Progress towards a *Pseudopleuronectes americanus* liver microarray

Peter F. Straub[1, 2], Mary L. Higham,[2] William C. Phoel[2] & Ashok D. Deshpande[1]
[1] Marine Chemistry Branch, National Oceanographic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Highlands, NJ 07732
[2]Biology Program, Richard Stockton College, Pomona, NJ 08240

Winter flounder, *Pseudopleuronectes americanus,* liver pathology has been shown to be a sensitive indicator of habitat degradation related to anthropogenic contamination[1]. To further investigate the molecular basis of this sensitivity, suppression subtractive hybridization (SSH) cDNA libraries from the livers of wild caught winter flounder[2] and from oil treated winter flounder[3] were previously constructed and sequenced to identify candidate marker genes. Common transcripts seen in the two studies included: cytochrome P450 1A, complement component C-3, C-type lysozyme, fibrinogen, antifreeze protein, gastrulation specific protein and ceruloplasmin. In addition, transcripts that were regulated with oil treatment included: defender against death cell protein, retinoid-x receptor-alpha, ribophorin1, GABA receptor associated protein, complement components C-5 and C-8, cytochrome P450 24A, hepatic glucose transporter, antithrombin and cardiac morphogenesis protein ES/130. Approximately 600 winter flounder transcripts from the above studies plus 350 transcripts from European flounder (*P. flesus*) (supplied by A. Tanguy of the University of Brittany, France) were used to produce a targeted microarray to investigate the effects of habitat contamination on the liver of winter flounder.

A flounder microarray was produced with 952 cDNA amplicon probe elements printed in 5 replicates on each slide (ca. 5000 elements per slide). Each element was produced by PCR amplification of a flounder plasmid cDNA clone using the T-7 and Sp6 primer sites of the pGEM-T vector. Each amplicon was purified using a 96 well PCR cleanup kit (Promega), checked for purity on a 96 well E-gel agarose gel system (Invitrogen), precipitated with ethanol and brought up in Corning "Pronto" microarray spotting solution. After transfer of amplified clones to a 384 well plate, the arrays were printed on a GeneMachines "OmniGrid-Accent" microarray printer at the Mt. Desert Island Biological Laboratory (MDIBL) core gene expression facility using 12 pins and Corning CMT-GAPS microarray prepared slides. Each array was set up as 12 blocks of 8 rows x 10 columns with 400µm spacing and replicated 5 times across each slide. Pins were cleaned by sonication after each sample and were blotted 10 times to glass before sample printing. After printing, the slides were dried in a vacuum desiccator and then cross linked with 300mJ UV in a "StrataLinker" oven (Stratagene).

For this preliminary study, winter flounder liver sample RNA's were from pools of individuals collected from relatively contaminated habitats (Hudson-Raritan) or from relatively clean habitats (Southern New Jersey coast).[2] These total RNA pools were extracted from flash frozen liver tissue using Trizol reagent (Invitrogen) and a mechanical homogenizer. The mRNA was purified from total RNA using a PolyAttract mRNA kit (Promega). Total and mRNA were quantified and checked for quality using an Agilent Bioanalyzer with an RNA Nano Lab chip. Labeled RNA for microarray hybridization was produced using an indirect (amino-allyl) reverse transcription labeling and purification kit (Invitrogen) followed by Cy-3 or Cy-5 (Amersham) fluorescent tag coupling. Equal amounts of Cy3 and Cy-5 labeled product, including dye swaps, from each of the test contaminated and control clean groups (ca. 50 pmole for total RNA or 12 pmole for mRNA) were denatured at 95 C in 50 µl hybridization buffer and added to prehybridized microarray slides under 24 x 60 mm coverslips. Microarrays were hybridized 16-18 hrs at 42 C in Corning microarray hybridization

chambers. Prehybridization, hybridization and subsequent wash protocols used the "Pronto" microarray system (Corning).

Hybridized microarrays were centrifuged briefly and then scanned using a GenePix 4000B scanner (Molecular Devices) at the MDIBL core gene expression facility or at the Public Health Research Institute, Center for Advanced Genomics (PHRI-CAG) in Newark, NJ. Data was analyzed using GenePix Pro 4.0 (Molecular Devices) software for acquisition of the Cy3 and Cy5 signal channels and Acuity 3.1 (Molecular Devices) for clustering and statistical analysis after linear, ratio-based normalization of individual microarrays. In initial experiments, winter flounder total RNA from the two sources, clean control and contaminated collection sites, was used as a template in the labeling reactions. Follow-up experiments using mRNA as a template for labeling seemed to produce higher and more reproducible signals on the microarrays.

Excellent resolution of the array elements was noted for many of the probes with good replication although weakly hybridizing areas may indicate that DNA concentration was too low for some of the gene probes (Figure 1). To reduce the chance of bias in dye incorporation, indirect labeling methods were used with dye swaps to control bias of incorporation in the dyes. The microarrays in figure 1 are dye-swap replicates and the intensity and color of the spots in the enlarged region are consistent with even incorporation of the dyes. The microarrays in figure 1 and their replicates from an experiment comparing mRNA extracted from flounder livers from more contamination impacted vs., less impacted control sites are analyzed and clustered by expression pattern in figure 2 and represent only those gene probes that showed a two-fold or greater increase or decrease in expression ratio over the 4 microarray slides (two replicate slides (each with 5 arrays) x two dye swaps). Table 1 shows 65 gene probes that met the selection criteria and were congruent with previous results seen with SSH cDNA cloning and quantitative PCR[2,3]. In particular, the microarray analysis detected an up-regulation in the fish from more contamination impacted sites of well known phase I and II liver detoxification enzymes such as cytochrome P450's 1A and 24 and glutathione-s-transferase. Several acute-phase, immune- reactive proteins such as differentially regulated trout protein-1, complement component C-8, hepcidin and coelomic cytolytic factor were up-regulated while complement regulatory protein was down-regulated. Several signals proteins were up-regulated including hepatocyte growth factor or down-regulated including cardiac morphogenesis protein ES/130, fetuin and catecholamines up protein. Overall, the measurement of differential gene expression by microarray appeared to have given similar results to the SSH cloning and quantitative PCR results observed so far.[2,3] These results point to measurable differences in the qualitative and quantitative levels of genes expressed in the liver in response to increased anthropogenic habitat disturbance.

The advantage of microarray technology is that many genes can be looked at simultaneously to detect expression patterns that can help decipher the condition of the organism. These patterns may serve as sensitive indicators for monitoring the effects of waterborne and sediment contaminants on the general health of the winter flounder and its ecosystem. Measurable sub-lethal effects may correlate to cumulative effects on growth, behavior, reproduction, recruitment and disease susceptibility and the ability to quantify these subtle effects can lead to better ecosystem and risk management decisions. In addition, insights gained into the molecular responses to sub-lethal exposure to contaminants in fish may serve as a model for the health of human populations exposed via recreational activities or through dietary consumption.

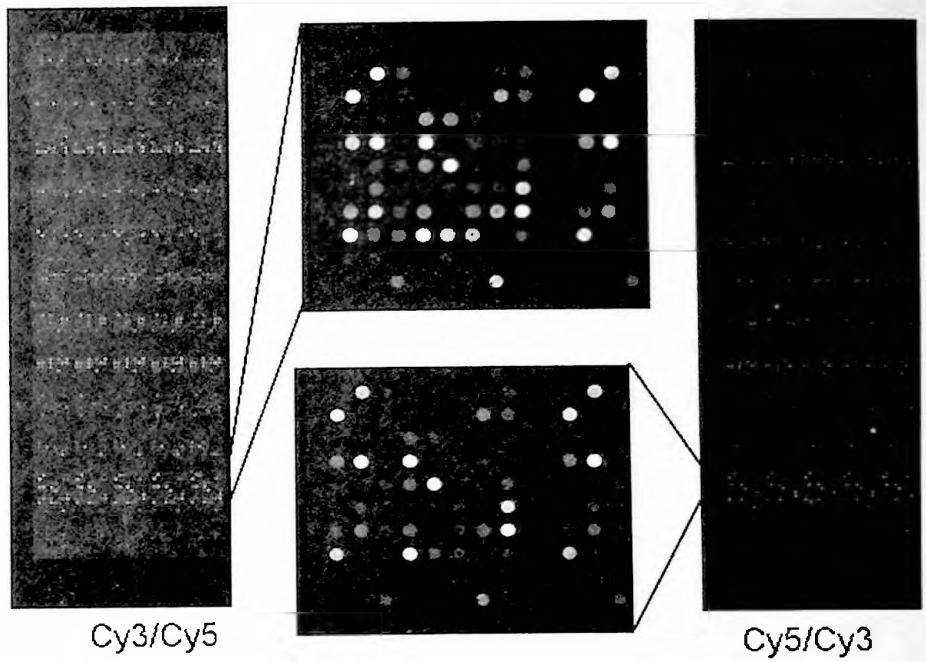

Fig. 1. Dye swapped winter flounder microarrays. The left array was hybridized with a Cy3 (green) labeled clean control and a Cy5 (red) labeled contamination impacted cDNA produced from mRNA. The array on the right was hybridized with a Cy5 (red) labeled control and a Cy3 (green) labeled pollution impacted cDNA produced from mRNA. The expanded middle panels are the same block with the color swap evident in the primarily green and red spots. Yellow spots are hybridizing equally to red and green dye and are not differentially expressed

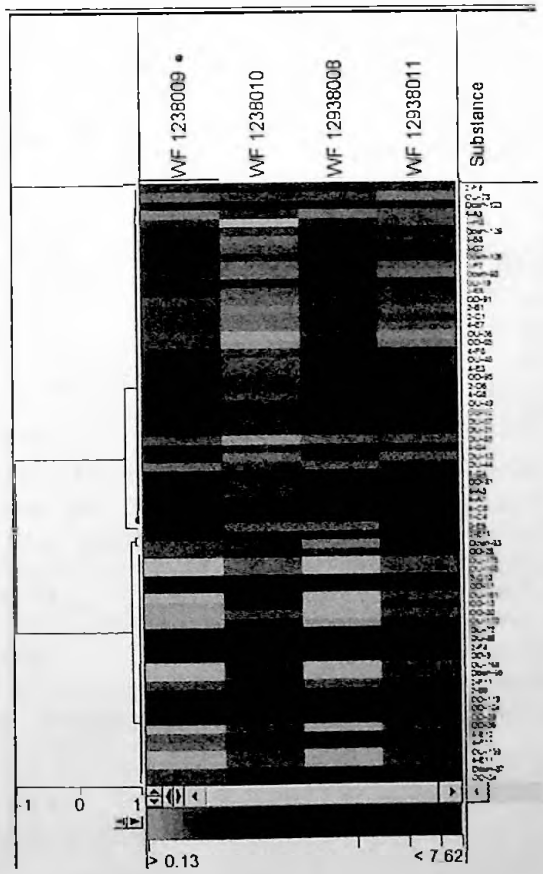

Fig. 2. Hierarchical clustering of mean value of ratio of medians for 4 microarray slides (each with 5 replicate arrays) in Acuity 3.1 software (Axon Corp). Microarrays WF1238008 and WF1238009 are replicates in which red indicates up- and green indicates down-regulated genes. WF1238010 and WF1238011 are replicate dye swaps respectively of the first two with green indicating up- and red indicating down-regulation. Only microarray elements showing greater than two fold increases or decreases on replicate dye-swapped microarrays are shown here.

Table 1. Winter flounder genes found to be up or down regulated 2 fold or greater upon microarray analysis. This is a comparison of mRNA from liver samples collected from habitats with high anthropogenic contamination (Hudson-Raritan estuary) versus low anthropogenic contamination (Southern New Jersey coastal estuaries). Fold changes are the mean (ratio of median) for four replicate slides each with five replicate arrays for an n=20 spots. Standard deviation = sd.

Up Regulated					Down Regulated				
			Fold Up					Fold Down	
ID	Annotation	Function	mean	sd	ID	Annotation	Function	mean	sd
2-D6	unknown expressed sequence		2.3	0.4	OU-175	Fertilization envelope outer layer protein	Reproduction	29.3	14.9
4-G4	TBT binding protein	Signal	2.4	0.3	4-E11	unknown expressed sequence		16.2	7.0
OU-12	glyoxylase II	Metabolism	2.3	0.2	OU-165	ribophorin I	Metabolism	26.1	13.7
OU-19	complement component C8 β chain	Immune	2.1	0.3	OU-159	unknown expressed sequence		13.4	3.8
Down-123	regucalcin	Metabolism	2.2	0.3	Down-95	Catecholamines Up Protein (Catsup)	Signal	9.4	3.3
OU-49	unknown expressed sequence		2.3	0.2	OD-36	fetuin (α2sH glycoprotein)	Signal	8.6	1.3
1-G4	unknown expressed sequence		2.4	0.2	OU-168	unknown expressed sequence		8.2	1.7
Down-106	unknown expressed sequence		2.1	0.4	OU-161	unknown expressed sequence		7.1	3.1
OU-57	unknown expressed sequence		2.6	0.3	OD-30	apolpoprotein B	Transport	10.5	5.3
OU-46	unknown expressed sequence		2.8	0.5	OD-13	chitinase (acidic)	Metabolism	5.7	2.4
3-G7	unknown expressed sequence		2.6	0.5	OU-170	cardiac morphogenesis protein ES/130	Signal	5.2	1.4
4-G6	glutathione-s-transferase subunit 13	Detox/Metabolism	2.7	0.4	4-F11	cytochrome C oxidase subunit I	Metabolism	4.1	0.5
3-B8	trypsinogen-2 precursor	Metabolism	2.8	0.8	4-E12	unknown expressed sequence		3.3	0.8
4-F8	hepcidin	immune/iron	2.6	0.3	2-F11	ribosomal protein S-25	Metabolism	3.3	1.3
4-E3	2-isopropylmalate synthase	Metabolism	2.7	0.4	OD-34	complement regulatory plasma protein	Immune	2.6	1.1
4-A8	coelomic cytolytic factor 1	immune	2.8	0.3	Down-93	cytochrome P-450 7A	Detox/Metabolism	3.6	1.8
3-E8	hepatocyte growth factor activator	Signal	2.8	0.4	OD-26	transferrin	Metabolism	2.6	0.8
1-C4	unknown expressed sequence		2.2	0.4	OD-5	unknown expressed sequence		2.7	0.3
3-E6	guanine nucleotide binding protein β subunit	Signal	3.9	3.1	2-F4	ribosomal protein large P2	Metabolism	2.0	0.3
2-F7	ribosomal protein S7	Metabolism	3.1	0.7	OD-119	unknown expressed sequence		2.6	0.6
4-A7	ferritin GF2	Metabolism	2.7	0.2	OU-72	unknown expressed sequence		2.0	0.3
2-F9	ribosomal protein S19	Metabolism	2.5	0.4	2-E11	ribosomal protein L13a	Metabolism	1.8	0.4
OU-21	cytochrome P450 1A1	Detox/Metabolism	2.9	0.5	2-B9	unknown expressed sequence		1.7	0.5
4-C8	40S ribosomal protein S12	Metabolism	2.5	0.7	2-G10	40S ribosomal protein S6	Metabolism	1.8	0.4
Down-98	RUSH 1-beta	Signal	3.5	0.7	OU-88	transcription factor BTF3a	Signal	1.9	0.4
OD-81	cytochrome C oxidase subunit VIa precursor	Metabolism	3.2	0.3	OD-39	fetuin (a2sH glycoprotein)	Signal	2.3	0.1
2-C1	unknown expressed sequence		3.3	0.4	OD-104	biotinidase precursor	Metabolism	2.3	0.1
2-B1	unknown expressed sequence		3.6	0.6					
4-E7	Differentially regulated trout protein-1	Immune	3.6	0.3					
OU-13	similar to fertilization envelope outer protein	Reproduction	4.0	0.4					
3-H8	unknown expressed sequence		4.1	1.5					
OD-91	ribosomal protein L23a	Metabolism	3.6	0.5					
OU-36	cystathionine-β-synthase	Metabolism	4.6	0.7					
OD-85	eukaryotic translation initiation factor 3	Signal	4.4	0.6					
OU-28	cytochrome P450 24A1	Detox/Metabolism	4.2	1.1					
OU-55	vitamin K dependent γ- glutamyl carboxylase	Metabolism	5.8	1.7					
OU-44	ribosomal protein L10a	Metabolism	6.6	4.3					
4-A9	transcription factor ESE3A	Signals	8.9	7.5					

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1. **Johnson, L. L., J. E. Stein, T. K. Collier, E. Casillas, B. McCain, U. Varanasi**. (1992) Bioindicators of contaminant exposure, liver pathology, and reproductive development in prespawning female winter flounder (*Pseudopleuronectes americanus*) from urban and nonurban estuaries on the northeast Atlantic coast. U.S. Dept of Commerce/ NOAA/ NMFS/ NWFSC *NOAA Technical Memorandum* NMFS-NWFSC-1.
2. **Straub, P.F, M.L. Higham, A. Tanguy, B.J. Landau, W.C. Phoel, L.S. Hales, T.K.M. Thwing**. (2004). Suppression Subtractive Hybridization cDNA Libraries to Identify Differentially Expressed Genes from Contrasting Fish Habitats. *Marine Biotechnology* 6:386-399.
3. **Straub, P. F., M.L Higham and W.C. Phoel**. (2005). Suppression subtractive hybridization (SSH) cDNA library construction from fuel oil treated winter flounder (*Pseudopleuronectes americanus*). *Bulletin of the MDI Biological Laboratory*. 44: 121.