowed separation of the control and exposed samples for each chemical and also for the composite data set, suggesting a common transcriptional response to exposure. Genes identified as changing in expression level (by the least stringent test for significance) following exposure to two chemicals indicated a substantial number of common genes (>127). The three compound overlapping gene set, however, comprised only 25 genes. We suggest that the low commonality in transcriptional response may be linked to the chronic concentrations (~10% EC_{50}) and chronic duration (28 days) used. Annotations of the three compound overlapping gene set indicated that genes from pathways most often associated with responses to environmental stress, such as heat shock, phase I and II metabolism, antioxidant defense, and cation balance, were not represented. The strongest annotation signature was for genes important in mitochondrial function and energy metabolism.

Introduction

In (eco)toxicology, a major research theme is to identify the mechanisms that allow species to maximize fitness under chemical stress. To identify such mechanisms, a first step should be to identify the common and distinct biochemical pathways affected by chemical exposure. Such information can provide information on the molecular basis of toxicity to chemicals with different modes of action, including which biochemical pathways are uniquely and commonly affected (*1, 2*).

The current state of the art for analyzing molecular responses to environmental stress is to determine global transcript, protein, or metabolite profiles and then mine these to identify responses indicative of exposure and/or effect (*3–5*). Here we use a custom-made cDNA microarray of ~8000 transcripts (*6*) to measure transcriptional responses of an ecologically important soil-dwelling organism (the earthworm *Lumbricus rubellus*) to comparatively low concentrations (~EC_{10} for reproduction) of three chemicals from important pollutant groups. These are the nonessential trace metal cadmium (Cd); the narcotic nonpolar polycyclic aromatic hydrocarbon (PAH) fluoranthene (FA), and the photosynthesis-inhibiting herbicide atrazine (AZ). The aim of this study was not to investigate the specific mechanistic responses of the exposed worms to each chemical. That aspect is covered in an initial article upon which this work builds (*6*). Instead, here we specifically investigate the nature of shared transcriptional changes associated with low-dose (~EC_{10}) exposure.

Evidence that there is a shared transcriptional response to environmental stress has emerged from studies in the yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These identified the presence of a potential group of "common environmental response (CER)" genes, the expression of which was ubiquitously altered by a range of environmental stresses such as heat shock, acidity, alkalinity, salt, hypoxia, and osmotic stress (*7, 8*). Many of the identified CER genes are involved in known stress-responsive pathways like heat shock, organic compound metabolism, and oxidative stress (*9*) as well as in protein turnover, metal transport, thioredoxin/glutathione regulation, and energy generation (*7, 8*). By comparing transcriptional responses of earthworm following separate chronic exposures, we sought in this study to establish whether a set of common chemical stress genes exists for earthworms that matches those found in yeast, or alternatively, whether the diverse toxins studied produce unique transcriptional changes.

Materials and Methods

Design of Exposures and Selection of Biological Replicates for Transcriptional Profiling. Exposures were conducted with adult *L. rubellus* in a commercially available clay loam soil (Broughton Loams, Kettering, U.K.) amended with 3% organic matter following a 28-day protocol (*10*). Earthworms were kept at a series of soil concentrations ranging from unexposed (controls) to just below lethal levels. Exposure concentrations used were 0, 13, 43, 148, and 500 mg of Cd/kg; 0, 13.8, 46, 158, and 533 mgof FA/kg; and 0, 9.4, 20.7, 35, and 59 mg of AZ/kg. Eight control replicates were used to

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provide a clear picture of baseline variation of life-cycle and transcriptional responses. Five replicates were used for all remaining concentrations.

Each replicate container held 1.4 kg of dry soil in 180 × 180 × 93 mm polypropylene boxes. Test soil was spiked with Cd (as chloride salt) in water and then left for 14 days to reach an initial speciation equilibrium. FA and AZ were spiked into the test soil in pure acetone and ethanol, respectively. The same volume of solvent was added to all soils (including controls). Soils were ventilated for at least 7 days to ensure there was no solvent remaining, before the soil was wetted to 60% of water-holding capacity as used in all tests.

Adult *L. rubellus* were supplied from an uncontaminated field site (Neptune Ecology, Ipswich, U.K.) and then kept on a medium consisting of a 1:1:1 mix of loam soil:peat: composted bark for at least 4 weeks. Seven days before each experiment, sufficient worms were collected from culture and transferred to fresh culture medium kept under test conditions. To set up each replicate, eight adult worms were selected, weighed as a batch, and added to the soil. Five grams dry weight of horse manure, spiked to the corresponding chemical concentrations and rewetted to 80% water-holding capacity, was then added to the soil surface as food. All boxes were then covered to prevent water loss and kept at 12 °C under a 16 h light/8 h dark regime for 28 days.

After 14 days, any remaining manure was removed and boxes were assessed for earthworm survival. Soil and worms were then returned to the box and a fresh 5 g dry weight of spiked and wetted manure was added. After 28 days and at the same time (midday ± 30 min), all worms were retrieved from the soil, weighed, and visually inspected for phenotypic characteristics (presence of skin lesions, scarring of the clitellum, presence of body constrictions, loss of turgor, and reduced vigor) and given a "condition index" score ranging from 1 (pristine) to 5 (very poor for many characters). Within a maximum of 5 min, all individuals were frozen in liquid nitrogen and stored at −80 °C. All test soils were sieved and the number of cocoons was counted and used to calculate a cocoon production rate (cocoons per worm per week).

Global Transcript Profiling by Use of cDNA Microarrays. *Lumbricus rubellus* has been the subject of an extensive expressed sequence tag (EST) project. This has established a database of over 17,000 ESTs from unexposed and chemically exposed earthworms. All sequences have been assembled into clusters and annotated by use of PartiGene (*11*). Annotation for the sequence was established by BLAST homology as recoded in LumbriBASE (www.earthworms.org). To fabricate the glass slide cDNA microarray, a representative EST (usually the longest) of each sequence cluster was selected from clone stocks (*6*). This sequence was amplified by polymerase chain reaction (PCR), and aliquots (5 μL) of each concentrated product mixed in with an equal volume of dimethyl sulfoxide (DMSO). The cDNAs selected for each cluster were then printed singly onto Ultra-GAP glass slides (Corning) by use of 48 SMP3 pins (Telecham) mounted in a Spotarray 72 (Perkin-Elmer). Landmarks were introduced into each subarray as five replicates of the Lucida Scorecard (Amersham) gene reporters, which show no cross reactivity to earthworm transcripts. All reporters were cross-linked by baking at 80 °C for 2 h and UV exposure (*6*).

For each replicate, the three worms maintaining highest condition were pooled to make the sample for hybridization. High-condition worms were selected to avoid inclusion of diseased or parasite-infected individuals that may have confounded results. An issue with this approach could have been that it excluded transcript profiles from sensitive individuals showing the greatest condition loss. In fact, however, condition was only weakly affected by exposure (Supporting Information, Figure 1), meaning such selection bias was unlikely. All selected worms were combined and ground to powder under liquid nitrogen. Total RNA from each composite sample was then isolated (*12, 13*).

Lucida Scorecard test spike (Amersham Life Sciences) was added to 10 μg of total RNA prior to oligo-dT reverse transcription and coupling to Cy3 by an indirect aminoamyl procedure. Clean-up of labeled targets, yield, and integrity were assessed by separation on a 2% agarose gel and visualization. Only labeled samples where fluorescence incorporation exceeded 20 pmol ofCyDye/μg of cDNA in a wide size range of transcripts were hybridized. A reference design was used for all microarray experiments in which ~30 pmol of Cy3-labeled target RNA was hybridized against 1 pmol of reference. The reference used was a 65−70-mer oligonucleotide designed against the vector sequence between the amplification primer binding site and cDNA insert. The use of this universal reference design allowed comparison of slides to be made both within and between experiments. After hybrizidation (18 h), slides were washed and imaged according to Owen et al. (*6*).

Microarrays for eight control and five exposed replicates from the concentration most closely matching the cocoon production EC_{10} were used to identify chemically responsive genes. Array images were quality-controlled for artifacts such as background effects and spot morphology. The Lucida Scorecard calibration standards were analyzed to identify any slides showing nonlinear responses or where the detection limit was below 10 pmol. All array met these standards. Array images were analyzed with Imagene (Biodiscovery), using the default flagging and segmentation settings, and subsequently checked by eye (see ref *6*). Data were then imported into GeneSpring 7.3 (Agilent Technologies, Palo Alto, CA) and processed by background subtraction, and Cy3/Cy5 ratios were generated. Data were normalized by compound via median polishing (per gene and per chip) implemented with default GeneSpring settings. Processed data were visualized as box plots to compare median and quartile ranges. These confirmed the success of all hybridizations except for replicate 1 at 44 μg of Cd/g, which showed a divergent pattern. This sample was excluded from all further analysis. All data microarray collected was fully MIAMI compliant and has been submitted to ArrayExpress (E-MAXD-31, 34, 35, 36).

Statistical Analysis of Microarray Data. Normalized quality-checked data were filtered to include only spots flagged as present in at least 5 of the 38 successfully hybridized slides. This removed data from genes that were either poorly expressed or weakly detected in the majority of studies without removing genes only expressed in one condition. Filtered data were used to generate three gene lists for each chemical. These were (1) genes with significantly different ($p < 0.05$) expression between control and exposed samples following Benjamini and Hochberg false discovery rate correction (labeled as "significant with B&H"); (2) genes with significantly different expression between control and exposed without false discovery correction (labeled as "significant no FDC"); and (3) genes with a >2-fold difference in mean expression between control and exposed samples (labeled as ">2-fold"). Venn diagrams were used to identify genes overlapping between these lists and annotations categories inspected.

Pattern recognition analysis was conducted by importing the normalized data into SIMCA-P, version 10 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used for the individual experiments and for a composite experiment comprising all control and all exposed samples. PCA is unsupervised and so separations seen are likely to have a true biological basis (*14*); it may, however, fail to pick up biologically meaningful effects that do not represent a large proportion of variance. Supervised methods such as

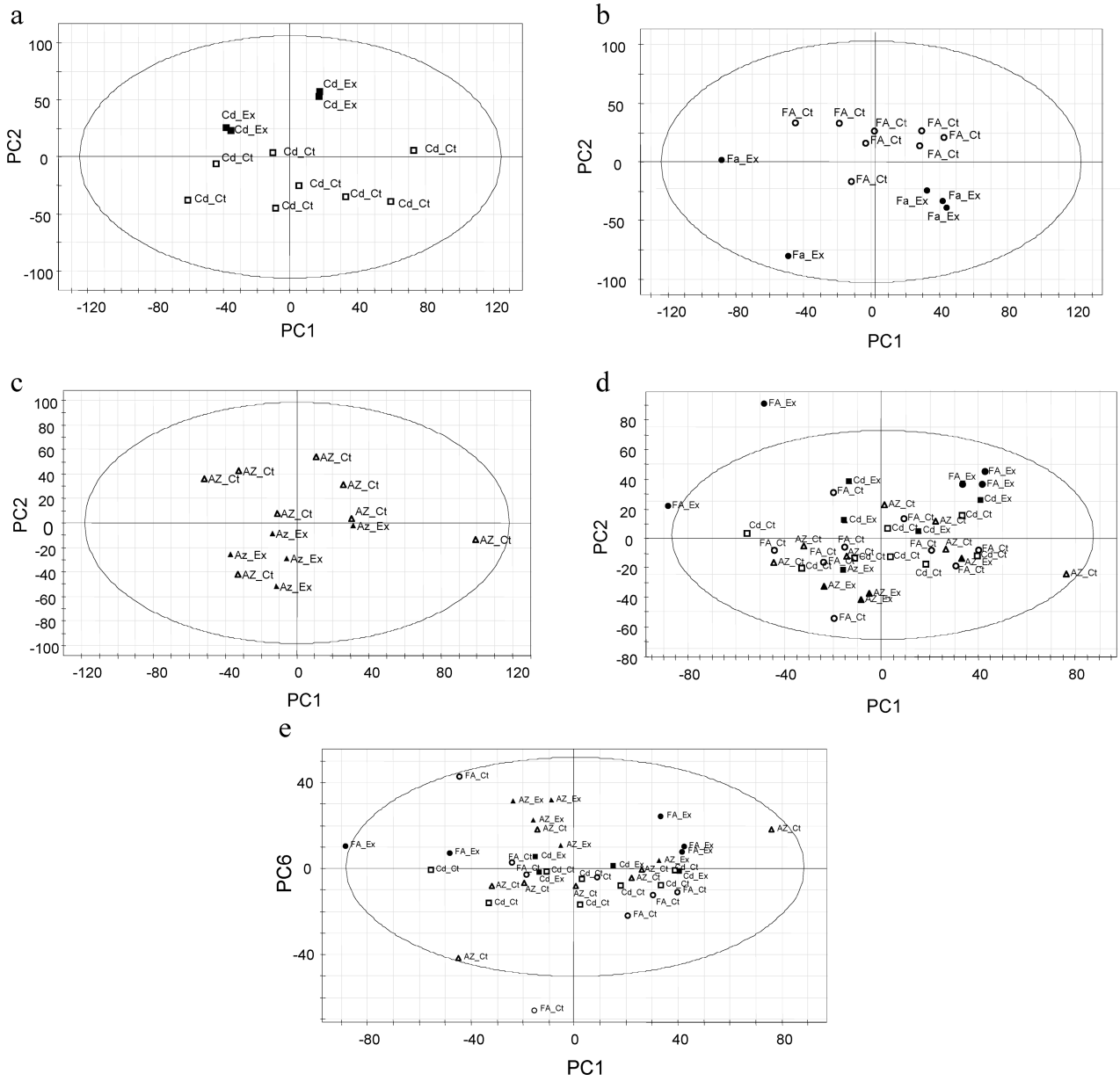

FIGURE 1. Scores plot for PC1 and PC2 from a principal component analysis of normalized cDNA microarray data for *L. rubellus* exposed to control soils and chronic concentrations of (a) Cd, (b) FA, (c) AZ, and (d) a composite of all control and exposed samples. (e) Scores plot for PC1 and PC6 from a principal component analysis of normalized microarray data for the composite set of all samples.

partial least-squares (PLS) regression have a strong track record for assisting in the further interpretation of "omics" data (*14, 15*). Therefore, in addition to PCA, we also used partial least-squares discriminant analysis (PLS-DA). All PCA and PLS-DA plots created in SIMCA-P were fitted with an ellipse calculated via Hotelling's T^2, a multivariate generalization of Student's *t*-test, which checks the multivariate normality of the data set. The area within the ellipse corresponds to the 95% confidence region of the model. Observations outside the ellipse may be considered outliers.

All PCA and PLS-DA models generated were cross-validated within SIMCA-P by use of an iterative procedure with a model constructed from $^6/_7$ of the data to predict the classes of the remaining samples. From this cross-validation, Q^2 (the fraction of the variation in both the *X*- and *Y*-matrices explained by the model), R^2X (the fraction of the sum of squares of the *X*-matrix explained by the model) and R^2Y (the fraction of the sum of squares of the *Y*-matrix explained by the model) values of between 0 and 1 can be calculated. A robust PCA has an $R^2 > 0.5$, while a robust PLS-DA Q^2 score is > 0.4 and $Q^2 > 0.7$ is highly robust (*16*).

Results

No effect of exposure on earthworm survival was found for any compound ($p > 0.05$) (Supporting Information, Figure 2a−c). A significant effect of exposure of worm condition was found only for FA ($p < 0.001$), with the condition at 533 μg of FA/g significantly lower than all other treatments (Supporting Information, Figure 1a−c). For all three compounds a significant concentration-dependent effect on reproduction was found ($p < 0.001$) (Supporting Information, Figure 2a−c). EC_{50}s were 153 (95% CI 50−470) μg of Cd/g, 182 (95% CI 106−311) μg of FA/g, and 59.42 (95% CI 47.3−74.5) μg of AZ/g. Replicates exposed to 44 μg of Cd/g, 47 μg of FA/g, and 20.7 μg of AZ/g were selected for the microarray experiment. These approximated the EC_{10} of each chemical having mean reproduction rates of 88%, 88%, and

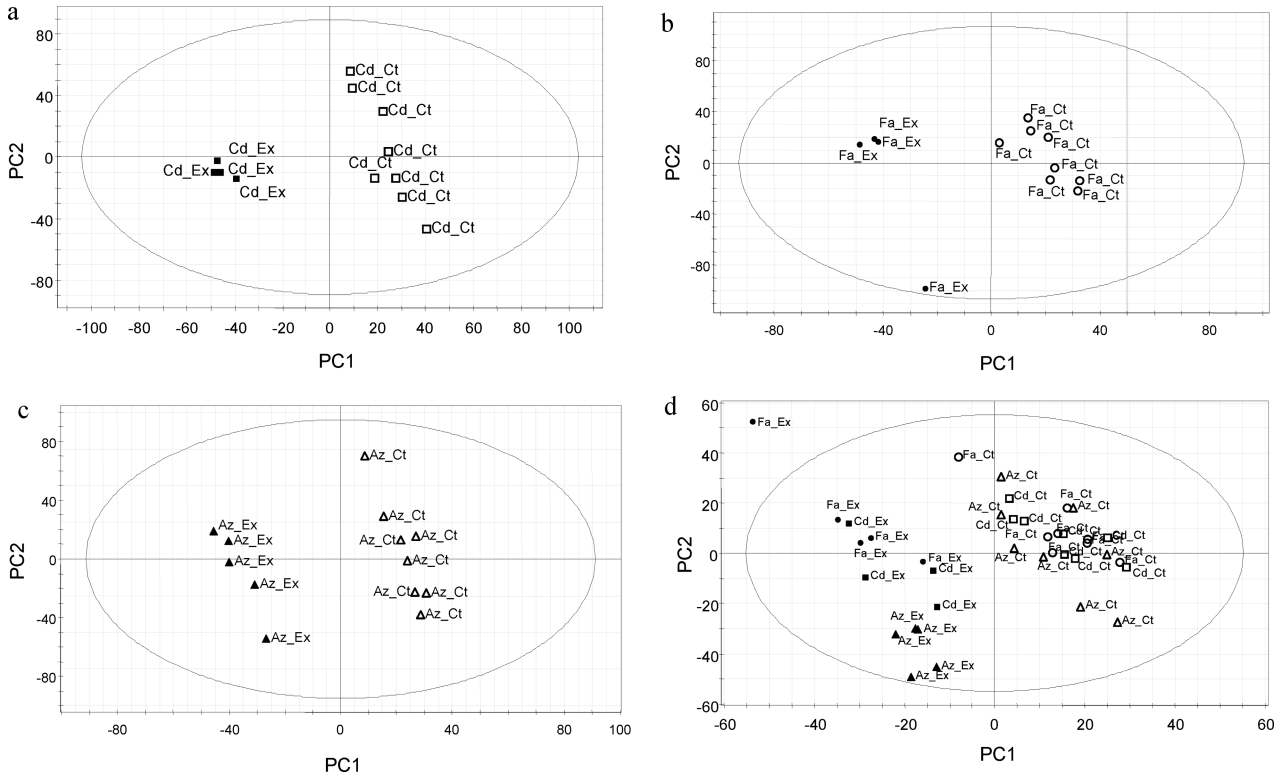

FIGURE 2. Scores plot for PC1 and PC2 of a partial least-squares discriminant analysis of normalized cDNA microarray data for earthworms exposed to control soils and chronic concentrations of (a) Cd, (b) FA, (c) AZ, (d) a composite of all samples.

87% of the relevant experimental control (solid bars on Supporting Information, Figure 2a−c).

All microarrays from each experiment that passed the quality criteria for hybridization were used for multivariate statistical analyses (total 38 microarrays of 13 per compound experiment with eight controls and five exposed at ~EC_{10}, except for Cd where one replicate was excluded; see Supporting Information, Figure 3a−c). Filtration based on presence in five out of 38 microarrays reduced the number of spots to 7406 out of 7870 total (94.1% passed filter). PCA on normalized data from these present spots, with controls only, indicated no separation along any of the five calculated PCs. Further, no PLS-DA model capable of separating control samples from the different experiments could be generated (data not shown). Failure to separate the different experimental controls indicates no systematic bias between experiments that could affect the outcome of any composite analysis.

Separate PCAs were conducted for each chemical and also for a composite of all control and exposed samples. Three (Cd, FA) and two (AZ) PCs could be fitted with R^2X for Cd of 0.542, FA of 0.527, and AZ of 0.369. Visualization by scores plot indicated partial or complete separation of control and treated worm along the second principal component (Figure 1a−c). Composite analysis of control and exposed samples produced a model with seven PCs and an R^2X of 0.544. Separation of exposed and treated samples was not seen along either of the first two principal components (Figure 1d); however, a separation along PC6 was seen, indicating a subtle generic chemical effect on transcript profiles (Figure 1e).

Highly robust PLS-DA models with three PCs and a Q^2 value of 0.822 for Cd and two PCs and a Q^2 of 0.792 for AZ could be calculated, with both showing clear separation of control and exposed samples on PC1 (Figure 2a,c). For FA, the initial PLS-DA allowed calculation of only the first PC. The Q^2 for this model was 0.36, indicating that the model was not robust. Analysis of the scores plot indicated that controls generally separated from exposed samples along the single PC. The exception to this was a single exposed sample (replicate 4) that was outside the response range of all other samples. Exclusion of this sample allowed a three-PC model with a robust Q^2 score of 0.487 to be calculated, within which control and exposed samples separated along PC1 (Figure 2b).

PLS-DA of the composite set of all control and exposed samples generated a model with two PCs. Q^2 for this model was >0.4, indicating the model was robust. Control samples separated from exposed samples along PC1 (Figure 2d). Further, among exposed samples, AZ replicates separated from those for FA and Cd along PC 2. This indicates a secondary compound specific effect on transcription overlaid onto the common exposure effect.

Similarities in transcriptional responses were compared by filtering the microarray data to generate three gene lists that were then compared in Venn diagram analyses to identified common differentially expressed genes. The numberd of genes identified within the single-compound and overlapping gene lists are summarized in Supporting Information, Table 1. The least stringent filter was "significant no FDC". This identified a list of between 864 and 901 significant genes for the three chemicals. By chance it would be anticipated that, of the ~7500 transcripts in the initial data set, 375 would pass this filter. Thus, around 42% of the genes in these single-chemical tests are likely to be false positives. The ">2-fold" filter identified between 148 and 394 up- or down-regulated genes. The most stringent filter was "significant with B&H". This failed to identify a single gene that passed this statistical test in either the Cd or FA experiments. For AZ, 29 genes were identified as significantly different according to the test criteria. Because of the small number of genes passing the "significant with B&H" filter based on the analysis of the list of present genes, analysis for overlapping genes could not be continued with these lists.

Construction of Venn diagram plots to determine overlap between the "significant no FDC" gene list identified

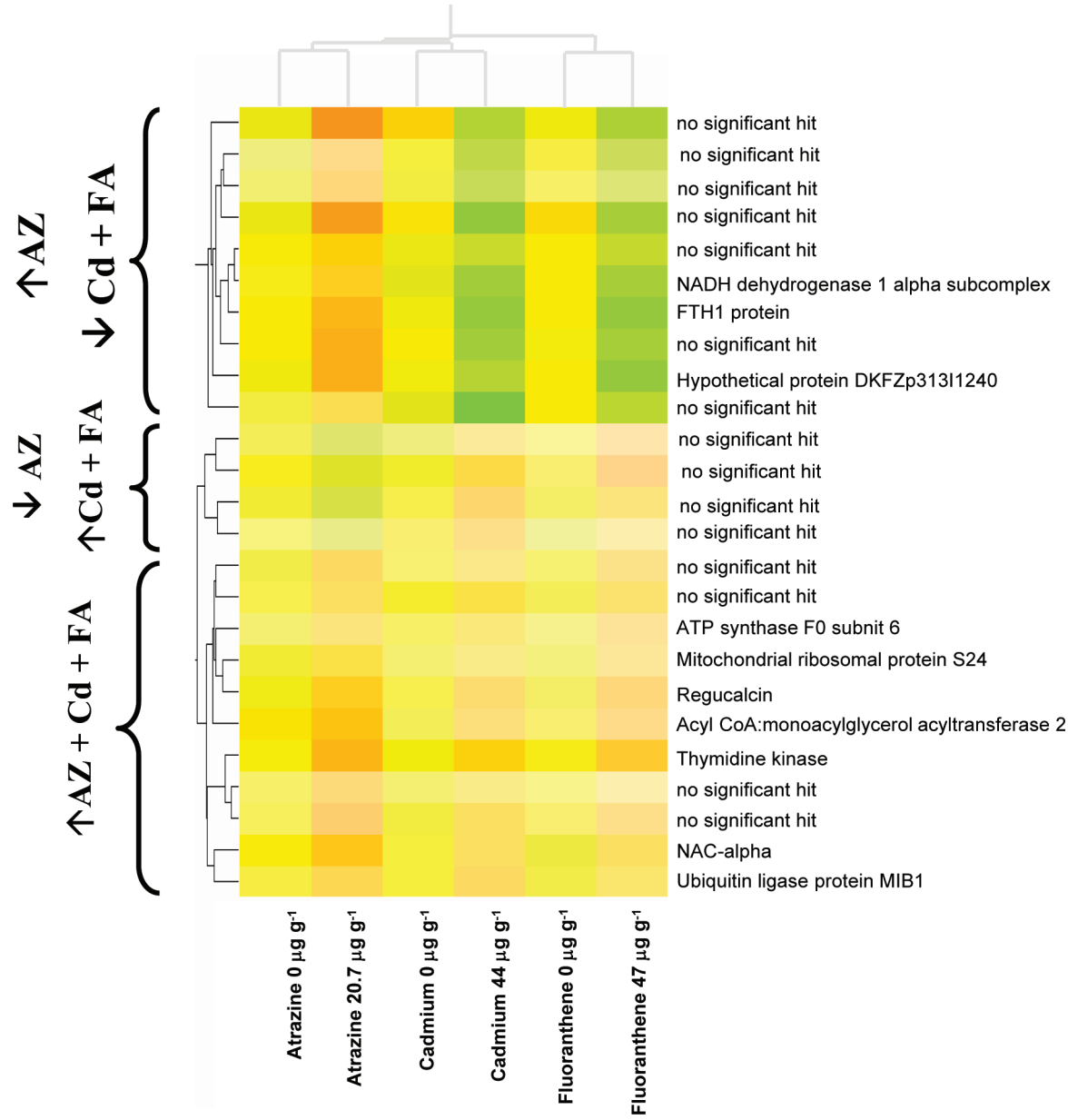

FIGURE 3. Gene-tree showing mean transcript expression of the three compound overlapping significant no FDC genes. Ontology indicates significant homology to a human sequence.

between 127 and 133 common genes for two-chemical combinations (against an expectation of ~18 by chance) and 25 common genes for the three-chemical combination (against an expectation of ~1 by chance). Overlaps of the "<2 fold" gene lists identified between 21 and 39 genes in the two-chemical combinations and three genes for the three-chemical combinationss.

Hierarchical clustering of the 25 common "significant no FDC" genes identified three main groupings (see Figure 3 and Supporting Information, Table 2 for Systematic IDs, GenBank accession numbers, LumbriBASE IDs, and Blast annotation). Ten genes were significantly up-regulated by AZ exposure but down-regulated by Cd and FA; two of which had significant human homology (NADH dehydrogenase 1 α subcomplex and FTH1 protein). Four genes were down-regulated by AZ exposure but up-regulated by Cd and FA. None had significant human homology. Finally, 11 genes were up-regulated in all exposures. Among these were seven transcripts with significant human homology, including transcripts associated with mitochondrial function and energy metabolism (e.g., ATP synthase F0 subunit 6, mitochondrial ribosomal protein S24, and acyl-CoA monoacylglycerol acyltransferase 2), as well as protein synthesis and DNA replication. The unannotated genes identified all correspond to unique clusters within LumbriBASE. The three common genes between the ">2 fold" gene lists did not include any genes with the same directional change and only one gene with a significant human hit. This was the mitochondrial protein COX1.

Discussion

Because they can measure the expression of multiple transcripts, DNA microarrays are increasingly being used in toxicology to cluster chemicals according to their modes of action (*17, 18*) and biological effects (*4, 19*). Increasingly, microarrays are being developed for nonmodel species (*20, 21*). Here we have used a high-density cDNA microarray for *L. rubellus* (*6*) to compare and contrast transcription responses of worms following chronic exposure (<13% reduction in reproduction) to three pollutants with the aim of establishing whether there was a shared transcriptional response.

PCA for each chemical identified partial or complete separation (along PC2) of control and exposed samples (Figure 1a−c). A composite analysis with all control samples, however, failed to show a clear separation (Figure 1d), except for PC6, which accounted for only a small percentage of total variation (Figure 1e). Further investigation by PLS-DA, a supervised method, allowed calculation of robust ($Q^2 > 0.4$) models that separated controls from exposed samples along PC1 for all three single chemicals and also the composite data set (Figure 2a−d). The separation of control and exposed samples by PLS-DA indicates a common effect of exposure on the transcriptome. That the unsupervised PCA failed to identify such clear separation, however, indicates that these common transcriptional changes may be masked by compound-specific effects.

To identify the transcriptional changes that separate control and exposed samples within the PLS-DA model, compound-specific gene lists generated by filtering on significance and fold change were compared. Although the number of genes in the overlapping gene list exceeded expectation by chance (e.g., 25 genes in the "significant without FDC" data sets against an expectation of 1), the number of genes showing common differential expression in all exposures was comparatively small. Of the 25 common "significant no FDC" genes, only 11 showed similar directional expression change. None of the three genes in the ">2-fold" overlap set showed a similar regulatory change.

Annotation of the 25 common "significant no FDC" genes identified multiple transcripts associated with energy metabolism and mitochondrial function. Genes associated with this biological process and cellular compartment were among the CER genes identified in *S. cerevisiae* and *S. pombe* (*7, 8*). Genes associated with other protective functions (such as heat-shock proteins, metallothioneins, cytochrome P450s, glutathione S-transferase, antioxidant defense, and mitogen-activated kinases) also identified as common responses to environmental stress (*7, 9*), were not, however, represented in the earthworm common list. This indicates that although consistent patterns in transcriptional response to exposure are identified by the PLS-DA model, the nature of this common response at the investigated effect level is not consistent with those identified for studied yeast species.

Failure to identify a conserved response to chemical stress in earthworms that is consistent with those found for other organisms raises interesting questions regarding the basis of these differences between taxa. One explanation for the divergent responses could be that technical and biological variation in the exposure and microarray analysis for *L. rubellus* increases data variability and, thereby, precludes identification of common responses. Certainly in our work, the earthworms were exposed to each chemical in a complex medium (soil) that is inherently more variable than the nutrient media on which the species used in previous work (e.g., *S. cerevisiae* and *S. pombe*) and other laboratory models (*Caenorhabditis elegans* and *Drosophila melanogaster*) are reared and exposed. Nonetheless, each experiment identified that between 2% and 5% of the earthworm genome was up or down-regulated at least 2-fold. This observation is somewhat below the 10% remodeling of the *S. cerevisiae* genome found, for example, by Causton et al. (*7*) but not greatly outside expectation. Thus it is unlikely that experimental variability alone prevents identification of a conserved response.

Two further characteristics of our exposure may offer plausible explanations for the small number of common responsive transcripts identified. First, while the exposure concentrations used were clearly sufficient to cause remodeling of the transcriptome (as shown by PCA and PLS-DA), the extent of toxic effect was comparatively small (<13% effect on reproduction). The changes in expression associated with chronic effects may be more compound-specific than those associated with acute toxicity. Second, compared to the short exposure periods (24−48 h) used in many previous studies (*7, 8*), our exposure was comparatively long-term (28 days). Time-series studies have established that many of the most well-established changes associated with environmental stress, such as heat-shock protein induction, can be transient (*9, 22, 23*), with the organism later able to down-regulate these systems and return to a more stable physiological state (*9*). Thus, while over the short term stress responses such as heat shock, cation trafficking, and antioxidant defense may be up- or down-regulated (*24*), these responses may be transient, and over time the transcriptional changes needed to permit optimum functioning following exposure to chemicals of different modes of action may diverge, leaving only a limited overlap between transcriptional profiles.

While transcriptional profiling is clearly valuable for assessing gene expression (*25*), microarrays inevitably provide only a snapshot of what is a dynamic system. This raises concerns regarding the application of transcriptional profiling within applied environmental science. Initial work to incorporate measurements of changes in gene or protein expression into environmental monitoring or regulatory policy have focused on single biomarkers (*26*). This may be appropriate when the specific mode of action of the chemical is known (e.g., vitellogenin induction by environmental estrogens) but not for more general chemical modeling. Here, although the overlap between chronic transcriptional response profiles was relatively small, PLS-DA models could be built that separated control and exposed worm samples independent of the nature of the exposure. This indicates that, despite recent debates about the value of the environmental "biomarker" approach (*27*), transcriptional response profiles can theoretically be used to identify earthworms exposed to diverse chemicals. Further work is of course needed to confirm whether such separations can be maintained when responses to further chemicals from additional classes are considered.

Acknowledgments

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Supporting Information Available

Three figures and two tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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