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To cite this version:

Natàlia Garcia-Reyero, Ira Adelman, Li Liu, Nancy Denslow. Gene expression profiles of Fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota. Marine Environmental Research, 2008, 66 (1), pp.134. 10.1016/j.marenvres.2008.02.073 . hal-00563027

HAL Id: hal-00563027

https://hal.science/hal-00563027v1

Submitted on 4 Feb 2011

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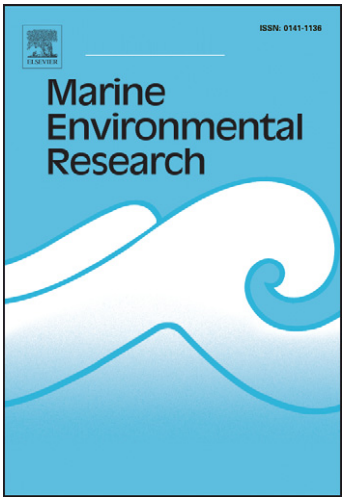

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PII: S0141-1136(08)00050-0
DOI: 10.1016/j.marenvres.2008.02.073
Reference: MERE 3202

To appear in: *Marine Environmental Research*

Please cite this article as: Garcia-Reyero, N., Adelman, I., Liu, L., Denslow, N., Gene expression profiles of Fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota, *Marine Environmental Research* (2008), doi: 10.1016/j.marenvres.2008.02.073

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Gene expression profiles of Fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota

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Abstract

Water treatment plants are often not effective in removing pharmaceuticals, personal care products or natural hormones from their effluents. To test the effects of these effluents on fish, we exposed male fathead minnows (*Pimephales promelas*) for 48 hours to effluents at two sites, one up-stream and the other down-stream from a water treatment plant. Gene expression profiling showed that significant changes occurred in the gonad of fish exposed below, compared to above the treatment plant and to laboratory control fish. Among the biological processes affected were the innate immune response, response to stress, control of homeostasis, control of transcription, metabolism, and cell communication. This work suggests that fish are impacted by

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exposures to the sewage treatment effluents and effects can be detected rapidly by gene expression profiling.

Keywords: Gene expression profiling; Sewage treatment plants; Pharmaceuticals

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Pharmaceuticals, natural hormones and personal care products in municipal effluents and surface waters around the world may cause hazards, which have not yet been characterized (Huggett et al., 2002; Daughton and Ternes, 1999). These waterways are complex soups of compounds, ranging from antibiotics to acetaminophen to soaps and other personal care products, many of which may be affecting aquatic life. Many of these pollutants are endocrine active compounds that disrupt reproduction at different levels along the hypothalamus-pituitary-gonadal axis. In this work, we use expression profiling on fathead minnows to determine changes in gene expression in fish exposed to river waters up-stream and down-stream from a municipal sewage treatment facility located in Minnesota.

Fathead minnows (FHM) were placed in wire mesh minnow traps that were anchored to the bottom in the stream current. Fish were removed from the traps after 48 hours and transported back to the laboratory in stream water. Gonads were removed from four males for total RNA isolation, using Stat-60 reagent (Tel-test, Friendswood, TX), as previously described (Sabo Atwood et al., 2004). Total RNA was treated with an RNase inhibitor (RNASecure, Ambion) and with DNase (DNA-free, Ambion) to remove traces of DNA. RNA integrity and quantity were measured on the Agilent bioanalyzer and the nanodrop spectrophotometer, respectively.

Microarray analyses were performed on the individual samples using competitive hybridization on the 4 X 44K FHM microarray (EcoArray, Alachua, FL). These microarrays are oligonucleotide based and contain 22,000 genes. A reference design was used, where the reference sample, consisting of equal aliquots of control and treated samples, was labeled with the Cy3 dye, while experimental samples were labeled with the Cy5 dye, following Agilent protocols.

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Microarrays were analyzed by ANOVA followed by Tukey's Honestly Significantly Difference post-hoc test to determine the set of differentially regulated genes at site #12 compared to site #11. Hierarchical clustering was performed for genes with a P value < 0.05. To determine biological processes that were specifically changed in fish at site 12, we analyzed for Gene Ontology (GO) categories that are differentially enriched at site #12 compared to site #11, using Fisher's exact test and also calculated the false discovery rate.

The two sites in our study are located on the Des Moines River, one several miles upstream (site #11) and the other, immediately downstream (site #12) from the Windom sewage treatment plant. The upstream site # 11 drains extensive agricultural land, although few animal feeding operations are within 5 miles of the site. These sites were characterized by Lee et al. (1999) who found significant effects in resident male carp at site #12, including the presence of plasma vitellogenin (< 10 ug/ml), decreased levels of 11-keto testosterone, and higher amounts of ceroid/lipofuscin deposits in the gonads, which correlate with cellular abnormalities.

In our study of the caged FHM, we analyzed differentially expressed genes by hierarchical clustering for the gonad (Fig. 1). The individual gonad samples clustered together for the two sites and the expression patterns were quite distinct from each other. If we add laboratory controls to the hierarchical clustering, they cluster with site #11, suggesting that fish at site # 11 are more closely related to controls (data not shown). In the gonad, 119 genes were up-regulated and 128 genes were down-regulated at P < 0.05.

We determined the molecular pathways most affected in the gonad by the downstream effluent by calculating P values for each GO category for Biological Process. GO categories are plotted as a function of increasing P value (Fig. 2) and identified in Fig. 2B, along with their P values. Interestingly, there are more pathways represented in the list of down-regulated genes

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(30 categories) than in the list of up-regulated genes (3 categories) (Fig 2B). The main pathways up-regulated by the exposure include cell communication, signal transduction, and the protein kinase cascade. Among the pathways that are down-regulated we note at the top of the list innate immune response and response to stress. Other pathways include control of homeostasis, control of transcription, metabolism, and embryonic development.

These results suggest that exposure of fish to the downstream effluent results in changes in gene transcription that may adversely affect reproduction, growth and susceptibility to disease. The documented changes in gonad structure in carp collected at this site by Lee et al. (1999) are consonant with the changes in the pathways we have measured. To date there are no analytical measurements of contaminants in the downstream waters, but these findings, together with the phenotypic changes seen in carp collected at the site, strongly indicate that such measurements should be carried out and that this site should be evaluated more thoroughly by traditional assessments to determine the risk to fish.

The use of gene expression changes to evaluate exposure of fish to toxicants is still in its infancy. We have shown that a 48-hour exposure of caged fish to the effluent is sufficiently long to change gene expression in a robust and specific way. As more controlled laboratory experiments are performed with individual chemicals, gene expression signatures will be identified that relate back to individual chemicals.

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Acknowledgements

This work is the result of research sponsored by the Minnesota Sea Grant College Program supported by the NOAA Office of Sea Grant, United States Department of Commerce, under grant No. NA03OAR4170048. The U.S. Government is authorized to reproduce and

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Figure captions:

Fig. 1. Hierarchical clusters for genes that are differentially expressed in the gonad of fathead minnows exposed to water at sites 11 and 12. Expression data was analyzed by ANOVA and then z-transformed. Genes used in the cluster were significant at $P < 0.05$.

Fig. 2. (A) Differential expression of biological function pathways in fish exposed to site 12 compared to site 11. P values refer to the probability that genes in the selected GO pathways are enriched at site 12 compared to site 11. (B) Identification of biological process categories differentially expressed in the gonad of fish exposed to site 12 compared to site 11.

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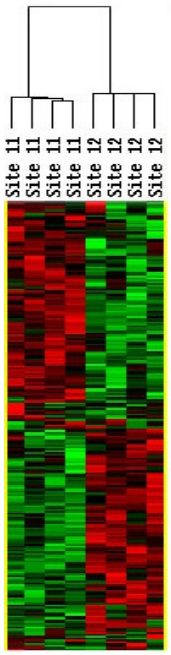

Fig. 1. Gonad

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GO category Biological Process

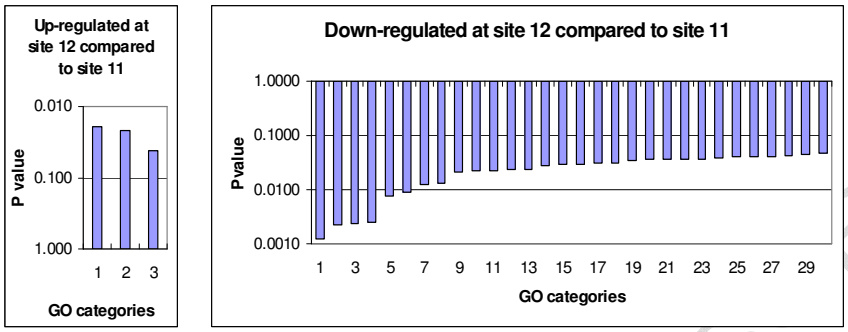

Fig. 2A

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Fig 2B. Biological process categories significantly enriched at site #12 compared to site # 11

GO Categories that are significantly up regulated (P < 0.05)

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0007154	cell communication	15	1692	0.019
GO:0007165	signal transduction	14	1562	0.022
GO:0007243	protein kinase cascade	3	149	0.041

GO Categories that are significantly down regulated (P < 0.05)

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0045087	innate immune response	3	31	0.0013
GO:0006950	response to stress	10	538	0.0023
GO:0007582	physiological process	58	7279	0.0024
GO:0050875	cellular physiological process	58	7292	0.0025
GO:0030003	cation homeostasis	3	61	0.0077
GO:0006873	cell ion homeostasis	3	64	0.0088
GO:0044237	cellular metabolism	43	5169	0.0126
GO:0050801	ion homeostasis	3	74	0.0128
GO:0019725	cell homeostasis	3	90	0.0211
GO:0008152	metabolism	45	5631	0.0219
GO:0044238	primary metabolism	40	4858	0.0225
GO:0006955	immune response	5	251	0.0231
GO:0009611	response to wounding	4	167	0.0231
GO:0006464	protein modification	15	1386	0.0278
GO:0009613	response to pest, pathogen or parasite	4	180	0.0293
GO:0051242	positive regulation of cellular physiological process	3	103	0.0297
GO:0044267	cellular protein metabolism	22	2321	0.0303
GO:0009987	cellular process	60	8256	0.0312
GO:0019222	regulation of metabolism	15	1423	0.0341
GO:0044260	cellular macromolecule metabolism	22	2357	0.0354
GO:0042592	homeostasis	3	111	0.0357
GO:0043412	biopolymer modification	15	1434	0.0362
GO:0009792	embryonic development (sensu Metazoa)	3	112	0.0365
GO:0050896	response to stimulus	11	948	0.0383
GO:0006952	defense response	5	291	0.0395
GO:0009790	embryonic development	4	200	0.0404
GO:0050794	regulation of cellular process	19	1982	0.0407
GO:0008283	cell proliferation	4	205	0.0435
GO:0006350	transcription	14	1351	0.0458
GO:0009605	response to external stimulus	4	209	0.0461

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