Gene Expression Affected by the Endocrine Disrupting Chemical 4-Nonylphenol in Winter Flounder (*Pseudopleuronectes americanus*)

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Introduction

4-Nonylphenol (4-NP) is a primary breakdown product of alkylphenol ethoxylates, which are highly cost-effective non-ionic surfactants important in industrial and institutional formulations (1). 4-NP is not a single chemical compound, but rather a family of compounds all with a benzene ring and a nine-carbon side chain. 4-NP is a member of this family and makes up about 90% of commercial NP (2). 4-NP is used in the production of textile, pulp and paper processing, steel manufacturing, oil and gas recovery, and power generation. 4-NP can also be found in paints, plastics, resins protective coating in pest control products, institutional cleaning agents, agricultural chemicals, and household cleaning products (3). 4-NP has been used for decades as a stabilizer in certain plastic products such as polyethylene. It has been demonstrated that 4-NP can leech out of plastic products at high temperatures and absorbed into foods since 4-NP is lipophilic (4). In the year 2000 alone, 240 million pounds of this product were produced in the United States and, at a 2% annual increase; it is projected that 260 million pounds of 4-NP will be produced in 2004 (4). Industries claim NP’s as a trade secret since they are used as inert and it is difficult to say exactly how much are used, but it is estimated that half of the produced 4-NP goes down the drain because of it’s poor biodegradation.

4-NP has been found in sewer sludge, sludge form municipal and industrial waste water treatment plants, soil on which the sludge has been spread over rivers, lakes, and estuaries (3). 4-NP enters the environment primarily through industrial and municipal wastewater, but also from their application in pesticides (5). 4-NP is used in pesticides to make them more potent by increasing their bioavailability. Approximately 60% of
nonylphenols produced end up in water (6), and since 4-NP is an endocrine disrupting chemical (EDC), it can interfere with the body’s reproductive system.

EDC’s are environmental estrogens that work by activating nuclear receptors then cause chemical reactions in the body that would normally be set off by natural hormones, such as estrogens and androgens. EDC’s include dichloro-diphenyl-trichloroethane (DDT), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), polychlorinated biphenyls (PCB), bisphenol-A, tributyltin (TBT), dichlorophenylchloroethylene (DDE), phthalates and alkylphenols (7). EDC’s are associated with a variety of adverse biological effects in wildlife and humans such as reproductive and behavioral problems. They have little affect on the exposed organism but are found to be tetratogens (producing birth defect) that produce distorted sex organ development, infertility, undescended testicles, smaller penis or testicles, lower sperm count, reproductive abnormalities and thyroid dysfunction (8). A number of case studies have shown a connection between reproductive abnormalities in wildlife and environmental estrogens especially aquatic animals. For example the pesticides DDT and DDE, available following a spill into Lake Apopka, Fl. are suspected of causing male alligators to have smaller penis size, poorly organized testes and lower plasma testosterone levels (9). EDC’s found in the Kalamazoo River were linked to reproductive decline in bald eagles. In efforts to restore the population nesting attempts were done. Two out of seventeen attempts were successful, unhatched eggs were taken and tested for PCB’s. The resultant PCB concentrations in the unhatched eggs were the highest ever found (10). Though humans are not exposed to as many EDC’s as wildlife, environmental estrogens may still cause problems like
premature puberty, lower sperm counts, undescended testicles, and thyroid dysfunction (8).

4-NP is an endocrine disrupting chemical (EDC) that binds to the estrogen receptor, mimics the action of the naturally produced hormone estradiol, and as such it interferes with the body’s endocrine system. In vivo studies showed an increase of vitellogenin (VTG) in male rainbow trout exposed to 4-NP. Vitellogenin is an egg yolk protein precursor that is under the control of estradiol usually found only in females, and thus should not be produced in males. In England, fish populations in the Thames River have high VTG, attributed to 4-NP, and hermaphroditism in roaches appeared near effluents from sewage-treatments works (11). Furthermore, human male populations along the Thames River showed a decrease in sperm count potentially due to 4-NP and other estrogenic chemicals found in the water (12). In addition, female Daphnia magna, a small freshwater crustacean, exposed to 4-NP showed reduced reproduction, and reduced elimination of steroids especially in the second generation (13). Neonates of exposed maternal Daphnia magna showed abnormalities consistent with partial arrest of early embryonic development, including reduced shell spine and 1st antennal development (14).

4-NP also binds to the orphan nuclear receptor, the Pregnane-X-Receptor (PXR). Orphan nuclear receptors are receptors that regulate transcription in a ligand-dependent fashion, but the endogenous ligand is unknown. PXR regulates the expression of CYP3A, UDPGT, and SULT I, and MRP2 and other detoxification genes found in the liver and intestine. 4-NP binds to the PXR, which then dimerizes, with the RXR binds to DNA, and then induces the CYP3A enzyme, as well as other transcripts. CYP3A is a
Gene Expression Affected By 4-Nonylphenol

hydroxylase for estradiol and testosterone and can alter the metabolism of these hormones if induced.

The purpose of this research is to assess altered gene expression caused by 4-NP in winter flounder, Pseudopleuronectes americanus. Winter flounder was used because they are found in estuaries, associated with sediments, and thus exposed to many toxicants. Furthermore, humans consume them. Distinguishing genes altered may help identify physiological perturbations in flounder caused by 4-NP, and provide novel biomarkers of 4-NP exposure. We hypothesize that 4-NP will alter the expression of genes controlled by the PXR and ER such as vitellogenin, cell cycle proteins, and CYP3A in winter flounder.

Methods and Materials

Winter Flounder

Winter flounder were obtained in Maine, USA and were allowed to acclimatize for a period of seven days prior to treatment. A total of eight flounder were randomly assigned to two 250-gallon tanks. Four exposed fish received 4-NP injections of 100mg/kg body weight in approximately 160µl of corn oil, for two days. Four untreated fish were injected with 160µl of corn oil. Fish were euthanized on day two of the experiment with MS-222 and decapitated. Blood was collected from the tail veins, and livers were excised and separated into five different tubes for RNA extraction and protein preparation.
Subtractive Hybridization

RNA was extracted from livers using Trireagent (Sigma, Chemical Co., St. Louis, Mo.). Clontech Nucleotrap™ method was used to isolate mRNA from the total RNA. The mRNA was then used for subtractive hybridization to amplify and isolate genes that were potentially differentially expressed (Clontech Kit, BD Biosciences, and Palo Alto.) Forward and reverse subtractions were performed in order to isolate genes that were over expressed and under expressed following 4-NP treatment. Genes that were not subtracted after subtractive hybridization were inserted into pCR2.1 plasmids from Invitrogen® (Carlsbad, CA.). PCR2.1 plasmids contain the luciferase gene.

Insertion into Cells

Plasmids were then transfected into competent E.coli bacteria by thawing out the appropriate number of competent cells on ice, then adding 2µl of 0.5M Beta-Mercaptoethanol to each vial mixing with the pipette tip. After mixing, 2µl of the ligation reaction was added to the cells incubated on ice for 30 minutes, and then heat shocked at 42°C for 30 seconds prior to another 2 minutes on ice. Sterile SOC medium was added (250µl) and the vials were placed in Environ Shaker for an hour at 37°C at 225 rpm. X-gal was spread over the Ampicillin/Kanamycin plates and allowed to dry before plating 100µl of the cell culture on each plate. The prepared plates were then incubated overnight at 37°C to allow for cell growth. Colonies then appeared white or blue. Blue colonies were not picked because they contained an intact luciferase gene and thus lacked an insert. Colonies that were white were picked, labeled and grown in 3ml of LB ampicillin
broth overnight in a shaker at 225rpm at 37°C. A total of 134 colonies were picked. Plasmids were purified using the QiaPrep® Kit (Qiagen, Valencia, CA.). Briefly, bacteria grown in broth were put in two 1.5ml centrifuge tubes and centrifuged at 400XE for three minutes. The supernatant was disposed, the pellet was dissolved in 250µl of P1 buffer, 250µl of P2 buffer was added and samples were inverted to mix. 350µl of N3 was added to samples and all were centrifuged at max speed for ten minutes. The supernatant was transferred to QiaPrep spin columns and centrifuged for 60 sec. The columns were washed twice, and the plasmid dissolved in 50µl of Tris-HCL prior to centrifuging the plasmid from the column.

**cDNA Amplification**

Polymerase Chain Reaction (PCR) was performed to amplify genes that were inserted into cells. A PCR master mix was prepared by using 39.2µl PCR nucleotide mix, 196µl PCR buffer mix, 1489.6µl of water, 19.6µl of DNA polymerase mix 50x, and 58.8µl of Primer 1 and Primer 2. Nineteen micro liters of master mix was added to 1.0µl of purified plasmid samples. Samples were PCR’d for 23 cycles at 95°C for 15 seconds to denature the DNA, then at 68°C for 3 minutes during which both primer annealing and extension occurred. All of the samples were placed on an electropherisis gel for selection. Samples were automatically eliminated if they did not materialize on the gel; further elimination was based on samples that appeared to have the same molecular weight according to the gel. A total of 94 samples were chosen to place on a 96 well cDNA array, along with 2 housekeeping genes (B-actin and 16S).
cDNA Array

The cDNA arrays were used to confirm differential gene expression. Ninety-six genes were placed on nitrocellulose paper. The samples were prepared by combining 5µl of 0.6M NaOH solution with 5µl of PCR product in new tubes. After placing 2µl of the new sample mixture on nitrocellulose paper they were soaked in 0.5M Tris-HCl solution for 3 minutes to neutralize the NaOH. The sheets were cross-linked, adhering cDNA to the nitrocellulose paper. A total of four arrays were prepared, two for reverse and two for forward-subtracted cDNA. Table 1 provides a map of the PCR products spotted on the array.

Radioactive probes were prepared using the subtracted libraries. Briefly, [\(^{33}\)P] ATP, CTP, TTP and GTP were added to a mix containing buffer and klenow enzyme to produce a radio labeled copy of each gene in the subtractive library. Probes were then hybridized onto the array in Hybrisol (Clontech) and left rotating overnight at 72°C. The arrays were then washed in warm low stringency buffer (2X SSC and 0.5% SDS) four times for 20 minutes at 68°C and then high stringency buffer (0.2x SSC and 0.5% SDS) two times for 20 minutes at 68°C and placed on a K-screen for three days to absorb radiation, making it possible to distinguish what genes have been either up-regulated or down-regulated (Figure 1). The blot was then exposed to x-ray film for two weeks with an intensifying screen at -80°C. Through the array, genes that showed possible differentiation were selected for sequencing.
Sequencing of Samples

The selected gene samples were sent to Macrogen Corp, Inc. for DNA sequencing in Seoul, Korea. Once samples are sequenced Blast searches were performed using GenBank to identify the genes in question (17).

Results

Production of Plasmid Clones

Plasmid insertion produced approximately 150 clones, of which 134 plasmid clones were grew and were purified. Ninety-four of these clones were amplified via PCR, and then spotted onto nylon paper to make cDNA arrays. Two additional genes, 16S rRNA and β-actin, were added as housekeepers in the 96-well arrays.

cDNA Arrays

The arrays were compared to each other in order to identify which of the samples showed differentiation. The dark spots were compared; if they showed to be down regulated they would appear on the down regulated array and not on the up regulated array. Figure 1A shows the down regulated genes in comparison to Figure 1B where they did not appear. In addition, other clones appear only in the up-regulated section of the array and thus appear to be induced. These genes are spots below the line on Figure 1. Genes or spots that appear on both arrays are not differentially expressed, as are genes
that do not appear on either array. None of the housekeeping genes had spots following array hybridization, indicating that they had been subtracted as expected.

This is how differentiation of gene expression after 4-Nonylphenol treatment was confirmed using the arrays. Samples that were reverse subtracted, or down regulated, were labeled as 1’s. Forward subtracted, or up-regulated, samples were labeled as 2’s. 4-NP appeared to down regulate 9 clones and up regulate 16 clones (Figure 1).

*DNA Sequencing*

DNA sequences were used to perform blastn (nucleotide) and blastp (protein) searches on GenBank. Blastn searches compared the samples to all nucleotide sequences from other species that have been submitted to GenBank in attempts to match and identify the genes. The sequences were also used to do blastp searches. The blastp database on GenBank translates the sequences and compares them to protein sequences that have been identified and submitted to GenBank. After performing the Blast searches we can see what genes the samples are significantly similar to according to the e-value, as seen in Table 2.

*Discussion*

The purpose of this study was to determine the effects of 4-nonylphenol on gene expression in the liver. Several genes were found to be altered including many ESTs, a signal recognition particle, complement component C3, ApoA1 and a glucose transporter. Complement component C3 is an acute phase reactant and thus is induced during an
acute inflammatory reaction. This is somewhat typically following a toxic insult. Many of the other genes induced are involved in cell growth. This may be explained by 4-NP’s estrogenic nature. Interestingly, vitellogenin was not one of the genes altered by 4-NP. No genes were found that implicated the PXR in 4-nonylphenol’s actions in winter flounder. However, there are several more genes that must be sequenced and confirmation of altered gene expression will be done by quantitative PCR in the future. The importance of this is to see the effects 4-NP on winter flounder at a genetic level. After observing what genes 4-NP alters, we can then begin to establish how the fish are affected physiologically, and study those parameters.

**Acknowledgements**

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References

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17) Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. &

**TABLE 1:** Map of PCR sample placement on array. Assigned location of subtracted clones on cDNA array. All clones were placed in the same location on all four arrays.

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HK-A= Housekeeper Gene B-actin; HK-16S= Housekeeper gene 16S rRNA.
### TABLE 2: Gene similarity by nucleotide and protein blast.

Shows differential expression of genes on the subtractive arrays. Below are the genes that were found to be differentially expressed on the subtractive arrays and then blasted on GenBank. Nucleotide (blast n) and protein blast (blastp) e-values are reported for genes showing significant similarity to genes already submitted to GenBank.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene Similarity</th>
<th>E-Value(n)</th>
<th>E-Value (p)</th>
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<td>NA</td>
<td>NA</td>
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<td>1-44</td>
<td>EST</td>
<td>NA</td>
<td>NA</td>
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<td>1-47</td>
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<td>5e-07</td>
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<td>EST</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>2-88</td>
<td>sequence is poor</td>
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<td>NA</td>
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<td>2-90</td>
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<td>9e-62</td>
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<td>1e-57(^a)</td>
<td>e-134(^a)</td>
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<td></td>
<td>IGF-1</td>
<td>4e-19(^b)</td>
<td>3e-60(^b)</td>
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<td>signal recognition particle 72</td>
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EST: expressed sequence tag: no similarity found in GenBank
NA: not applicable
\(^a\)3’-end of clone
\(^b\)5’-end of clone

**Figure 1:** Subtractive arrays: cDNA arrays were performed on forward and reverse subtracted samples. Forward subtraction generally produced up-regulated genes and reverse subtractions generally produced down-regulated genes.

(A) Reverse subtracted and (B) Forward subtracted cDNA arrays; Black dots above the line in (A) that are not on array (B) were considered down-regulated, and black dots on (B) below the line on (B) that are not present on (A) are considered up-regulated by 4-Nonylphenol.

**Down-Regulated Genes (A)**

**Up-Regulated Array (B)**
Gene Expression Affected
By 4-Nonylphenol