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Initial development of a multigene 'omics-based exposure biomarker for pyrethroid pesticides

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ABSTRACT

Omics technologies have long since promised to address a number of long standing issues related to environmental regulation. Despite considerable resource investment, there are few examples where these tools have been adopted by the regulatory community, which is in part due to a focus of most studies on discovery rather than assay development. The current work describes the initial development of an omics based assay using 48 h *Pimephales promelas* (FHM) larvae for identifying aquatic exposures to pyrethroid pesticides. Larval FHM were exposed to seven concentrations of each of four pyrethroids (permethrin, cypermethrin, esfenvalerate and bifenthrin) in order to establish dose response curves. Then, in three separate identical experiments, FHM were exposed to a single equitoxic concentration of each pyrethroid, corresponding to 33% of the calculated LC50. All exposures were separated by weeks and all materials were either cleaned or replaced between runs in an attempt to maintain independence among exposure experiments. Gene expression classifiers were developed using the random forest algorithm for each exposure and evaluated first by cross-validation using hold out organisms from the same exposure experiment and then against test sets of each pyrethroid from separate exposure experiments. Bifenthrin exposed organisms generated the highest quality classifier, demonstrating an empirical Area Under the Curve (eAUC) of 0.97 when tested against bifenthrin exposed organisms from other exposure experiments and 0.91 against organisms exposed to any of the pyrethroids. An eAUC of 1.0 represents perfect classification with no false positives or negatives. Additionally, the bifenthrin classifier was able to successfully classify organisms from all other pyrethroid exposures at multiple concentrations, suggesting a potential utility for detecting cumulative exposures. Considerable run-to-run variability was observed both in exposure concentrations and molecular responses of exposed fish across exposure experiments. The application of a calibration step in analysis successfully corrected this, resulting in a significantly improved classifier. Classifier evaluation suggested the importance of considering a number of aspects of experimental design when developing an expression based tool for general use in ecological monitoring and risk assessment, such as the inclusion of multiple experimental runs and high replicate numbers.

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1. Introduction

Current approaches for estimating chemical risk and identifying causes of ecosystem impairment rely upon measured or estimated environmental concentrations of a specific or limited set of toxicants. Occurrence values are compared to toxicity values generally obtained from highly controlled laboratory studies conducted with a few model species. This approach suffers from a number of limitations which can lead to difficulty in estimating the risk. For instance, the approach uses an occurrence value rather than a systemic exposure value, and typically does not account for interactions among co-occurring chemical constituents, such as other contaminants not targeted for analysis. Other components that are naturally present at varying levels, such as dissolved organic matter, often modify the toxicity of a given concentration of toxicant in unanticipated ways (Denton et al., 2003). For example, low dissolved oxygen levels will increase respiration rates in aquatic species, increasing systemic exposure rates as more water is moved over the gill per unit time (McKim and Goeden, 1982). The traditional ecological risk estimation paradigm is not well-suited to account for uncertainty arising from interactions in the complex chemical mixes typical in environmental matrices, complicating the

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estimation of systemic exposure and the potential for adverse outcome. Further limiting the linkage of exposure and effect, especially when applied to ecological forensics, is the reliance on apical endpoints of regulatory interest, such as growth, reproduction and mortality, which have clear ecological relevance yet are relatively uninformative with regards to identifying causative agents because they are directly or indirectly impacted by a multitude of stressors.

Focusing on early, sub-lethal responses which occur at the molecular level may prove to be faster, less costly, and more informative than whole organism apical endpoints (Ankley et al., 2006, 2010). In general, differential expression of genes leads to altered abundance of proteins which are often involved in the clearance and metabolism of the stressor. These endpoints may also act as early initiating events for adverse apical outcomes resulting from activation of a particular mode of action (MOA). Changes in gene expression are among the earliest responses to a chemical and often occur as the direct result of an exposure. Therefore, the suite of genes that are differentially expressed may be considered specific for a relatively small structural class of stressors or MOA. Moreover, because these are responses within the organism reflecting modulation of particular physiological pathways, they reflect true systemic exposure levels, as well as physiological interactions between constituents of mixtures as well as interactions between chemical contaminants and other water quality characteristics, such as DO levels or pH.

Microarrays and next generation sequencing can screen for changes in tens of thousands of genes, reflecting the activity of nearly all the genes in an organism, in a single assay. The large number of physiological pathways represented in these types of experiments, including those involved in detoxification, stress adaptation, and apical endpoint manifestation, suggest the possibility of developing "fingerprints" for particular chemicals, MOAs, or for the overall health status of exposed organisms. As additional fingerprints are developed they can be applied in concert, in principle making it possible to screen for exposure to tens to thousands of potential toxicants in a single assay, while reflecting important effects of co-occurring substances, and facilitating predictions of the likelihood of eliciting effects on growth, fecundity, or survival.

The study of transcriptional changes as a consequence of chemical exposure has been ongoing for well over a decade. The focus of most early transcriptional studies was on endocrine active chemicals (Ankley et al., 2009). Although this work advanced the science and thinking about the utility of molecular indicators of exposure and effect, there are few examples of transcriptional indicators being employed for ecological protection. A notable exception is the use of vitellogenin as an indicator of estrogen exposure (Sumpter and Jobling, 1995). *Vitellogenin 1* (*vtg1*), an egg protein precursor gene, is transcribed and translated in the liver of reproducing female fish and the resulting protein is transported to the ovary and deposited into the developing oocyte. Both *vtg1* mRNA and protein are usually undetectable in males; however, upon exposure to estrogens, both are rapidly and highly expressed (Flick et al., 2014). The on/off nature of the *vtg*1 gene in males, the large dynamic range of the response, along with the fact that it is a single gene, has made it a convenient and well accepted tool for the identification of estrogenic exposures in aquatic systems, yet these attributes also make it somewhat exceptional. It has been used extensively in field studies in a wide variety of fish species and its up-regulation in males is regarded as a reliable indicator of exposure to estrogenic compounds (Biales et al., 2007, 2015; de Vlaming et al., 2007; Kidd et al., 2007).

While the simplicity of the *vtg1* response has suggested the possibilities for expression-based biomarker use on a broad scale, incorporation of 'omics-based biomarkers into environmental decision making has been slow to occur. One reason for this is that most gene expression studies were not designed for developing diagnostic biomarkers, but rather for elucidating physiological pathways underlying toxicity. Fortunately, there are analogous efforts to develop biomarkers for the characterization of disease states and guiding treatment for cancer for use in human health that are comparably advanced. The literature describing the development of these biomarkers (Monzon et al., 2009; Nystrom et al., 2012; van 't Veer et al., 2002), together with a number of guidance documents outlining best practices (McShane et al., 2013; Micheel et al., 2012), serve as a potential roadmap for adapting this technology for environmental protection. This literature directly addresses issues that potentially affect the reproducibility of global gene expression-based methods, such as microarrays. It suggests the need for high levels of biological replication during biomarker development, adjustment for the large number of hypotheses being tested, careful evaluation of reproducibility of laboratory results, and using real-world samples.

Many recently published research efforts employ omics-based measures to characterize environmental stressor exposures. Usually these studies infer causal relationships based on observed associations between exposure, gene expression and apical endpoint. However, the performance characteristics, such as sensitivity, specificity, and reproducibility, of these associations are seldom reported, even though the reliability of these types of associations in biomedical studies has been reported to be low (Ein-Dor et al., 2006). The lack of any performance characterization or consideration of the limitations of the experimental design used to generate omics-based biomarkers clouds their interpretability and has the potential to lead to erroneous conclusions. Often hundreds to thousands of genes are identified as differentially expressed, the majority of which lack adequate characterization, a situation exacerbated by the use of non-model species. Thus interpretation is limited based on the few genes or pathways that have been significantly characterized.

The current work represents an initial effort to develop a library of validated gene expression biomarkers that can be used in non-targeted screening approaches for exposure to a wide array of toxicants. This work is also intended to highlight critical components of experimental design and analysis that will be useful in characterizing the performance of omics measures and will facilitate their implementation and interpretability. The most proximate goal of the study is the development of a practical, accessible tool that has the potential to be used in number of applications, such as environmental monitoring or forensics. To this end, we balanced the potential power and scope of the assay against real-world considerations, such as sample volume requirements and total assay cost. This balance suggested the selection of a single test species, optimal life stage and level of biological organization (e.g. whole organism, tissue, etc.)

Species exhibit a range of sensitivities to toxicants. For example, because most pharmaceuticals are specifically designed for use in vertebrates, a vertebrate species will often times be more sensitive than an invertebrate (Langheinrich, 2003), whereas the opposite is true for an insecticide. Similarly, sensitivities differ across life stages (Liney et al., 2005). Therefore, there is no ideal species or life stage for all toxicants. In cases where the drivers of impairment are unclear, using multiple species would be expected to provide better sensitivity to a broader range of contaminants, yet would come at the expense of cost and complexity. The fathead minnow (*Pimephales promelas*, FHM) was chosen as a test species for this experiment for several reasons. It is easily reared, relatively small, has a broad native range across the US and a long history of use for traditional toxicity testing (U.S.E.P.A., 2002). The selection of a vertebrate species was driven by the desire to develop a tool useful for bridging ecological and human health risk estimation, since we expect that responses and sensitivity would better approximate those of humans compared to invertebrate models.