Analysis of endocrine disruption in southern California coastal fish using an aquatic multi-species microarray

Michael E. Baker[1], Barbara Ruggeri[2,3], James Sprague[2], Colleen Eckhardt[2], Jennifer Lapira[2], Ivan Wick[2], Laura Soverchia[2,3], Massimo Ubaldi[3], Alberta Maria Polzonetti-Magni[3], Doris E. Vidal-Dorsch, Steven M. Bay, Joseph R. Gully[4], Jesus A. Reyes[5], Kevin M. Kelley[5], Daniel Schlenk[6], Ellen C. Breen[7], Roman Šášik[2,8] and Gary Hardiman[1,2]

Abstract

Endocrine disruptors include plasticizers, pesticides, detergents and pharmaceuticals. Sentinel fish such as turbot are used to characterize the presence of chemicals in the marine environment. Unfortunately, there are relatively few genes of turbot and other flatfish in GenBank, which limits the use of molecular tools such as microarrays and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to study disruption of endocrine responses in sentinel fish monitored by water quality agencies. A multigene cross species microarray was developed as a diagnostic tool to screen the effects of environmental chemicals in fish for which there is minimal genomic information. The array included genes that are involved in the actions of adrenal and sex steroids, thyroid hormone, and xenobiotic responses. This will provide a sensitive tool for screening for the presence of chemicals with adverse effects on endocrine responses in coastal fish species. A custom multi-species microarray was used to study gene expression in wild hornyhead turbot, collected from reference and contaminated coastal waters, and in laboratory male zebrafish following exposure to estradiol and 4-nonylphenol. Gene-specific expression in turbot liver was measured by qRT-PCR and correlated to microarray data. Microarray and qRT-PCR analyses of livers from turbot collected from contaminated areas revealed altered gene expression profiles compared to those from non-impacted areas. The agreement between the array data and qRT-PCR analyses validates this multi-species microarray. The microarray measurement of gene expression in zebrafish, which are phylogenetically distant from turbot, indicates that this multi-species microarray will be useful for measuring endocrine responses in other fish.

Introduction

The European Community in 1996 defined endocrine disruptors as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism or its progeny or subpopulations". Some endocrine disruptors interfere with normal endocrine responses because the chemical has structural similarities to hormones such as steroids. As a result the endocrine disruptors bind to a hormone receptor or to an enzyme that catalyzes hormone synthesis or degradation (Atanasov *et al*. 2005, Baker 2001). Endocrine disruptors of concern include plasticizers such as phthalates and alkylphenols, pesticides, fungicides, detergents, dioxin, polychlorinated biphenyls and pharmaceuticals such as the synthetic estrogen 17α-ethynylestradiol. These xenobiotics are discharged into rivers, lakes and the

[1] *University of California, School of Medicine, La Jolla, CA*
[2] *University of California, School of Medicine, BIOGEM, La Jolla, CA*
[3] *University of Camerino, Department of Experimental Medicine and Public Health, Camerino, Italy,*
[4] *Los Angeles County Sanitation Districts, Whittier, CA*
[5] *California State University, Department of Biological Sciences, Environmental Endocrinology Laboratory, Long Beach, CA*
[6] *University of California, Department of Environmental Sciences, Riverside, CA*
[7] *University of California, School of Medicine, Division of Physiology, La Jolla, CA*
[8] *University of California, School of Medicine, Moore's Cancer Center, La Jolla, CA*

ocean, where they accumulate in aquatic species.

Amongst endocrine disruptors, alkylphenol ethoxylates, such as nonylphenol, have been widely studied owing to their wide diffusion in the environment through the use of alkylphenols in the plastics industry and in detergents, paints, herbicides and pesticides (Soto *et al*. 1991). It has been estimated that 60% of man-made alkylphenols enter the aquatic environment (Naylor *et al*. 1992), with most entering via sewage treatment works, where they are readily degraded to form relatively stable metabolites (Ahel *et al*. 1987). Nonylphenol is the predominant degradation product of the alkylphenols encountered in the aquatic environment (Giger at al. 1984). Exposures of cell cultures and laboratory animals to nonylphenol have demonstrated that it competes with estradiol for binding to the estrogen receptor (ER), but has only weak estrogenic activity. As a result there are concerns that exposure of humans and fish to nonylphenol will disrupt male and female reproduction and development.

It also is clear that xenobiotics affect other hormone pathways, such as thyroid hormone (Boas *et al*. 2006, Zhou *et al*. 2000), among others. Thus, there is a need for a tool that can screen many endocrine responses in fish taken from polluted water and monitor harmful effluents entering the ecosystem. Ideally suited for this purpose are microarrays, which can simultaneously measure the level of expression of hundreds of genes from a single tissue sample in each animal, collected from a polluted environment (Benson and Di Giulio 2008, Hardiman 2004, Hardiman and Carmen 2007, Marton *et al*. 1998). Microarray analysis of alterations – either up or down – in the levels of genes involved in physiological responses to estrogens, androgens, glucocorticoids, thyroid hormone, and detoxification of chemicals provides a powerful tool for obtaining a more complete diagnosis of endocrine disruption in fish.

The development of the microarray described here was motivated by the results of recent research on endocrine disruption in local species of flatfish. Hornyhead turbot collected in the vicinity of the Orange County Sanitation District's ocean outfall showed alterations in hormones and vitellogenin (egg yolk protein precursor) that were suggestive of contaminant-related alterations in hormone systems (Deng *et al*. 2007, Rempel *et al*. 2006). Microarray and quantitative polymerase chain reaction (q-PCR) analysis of turbot organs would provide enhanced sensitivity to xenobiotics in the marine environment, and facilitate control of toxic effluents. Unfortunately, few genes of turbot and other flatfish have been sequenced, which limits the use microarrays and qRT-PCR to study disruption of endocrine responses.

In this study we present data from a multi-gene cross species microarray, which was used to analyze gene expression in hornyhead turbot collected in the coastal waters of Orange County and Los Angeles County in California. Parallel experiments with qRT-PCR verify the microarray data. The multi-species microarray also was used to study gene expression in livers of zebrafish exposed to estradiol and nonylphenol. The use of the multi-species microarray to study gene expression in hornyhead turbot and zebrafish, which are phylogenetically distant, suggests that this array will be a useful diagnostic screening tool to monitor responses to contaminants in *Perciformes*, *Pleuronectiformes* and fish from other taxa for which there is limited genomic sequence.

METHODS

Approximately eighty male zebrafish were divided evenly between three 80-L glass aquaria. Each aquarium was individually heated to maintain a temperature of 26 -/29 °C with a light-dark cycle ratio of 14:10 hour. Fish were acclimated for one week prior to commencing the experiment. One tank contained only tap water (negative control) and the others contained water with either 10^{-7} M estradiol or 4-nonylphenol. All exposures utilized a continuous flow-through system, to maintain constant concentrations of the chemicals during the two-week experiment, after which the fish were anaesthetized with 3-aminobenzoic acid ethyl ester (10 g/L) and liver samples harvested, frozen in liquid nitrogen and stored at -70°C.

Male hornyhead turbot were collected off of the coast of Southern California collected as part of the Southern California Bight 2003 regional monitoring survey (Bight'03 Field Sampling and Logistics Committee 2003). Livers from three individual fish from a monitoring station near the Orange County Sanitation District outfall (OCSD) and four individuals from a monitoring station near Los Angeles County Sanitation Districts outfall (LACSD) were used for microarray analysis. These fish exhibited morphological abnormalities, high levels of vitellogenin and estradiol, low levels of cortisol, and histological abnormalities, such as the presence of immature oocytes (eggs) within the testis (Table 1;

Deng *et al*. 2007, Rempel *et al*. 2006). Control fish were obtained from a monitoring station offshore of Dana Point, CA, an area considered relatively non-impacted and maintained in a clean-water laboratory setting for four weeks. Measurement of vitellogenin in the control male turbot with an Enzyme-Linked Immunosorbent assay (ELISA) found 0.0037 ng/µg protein. This is from 27- to 700-fold lower than the vitellogenin levels in the turbot collected from the LACSD and OCSD sites (Table 1). This indicates that the control male turbot were not exposed to an estrogenic compound, which validates the use of their liver RNA as a control for the microarray and the qRT-PCR analyses. Fish were sacrificed immediately following capture and the livers were harvested, frozen, and stored at -70°C. All the animals were treated humanely and with regard for alleviation of suffering.

Hornyhead turbot plasma vitellogenin was measured using an ELISA optimized for this species (Rempel *et al*. 2006). Plasma concentrations of 17Î²-estradiol, testosterone, and cortisol were measured by specific radioimmunoassays using ^{125}I-labeled steroid and polyclonal rabbit antisera obtained from DSL/Beckman Coulter (Webster, TX). Methods for the assays are described in Baker *et al*. (2008).

Multi-species Microarray

To overcome the scarcity of sequence data in GenBank for hornyhead turbot we constructed a 65-mer oligonucleotide-based microarray containing conserved sequences from genes of interest. Oligo probes were designed by collecting available fish sequences in GenBank for a given gene (e.g. *ESR1/Era, Vtg, CYP3A* and *FXR*) using BLAST (Altschul *et al*. 1990). Sequences from Tetraodoniformes (*Fugu, Tetraodon*) and *Perciformes* (cichlid, tilapia, sea bass, seabream), which are close from a phylogenetic perspective to Pleuronectiformes (hornyhead turbot, California halibut) were selected (Figure 1). Available sequences from medaka, stickleback and zebrafish also were used, in addition to some hornyhead turbot-specific cDNA sequences obtained by degenerate PCR cloning. Clustal X was used to construct multiple alignments to uncover conserved regions (Thompson *et al*. 1997) and nucleotide sequences within 1200 bases from the mRNA 3' end were identified and analyzed using Oligowiz (Nielsen *et al*. 2003, Wernersson and Nielsen 2005) to design 65-mer microarray probes. Each copy of an individual gene in several fish was subjected to a pair-wise BLAST comparison with the corresponding gene from other fish to insure that the DNA sequence was between 80 and 90% identical thereby increasing the likelihood that the homologous turbot sequence would contain at least 85% identity to one of the oligonucleotides. The gene names and corresponding accession numbers are provided in Baker *et al*. (2008). Oligonucleotides were synthesized to contain a 5' amine group and desalted by Operon Technologies (Alameda, CA) and Invitrogen (Carlsbad, CA), and used without further purification. Oligonucleotides were printed on amine-reactive silanized glass slides (Surmodics, Inc., Eden Prairie, MN) as described previously at the UCSD BioMedical Genomics Microarray Facility (BIOGEM; http://microarrays.ucsd.edu; Hardiman *et al*. 2003).

This study amplified partial turbot transcripts using conserved sequences from other fish species to guide the choice of primer design. Gene-specific

Table 1. Characteristics of hornyhead turbot sampled. Individuals with endocrine or morphological abnormalities induced were chosen for microarray experiments. Cortisol, estradiol, insulin like growth factor (IGF-1), thyroxine (T4), vitellogenin (VTG) levels are indicated. Morphological lesions (not caused during capture) and maturity stage are noted.

Location	Station ID	Sample ID	Sex	Cortisol (ng/ml)	Estradiol (pg/ml)	IGF (ng/ml)	T (ng/ml)	VTG (ng/µg)	Morpholology Diagnosis	Lesion Grade	Maturity Stage
OCSD	4041	3	M	8.0	58.1	16.0	0.7	0.1	Oocytes	Minimal	Stage 1
OCSD	4041	4	M	18.6	134.0	20.2	2.5	0.2	Oocytes, Macrophage Aggregates	Minimal	Stage 1
OCSD	4041	6	M	89.4	90.8	17.1	0.5	0.4	None	NA	Stage 1
LACSD	4086	1	M	20.7	2.2	16.3	0.4	1.5	Fibrous Septa	Minimal	Stage 1
LACSD	4086	2	M	15.1	2.2	17.0	0.4	2.6	Fibrous Septa	Moderate	Stage 2
LACSD	4086	3	M	1.1	21.3	15.9	0.4	1.0	None	NA	Stage 2
LACSD	4086	4	M	8.4	2.2	17.4	0.5	1.6	Oocytes, Fibrous Septa	Moderate	Stage 1

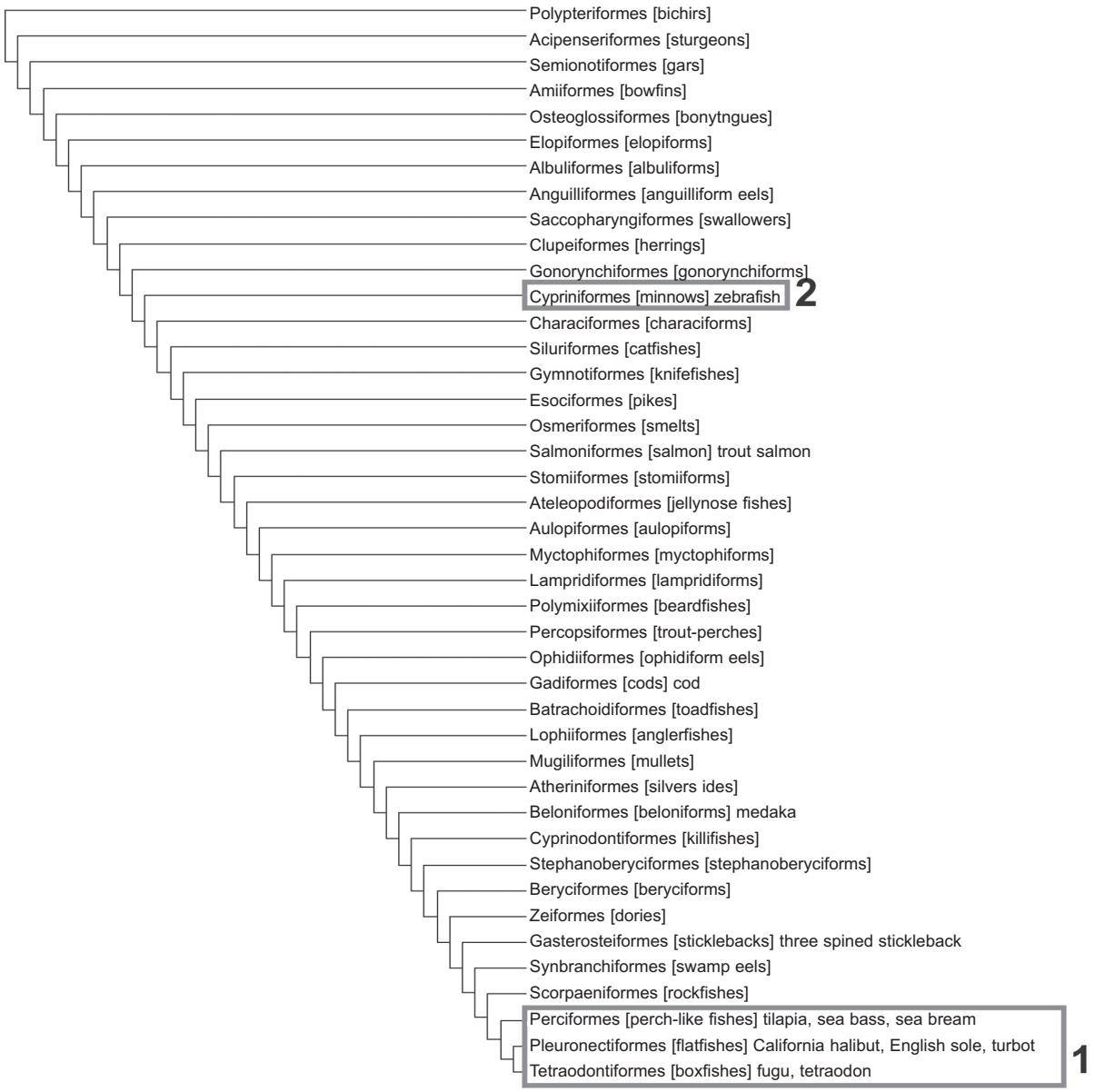

Figure 1. Flatfish (*Pleuronectiformes*) in an evolutionary context. Adapted from the phylogeny at http://cichlidresearch.com/fish_html/cactinop.html (Nelson 2006). *Tetraodontiformes* (*Fugu*, *Tetraodon*) and *Perciformes* (cichlid, tilapia, sea bass, seabream, perch) are close phylogenetic relatives of *Pleuronectiformes* (turbot, halibut, sole; box 1). *Cypriniformes* (zebrafish) are distant phylogenetic relatives (box 2).

primers were designed using Primer3 software (Rozen and Skaletsky 2000). All the amplicons were directly sequenced using the respective forward and reverse PCR primers. All of the sequencing reads were subjected to a series of quality control measures, including a phred quality score >20, and manual trace inspection. The identity of each sequence was confirmed by performing BLAST searches of GenBank.

For this study, 500 ng of total RNA were converted into fluorescently labeled Cy 3 or 5 cRNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescent targets were purified to remove unincorporated nucleotides using RNeasy (Qiagen). Absorbance (OD) at 260 nm was used to quantify the cRNA concentrations, and absorbance at 550 nm and 650 nm was used to measure the efficiency of Cy3 and Cy5 dye incorporation. Hybridization was carried out for 18 hours at 42°C in a shaking incubator at 100 rpm. The microarrays were washed with 1x SSC/0.2% SDS for 5 minutes at room temperature followed by two 5-minute washes with 0.1x SSC/0.2% SDS at room temperature. The microarray was rinsed briefly with water and dried by centrifugation at 800 rpm for 5 minutes. Technical replicate hybridizations were

carried out with each sample. Slides were scanned using an Axon 4000A scanner (Molecular Devices, Sunnyvale, CA) at the photomultiplier tube (pmt) settings of 500 for Cy3 and 600 for Cy5.

Microarray Data Analysis

Array data has been deposited in the EBI Array Express Database (accession number pending). Statistical analysis of the microarray experiment involved two steps: normalization of microarray data, and sorting of the genes according to interest. We normalized all samples simultaneously using a multiple-loess technique described previously (Šášik *et al*. 2004). In designing the interest statistic we borrowed ideas from the software package *Focus* (Cole *et al*. 2003). The interest statistic reflects a biologists understanding that a gene with a greater fold change (in absolute value) than other genes is potentially more interesting. Also, given two genes with the same fold changes, it is the gene with a higher expression level (and therefore higher absolute change) that is more relevant. This approach is described in greater detail elsewhere (Ogawa *et al*. 2004). To investigate alterations in gene expression of controls and exposed fish, two independent analytical methods were used: differential gene expression vs. signal intensity (MA) plots and normal quantile (q-q) plots, as described in detail in Baker *et al*. (2008).

Quantitative Real-time PCR Analysis

Relative turbot mRNA transcript levels were measured by real-time quantitative RT-PCR in a LightCycler 480. Total RNA was extracted from hepatic turbot samples as described above and reverse-transcribed using the Roche Transcriptor kit and 50 ng cDNA were quantified using LightCycler 480 SYBR Green Master kit. The 18S rRNA served as an internal control for normalization. Each sample was run in triplicate and mean values were reported. Normalized gene expression values were obtained using LightCycler Relative Quantification software. Relative gene copy numbers were derived using the formula $2^{\Delta CT}$ where ΔCT is the difference in amplification cycles required to detect amplification product from equal starting concentrations of turbot liver RNA.

Results

Development of the multi-species microarray was motivated by the mission of the participating sanitation districts, university research groups and SCCWRP to monitor the endocrine status of turbot, halibut and other flatfish at different sites in coastal southern California. A broad measure of the effects of chemicals on a variety of endocrine responses in fish was determined to be most useful. Thus, the microarray included probes for genes encoding receptors for estradiol (*ESR1/ Erα; ESR2/ Erβ*), progesterone (*PR*), testosterone (*AR*), cortisol (*GR*), aldosterone (*MR*), thyroid hormone (*THRA/TRα;THRB/TRβ*), retinoids (*RAR, RXR*) and vitamin D (*VDR*), and other nuclear receptors: farnesoid X receptor (*FXR*), and pregnane X receptor (*PXR*), hydroxysteroid dehydrogenases and the enzymes involved in detoxification (*CYP1A1, CYP3A*) were represented (Baker *et al*. 2008). This provided a diagnostic tool for measuring altered expression of genes that are important in several endocrine pathways in fish, which increased the scope of detection for the presence of endocrine disruptors in coastal waters off of southern California.

A challenge in developing this platform was the paucity of available sequence information for hornyhead turbot in GenBank. To overcome this problem, GenBank was searched for sequences in other fish to find regions of sequence conservation that could beused to construct a microarray slide that could detect altered gene expression in multiple fish species. Fortunately, the genomes of two *Tetraodontiformes*, *Fugu* and *Tetraodon*, have been sequenced. Moreover, many genes from various *Perciformes* were in GenBank. *Tetraodontiformes* and *Perciformes* are phylogenetically close to *Pleuronectiformes* as shown in Figure 1. Also of importance was the extensive catalog of sequenced genes from zebrafish, which is distant from of *Pleuronectiformes*. Sequences conserved in zebrafish, *Fugu*, *Tetraodon* and various *Perciformes* were likely to be conserved in turbot. A schematic representation of the design and application of the multi-species microarray test to monitor xenobiotic exposure is presented in Figure 2.

Differential Expression and Signal Intensity Measurements Using the Multi-species Microarray

Male hornyhead turbot sampled at two California monitoring stations in Orange and Los Angeles Counties were assessed for exposure to xenoestrogens using the multi-species microarray. Figure 3 shows the measurements obtained using the

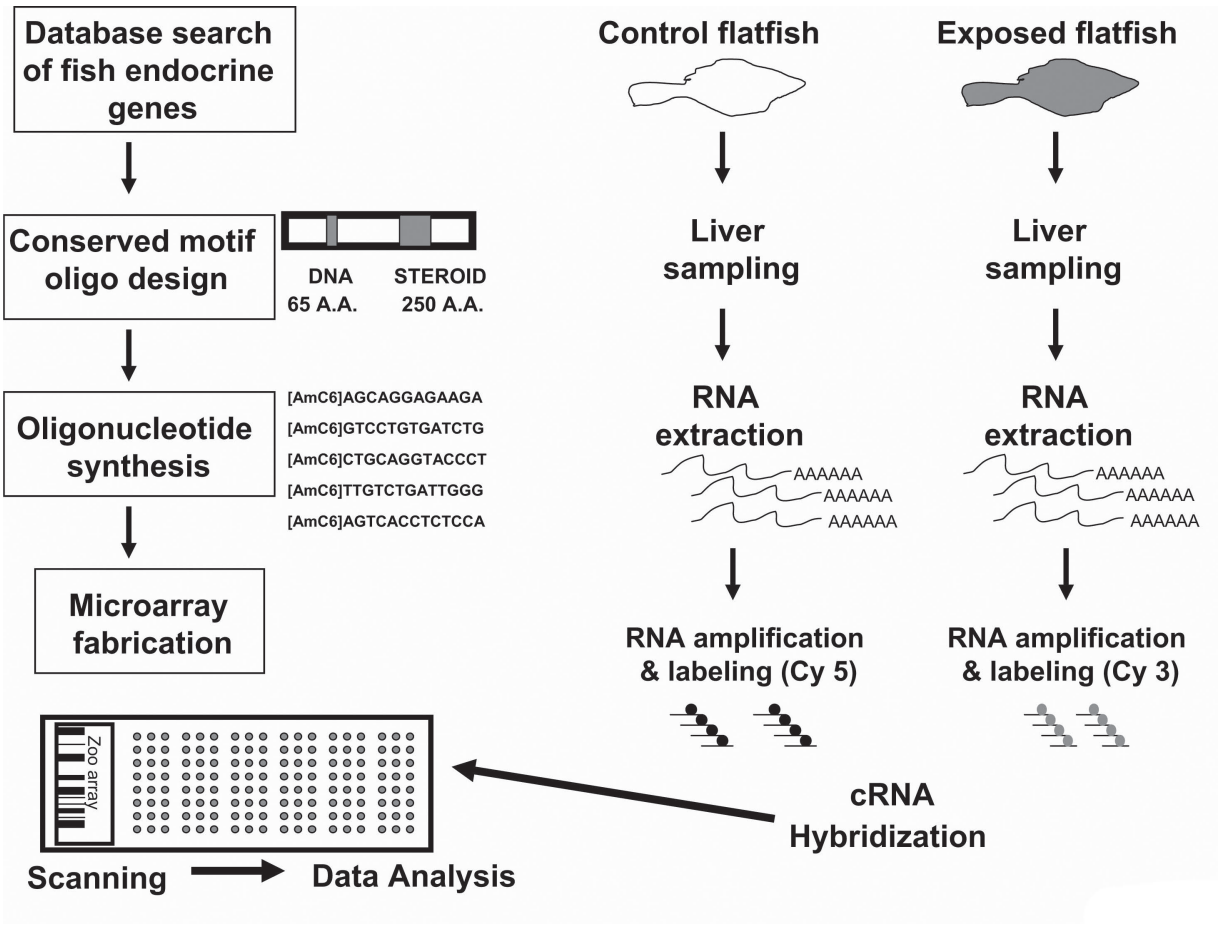

Figure 2. Development of a Multi-Species Endocrine Microarray for environmental monitoring. Schematic representation of the design and application of a microarray based test to monitor xenoestrogen exposure.

multi-species microarray to examine control and exposed fish. Alterations in gene expression in hornyhead turbot liver relative to control fish were determined by using a threshold of log2 intensity ratio of 2 or greater. The MA plots revealed differential gene expression profiles between exposed and control turbot taken from sites in LACSD (panels a - d) and OCSD (panels e - g).

Normal quantile plots were used to examine more closely differences in expression between the control and exposed fish (Figure 4). The q-q plots in Figure 4 examined the distribution of the log2 (exposed/control) fold changes and the deviation from a normal Gaussian distribution. When a data set is derived from the Gaussian distribution, the normal-quantile plot is a straight line. The nature of plots in panels h - k shows that the observed log2 ratio between control fish, both pooled and individual controls is reasonably close to a Gaussian. This distribution is due to individual variation in fish combined with unavoidable random experimental errors.

When the log2 ratio is taken between exposed and control fish (panels a - g), the curved ends of the q-q line indicate the presence of heavy tails in the distribution of log2 (exposed/control). The exposed samples clearly differ from the control samples. Specifically, the sharp increase in the quantile curve at log2 ratio of about 2 suggests that genes with |log2 (exposed/control)| >2 show significant regulation in the LACSD (panels a - d) and OCSD (panels e - g) exposed fish compared to controls, which is in agreement with the MA plots in Figure 3.

A heat map of selected genes that were either strongly down-regulated or up-regulated in fish collected near the Orange County Sanitation District and Los Angeles County Sanitation District outfalls relative to controls is presented in Figure 5. Fish sampled at both impacted sites exhibited strong increases in the expression of *CYP3A*, *RXR*, *ERβ*, *Vtg2* and *MR* relative to control fish. The *VDR* was also up-regulated compared to control fish. Transcripts encoding *FXR* and *PPARα* were down-

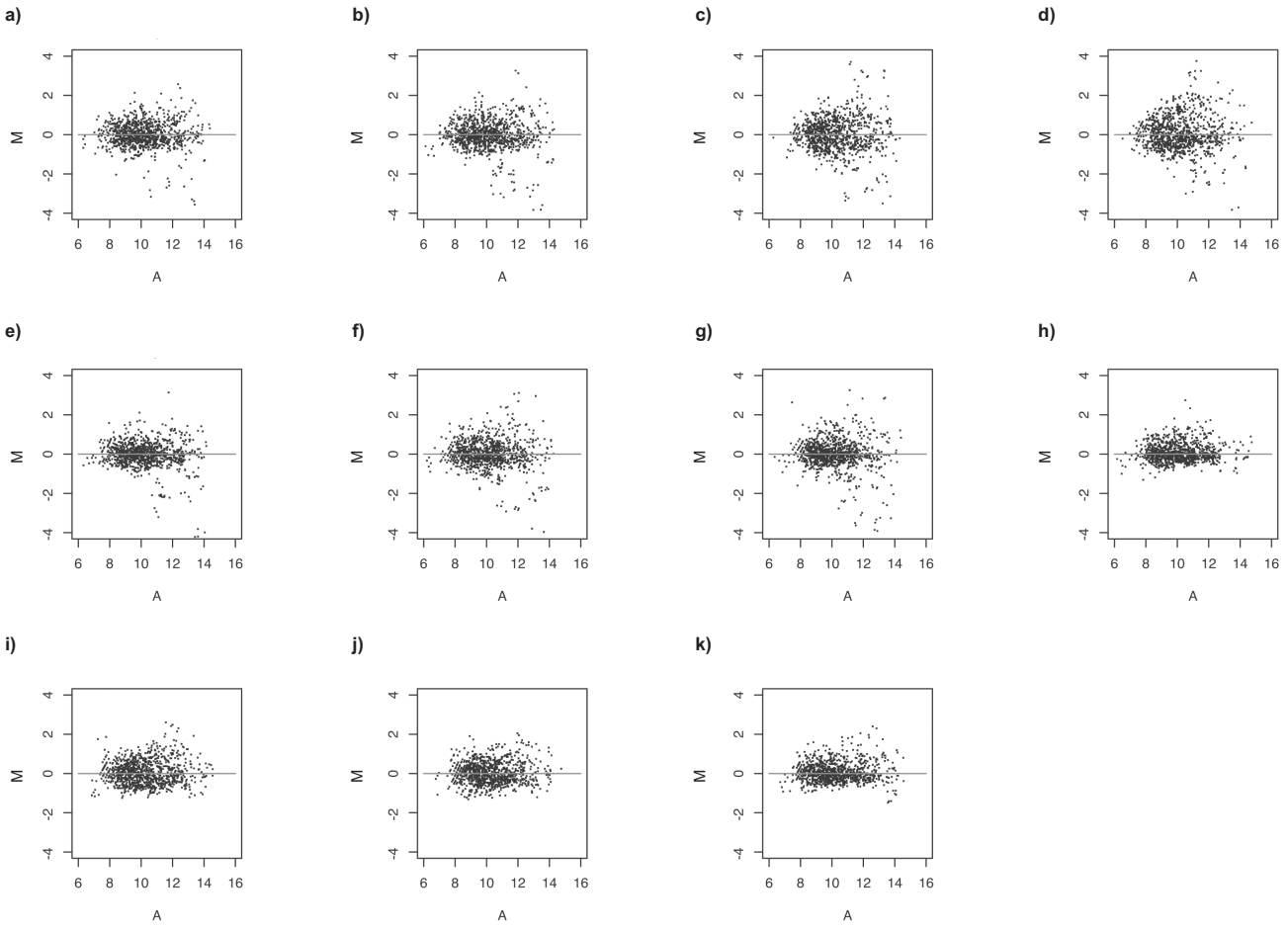

Figure 3. Differential expression and signal intensity measurements using the Multi-Species Endocrine Microarray for control and exposed fish. Individual LACSD exposed fish versus pooled controls (a - d); Individual OCSD exposed fish versus pooled controls (e - g); Pooled controls versus pooled controls (h); individual control fish versus pooled control (i - k). Each point represents data from a single 65-mer oligonucleotide probe. M is a measure of differential gene expression (log2 (exposed /control)) in plots a - g, or absence of significant differential gene expression in the self-self plots (log2 (control / control intensity)) in plots h - k. A is a measure of signal intensity (0.5 log2 exposed intensity + 0.5 log2 control intensity) in plots a - g and (0.5 log2 control intensity + 0.5 log2 control intensity) in h - k.

regulated compared to control fish. Additionally, thyroid receptor α and β mRNAs were down-regulated.

Quantitative RT-PCR Analysis of Turbot Gene Expression

In order to use qRT-PCR to determine if the microarray data was accurately monitoring changes in hepatic gene expression in the turbot, partial fragments corresponding to highly conserved regions in the 28S rRNA, *CYP3A*, *TRβ*, *Vtg1* and *Vtg2* were cloned using reverse transcriptase PCR. Identities of the fragments were confirmed by DNA sequencing and sequence data has been deposited in the National Center for Biotechnology Information (NCBI) Database (accession numbers pending). These short turbot-specific sequences were used for SYBR green qPCR experiments on individual turbot from impacted regions. As shown in Figure 6, the greatest differences were observed with the *Vtg1* and *Vtg2* transcripts, with greater than a 15-fold induction observed in one turbot. TRβ was down-regulated in two control fish examined. *CYP3A* was up-regulated in three fish. Thus, these qRT-PCR data validate the microarray analysis for these genes.

Moreover, vitellogenin 1 (*Vtg* 1) in control turbot is 0.0037 ng/µg protein, as determined by an ELISA. Thus control male turbot have from 27- to 700-fold lower vitellogenin levels than those found in turbot collected from contaminated sites in Orange County and Los Angeles County (Table 1). The agreement between vitellognenin measured with the ELISA and

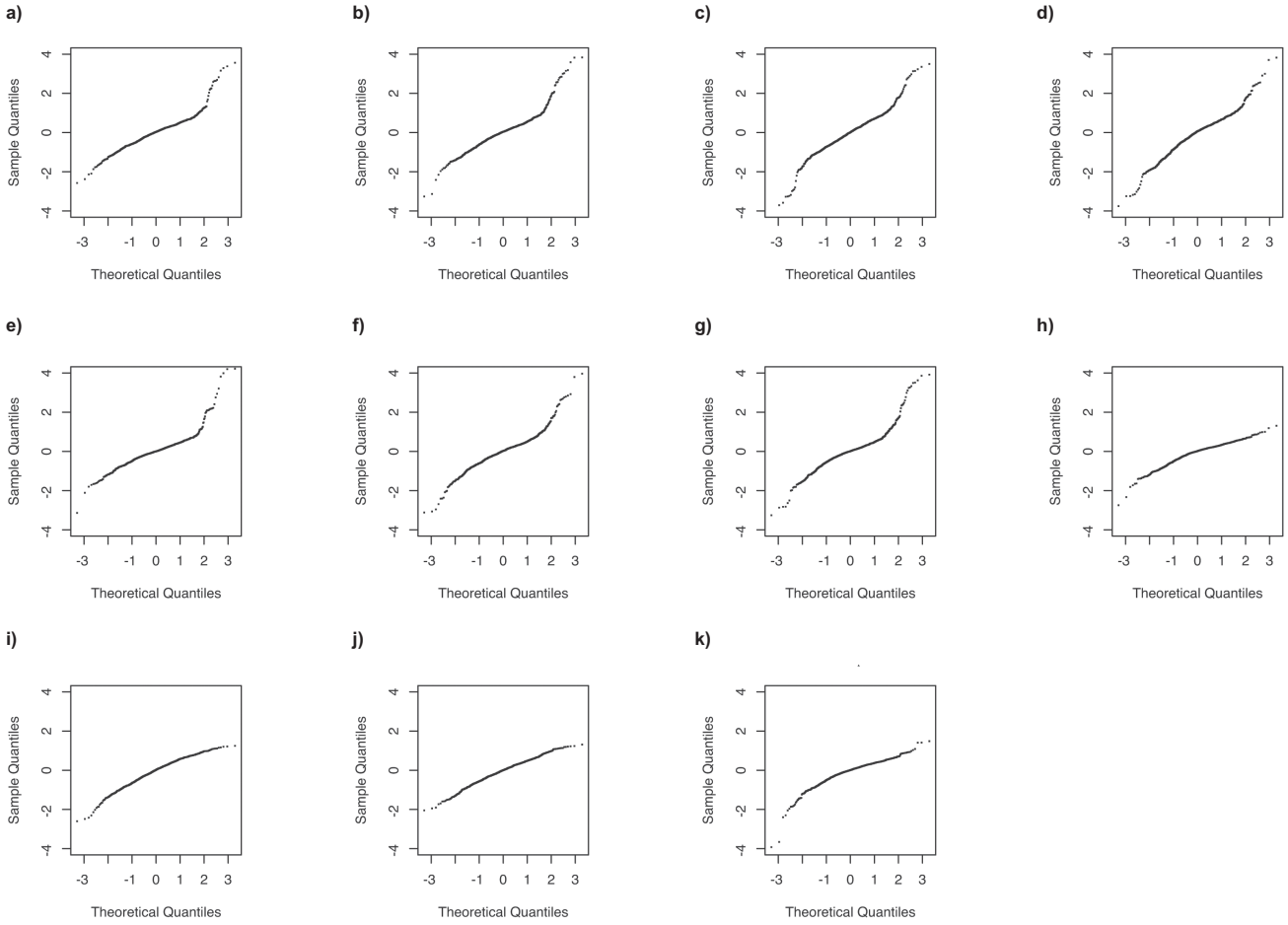

Figure 4. Normal-quantile-quantile (Q-Q) plots of Multi-Species Endocrine Microarray data. Q-Q plots were constructed to determine if control and exposed fish data sets derived from populations have a common distribution. Individual LACSD exposed fish versus pooled controls (a - d); Individual OCSD exposed fish versus pooled controls (e - g); Pooled controls versus pooled controls (h); individual control fish versus pooled control (i - k). The Q-Q plots examined the distribution of the log2 (exposed/control) fold changes and the deviation if any from a normal Gaussian distribution. A 45-degree reference line was plotted. When the two data sets derived from a population with the same distribution, the points fall approximately along this reference line, as is the case with the control samples data populations, both pooled and individual (i - k). When the two data sets derived from populations with different distributions, the data deviated from this reference line (a - g). Exposed samples differ from the control, with a sharp rise observed in the quantile curve at log2 ratio values of 2, indicating the presence of large log2 ratios and differences in gene expression.

with the qRT-PCR analysis (Figure 6) indicates confidence in qRT-PCR analyses of other genes in male turbot taken from contaminated sites.

Gene Expression Patterns in Zebrafish Exposed to Estradiol and 4-nonylphenol

Alterations in gene expression in zebrafish liver were investigated using the multi-species microarray after exposing fish for two weeks to either the xenoestrogen 4-nonylphenol or estradiol at 10^{-7} M. These experiments were carried out to determine if probes designed from conserved sequences from *Tetraodoniformes* and *Perciformes* were able to hybridize to genes in zebrafish (*Cypriniformes*), which are distant phylogenetic relatives of *Pleuronectiformes* (Figure 1).

The data in the heat map in Figure 7 show that estradiol produced a broader and higher response than 4-nonylphenol. Both treatments induced strong up-regulation of vitellogenin, several nuclear receptors, proteins involved in oogenesis and in steroid metabolism. Amongst these, the *FXR* transcript was the most up-regulated. Other transcripts that were strongly up-regulated included the *PR*, the *MR*, *PPARα*, *PXR*, *RXRα*, *TRα* and *GR*.

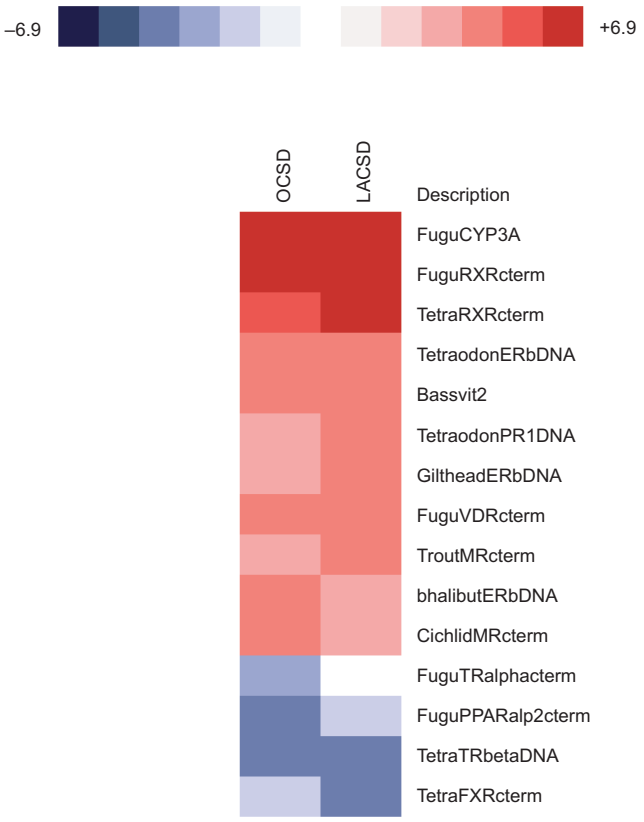

Figure 5. Gene expression profiling of turbots from coastal sites. Gene expression changes were investigated in male turbot liver collected in two coastal monitoring stations in Orange County (OCSD) and Los Angeles County (LACSD) in California that are considered contaminated. Control fish were obtained from a monitoring station in Dana Point a relatively non-impacted area and maintained in a clean-water laboratory for four weeks. Fold changes observed between exposed and control fish are depicted as a heat map. LACSD and OCSD data derived from four and three independent biological replicate microarray experiments, respectively.

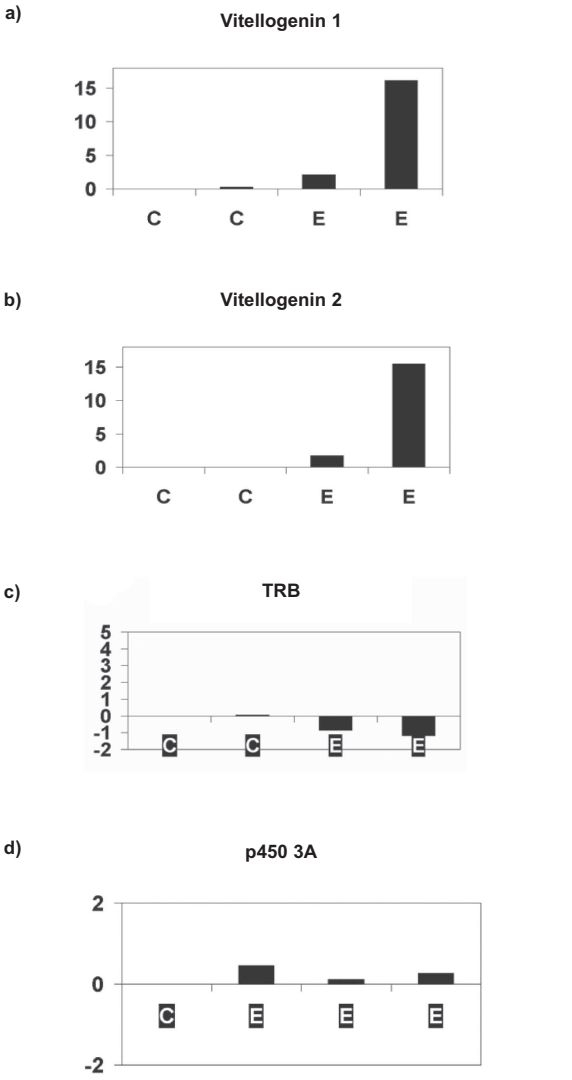

Figure 6. Validation of Multi-Species Endocrine Microarray. SYBR green qPCR for Vitellogenin 1 (a), Vitellogenin 2 (b), Thyroid hormone receptor *b* (c), and CYP3A (d) specific transcripts in livers from control C and exposed E hornyhead turbot. The 18S rRNA served as an internal control for normalization. Mean values from triplicate measurements were plotted. Fold changes relative to control fish are plotted. Vitellogenin 1 and Vitellogenin 2 transcripts were strongly up-regulated (>15 fold) in one exposed fish. TR*b* was down-regulated in two control fish examined. *CYP3A* was up-regulated in three fish and down-regulated in one fish.

Other transcripts were down-regulated by treatment with estradiol and 4-nonylphenol including *ERα*, the glucose transporter (*GLUT*), an insulin-like growth factor binding protein (*IGFBP*; Kelley *et al*. 2002) and the 11β-hydroxysteroid dehydrogenase-type3 (*11βHD3*; Baker 2004). Estradiol also induced a strong decrease in both *CYP3A* and *ERα* expression. Although *CYP3A* expression was down-regulated in 4-nonylphenol treated fish, the response was muted compared to estradiol.

Discussion

Microarrays are ideally suited for the comprehensive assessment of contaminant effects in aquatic organisms because they can detect changes in many genes in a single tissue sample, providing snapshots of alterations in endocrine pathways in normal and contaminated fish. This knowledge identifies gene families and biochemical pathways that are affected, in addition to those identifying those that remain unaffected (Vilo and Kivinen 2001). A challenge in developing a microarray platform to study alterations

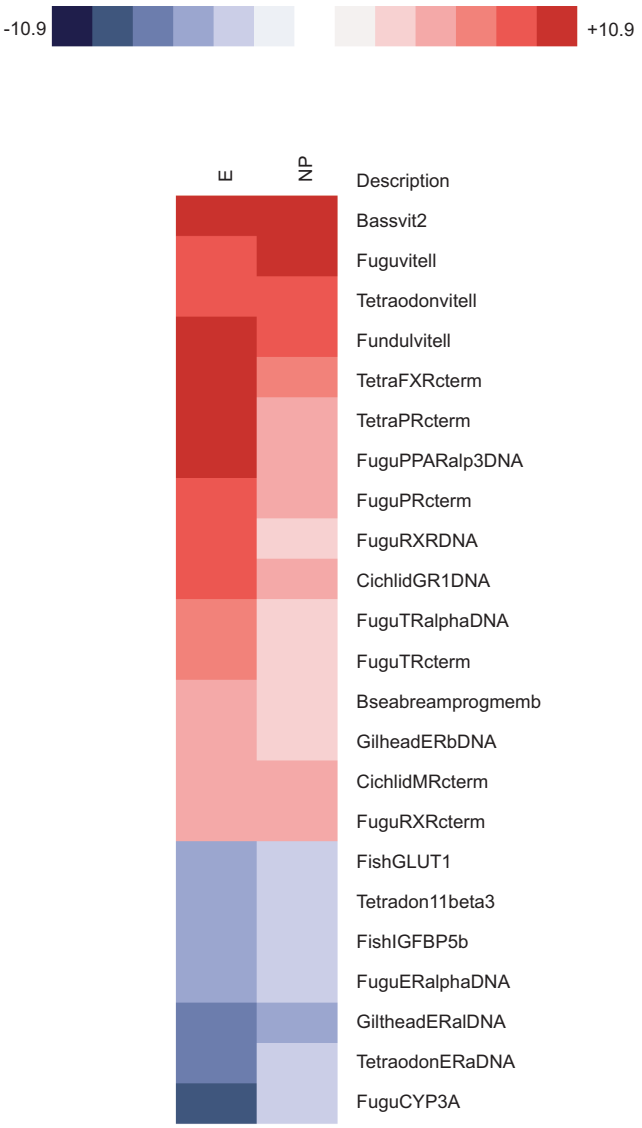

Figure 7. Cross species applicability of the Multi-Species Endocrine Microarray. Alterations in gene expression in zebrafish liver after a two week exposure to either 4-nonylphenol or estradiol using the multi-species endocrine microarray. Fold changes between exposed and control fish are depicted as a heat map. Data derived from four independent biological replicate microarray experiments.

in gene transcription in sentinel coastal species was the paucity of genomic sequence data. The novelty of this platform is that it used highly conserved probes from several fish species, permitting application of the array to studies involving turbot and zebrafish. 65-mers were used to accommodate sequence differences, polymorphic regions, and species specific codon usage. It was reasoned that this approach would be successful, as it has been previously demonstrated that oligonucleotides 50 nucleotides in length can hybridize to RNA sequences that differ by 15% in their overall sequence (Li *et al*. 2005, Nielsen *et al*. 2003).

Microarray Analysis of Turbot Exposed to Pollutants

Microarray analysis detected differences in hepatic gene expression patterns in exposed turbot from the two monitoring areas compared to control individuals. Exposed turbot showed up-regulation of *CYP3A* and *RXR*. Interestingly, thyroid receptors α and β were down-regulated in fish from both monitoring areas indicating the presence of compounds that are able to interfere with the thyroid response.

Another example of the utility of microarray analysis of fish from polluted areas can be seen in a study of male flounder collected from an impacted site (Tyne) and a reference site (Alde) in the United Kingdom (Williams *et al*. 2003). Eleven transcripts were differentially expressed between male flounder collected from an impacted site relative to a reference site. Seven transcripts were more highly expressed in the Tyne male fish (*CYP1A1*, *UDPGT*, α-2HS-glycoprotein, dihydropyrimidine dehydrogenase, Cu/Zn SOD, aldehyde dehydrogenase and paraoxonase), while four transcripts (Elongation factor 1 (*EF1*), *EF2*, *Int-6* and complement component C3 mRNA) were found to be significantly less abundant in the Tyne male fish (Williams *et al*. 2003).

Microarray Analysis of Zebrafish Exposed to Estradiol and 4-nonylphenol

Differences observed between estradiol and 4-nonylphenol in the strength of the estrogen-like response, which we attribute to 4-nonylphenol having a low affinity for the ER (Kuiper *et al*. 1997). The data on gene expression in zebrafish provide another validation of the multi-species microarray and demonstrate its potential for investigating gene expression in *Pleuronectiformes* and *Perciformes*.

The study found up-regulation of vitellogenin, *PR*, *RXR* and *ERβ* transcripts in male zebrafish exposed to both estradiol and 4-nonylphenol. Up-regulation of vitellogenin and *PR* are well-established responses to estrogens. And *RXR* has been shown to be upregulated by bisphenol A, an estrogenic chemical in murine embryos (Nishizawa *et al*. 2005). *ERβ* expression, has previously been shown to be increased upon exposure to xenoestrogens in zebrafish (Islinger *et al*. 2003) and to 4-nonylphenol in many fish species (Soverchia *et al*. 2006).

Up-regulation of *ERβ* was reported following exposure to alkylphenols in juvenile goldfish, medaka, rainbow trout and zebrafish (Inui *et al*. 2003, Islinger *et al*. 2003, Soverchia *et al*. 2006, Vetillard and Bailhache 2006) indicating that that it can be considered a biomarker for xenoestrogen exposure.

ERα expression was repressed with both estradiol and 4-nonylphenol treatments. Distinct patterns of expression for *ERα* and *ERβ* have been documented in fish (Choi and Habibi 2003), but differences in the interaction of xenoestrogens with the two subtypes of estrogen receptors have not been well characterized.

Following exposure to 4-nonylphenol a modest repression of *CYP3A* was observed in zebrafish. A strong repression of *CYP3A* mRNA however was observed following estradiol exposure. Similar results have been reported in trout (Pajor *et al*. 1990), suggesting an important role of the sex hormones in *CYP3A* expression, which is further supported by the sexually dimorphic expression of *CYP3A* genes reported in many fish species (Hasselberg *et al*. 2004, Hegelund and Celander 2003).

Interestingly, expression of the *PXR*, a sensor for xenobiotics (Moreau *et al*. 2008, Xie *et al*. 2000), was induced by both compounds. *PXR* mediates the effects of 4-nonylphenol on the activation of CYP3A genes in mouse, rat and humans (Masuyama *et al*. 2000, Masuyama *et al*. 2002). In juvenile Atlantic salmon, increases in *PXR* and *CYP3A* transcript levels were observed following 4-nonylphenol exposure suggesting a similar mechanism to that reported in the mammalian systems (Meucci and Arukwe 2006). Furthermore hepatic expression of *CYP3A* is induced by the organochlorine pesticide methoxychlor in male largemouth bass Micropterus salmoides (Blum *et al*. 2008).

It was also found strong activation of *FXR*, *PPAR* and *RXRα* expression in zebrafish exposed to estradiol or 4-nonylphenol, which indicates xenoestrogens can affect a variety on physiological pathways. *PPAR* is involved in the regulation of lipid metabolism-related genes and its interaction with xenobiotic compounds is thought to be responsible for alterations in adipogenesis and diseases such as obesity in humans (Grun and Blumberg 2006). *FXR* is strongly activated by bile acids and serves as a central coordinator for bile acid biosynthesis metabolism and transport. Possible interaction of xenobiotics with *FXR* could lead to changes in bile acid homeostasis and hepatic toxicity. The finding that the *FXR* was up-regulated in zebrafish exposed to estradiol and 4-nonylphenol has not been reported previously in any fish. This finding demonstrates the utility of microarrays in uncovering the effects of hormones and chemicals, which can subsequently be used to construct a profile for exposure to a given chemical.

Of practical importance for the analysis of zebrafish exposed to estradiol and 4-nonylphenol is that zebrafish belongs to the order *Cypriniformes* and is phylogenetically distant from *Tetraodoniformes*, *Perciformes* and *Pleuronectiformes*, whose sequence information were used to guide the design of the array probes. Thus, the data obtained from the zebrafish experiments indicated that the multi-species microarray possesses cross-species utility.

Literature Cited

Ahel, M., J. Conrad and W. Giger. 1987. Persistent organic chemicals in sewage effluents. *Environmental Science & Technology* 21:697-703.

Altschul, S.F, W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.

Atanasov, A.G., L.G. Nashev, S. Tam, M.E. Baker and A. Odermatt. 2005. Organotins disrupt the 11beta-hydroxysteroid dehydrogenase type 2-dependent local inactivation of glucocorticoids. *Environmental Health Perspectives* 113:1600-1606.

Baker, M.E. 2001. Adrenal and sex steroid receptor evolution: Environmental implications. *Journal of Molecular Endocrinology* 26:119-125.

Baker, M.E. 2004. Evolutionary analysis of 11beta-hydroxysteroid dehydrogenase-type 1, -type 2, -type 3 and 17beta-hydroxysteroid dehydrogenase-type 2 in fish. *Federation of European Biochemical Society (FEBS) Letters* 574:167-170.

Baker, M.E., B. Ruggero, J. Sprague, C. Eckhardt, J. Lapira, I. Wick, L. Soverchia, M Ubaldi, A.M. Polzonetti-Magni, D. Vidal-Dorsch, S. Bay, J.R. Gully, J.A. Reyes, K.M. Kelley, D. Schlenk, E.C. Breen, R. Sásik and G. Hardiman. 2008. Analysis of endocrine disruption in Southern California coastal fish using an aquatic multi-species microarray. *Environmental Health Perspectives* doi: 10.1289/ehp.11627.

Benson, W.H. and R.T. Di Giulio. 2008. Genomic approaches for cross-species extrapolation in toxicology. CRC Press. Boca Raton, FL.

Bight'03 Field Sampling and Logistics Committee. 2003. Field Operations Manual. Southern California Coastal Water Research Project. Westminster, CA.

Blue, J.L., B.A. Nyagode, M.O. James and N.D. Denslow. 2008. Effects of the pesticide methoxychlor on gene expression in the liver and testes of the male largemouth bass (*Micropterus salmoides*). *Aquatic Toxicology* 86:459-469.

Boas, M., U. Feldt-Rassmussen, N.E. Skakkebaek and K.M. Main. 2006. Environmental chemicals and thyroid function. *European Journal of Endocrinology* 154:599-611.

Choi, C.Y. and H.R. Habibi. 2003. Molecular cloning of estrogen receptor alpha and expression pattern of estrogen receptor subtypes in male and female goldfish. *Molecular and Cellular Endocrinology* 204:169-177.

Cole, S.W., Z. Galic and J.A. Zack. 2003. Controlling false-negative errors in microarray differential expression analysis: a PRIM approach. *Bioinformatics* 19:1808-1816.

Deng, X., M.A. Rempel, J. Armstrong and D. Schlenk. 2007. Seasonal evaluation of reproductive status and exposure to environmental estrogens in hornyhead turbot at the municipal wastewater outfall of Orange County, CA. *Environmental Toxicology* 22:464-471.

Giger, W., P.H. Brunner and C. Schaffner. 1984. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. *Science* 4662:623-625.

Grun, F. and B. Blumberg. 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* 147:S50-855.

Hardiman,. G, Y. Tran, K. Franz-Bacon, R. Clover, B. Reardon and R. Wisotzkey, Andrew Watson, A. Mazumder and S. Balasubramanian. 2003. Analysis of gene expression in *Caenorhabditis elegans* using microarrays of covalently attached 50mer oligodeoxyribonucleotides. pp. 205-230 *in*: G. Hardiman (ed.), Microarray Methods and Applications. DNA Press. Glendale, CA.

Hardiman, G. 2004. Microarray platforms-comparisons and contrasts. *Pharmacogenomics* 5:487-502.

Hardiman, G. and A. Carmen. 2007. DNA Biochips – Past, Present and Future: An Overview. pp. 1-13 *in*: A. Carmen and G. Hardiman (eds.), Biochips as Pathways to Drug Discovery. Taylor & Francis Group, CRC Press. Boca Raton, FL.

Hasselberg, L., S. Meier, A. Svardal, T. Hegelund and M.C. Celander. 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). *Aquatic Toxicology* 67:303-313.

Hegelund, T. and M.C. Celander. 2003. Hepatic versus extrahepatic expression of CYP3A30 and CYP3A56 in adult killifish (*Fundulus heteroclitus*). *Aquatic Toxicology* 64:277-291.

Inui, M., T. Adachi, S. Takenaka, H. Inui, M. Nakazawa, M. Ueda, H. Watanabe, C. Mori, T. Iguchi and K. Miyatake. 2003. Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). *Toxicology* 194:43-50.

Islinger, M., D. Willimski, A. Volkl and T. Braunbeck. 2003. Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. *Aquatic Toxicology* 62:85-103.

Kelley, K.M., J.T. Haigwood, M. Perez and M.M. Galima. 2001. Serum insulin-like growth factor-binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 129:229-236.

Kelley, K.M., K.E. Schmidt, L. Berg, K. Sak, M.M. Galima C. Gillespie, L. Balogh, a. Hawayek, J.A. Reyes and M. Jamison. 2002. Comparative endocrinology of the insulin-like growth factor-binding protein. *Journal of Endocrinology* 175:3-18.

Kuiper, G.J.M., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad S. Nilsson and J.-A. Gustafsson. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of

estrogen receptors alpha and beta. *Endocrinology* 138:863-870.

Li, X., Z. He; and J. Zhou. 2005. Selection of optimal oligonucleotide probes for microarrays using multiple criteria, global alignment and parameter estimation. *Nucleic Acids Research* 33:6114-6123.

Marton, M.J., J.L. DeRisi, H.A. Bennett, V.R. Iyer, M.R. Meyer, C.J. Roberts, R. Stoughton, J. Burchard, D. Slade, H. Dai, D.E. Bassett, Jr., L.H. Hartwell, P.O. Brown and S.H. Friend. 1998. Drug target validation and identification of secondary drug target effects using DNA microarrays. *Nature Medicine* 4:1293-1301.

Masuyama, H., Y. Hiramatsu, M. Kunitomi, T. Kudo and P.N. McDonald. 2000. Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate pregnane X receptor-mediated transcription. *Molecular Endocrinology* 14:421-428.

Masuyama, H., H. Inoshita, Y. Hiramatsu and T. Kudo. 2002. Ligands have various potential effects on the degradation of pregnane X receptor by proteasome. *Endocrinology* 143:55-61.

Meucci, V. and A. Arukwe. 2006. The xenoestrogen 4-nonylphenol modulates hepatic gene expression of pregnane X receptor, aryl hydrocarbon receptor, CYP3A and CYP1A1 in juvenile Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 142:142-50.

Moreau, A., M.J. Vilarem, P. Maurel and J.M. Pascussi. 2008. Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Molecular Pharmacology* 5:35-41.

Naylor, C.G., J.P. Mieure, W.J. Adams, J.A. Weeks, F.J. Castaldi, L.D. Ogle and R.R. Romano. 1992. Alkylphenol ethoxylates in the environment. *Journal of the American Oil Chemists' Society* 69:695-703.

Nelson, J.S. 2006. Fishes of the World. 4th edition John Wiley and Sons. New York, NY.

Nielsen, HB., R. Wernersson and S. Knudsen. 2003. Design of oligonucleotides for microarrays and perspectives for design of multi-transcriptome arrays. *Nucleic Acids Research* 13:3491-3496.

Nishizawa, H., M. Morita, M. Sugimoto, S. Imanishi and N. Manabe. 2005. Effects of in utero exposure to bisphenol A on mRNA expression of arylhydrocarbon and retinoid receptors in murine embryos. *The Journal of Reproduction and Development* 51:315-324.

Ogawa, S., J. Lozach, K. Jepsen, D. Sawka-Verhelle, V. Perissi R. _á_ik, D.W. Rose, R.S. Johnson, M.G. Rosenfeld and C.K. Glass. 2004. A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation. *Proceedings of the National Academy of Sciences (PNAS) USA* 101:14461-14466.

Pajor, A.M., J.J. Stegeman, P. Thomas and B.R. Woodin. 1990. Feminization of the hepatic microsomal cytochrome P-450 system in brook trout by estradiol, testosterone, and pituitary factors. *Journal of Experimental Zoology* 253:51-60.

Rempel, M.A., J. Reyes, S. Steinert, W. Hwang, J. Armstrong, K. Sakamoto, K. Kelly and D. Schlenk. 2006. Evaluation of relationships between reproductive metrics, gender and vitellogenin expression in demersal flatfish collected near the municipal wastewater outfall of Orange County, California, USA. *Aquatic Toxicology* 77:241-249.

Rozen, S. and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. pp. 365-386 *in*: S. Krawetz and S. Misener (eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press. Totowa, NJ.

Sásik, R., C.H. Woelk and J. Corbeil. 2004. Microarray truths and consequences. Journal of *Molecular Endocrinology* 33:1-9.

Soto, A.M., H. Justicia, J.W. Wray and C. Sonnenschein. 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environmental Health Perspectives* 92:167-73.

Soverchia, L., B. Ruggeri, F. Palermo, G. Mosconi, G. Cardinaletti, G. Scortichini, G. Gatti and A.M. Polzonetti-Magni. 2006. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17-beta estradiol and nonylphenol. *Toxicology and Applied Pharmacology* 209:236-243.

Thompson, J.D., T.J. Gibson, .F Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25:4876-4882.

Vetillard, A. and T. Bailhache. 2006. Effects of 4-n-nonylphenol and tamoxifen on salmon gonadotropin-releasing hormone, estrogen receptor, and vitellogenin gene expression in juvenile rainbow trout. *Toxicological Sciences* 92:537-544.

Vilo, J. and K. Kivinen. 2001. Regulatory sequence analysis: application to the interpretation of gene expression. *European Neuropsychopharmacology* 11:399-411.

Wernersson, R. and H.B. Nielsen. 2005. OligoWiz 2.0--integrating sequence feature annotation into the design of microarray probes. *Nucleic Acids Research 33 (Web Server issue)*:W611-615.

Williams, T.D., K. Gensberg, S.D. Minchin and J.K. Chipman. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). *Aquatic Toxicology* 65:141-157.

Xie, W., J.L. Barwick, C.M. Simon, A.M. Pierce, S. Safe, B. Blumberg, P.S. Guzelian and R.M. Evans. 2000. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes and Development* 14:3014-3023.

Zhou, T., H.B. John-Alder, J.S. Weis and P. Weis. 2000. Endocrine disruption: thyroid dysfunction in mummichogs (*Fundulus heteroclitus*) from a polluted habitat. *Marine Environmental Research* 50:393-397.

Acknowledgements

This work was supported in part by NIH/NIDDK - Award 1 P30 DK063491-03, a contract from LACSD administered via SCCWRP and USC Sea Grant Program (NOAA grant #NA06OAR4170012, CFDA No. 11.417, Project CE-17). The multi-species microarray was jointly developed in the Baker (mbaker@ucsd.edu) and Hardiman (gthardiman@ucsd.edu) laboratories. The authors thank members of both laboratories for useful discussions.