Evaluation of water quality threats to the endangered Okaloosa darter, *Etheostoma okaloosae*, in East Turkey Creek on Eglin Air Force Base

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Abstract:

The federally endangered Okaloosa darter (*Etheostoma okaloosae*) is found almost exclusively on the Eglin Air Force Base in the Choctawhatchee Bay watershed of Florida. Portions of this limited habitat are threatened by smothering with erosional soils, altered hydrology, and impaired water quality. Taken together, these factors have contributed to the endangered status of this fish. The U.S. Fish and Wildlife Service has been working to remove impediments to the recovery of the darter through threat identification and stream segment restoration. One stream reach in particular, East Turkey Creek, has demonstrated potential water quality problems. Preliminary data indicating possible problems have included poor invertebrate bio-assessment scores (IBI), uncharacteristically high conductivity values, and low numbers of Okaloosa darters. For these reasons, the influence of adjacent land uses, including a wastewater spray field, was assessed. Differences in ambient water quality were found between East Turkey Creek and the reference site. 48-h fish exposure experiments showed that critical pathways of toxicity were induced in laboratory-exposed fathead minnows, suggesting that these exposures could interfere with reproduction and growth of

endogenous fish. To better understand the ecological significance of these preliminary findings, a longer exposure regimen with surrogate fish showing reproductive outcomes (21 d reproductive test), changes in plasma levels of sex steroid hormones and vitellogenin, and changes in gonad histology would be paramount. The data presented here will be used to focus future analysis and to adapt management strategies to address water quality issues to further recovery of the Okaloosa darter.

Keywords: Okaloosa darter, *Etheostoma okaloosae*, species recovery, wastewater effluent, microarrays, passive samplers

Introduction

The Okaloosa darter *(Etheostoma okaloosae)* is a small, elongate, slightly compressed darter, with adults ranging in size from 27-49 mm standard length (Metee and Crittenden 1979; Ogilvie 1980, Figure 1). The U.S. Fish and Wildlife Service (Service) included this fish on the List of Endangered and Threatened Wildlife and Plants as endangered, because it only occurs in six stream systems, located in Okaloosa and Walton Counties, Florida (June 4, 1973, 38 FR 14678). The extremely limited range of the darter and the amount of its habitat degraded by road and dam construction, as well as siltation from land clearing, were primary factors in the initial listing (USFWS 1998a). Continued threats include water quality impacts, ongoing increases in urbanization, ground and surface water withdrawal, and vulnerability to catastrophic, hazardous material spills (USFWS 1998a).

Figure 1. The Okaloosa darter *(Etheostoma okaloosae).*

Preferred Okaloosa dater habitat is in the margins of clear flowing streams among vegetation and woody debris (Figure 2). Its entire range totals only 242.8 stream miles. Population monitoring has highlighted areas within the darter's range with

declining numbers of fish or with a consistently low number of individuals compared to creeks of similar character and watershed (USFWS 1998a). As directed by the Recovery Plan, we examined potential water quality limitations to darters in areas with unexpectedly low numbers based on physical stream characteristics (e.g. East Turkey Creek). Suspected contributors to water quality degradation in East Turkey Creek include the infiltration of municipal wastewater effluent from a spray field adjacent to the creek. Preliminary ambient water quality data show elevated conductivity relative to background (Thom and Herod 2005). Additionally, East Turkey Creek has routinely failed the Florida Department of Environmental Protection's stream biotic integrity standard (Ray, 2001).

Figure 2. Preferred Okaloosa darter habitat.

The impact of municipal wastewater effluents on water quality have been long recognized, coming to the forefront with the amendments to the Clean Water Act in 1977. After the impact to human health had been addressed, it was clear that the complexity of municipal effluents would present challenges to the ecosystems that

inhabit effluent receiving systems. Comparative studies of methods for measuring toxicity in municipal and industrial effluents have provided information about the relative strengths of certain tests and the variability of effluents (Garric *et al.* 1996; Middaugh *et al.* 1997; Doherty *et al.* 1999; Kosmala *et al.* 1999, Alvarez *et al.* 2009). Today, metals, anthropogenic hormones, and various consumer products such as plastics, detergents, antibacterial agents, and pharmaceuticals including antidepressants, heart medications, and birth control products are now routinely found in wastewater effluents at effective concentrations (reviewed in Fent el al., 2006). These products are the source of both natural and synthetic substances including, but certainly not limited to, polychlorinated biphenyls, phthalates, pesticides, heavy metals, alkylphenols, polycyclic aromatic hydrocarbons, 17β-estradiol, 17α-ethinylestradiol, and bisphenol A (Pait and Nelson 2002, Aguayo *et al.* 2004, Nakada *et al.* 2004, Iwanowicz *et al.* 2009, Björkblom *et al.* 2009).

This assessment was designed to evaluate potential negative influences from spray field-related municipal effluent on water quality in East Turkey Creek. Preliminary reports have indicated possible impacts on fish abundance, water quality (elevated conductivity) and macroinvertebrate biodiversity (USFWS 1998a). During this evaluation (1) ambient water quality was remotely sampled using passive sampling devices for the examination of non-polar and polar organic pollutants, (2) fish metal burdens were examined in a native species similar in size and habit to the darter, and (3) microarray responses were examined from gonad and liver tissues after laboratory exposure of fathead minnows to stream waters. The data derived from this study will be used in management discussions to better understand darter population status, previously

obtained water quality data, and trends information. Together this will move us forward toward the identification of problem sources to help prevent future declines in Okaloosa darter populations. Better understanding the stressors originating from these spray fields will help prevent continued declines in Okaloosa darter populations, achieve recovery objectives outlined in the recovery plan (Objectives 2.2, 3.2, 3.2.2), and meet a critical delisting criterion (1F, USFWS 1998a).

Materials and Methods

Site selection

Sites were selected based on Okaloosa darter population monitoring locations, proximity to the wastewater treatment spray fields, and watershed size (Figure 3). In East Turkey Creek, two sites were selected that corresponded with historical Okaloosa darter monitoring locations (TC01/E and TC03/no water for lab exposure). A third East Turkey Creek site(TC02/A) was selected between these two sites, despite a lack of darter monitoring data available for the site. The watershed for East Turkey Creek is a 12.3 km^2 drainage. The reference sites in Long Creek were contained within a watershed 8.8 km^2 in size. Both locations in Long Creek have historically been used as Okaloosa darter monitoring sites (LC01/D) and LC02/ no water for lab exposure). Two water samples were taken for lab exposures at the headwater areas of the streams (“B” for East Turkey Creek and “C” for Long Creek) in an attempt to collect waters “upstream” of the sprayfield influence. Although there was insufficient flow at these sites for passive samplers and water quality loggers, taking water for laboratory

exposures was possible. As a result, waters were not taken from the site furthest downstream in each creek (TC03 for East Turkey Creek and LC02 for Long Creek).

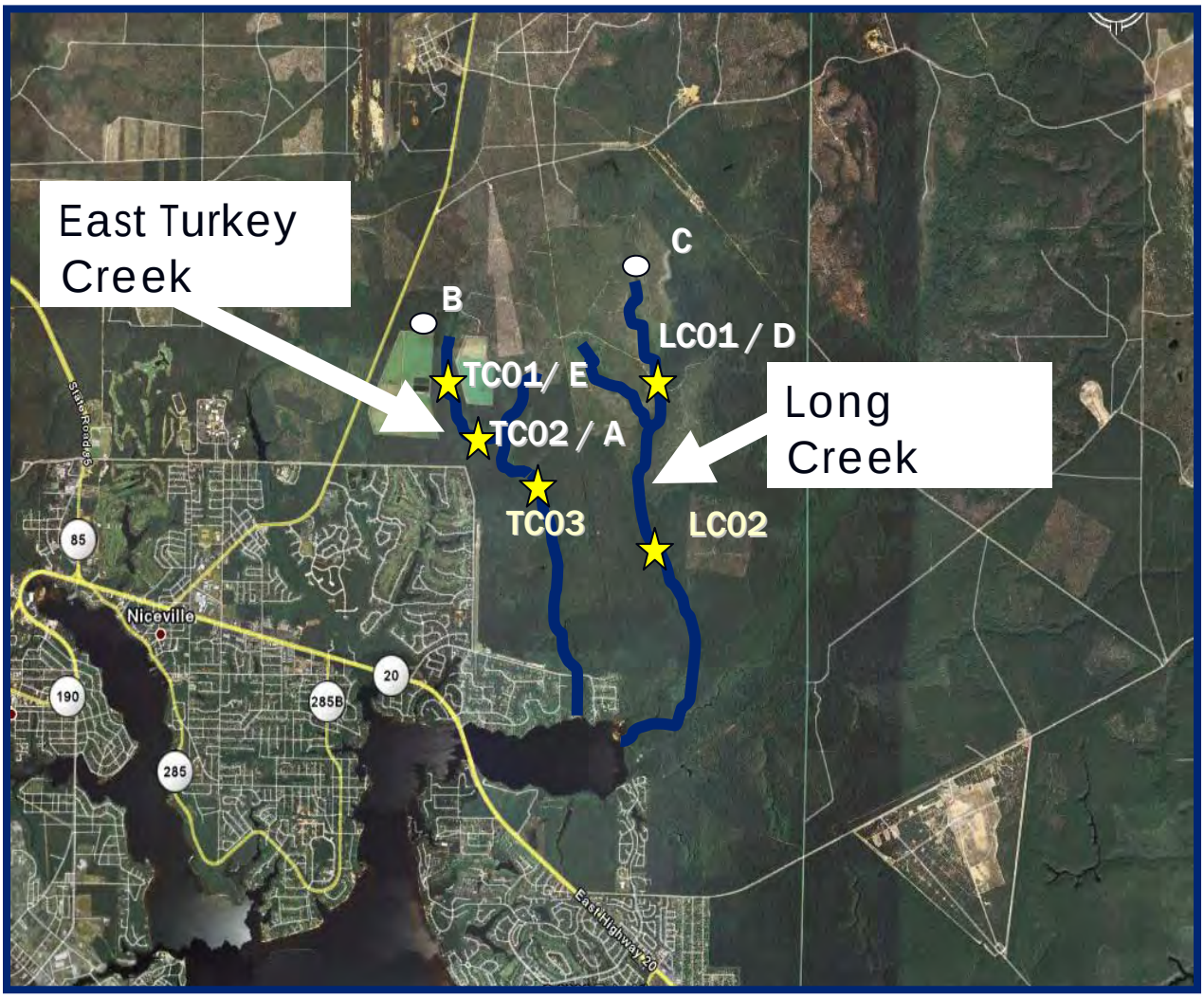


Figure 3. Evaluation sites in Okaloosa darter habitat on Eglin Air Force Base.

Ambient Water Quality

Ambient water quality was monitored at one site in East Turkey Creek (TC01) and at one site in Long Creek (LC01). Water quality parameters included dissolved oxygen (mg l^{-1}), temperature (°C), pH (SU), specific conductance (µS cm^{-1}@25°C), chlorophyll *a* concentration (µg l^{-1}) as calculated from fluorescence, and turbidity (NTU). All were monitored in the field using a Yellow Springs Instruments (YSI) Model 6600 multiparameter data logger (Figure 4). The instrument included a rapid pulse dissolved oxygen probe, conductivity/temperature probe, fluorescence derived chlorophyll probe, nephlometric turbidity probe, and pH probe. Readings were taken at the streambed level at both sites. The data were recorded to the YSI 6600 models every 40 minutes from August 2007 to June 2008. The data was uploaded weekly to the hand-held YSI 650 multiparameter display system for transfer of the comma delimited files to the office desktop computer via EcoWatch software (YSI, EcoWatch 3.18, 2006). Multiprobe calibration was carried out approximately every three weeks.

The data loggers were placed in similar locations in both creeks. Within the creeks, locations were chosen based on water depth and at sites where the loggers were least likely to be compromised by woody debris, flocculent substrate or algae. Once a suitable site was identified, the multiprobes were activated and then placed in a locked PVC housing which was then secured to a tree. The PVC housing was constructed by drilling ½” holes into a 4” diameter pipe with one end of the pipe permanently closed and the other end closable by a screw top with a key lock. The housing was developed in order to prevent the loggers from getting smothered by sand during unattended data collection and to provide a means for protection against theft.

Figure 4. Site monitoring with YSI Model 6600 multiparameter data logger.

Rain gauge data was coupled with the collected water quality data in order to reference the effects of precipitation on the measurements. Precipitation information was collected and made available courtesy of the 46th Weather Squadron on Eglin Air Force Base.

Statistical analyses on ambient water quality data were performed using R 2.9.0 (R Project 2009). Statistically significant differences were accepted at $\alpha=0.05$. Ambient water quality data were analyzed with a Two Sample T-Test when assumptions of normality and homogeneity were met. When parametric assumptions were not met, Two Sample Wilcoxon Rank Sum Test was used to analyze the data. Associations were examined via Pearson Correlation Analysis for parametric data and Spearman Correlation Analysis for non-parametric data. Data are presented as means plus one standard deviation.

Passive Samplers

In addition to the general ambient water quality sampling described above, nonpolar organic chemicals and polar organic chemicals were sampled to provide information about potential stream flow contaminants. Semi-permeable membrane devices (SPMDs), supplied by Environmental Sampling Technologies, Inc. (EST; St. Joseph, MO), were used for non-polar contaminant collections from water at three sites (TC01, TC02, and TC03) in East Turkey Creek and at the two reference sites (LC01 and LC02) in Long Creek. These membranes, made of low density polyethylene tubing containing thin layers of triolein, are designed to mimic tissues found in humans and fish. The analytical advantage of SPMDs over living tissues is that SPMDs do not metabolize the analytes as live tissue would and the analytes are easier to extract from triolein. Additionally, because SPMDs are exposed to site waters for a period of time, in this case one month, they sample a larger portion of the waters across the entire deployment period and collect a more complete profile of non-polar contaminants such as pesticides at magnified concentrations compared to contaminants collected using a traditional grab sample.

The SPMD membranes were secured to stainless steel suspension devices and enclosed in flow-through stainless steel canisters (Figure 5). The canisters were then deployed in the creek at the creekbed and secured to logs or roots with cotton line (Figure 5). Three replicate SPMDs were deployed at all sites (3 in East Turkey Creek, 2 in Long Creek) with similar constraints as described above for the water quality data logger placement. At four sites the membranes were deployed for 31 days; at the fifth site (TC03) the membranes were deployed for 29 days. After recovery from the creek

locations, the membranes enclosed in an airtight can with sealed lid to avoid additional exposures via air. These sealed cans were immediately placed in chilled coolers and sent for extraction to EST. Transfer of contaminants from sources other than the creek water exposures to the membranes were accounted for by the SPMD field blank that traveled to the sites during deployment and recovery and was shipped along with the site membranes to EST.

Figure 5. Semipermiable Membrane Devices (SPMDs).

Polar Organic Chemical Integrative Samplers (POCISs), also obtained from EST, were deployed along with the SPMDs in order to assess the presence of hydrophilic organic contaminants such as pharmaceuticals (Figure 6). These flat membrane disks

(n=3) were secured in the same stainless steel canisters as the SPMDs and were deployed for the same durations at the same sites as described previously. After recovery, these membrane disks were also placed in sealed cans and sent in chilled coolers to EST for extraction. A POCIS field blank, used to track sources of contamination other than the site waters during deployment and recovery, was shipped along with the site membranes to EST.

Figure 6. Polar Organic Chemical Integrative Samplers (POCISs).

At EST, contaminants were removed from SPMD membranes via dialysis. The dialysates from each set of triplicate membranes were combined into one composite

sample and concentrated by Kuderna-Danish prior to cleanup by gel-permeation chromatography (GPC). The final SPMD extract for each site had an approximate volume of 1.25 mL in hexane and was stored at -20º C in a sealed ampule. One dialysis blank and the field blank were prepared alongside the site samples.

Polar contaminants were extracted by EST staff into methanol from the POCIS membrane disks of each site using a chromatography column. The resulting composite extract for each site had an approximate volume of 1 mL in methanol and was stored at -20º C in a sealed ampule. One extraction blank and the field blank were prepared alongside the site samples. All blanks, SPMD, and POCIS extracts were transferred on ice to the ATCL for contaminant analysis.

For greater sensitivity, SPMD extracts were concentrated under nitrogen to 0.3 mL before analysis for 30 organochlorine pesticides (OCP), 16 polyaromatic hydrocarbons (PAH), and total polychlorinated biphenyls (PCB; sum of mono-substituted to octa-substituted chlorinated biphenyls). Analytical grade standards for the following compounds were purchased from the sources indicated: aldrin, α-BHC, β-BHC, lindane, δ-BHC, p,p'-DDD, p,p'-DDE, p,p'-DDT, dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone (also called chlordecone), methoxychlor, mirex, cis-nonachlor, and trans-nonachlor from Ultra Scientific (Kingstown, RI); cis-chlordane, trans-chlordane, and the 525, 525.1 PCB Mix from Supelco (Bellefonte, PA); oxychlordane from Chem Service, Inc. (West Chester, PA); o,p'-DDD, o,p'-DDE, o,p'-DDT from Accustandard (New Haven, CT); and toxaphene from Restek Corp. (Bellefonte, PA). Analysis was performed using a Hewlett Packard HP-6890 gas

chromatograph (Wilmington, DE) with split/splitless inlet operated in splitless mode. The analytes were introduced in a 1 µL injection and separated across the HP-5MS column (30 m x 0.25 mm; 0.25 µm film thickness) under a temperature program that began at 60º C, increased at 10º C/min to 270º C, was held for 5 min, then increased at 25º C/min to 300º C and was held for 5 min. Detection utilized an HP 5973 mass spectrometer in electron impact mode. Identification for all analytes and quantitation for toxaphene were conducted in full scan mode, where all ions are monitored. To improve sensitivity selected ion monitoring was used for quantitation of all analytes other than toxaphene. Target analytes were quantified against corresponding standard curves containing at least five points ($R^2 \geq 0.995$). To normalize for instrumental variability, each standard and sample was fortified with a deuterated internal standard, 5 µL of US-108 (120 µg/mL; Ultra Scientific) prior to analysis. Sample extract concentrations were converted to estimated concentrations in site waters using the EST SPMD Water Concentration Estimator v4.1 (EST, personal communication).

For greater sensitivity, POCIS extracts were derivatized and concentrated to a final volume of 0.2 mL before analysis of estrogenic compounds. Analytical grade standards for 17β-estradiol, estriol, and estrone were obtained from Sigma-Aldrich Co. (Milwaukee, WI), 17α-ethinylestradiol from Steraloids Inc. (Newport, RI), and 17α-ethinylestradiol-d4 from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Each POCIS extract was concentrated to a residue under nitrogen, then dansylated via incubation (10 min at 60 ºC) in 0.1 mL of 0.1 M bicarbonate (pH 10.5) and 0.1 mL dansylchloride (2 mg/mL in acetone). The derivatized products were analyzed using a Hewlett-Packard HP1100 liquid chromatograph (Wilmington, DE) with tandem mass

spectrometric detection (LCQ Ion Trap Mass Spectrometer; Finnigan MAT, San Jose, CA) in a method modified from Nelson et al. (2004). Analytes were introduced in a 50-µL injection and separated across an Adsorbosphere HS C18 column (250 mm x 4.6 mm x 5 µm; W.R. Grace & Co., Columbia, MD) under gradient conditions at a flow rate of 0.60 mL/min. Mobile phase A was 5: 95 acetonitrile: water with 0.1% formic acid. The gradient began at 50% mobile phase B (95: 5 acetonitrile: water with 0.1% formic acid), held for 2 min, increased to 95% B over 10 min, decreased back to 50% B over 5 min, and was allowed to equilibrate for 8 min. Detection utilized MS/MS via APCI in positive ion mode. Although dansylation improved separation and ionization efficiency, greatest sensitivity was obtained by monitoring the unique ions belonging to each analyte. The transitions and collision energies monitored for estriol, estrone, 17β-estradiol, 17α-ethinylestradiol, and 17α-ethinylestradiol-d4 were 522.4 to 440 at 34%, 504 to 422 at 34%, 506 to 442 at 34%, 530 to 448 at 34%, and 534 to 470 at 36%, respectively. Target analytes were quantified against a standard curve of at least six points having a correlation coefficient of at least 0.995. All standards and samples contained a surrogate (0.5 µg/mL of 17α-ethinylestradiol-d4) that was fortified prior to derivatization, so that quantitation was against a ratio of analyte to internal standard response. Sample extract concentrations were converted to estimated concentrations in site waters using the EST POCIS Turbulent Systems Estimator (EST, personal communication).

Metals assessment

In order to assess the darter's potential for exposure to the thirteen USEPA priority pollutant metals (Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, Zn) in East

Turkey and Long Creeks, Sailfin shiners, *Pternotropis hypselopterus*, were collected at each study site and one reference site (LC02) using an 8' seine net. This species of shiner is commonly found in the study areas, is about the same size as the darter and shares similar habit. Collected fish were placed in plastic bags and transported on ice to the ATCL, where they were evaluated for size and appropriateness before being shipped to Columbia Analytical Services (Kelso, WA) for analysis by USEPA methods. Whole fish composites from reference site LC01 and experimental sites TC01, TC02, and TC03 contained 21, 12, 15, and 23 individual *Pteronotropis hypseleotris* and weighed 26.2 g, 19.8 g, 22.4 g, and 32.7 g, respectively. Three sub-samples were analyzed from the composite of each site. Extraction and analysis methods, along with method reporting limits (MRL) and method detection limits (MDL) for each target element in this matrix are presented in Table 1.

Table 1: USEPA methods, method reporting limits (MRL), and method detection limits (MDL) for priority pollutant metals in composite fish tissue (EPA Method 6020, ICP-MS; EPA Method 1631E, P&T-CVAFS; EPA Method 60210B, ICP-OES; EPA Method 7742, Hydride AAS).

Priority Pollutant	**EPA Method (Extraction/Analysis)**	**Dry Wt Tissue Concentrations**		
		MRL	**MDL**	**Units**
Mercury	1631	2.5	0.5	ng/g
Antimony	200.8 / 6020	0.04	0.01	mg/kg
Arsenic	200.8 / 6020	0.43	0.07	mg/kg
Beryllium	200.8 / 6020	0.017	0.006	mg/kg
Cadmium	200.8 / 6020	0.02	0.01	mg/kg
Chromium	6010B-LL	0.17	0.06	mg/kg
Copper	200.8 / 6020	0.09	0.04	mg/kg
Lead	200.8 / 6020	0.017	0.003	mg/kg
Nickel	200.8 / 6020	0.17	0.04	mg/kg
Selenium	7742	0.09	0.04	mg/kg
Silver	200.8 / 6020	0.017	0.007	mg/kg
Thallium	200.8 / 6020	0.017	0.003	mg/kg
Zinc	200.8 / 6020	0.17	0.04	mg/kg

Fish Exposure and Tissue Collection

To facilitate exposures to waters from the study sites (B-headwater, TC01/E, and TC02/A) in East Turkey Creek and the reference sites (C-headwater and LC01/D) in Long Creek while controlling for the importance of temperature homogeneity, exposures were performed at the University of Florida under controlled conditions. Water samples were taken by glass vessel and composited in 30 gallon drums lined with fiberglass to prevent metal intrusion. Water was immediately transported to the University of Florida for fathead minnow exposure over 48 h with static renewal.

Reproductively-mature, pond-reared FHM were purchased from Andersen Minnow Farm, AR, 4 days prior to starting the experiment. Upon arrival, the fish were treated for parasites and bacteria by a prophylactic salt-water dip (3%, 1 min). Males with pronounced secondary sex characteristics (fat pads, tubercles, markings) were separated from the population the following day, and acclimated in the treatment aquaria for 48 h. The control water used for this study was carbon-filtered, dechlorinated tap water.

The exposure system consisted of 40 L glass aquaria supplied by a magnetic drive pump and Chemfluor PTFE tubing (Cole-Parmer). Each treatment was conducted in triplicate and each aquarium contained four male fathead minnows in 8 L of treatment water. Test waters from each location and a lab control for each treatment group (120L) were collected in fiberglass coated barrels and pumped to each test aquarium using EPA approved tubing during the exposure. Test water was changed a total of 5

times during the experiment (3 aquariums x 8 liters x 5 exchanges= 120 liters). Water changes occurred as follows:

Aquaria were equilibrated with test waters for 24 h prior to the introduction of fish (time 0 h). Test waters were renewed to 90% twice daily (12, 24, 36, and 48 h after start of the experiment). The positions of the treatment tanks were randomized and test initiation times were staggered to ensure an exposure/sampling interval of 48 h. The fish were not fed the day before and during the experiment. Temperature was maintained at 25°C with a photoperiod of 16 h light: 8 h dark.

Live fish were first anesthetized using MS-222 (3-aminobenzoic acid ethyl ester) in a glass beaker for approximately 20 s to so that blood could be collected from the caudal blood vessel with hematocrit tubes following the approved protocol by the University of Florida IACUC. Liver and gonad samples were collected, placed in plastic tubes and stored in liquid nitrogen for transport to the lab for further processing.

Microarray Procedures

RNA Extraction

Total RNA was isolated from 30-50 mg FHM gonadal tissue with the RNA Stat-60 reagent (Tel-test, Friendswood, TX), as previously described (Garcia-Reyero et al., 2006). Total RNA was treated with DNase and the quality assessed with an Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA), and the quantity determined on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was stored at -80°C until further use.

Microarrays

Fathead minnow microarrays manufactured by Agilent (Palo Alto, CA) were designed in the Denslow laboratory and were in the 8 X 15K format. The array consisted of 15,000 oligonucleotides bases long that corresponded to 15,000 FHM genes. The sequences of the genes were obtained by compilation and bioinformatics analysis of 250,000 ESTs publicly available in NCBI databases. Annotation of the genes was done by BLAST against both the NR and NT databases.

Array hybridizations were performed using a single color design. Four replicates consisting of four different individuals were analyzed for each of the treatments (University of Florida control, and each of the sites). The cDNA synthesis, cRNA labeling and hybridization were performed following the manufacturer's kits and protocols (Agilent Low RNA Input Fluorescent Linear Amplification Kit and Agilent 60-mer oligo microarray processing protocol; Agilent, Palo Alto, CA). The gonad samples were labeled with Cy_3. Once the labeling was complete, samples were hybridized to the microarray using conditions recommended by the manufacturer. After hybridizing for 17 h, microarrays were washed and then scanned with a laser-based detection system (Agilent, Palo Alto, CA). Text versions of the Agilent raw data have been deposited at the Gene Expression Omnibus website.

Statistical and pathway analysis of microarray data

Microarray data were imported into JMP Genomics software v3.1 (SAS, Cary, NC) for analysis. To begin, any rows not containing at least two data points for all treatment groups were deleted and the data were median-centered. ANOVA was performed using the median-centered dataset, and genes that differed significantly

among the groups were identified ($p<0.01$ or $p<0.05$ for further analyses, False discovery rate (FDR) = 5%). The program Cluster (Eisen et al. 1998) was used to perform hierarchical clustering analysis based on all genes significantly differentially regulated under a p-value cutoff of $p < 0.01$; the result was visualized in the program Java TreeView (Saldanha 2004). For this analysis, array data were median-centered by gene and a centered correlation was performed based on complete linkage. The FatiGO tool within the program Babelomics (Al-Shahrour et al. 2005) was used to perform functional enrichment analysis based on Gene Ontology (GO) terms. This analysis was based on Fisher's exact test and was used to identify over-represented terms in the list of differentially regulated genes. All terms with a nominal p-value of $p < 0.05$ (no *post hoc* correction) were considered to be enriched.

Results

Water quality

Differences in ambient water quality were found between East Turkey Creek and Long Creek (Table 2). Specific conductivity was markedly higher in East Turkey Creek (median over five times more) when compared to the reference, Long Creek. Large differences in pH (1.6 units higher) were also found, with East Turkey Creek again being higher than Long Creek. A smaller difference in temperature (0.6 ºC) showed East Turkey Creek to warmer overall. Although statistically significant because of the large sample sizes (up to n = 13,058), only very small differences in dissolved oxygen concentration, turbidity, and chlorophyll were observed.

Correlations between daily precipitation and ambient water quality were not conducted because of scant rainfall data. This will be attempted with ongoing monitoring data.

Table 2. Ambient water quality measures for East Turkey and Long Creeks, medians reported with data range in parentheses.

Parameter	Long (LC01)	East Turkey (TC01)
Specific Conductance (µS cm^{-1}@25°C)	24 (6-36)	128 (4-164)
pH (Standard Units)	6.6 (5.2-6.5)	5.0 (4.4-5.2)
Temperature (ºC)	20.1 (9.4-24.6)	20.7 (13.1-24.6)
Dissolved Oxygen (mg/L)	8.1 (4.2-12.3)	8.4 (4.3-12.1)
Turbidity (NTU)	1.7 (0-28.9)	2.4 (0.2-30)
Chlorophyll (ug/L)	1.5 (0-12.8)	1.3 (0-12.9)

Metals

Composited fish from the reference site (LC02) showed higher levels of Hg, Sb, Ag and Zn (Table 3) than did the test sites which varied little. Copper and Se (total) concentrations were not observed to vary across the test sites; as one would generally expect, levels from reference site fish was somewhat lower than test site fish. Arsenic (total) and Be were found at low or undetectable levels for all sites, except TC03, the most downstream site on East Turkey Creek. Cadmium and Ni, on the other hand, were determined at comparative levels among the reference and two downstream test sites (TC02 and TC03), but at higher levels for the most upstream test site (TC01). Chromium was measured at low concentrations in the two upstream test sites, at an intermediate level in the Long Creek reference site, and at a surprisingly high level for the most downstream test site (TC03). Determined concentrations for Pb varied across

the sites with no obvious pattern. Only Th exhibited the generally anticipated profile with fish showing increasing concentrations as the test sites progressed further downstream; levels in the composite from the reference site (LC02) were however somewhat higher than those from the most downstream test site.

Table 3. Concentrations of priority pollutants in composite whole fish (average ± standard deviation; n = 3).

Element	Dry Weight Concentrations by Site				Units
	LC02	TC01	TC02	TC03	
%M	27.6 ± 0.25	25.7 ± 0.90	26.9 ± 0.66	27.7 ± 0.59	%
Mercury (total)	581 ± 44.2	412 ± 35.7	431 ± 36.6	492 ± 20.5	ng/g
Antimony	0.07 ± 0.110	<0.01[a]	<0.01[a]	0.01 ± 0.006	mg/kg
Arsenic (total)	0.21 ± 0.081	0.19 ± 0.030	0.17 ± 0.026	0.66 ± 0.057	mg/kg
Beryllium	0.010 ± 0.0067	<0.006[a]	<0.007[a]	0.043 ± 0.0626	mg/kg
Cadmium	0.18 ± 0.020	0.38 ± 0.075	0.21 ± 0.099	0.21 ± 0.021	mg/kg
Chromium	0.39 ± 0.336	0.12 ± 0.056	0.13 ± 0.098	2.71 ± 4.199	mg/kg
Copper	2.25 ± 0.168	2.99 ± 0.200	2.70 ± 0.223	2.70 ± 0.296	mg/kg
Lead	0.360 ± 0.0955	0.504 ± 0.2301	0.251 ± 0.0707	0.427 ± 0.5397	mg/kg
Nickel	1.74 ± 0.216	1.91 ± 0.194	1.63 ± 0.032	1.72 ± 0.217	mg/kg
Selenium (total)	3.22 ± 0.602	4.63 ± 0.417	4.55 ± 0.646	4.62 ± 0.721	mg/kg
Silver	0.029 ± 0.0341	0.016 ± 0.0060	0.012 ± 0.0040	0.013 ± 0.0040	mg/kg
Thallium	0.028 ± 0.0370	0.006 ± 0.0006	0.013 ± 0.0021	0.023 ± 0.0025	mg/kg
Zinc	431 ± 6.7	416 ± 17.6	372 ± 22.0	409 ± 38.2	mg/kg

[a] All replicates were <MDL.

Passive samplers

The following chlorinated compounds were not detected in any SPMD sample from reference or test site waters at levels down to 0.7 pg/L: aldrin, α-BHC, β-BHC, lindane, δ-BHC, p,p'-DDD, p,p'-DDT, o,p'-DDD, o,p'-DDE, o,p'-DDT, dieldrin, endosulfan, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, kepone, methoxychlor, mirex, trans-chlordane,

oxychlordane, cis-nonachlor, trans-nonachlor, toxaphene, anthracene, benzo[g,h,i]perylene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, pyrene, and PCBs.

Three organochlorine pesticides were detected in the site waters of this study (Table 4). Hexachlorobenzene was identified at all sites, including both reference sites, at an estimated average concentration of 9.1 pg/L. p,p'-DDE was also detected at all sites at an estimated average concentration of 4.75 pg/L. Trace levels of cis-chlordane were identified at one reference (LC02) and one test (TC02) site; although detectable, the levels were below the limit of quantification (11.5 pg/L).

Two PAHs were detected at multiple sites and at quantifiable levels (Table 4). Acenaphthene was present at all test sites and at one reference site (LC01). Acenaphthylene was also present at all test sites and one reference site (LC02). Five additional PAHs (chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, and benzo[a]pyrene) were found only in samples from the most downstream test site (TC03). Estimated concentrations ranged from 33.8 pg/L to 120.3 pg/L in the site waters. Of all sites considered, the most impacted site was TC03.

Most POCIS samples did not show detectable levels for estrogens. One reference site (LC01) was estimated to have estrone at levels of 254 pg/L and one test site (TC02) was estimated to have 17 α-ethynylestradiol at levels of 51 pg/L.

Table 4. Estimated site water concentrations (pg/L) for polar and non-polar contaminants collected via SPMD and POCIS

Target Analytes	Site Identifications				
	LC01	LC02	TC01	TC02	TC03
SPMD					
cis-Chlordane	BDL[a]	<11.5	BDL	<11.5	BDL
p,p-DDE	4.68	4.68	4.68	4.68	5.00
Hexachlorobenzene	8.96	8.96	8.96	8.96	9.58
Acenaphthene	14.24	BDL	28.48	28.48	68.96
Acenaphthylene	BDL	17.37	52.12	52.12	33.48
Chrysene	BDL	BDL	BDL	BDL	33.81
Benz[a]anthracene	BDL	BDL	BDL	BDL	86.21
Benzo[b]fluoranthene	BDL	BDL	BDL	BDL	101.42
Benzo[k]fluoranthene	BDL	BDL	BDL	BDL	103.45
Benzo[a]pyrene	BDL	BDL	BDL	BDL	120.29
POCIS					
Estrone	254.05	BDL	BDL	BDL	BDL
17 α-Ethynylestradiol	BDL	BDL	51.12	BDL	BDL

[a] MDL (chlorinated compounds) = 0.7 pg/L; MDL (Estrone) = 0.11 pg/L; MDL (17 α-Ethynylestradiol) = 0.18 pg/L

Fish response

Exposure of FHM for 48 h to waters from each of the sites was sufficient to elicit a change in expression of genes involved in toxicity pathways. Male fish were used for the exposures and changes in gene transcription were evaluated in their livers and testis. The two organs respond differently to contaminants. The liver is the site of toxicant metabolism and clearance, and we expected to see changes in gene expression of enzymes involved in these processes. The gonad is the main site involved in steroidogenesis, and again we expected to see changes in key enzymes in this tissue. Both organs reflect different contaminant influences. In both the liver and the testis, there was fairly good correspondence in gene expression changes among the

fish at each site. These were compared to controls concurrently tested in dechlorinated tap water at the University of Florida.

Originally the selection of sites included two sites (C and D) on Long Creek that were deemed suitable as reference sites, and three sites (A, B, E) on Turkey Creek that were deemed to be impacted by spray fields and runoff. However, after careful analysis of the microarray data it became obvious that Site C was very different from Site D (and from University of Florida controls) and therefore was unlikely to serve as an appropriate reference site. Site C is located in a steephead at the headwaters for Long Creek but it is just downstream from an area used heavily for various military training activities and apparently may be impacted from these activities. Gene expression changes at Site C are compared to the University of Florida controls separate from the rest of the data, below.

Microarray analysis in liver

Microarray data for sites A, B, D and E were analyzed together as one set by ANOVA. Individual microarrays were performed for four separate fish for each of the sites. Genes that were significantly differentially expressed ($p < 0.05$) are shown in the heat map (Fig. 7). In this representation, each column is a separate biological sample and each row is a separate gene. Genes that up-regulated with respect to the University of Florida controls are colored red and those that are down-regulated are green. Genes that are black are not regulated at the site. A two-way unsupervised hierarchical cluster was performed, clustering sites that are most like each other on the x-axis and gene

expression patterns that are most similar on the Y-axis. Notably, all individuals in site A water cluster together and differently from sites B, D, and E, which form a single cluster. Although the water quality analysis did not identify contaminants that were significantly different between site A and the other three, sites B, D and E are located above the point at which water from the spray fields enters the watershed and it may contain compounds we did not analyze.

To better compare the gene expression fingerprints at the four sites, the expression data ($p < 0.05$; FDR =5%) was arranged from the most up-regulated to the most down-regulated genes for each of the sites (Fig 8). Expression values are listed as log (2). If a particular gene was not significant for a site, its expression value was set to log (2) = 0. Thus, the only expression values plotted are those that are significant. Of the four sites, it is clear that Site D (the reference site) had fewer differences with the University of Florida controls. The graphs are in order of their location on Turkey Creek. Site B is most similar to Site D, but it does contain an additional set of genes that are not differentially expressed in Site D. Site E, which is

down stream from Site B also contains many of the same genes as found in Site D and B, but in addition has another set that are expressed differentially at this site. And, Site A, appears to be the most influenced of these sites, with many more genes differentially expressed. This shows the influence of the spray fields, which provide run off waters that enter the stream at a point between site E and site A.

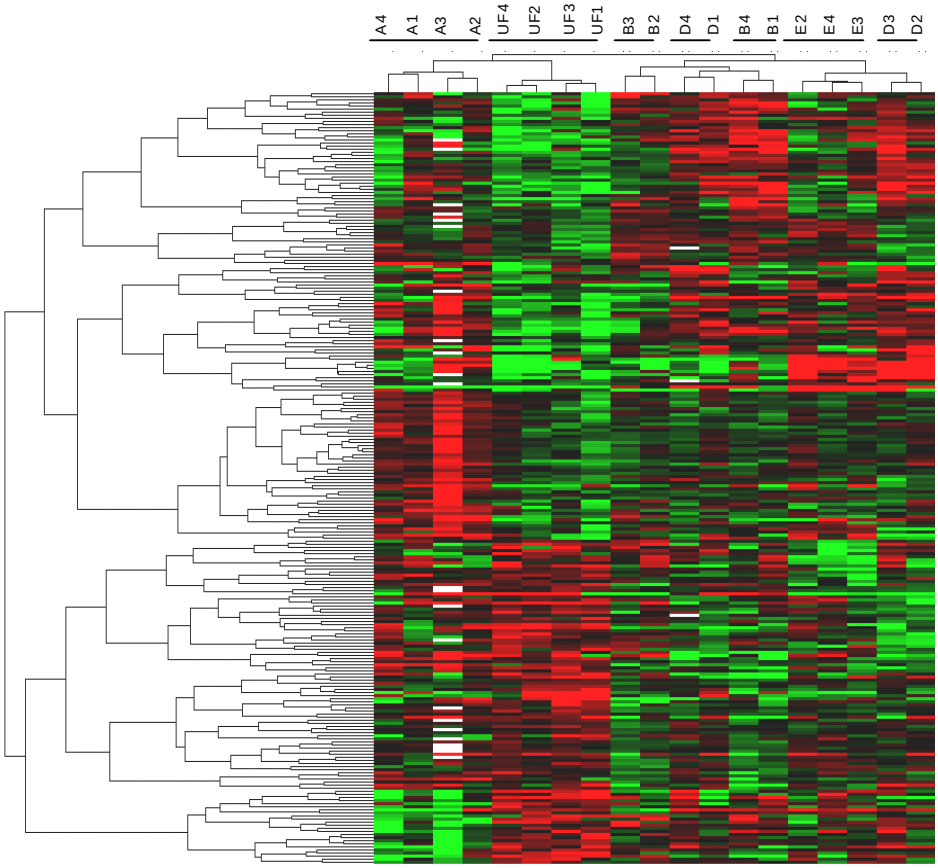


Figure 7. Bi-directional hierarchical cluster analysis of gene expression changes in the liver. Green indicates down-regulation relative to UF control and red indicates up-regulation relative to UF control. Fathead minnows were exposed for 48 h to waters from Sites A, B, D and E. Top, clustering was done by treatment; side, clustering was done by gene. Each column represents a different array

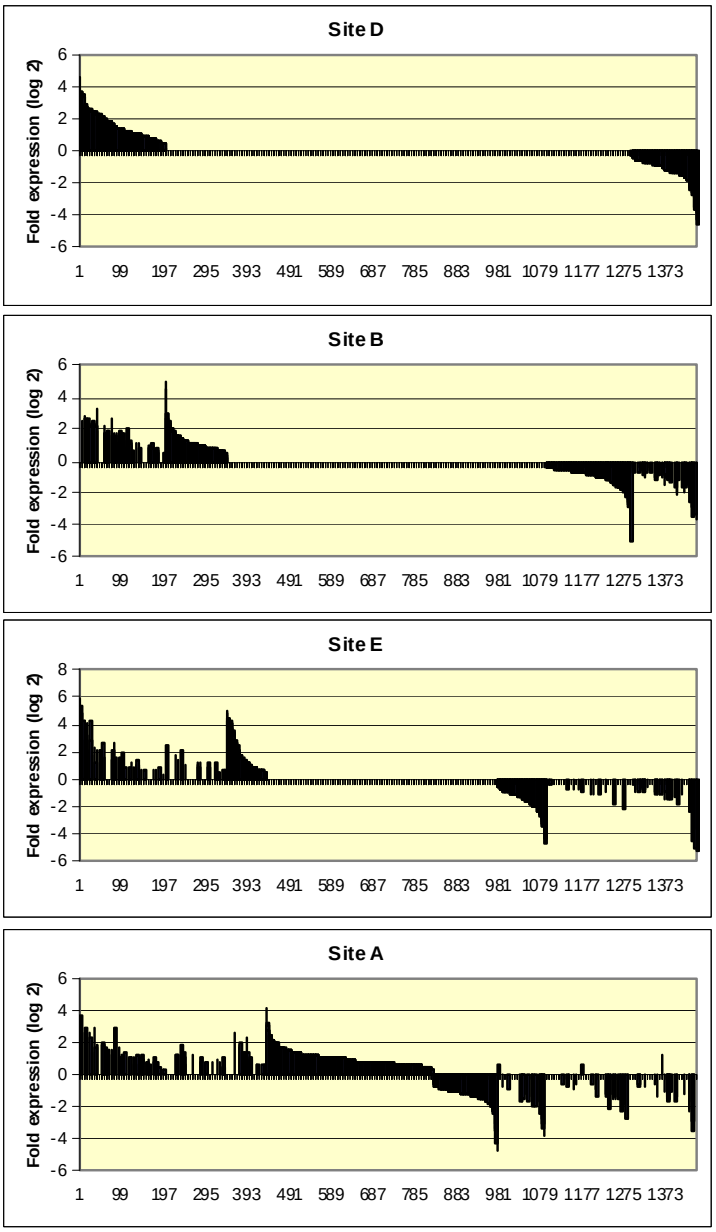


Figure 8: **Comparison of overall gene regulation in the liver.** (A) Site D, (B) Site B, (C) Site E and (D) Site A as determined by the array. The genes were ordered according to their expression levels at each of the sites and proceed from most up-regulated to most down regulated and represent median expression values of the four arrays for each condition ($p < 0.05$).

At a more stringent significance level ($p < 0.01$, FDR = 5%) we found 94 transcripts that were identified as differentially regulated between Turkey Creek Site A and UF control, 72 transcripts were differentially regulated between site B and UF control, 50 transcripts were significantly different between site E and UF control and 78 transcripts were identified as differentially regulated between Long Creek site D and UF control. To better understand the importance of these gene expression changes, they were analyzed further to get an indication of the biological processes that they may be involved in. Differentially expressed genes were assigned to different biological processes in terms of their gene ontology (GO) codes. Then, we used a Fischer exact test to determine whether specific biological processes contained genes that were over expressed in the regulated set compared to their representation on the array.

The GO categories significantly affected in the livers of fish exposed to water from sites B, D and E were primarily metabolic processes such as glucose homeostasis (Tables 5 and 6) and cell cycle control (Tables 5, 6, and 7), indicating that the liver may be undergoing a generalized stress response. The top biological processes that were altered by exposure to Site B waters included: “neurite development,” “gametogenesis” and “negative regulation of angiogenesis.” In addition,” “female meiois,” “ovulation,” “vitelline membrane formation” and “regulation of growth” were also altered, suggesting that long term exposure could affect reproduction and growth (Table 6). The natural steephead waters before the influence of sprayfield influence may not be suitable for reproduction and growth according to this non-indigenous surrogate species. However, darter populations in the reference creek (D) are more abundant relative to the Turkey Creek population despite having comparable natural steephead driven waters.

Fish exposed to Site E waters seemed to be more influenced than fish exposed to Site B or Site D waters. The top three Biological Processes influenced were “transcription from RNA polymerase 1 promoter,” “Mitotic anaphase,” and “arginine catabolic process” (Table 7). In addition, “vitelline membrane formation” and “gonad development” were also influenced, suggesting that long term exposure might compromise reproduction. These sites were within the expected influence of the sprayfields. The observed biomarker changes were considered to be above background levels from exposure to natural water and may lead to reproductive dysfunction of exposed fish.

Table 5. Biological processes GO categories that were significantly (P<0.05) by water collected at site D

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0042593	glucose homeostasis	2	6	0.007
GO:0006811	ion transport	9	174	0.019
GO:0007395	dorsal closure, spreading of leading edge cells	1	1	0.023
GO:0007435	salivary gland morphogenesis	1	1	0.023
GO:0007443	Malpighian tubule morphogenesis	1	1	0.023
GO:0015014	heparan sulfate proteoglycan biosynthetic process, polysaccharide chain biosynthetic process	1	1	0.023
GO:0019307	mannose biosynthetic process	1	1	0.023
GO:0019532	oxalate transport	1	1	0.023
GO:0030210	heparin biosynthetic process	1	1	0.023
GO:0030704	vitelline membrane formation	1	1	0.023
GO:0031054	pre-microRNA processing	1	1	0.023
GO:0035195	miRNA-mediated gene silencing	1	1	0.023
GO:0035196	miRNA-mediated gene silencing, production of miRNAs	1	1	0.023
GO:0035279	miRNA-mediated gene silencing, mRNA cleavage	1	1	0.023
GO:0042177	negative regulation of protein catabolic process	1	1	0.023
GO:0042476	odontogenesis	1	1	0.023
GO:0045604	regulation of epidermal cell differentiation	1	1	0.023
GO:0046080	dUTP metabolic process	1	1	0.023
GO:0046663	dorsal closure, leading edge cell differentiation	1	1	0.023
GO:0046838	phosphorylated carbohydrate dephosphorylation	1	1	0.023
GO:0050918	positive chemotaxis	1	1	0.023
GO:0051170	nuclear import	1	1	0.023
GO:0030163	protein catabolic process	2	11	0.025
GO:0008203	cholesterol metabolic process	2	12	0.03
GO:0006629	lipid metabolic process	4	59	0.045
GO:0000724	double-strand break repair via homologous recombination	1	2	0.046
GO:0001649	osteoblast differentiation	1	2	0.046
GO:0001944	vasculature development	1	2	0.046
GO:0007584	response to nutrient	1	2	0.046
GO:0008632	apoptotic program	1	2	0.046
GO:0008635	caspase activation via cytochrome c	1	2	0.046
GO:0009113	purine base biosynthetic process	1	2	0.046
GO:0009267	cellular response to starvation	1	2	0.046
GO:0015889	cobalamin transport	1	2	0.046
GO:0019276	UDP-N-acetylgalactosamine metabolic process	1	2	0.046
GO:0030206	chondroitin sulfate biosynthetic process	1	2	0.046
GO:0030879	mammary gland development	1	2	0.046
GO:0035265	organ growth	1	2	0.046
GO:0040004	cuticular attachment to epithelium (sensu Nematoda)	1	2	0.046
GO:0043089	positive regulation of Cdc42 GTPase activity	1	2	0.046
GO:0043161	proteasomal ubiquitin-dependent protein catabolic process	1	2	0.046
GO:0046415	urate metabolic process	1	2	0.046
GO:0008202	steroid metabolic process	2	15	0.046

Table 6. Biological processes GO categories that were significantly (P<0.05) by water collected at site B

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0031175	neurite development	2	3	0.003
GO:0007276	gametogenesis	2	4	0.006
GO:0016525	negative regulation of angiogenesis	2	4	0.006
GO:0001522	pseudouridine synthesis	2	5	0.009
GO:0042593	glucose homeostasis	2	3	0.013
GO:0007411	axon guidance	3	17	0.015
GO:0050930	induction of positive chemotaxis	2	7	0.018
GO:0000093	mitotic telophase	1	1	0.031
GO:0001302	replicative cell aging	1	1	0.031
GO:0001542	ovulation (sensu Mammalia)	1	1	0.031
GO:0007143	female meiosis	1	1	0.031
GO:0007143	dorsal closure, spreading of leading edge cells (GO:0007395)	1	1	0.031
GO:0007435	salivary gland morphogenesis	1	1	0.031
GO:0007443	Malpighian tubule morphogenesis	1	1	0.031
GO:0009087	methionine catabolic process	1	1	0.031
GO:0009156	ribonucleoside monophosphate biosynthetic process	1	1	0.031
GO:0015993	molecular hydrogen transport	1	1	0.031
GO:0016539	intein-mediated protein splicing	1	1	0.031
GO:0019307	mannose biosynthetic process	1	1	0.031
GO:0019852	L-ascorbic acid metabolic process	1	1	0.031
GO:0030704	vitelline membrane formation	1	1	0.031
GO:0040009	regulation of growth rate	1	1	0.031
GO:0042068	regulation of pteridine metabolic process	1	1	0.031
GO:0043039	tRNA aminoacylation	1	1	0.031
GO:0044249	cellular biosynthetic process	1	1	0.031
GO:0046663	dorsal closure, leading edge cell differentiation	1	1	0.031
GO:0046838	phosphorylated carbohydrate dephosphorylation	1	1	0.031
GO:0050860	negative regulation of T cell receptor signaling pathway	1	1	0.031
GO:0050918	positive chemotaxis	1	1	0.031
GO:0007059	chromosome segregation	2	10	0.037
GO:0051246	regulation of protein metabolic process	2	??	0.037
GO:0006396	RNA processing	4	10	0.044
GO:0007596	blood coagulation	3	28	0.046
GO:0006511	ubiquitin-dependent protein catabolic process	6	85	0.047

Table 7. Biological processes GO categories that were significantly (P<0.05) by water collected at site E

GO ID	GO Name	# of Genes Selected	# Genes / Array	Fisher p Value
GO:0006360	transcription from RNA polymerase I promoter	2	2	0.0004
GO:0000090	mitotic anaphase	2	3	0.001
GO:0006527	arginine catabolic process	2	3	0.001
GO:0030509	BMP signaling pathway	2	5	0.004
GO:0009953	dorsal/ventral pattern formation	3	26	0.014
GO:0050808	synapse organization and biogenesis	2	11	0.02
GO:0000093	mitotic telophase	1	1	0.021
GO:0006101	citrate metabolic process	1	1	0.021
GO:0006608	snRNP protein import into nucleus	1	1	0.021
GO:0007098	centrosome cycle	1	1	0.021
GO:0009087	methionine catabolic process	1	1	0.021
GO:0009414	response to water deprivation	1	1	0.021
GO:0009737	response to abscisic acid stimulus	1	1	0.021
GO:0009753	response to jasmonic acid stimulus	1	1	0.021
GO:0016080	synaptic vesicle targeting	1	1	0.021
GO:0016255	attachment of GPI anchor to protein	1	1	0.021
GO:0018153	isopeptide cross-linking via N6-(L-isoglutamyl)-L-lysine	1	1	0.021
GO:0019285	glycine betaine biosynthetic process from choline	1	1	0.021
GO:0019307	mannose biosynthetic process	1	1	0.021
GO:0030704	vitelline membrane formation	1	1	0.021
GO:0030951	establishment/maintenance microtub cytoskeleton polar	1	1	0.021
GO:0042135	neurotransmitter catabolic process	1	1	0.021
GO:0042985	negative regulation of amyloid precursor protein biosynthetic process	1	1	0.021
GO:0050860	negative regulation of T cell receptor signaling pathway	1	1	0.021
GO:0051300	spindle pole body organization and biogenesis	1	1	0.021
GO:0042254	ribosome biogenesis and assembly	2	14	0.032
GO:0000089	mitotic metaphase	1	2	0.041
GO:0006066	alcohol metabolic process	1	2	0.041
GO:0006997	nuclear organization and biogenesis	1	2	0.041
GO:0007584	response to nutrient	1	2	0.041
GO:0008406	gonad development	1	2	0.041
GO:0008615	pyridoxine biosynthetic process	1	2	0.041
GO:0009409	response to cold	1	2	0.041
GO:0015889	cobalamin transport	1	2	0.041
GO:0030968	unfolded protein response	1	2	0.041
GO:0040004	cuticular attachment to epithelium sensu Nematoda	1	2	0.041
GO:0043089	positive regulation of Cdc42 GTPase activity	1	2	0.041
GO:0045785	positive regulation of cell adhesion	1	2	0.041
GO:0050658	RNA transport	1	2	0.041
GO:0051085	chaperone cofactor-dependent protein folding	1	2	0.041
GO:0051297	centrosome organization and biogenesis	1	2	0.041
GO:0051321	meiotic cell cycle	1	2	0.041
GO:0007264	small GTPase mediated signal transduction	7	161	0.047

Functional enrichment analysis based on GO terms for biological process identified 44 significantly enriched terms within the list of differentially regulated genes in the fish exposed to water from Site A. Among these, tRNA aminoacylation (Table 8) for protein translation was the most significantly enriched (p = 2.51E-05). There are 47 genes on the array under this GO category, out of which one was down-regulated and 10 were significantly ($p < 0.05$) up-regulated, indicating an increased demand for protein synthesis in the liver. Genes involved in post-translational modification of proteins and protein transport were also significantly impacted by exposure to water from Site A. Interestingly, although the metal analysis in endogenous fish showed concentrations below toxicity thresholds for fish (USEPA 2004), the microarray data indicates that genes involved in metal ion transport were significantly (p= 0.039) impacted (Table 8). One possible reason is that although our list of metals analyzed was extensive, it was not exhaustive. And, metal analyses just down stream from this site were positive, suggesting that fish may be responding to low concentrations of metals, under the measurement threshold.

Table 8. Biological processes GO categories that were significantly (P<0.05) by water collected at site A.

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0006418	tRNA aminoacylation for protein translation	11	47	2.51E-05
GO:0008615	pyridoxine biosynthetic process	2	2	0.002
GO:0006897	endocytosis	6	40	0.006
GO:0007264	small GTPase mediated signal transduction	15	161	0.006
GO:0000075	cell cycle checkpoint	2	3	0.006
GO:0006432	phenylalanyl-tRNA aminoacylation	2	3	0.006
GO:0018279	protein amino acid N-linked glycosylation via asparagine	2	4	0.012
GO:0006486	protein amino acid glycosylation	5	35	0.018
GO:0006424	glutamyl-tRNA aminoacylation	2	5	0.019
GO:0015031	protein transport	14	175	0.026
GO:0019370	leukotriene biosynthetic process	2	6	0.027
GO:0030001	metal ion transport	3	17	0.039
GO:0000290	deadenylation-dependent decapping	1	1	0.045
GO:0001302	replicative cell aging	1	1	0.045
GO:0001682	tRNA 5'-leader removal	1	1	0.045
GO:0006101	citrate metabolic process	1	1	0.045
GO:0006112	energy reserve metabolism	1	1	0.045
GO:0006427	histidyl-tRNA aminoacylation	1	1	0.045
GO:0006429	leucyl-tRNA aminoacylation	1	1	0.045
GO:0006430	lysyl-tRNA aminoacylation	1	1	0.045
GO:0006433	prolyl-tRNA aminoacylation	1	1	0.045
GO:0006488	dolichol-linked oligosaccharide biosynthetic process	1	1	0.045
GO:0006668	sphinganine-1-phosphate metabolic process	1	1	0.045
GO:0006670	sphingosine metabolic process	1	1	0.045
GO:0006890	retrograde vesicle-mediated transport, Golgi to ER	1	1	0.045
GO:0009087	methionine catabolic process	1	1	0.045
GO:0009214	cyclic nucleotide catabolic process	1	1	0.045
GO:0009231	riboflavin biosynthetic process	1	1	0.045
GO:0009590	detection of gravity	1	1	0.045
GO:0015694	mercury ion transport	1	1	0.045
GO:0015813	glutamate transport	1	1	0.045
GO:0016078	tRNA catabolic process	1	1	0.045
GO:0016080	synaptic vesicle targeting	1	1	0.045
GO:0016320	endoplasmic reticulum membrane fusion	1	1	0.045
GO:0016926	protein desumoylation	1	1	0.045
GO:0019285	glycine betaine biosynthetic process from choline	1	1	0.045
GO:0019307	mannose biosynthetic process	1	1	0.045
GO:0040009	regulation of growth rate	1	1	0.045
GO:0042640	anagen	1	1	0.045

Table 8 continued. Biological processes GO categories that were significantly (P<0.05) by water collected at site A.

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0042743	hydrogen peroxide metabolic process	1	1	0.045
GO:0045604	regulation of epidermal cell differentiation	1	1	0.045
GO:0050773	regulation of dendrite development	1	1	0.045
GO:0050860	negative regulation of T cell receptor signaling pathway	1	1	0.045
GO:0050918	positive chemotaxis	1	1	0.045

Microarray analysis in the testis:

Using the same type of analysis, we also investigated gene expression changes in the testis of FHM. The gonads are the major sites of steroidogenesis and gamete maturation leading to reproduction in fish and thus is another organ that is essential for toxicity testing. As with the liver, gene expression data was analyzed by ANOVA and plotted as a two-way hierarchical cluster (Fig 9). In this organ, each of the individual samples clustered by site, suggesting that there was a significant site effect on gene expression. In the cluster analysis, the Long Creek reference site D clusters most closely with the Turkey Creek Site E. Turkey Creek Site B clusters with Turkey Creek Site A, which is closest to the spray fields clusters. The UF controls cluster together in the middle (Fig 9).

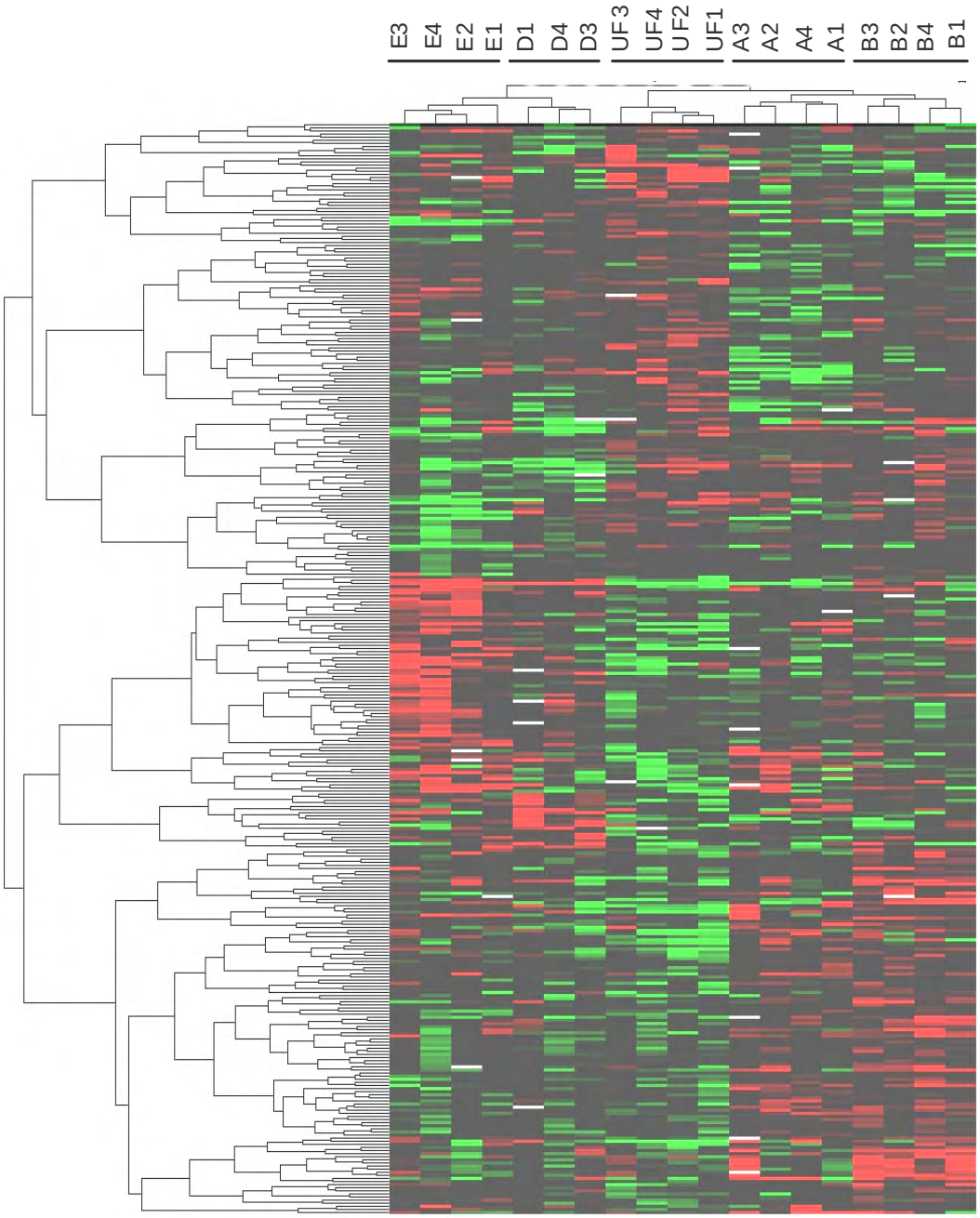


Figure 9. Bi-directional hierarchical cluster analysis of gene expression changes in the testis. Green indicates down-regulation relative to UF control and red indicates up-regulation relative to UF control. Fathead minnows were exposed for 48 h to waters from Sites A, B, D and E. Top, clustering was done by treatment; side, clustering was done by gene. Each column represents a different array.

Gene expression fingerprints for the four sites were constructed as described above for the liver using expression data ($p < 0.05$; FDR =5%) which was arranged from the most up-regulated to the most down-regulated genes for each of the sites (Fig 10).

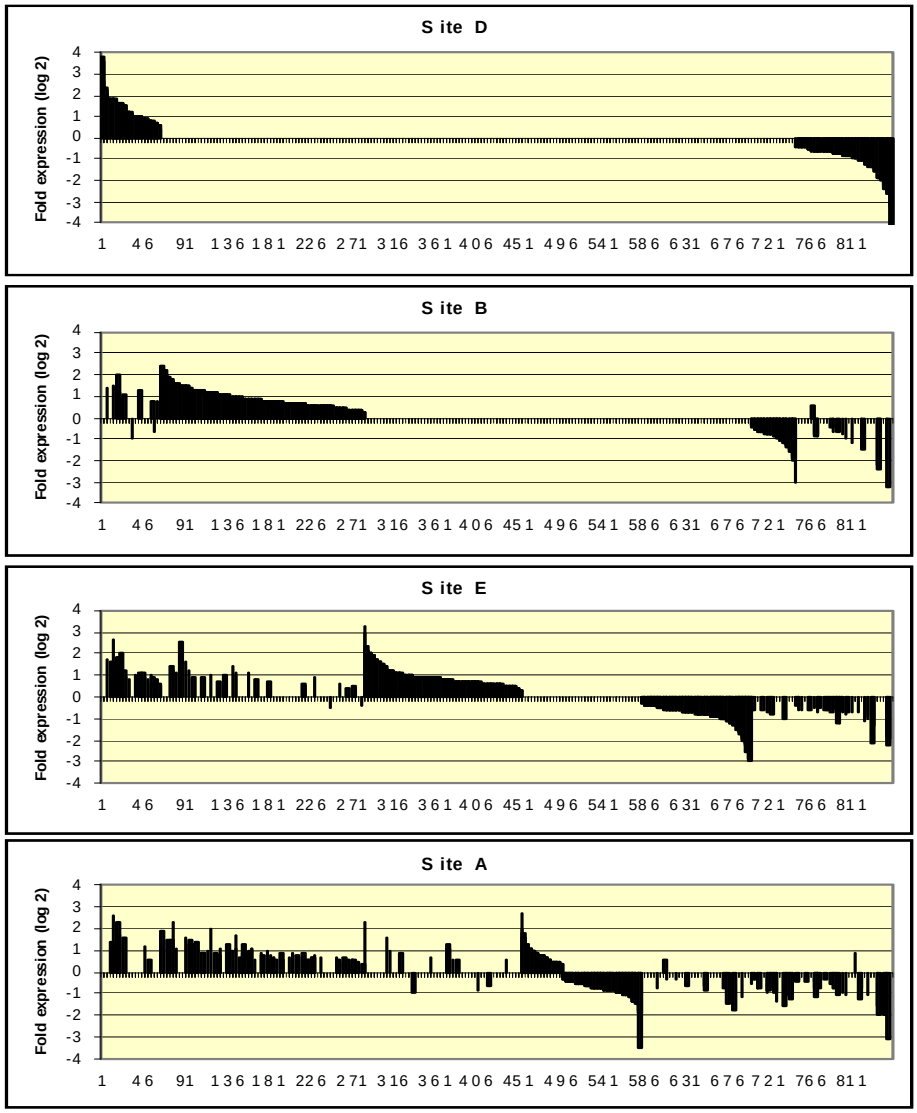


Figure 10: **Gene expression fingerprints in the testis.** (A) Site D, (B) Site B, (C) Site E and (D) Site A as determined by the array. The genes were ordered according to their expression levels at each of the sites and proceed from most up-regulated to most down regulated and represent median expression values of the four arrays for each condition. ($p < 0.05$)

Expression values are listed as log (2). Of the four sites, it is clear that Site D (the reference site) had the fewest differences with the University of Florida controls. The graphs are in order of their location on Turkey Creek. Site B consists of some of the genes altered in D plus an additional set of genes that are differentially expressed compared to Site D. Site E, which is down stream from Site B also contains many of the same genes as found in Site D and B, but in addition has another set that are expressed differentially at this site. And Site A, appears to be the most influenced of these sites, with many more genes differentially expressed. This shows the influence of the spray fields, which provide run off waters that enter the stream at a point between site E and site A.

As described above, gene expression in the testis was analyzed as a function of biological process. There were very few pathways in common among the various sites, although at the individual gene level, there were many that were present at all the sites as described above (Tables 9-12). In this comparison, the Long Creek reference stream site (D) was most similar to the Turkey Creek Site B, both close to headwaters on the respective streams. These two sites are not influenced by the sprayfield input. These sites had two biological processes in common after enrichment analysis. Those biological processes were somatic muscle development and regulation of S phase of mitotic cell cycle. These GO categories were each influenced by a single gene which was the same at each of the sites, but the genes remain unidentified. The Fischer exact test probabilities for both are similar (range $p = 0.029$-0.041).

The biological processes most highly represented in the Long Creek site (D) were those involved in embryonic heart tube development, angiogenesis caudal fin

morphogenesis and cell growth (Table 9). The Turkey Creek site B was most represented by metabolic processes, development and organ morphogenic processes and also included somitogenesis, zinc ion transport, response to pain, ovulation, programmed cell death and steroid hormone receptor signaling (Table 9).

Table 9. Biological processes GO categories of gonads that were significantly (P<0.05) by water collected at site D

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0001525	angiogenesis	3	12	0.004
GO:0035143	caudal fin morphogenesis	2	4	0.005
GO:0006607	NLS-bearing substrate import into nucleus	2	4	0.005
GO:0016049	cell growth	2	5	0.008
GO:0006508	proteolysis	17	349	0.014
GO:0006605	protein targeting	3	21	0.022
GO:0051026	chiasma formation	1	1	0.029
GO:0008362	chitin-based embryonic cuticle biosynthetic process	1	1	0.029
GO:0017004	cytochrome complex assembly	1	1	0.029
GO:0008065	establishment of blood-nerve barrier	1	1	0.029
GO:0015886	heme transport	1	1	0.029
GO:0007599	hemostasis	1	1	0.029
GO:0044241	lipid digestion	1	1	0.029
GO:0007141	male meiosis I	1	1	0.029
GO:0043060	meiotic metaphase I plate congression	1	1	0.029
GO:0045665	negative regulation of neuron differentiation	1	1	0.029
GO:0050860	negative regulation of T cell receptor signaling pathway	1	1	0.029
GO:0048011	nerve growth factor receptor signaling pathway	1	1	0.029
GO:0006374	nuclear mRNA splicing via U2-type spliceosome	1	1	0.029
GO:0007090	**regulation of S phase of mitotic cell cycle**[a]	1	1	0.029
GO:0007525	**somatic muscle development**	1	1	0.029
GO:0030182	neuron differentiation	2	10	0.032
GO:0007275	multicellular organismal development	11	202	0.033
GO:0007165	signal transduction	19	417	0.034
GO:0007160	cell-matrix adhesion	3	25	0.035
GO:0006289	nucleotide-excision repair	2	12	0.046
GO:0016568	chromatin modification	4	47	0.047

[a]Bold GO terms relate to biological processes that are found at more than one site.

Table 10. Biological processes GO categories of gonads that were significantly (P<0.05) by water collected at site B

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0006644	phospholipid metabolic process	5	16	0.0003
GO:0006353	transcription termination	17	195	0.006
GO:0016042	lipid catabolic process	4	19	0.006
GO:0051246	regulation of protein metabolic process	3	10	0.006
GO:0009887	organ morphogenesis	3	12	0.011
GO:0045449	regulation of transcription	19	252	0.015
GO:0001756	somitogenesis	4	24	0.015
GO:0006396	RNA processing	5	43	0.029
GO:0006829	zinc ion transport	26	438	0.034
GO:0045786	negative regulation of progression through cell cycle	4	31	0.036
GO:0000079	regulation of cyclin-dependent protein kinase activity	2	8	0.039
GO:0007213	acetylcholine receptor signaling, muscarinic pathway	1	1	0.041
GO:0048266	behavioral response to pain	1	1	0.041
GO:0006231	dTMP biosynthetic process	1	1	0.041
GO:0006106	fumarate metabolic process	1	1	0.041
GO:0006650	glycerophospholipid metabolic process	1	1	0.041
GO:0016574	histone ubiquitination	1	1	0.041
GO:0042472	inner ear morphogenesis	1	1	0.041
GO:0048016	inositol phosphate-mediated signaling	1	1	0.041
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	1	1	0.041
GO:0042985	neg reg of amyloid precursor protein biosynthet process	1	1	0.041
GO:0035021	negative regulation of Rac protein signal transduction	1	1	0.041
GO:0035024	negative regulation of Rho protein signal transduction	1	1	0.041
GO:0001542	ovulationsensu Mammalia)	1	1	0.041
GO:0009405	pathogenesis	1	1	0.041
GO:0019919	peptidyl-arginine methylation, to asymmetrical-dimethyl Arg	1	1	0.041
GO:0007202	phospholipase C activation	1	1	0.041
GO:0012501	programmed cell death	1	1	0.041
GO:0016925	protein sumoylation	1	1	0.041
GO:0007088	regulation of mitosis	1	1	0.041
GO:0007090	**regulation of S phase of mitotic cell cycle**	1	1	0.041
GO:0046498	S-adenosylhomocysteine metabolic process	1	1	0.041
GO:0007525	**somatic muscle development**	1	1	0.041
GO:0030518	steroid hormone receptor signaling pathway	1	1	0.041
GO:0007368	determination of left/right symmetry	3	20	0.046
GO:0009790	embryonic development	3	20	0.046
GO:0006412	translation	13	190	0.046
GO:0035023	regulation of Rho protein signal transduction	5	49	0.048

Sites E and A appeared to cluster together which makes sense based on their relative locations in Turkey Creek and the fact that both sites are probably influenced by run-off from the spraying fields. It is curious that these two sites cluster together in the testis analysis but not in the liver analysis as determined by microarrays. As mentioned above, liver genes will be mostly involved with metabolism whereas gonadal genes will be mostly representative of steroidogenesis and reproduction. This suggests that contaminants that come in from the sprayfields may influence gonadal reproduction more than liver function.

The most significant biological process altered at site E is embryonic eye morphogenesis. There are seven genes on the array belonging to this category, and three were influenced in FHM testis by exposure to water from site E. One gene was up-regulated while two were down regulated. At site A, two biological processes were most influenced: cellular protein metabolic process and meiosis, suggesting that site A waters could be affecting gamete maturation.

Table 11. Biological processes GO categories of gonads that were significantly (P<0.05) by water collected at site E

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0048048	embryonic eye morphogenesis	3	7	0.006
GO:0015824	proline transport	2	3	0.01
GO:0009401	**phosphoenolpyruvate-dependent sugar phosphotransferase**	3	10	0.017
GO:0007265	**Ras protein signal transduction**	3	10	0.017
GO:0007569	**cell aging**	2	4	0.019
GO:0030301	cholesterol transport	2	4	0.019
GO:0015808	L-alanine transport	2	4	0.019
GO:0043113	receptor clustering	2	4	0.019
GO:0006986	response to unfolded protein	2	4	0.019
GO:0001666	**response to hypoxia**	4	19	0.021
GO:0006270	DNA replication initiation	3	12	0.029
GO:0015074	DNA integration	2	5	0.03
GO:0015816	glycine transport	2	5	0.03
GO:0016071	mRNA metabolic process	3	14	0.044
GO:0007264	small GTPase mediated signal transduction	15	160	0.044
GO:0007267	cell-cell signaling	4	24	0.047

Table 12. Biological processes GO categories of gonads that were significantly (P<0.05) by water collected at site A.

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0044267	cellular protein metabolic process	12	14	0.003
GO:0007126	meiosis	15	20	0.009
GO:0051091	positive regulation of transcription factor activity	6	6	0.010
GO:0009617	response to bacterium	10	12	0.010
GO:0001666	**response to hypoxia**	14	19	0.015
GO:0007219	Notch signaling pathway	12	16	0.020
GO:0006541	glutamine metabolic process	5	5	0.022
GO:0001522	pseudouridine synthesis	5	5	0.022
GO:0030878	thyroid gland development	5	5	0.022
GO:0006955	immune response	37	63	0.034
GO:0009401	**phosphoenolpyruvate-dependent sugar**	8	10	0.034
GO:0048015	phosphoinositide-mediated signaling	8	10	0.034
GO:0007265	**Ras protein signal transduction**	8	10	0.034
GO:0016481	negative regulation of transcription	17	26	0.041
GO:0030178	negative regulation of Wnt receptor signaling pathway	6	7	0.042
GO:0051301	cell division	36	62	0.044
GO:0007049	cell cycle	75	139	0.045
GO:0007569	**cell aging**	4	4	0.047
GO:0007249	I-kappaB kinase/NF-kappaB cascade	4	4	0.047
GO:0006564	L-serine biosynthetic process	4	4	0.047
GO:0030241	muscle thick filament assembly	4	4	0.047
GO:0007548	sex differentiation	4	4	0.047
GO:0031119	tRNA pseudouridine synthesis	4	4	0.047

Turkey Creek sites E and A share four biological processes that were differentially regulated in the testis, “phosphoenolpyruvate-dependent sugar phosphotransferase,” “Ras protein signal transduction,” “cell aging” and “response to hypoxia.” Each of these biological processes was composed of multiple genes, strengthening the probability that elements within the pathway were indeed targeted by the treatments. Interestingly, the p value for the first three Biological Processes are lower at site E than at A, suggesting that these pathways are more influenced at site E (Fig. 11). The fourth element, “response to hypoxia” appears to be more influenced at

site A than at site E. All genes that were represented in site E were also represented in the GO enrichment of Site A and were regulated in the same direction.

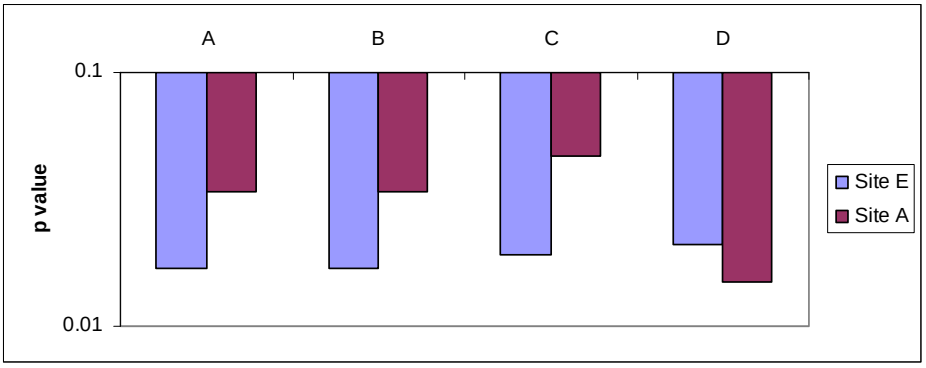


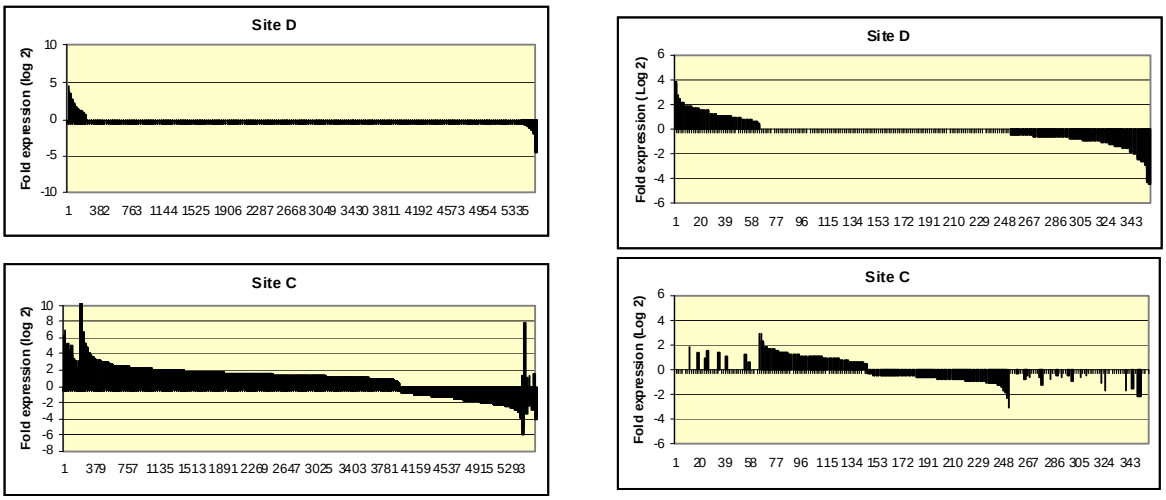
Figure 11. Change in p-value for GO Biological Processes as a function of location in Turkey Creek. (A), phosphoenolpyruvate-dependent sugar phosphotransferase system, (B), Ras protein signal transduction, (C) cell aging and (D) response to hypoxia.

Gene expression changes at Site C (headwaters of Long Creek)

Site C was initially picked as a reference site, especially since the site is located in a steephead at the headwaters for Long Creek. While this may appear at first glance to be a good site for a reference, there where a number of observations made that were inconsistent with the assumption that this would be an appropriate reference site. The use of the area surrounding the headwaters has included various military training activities involving munitions and assorted other mission related activities. This site is located in an area identified as “Closed to all forms of public access, with active military testing, training and administrative areas and/or known unexploded ordinance

contamination" (Eglin Airforce Base Map, 2005-2006). Microarray data of livers and testis of fish exposed to this site showed large numbers of genes changing after 48 h

(Figures 12 and 13, respectively), compared to fish exposed to Site D waters. In particular in the liver, 3455 transcripts changed, so many that it was not possible to



Figures 12 and 13. **Gene expression fingerprints in the liver and the gonad, respectively of FHM exposed to** (A) Site D or (B) Site C as determined by the array. The genes were ordered according to their expression levels at the two sites and proceed from most up-regulated to most down regulated and represent median expression values of the four arrays for each condition ($p < 0.05$).

analyze this site in the same ANOVA with the other sites. Most of the genes that were changed appeared to be involved in metabolism. The effects on gene expression in the testis were not as severe, suggesting that toxicants were mainly targeting the liver. For the purpose of this study, Site C was considered an outlier and not compared to the other sites.

We performed an enrichment analysis for gene ontology terms relating to biological process for each of the tissues. The results for the liver are presented in Table 13 and for the testis in Table 14. For the liver, the top processes regulated included “ATP synthesis, cellular organization and homeostasis” and many metabolic processes suggesting that the fish were trying to recover homeostasis.

For the testis, among the top processes that were regulated were processes such as “reactivation of latent virus”, “positive regulation of progression through cell cycle, respiratory gaseous exchange, embryonic development, cell aging, response to virus, gastrullation, and cell signaling,” among others Table 14. This list of processes suggests that fish may be impacted in early embryonic development and may be susceptible to viruses and bacterial infections.

Table 13. Biological processes GO categories that were significantly (P<0.05) by water collected at site C.

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p Value
GO:0015986	ATP synthesis coupled proton transport	23	40	6.24E-06
GO:0006457	protein folding	40	98	0.000162271
GO:0007015	actin filament organization	6	7	0.000983962
GO:0006744	ubiquinone biosynthetic process	12	20	0.00141867
GO:0006446	regulation of translational initiation	9	14	0.00142236
GO:0006511	ubiquitin-dependent protein catabolic process	33	86	0.00216365
GO:0048514	blood vessel morphogenesis	7	10	0.0025046
GO:0006904	vesicle docking during exocytosis	7	11	0.00548069
GO:0045454	cell redox homeostasis	19	46	0.00833654

Table 14. Biological processes GO categories of gonads that were significantly (P<0.05) by water collected at site C.

GO ID	GO Name	# of Genes Selected	# of Genes on Array	Fisher p value
GO:0019046	**reactivation of latent virus**	2	2	0.001004
GO:0045787	**positive regulation of progression through cell cycle**	2	2	0.001004
GO:0007585	**respiratory gaseous exchange**	2	3	0.002948
GO:0043254	**regulation of protein complex assembly**	2	3	0.002948
GO:0009790	**embryonic development**	4	20	0.003223
GO:0007569	**cell aging**	2	4	0.005773
GO:0015711	**organic anion transport**	2	4	0.005773
GO:0006397	**mRNA processing**	6	56	0.008333
GO:0030216	**keratinocyte differentiation**	2	5	0.009421
GO:0009615	**response to virus**	2	14	0.010846
GO:0045449	**regulation of transcription**	16	281	0.012614
GO:0007369	**gastrulation**	2	7	0.018967
GO:0030318	**melanocyte differentiation**	2	8	0.024765
GO:0046488	**phosphatidylinositol metabolic process**	2	8	0.024765
GO:0006310	**DNA recombination**	3	21	0.027567
GO:0006974	**response to DNA damage stimulus**	3	21	0.027567
GO:0001501	**skeletal development**	3	22	0.031188
GO:0000185	**activation of MAPKKK activity**	1	1	0.031732
GO:0006280	**mutagenesis**	1	1	0.031732
GO:0006987	**unfolded protein response, activation of signaling protein activity**	1	1	0.031732
GO:0007090	**regulation of S phase of mitotic cell cycle**	1	1	0.031732
GO:0007098	**centrosome cycle**	1	1	0.031732
GO:0007202	**phospholipase C activation**	1	1	0.031732
GO:0007213	**acetylcholine receptor signaling, muscarinic pathway**	1	1	0.031732
GO:0009086	**methionine biosynthetic process**	1	1	0.031732
GO:0009448	**gamma-aminobutyric acid metabolic process**	1	1	0.031732
GO:0035021	**negative regulation of Rac protein signal transduction**	1	1	0.031732
GO:0035024	**negative regulation of Rho protein signal transduction**	1	1	0.031732
GO:0042730	**fibrinolysis**	1	1	0.031732
GO:0045083	**negative regulation of interleukin-12 biosynthetic process**	1	1	0.031732
GO:0045600	**positive regulation of fat cell differentiation**	1	1	0.031732
GO:0045665	**negative regulation of neuron differentiation**	1	1	0.031732
GO:0048013	**ephrin receptor signaling pathway**	1	1	0.031732
GO:0048266	**behavioral response to pain**	1	1	0.031732
GO:0007265	**Ras protein signal transduction**	2	10	0.038173
GO:0030182	**neuron differentiation**	2	10	0.038173
GO:0001756	**somitogenesis**	3	24	0.039139
GO:0008380	**RNA splicing**	4	41	0.040123

Discussion

Water quality

Ambient water quality in East Turkey Creek was statistically significantly different from the waters of the reference creek, Long Creek as measured by one location over time. Potential meaningful differences were found for specific conductance where Turkey Creek was over five times greater. This may indicate the intrusion of wastewater effluent salts or hydrogeologic difference in the separate drainages. Further noteworthy differences were found with pH being considerably higher (1.6 units). This may be the result of the dissolved solids that drove the elevated specific conductance in East Turkey Creek. East Turkey Creek was slightly warmer than the reference over the course of sampling. However, it is not clear what the ecological significance of a temperature difference of less than one degree Celsius may be. Dissolved oxygen concentration, turbidity and chlorophyll measure differences were not thought to be ecological importance despite being statistically different. The actual differences were quite small and the high power of differentiation in the statistical analyses stemmed from the large sample size.

Ongoing monitoring includes sampling of the headwater steeps that were previously believed to lack the water-flow necessary for monitoring. If the ambient water quality of these headwaters is found to be different from downstream in East Turkey Creek, it may further support the effluent infiltration possibility. Hardness and alkalinity will also be examined. The influence of precipitation on ambient water quality will be examined via correlation analysis with this data.

Metals

Although the whole body burdens for priority pollutant metals were below concentrations at which overt symptomology or piscine toxicity would be anticipated, the presence and trends of especially the nonessential elements are suggestive of potential subchronic impact (USFWS, 1998b).

Taking into consideration the microarray findings and the relative location of an active military testing and training site, the metals profile should ideally have included Al and W. Aluminum has been a primary fuel component of composite propellants and stable oxidizer in explosives for many years. Tungsten, its alloys and composites replaced lead in most training rounds, small and heavy caliber munitions, and other explosive devices in 2000 (Clausen, 2009).

Initially considered benign, use of W was reduced in 2005 when negative environmental impacts such as decreased plant growth, reduced invertebrate reproduction, and the premature death of certain aquatic species were suspected to result from the activities of previously unrecognized W compounds (Clausen, 2009; Strigul, 2005). Weathered tungsten munitions fragments appear to be less stable, oxidizing and dissolving and forming various poly-oxyanionic and poly-tungstate species; more mobile, passing steadily through soils and entering ground waters; and more toxic, affecting phosphate regulated pathways, among others, than are the non-weathered W alloys, composites and anions previously studied (Clausen, 2009; Ringelberg, 2009; Strigul, 2005). Although the US has no current guidelines for W, the Russian Federation initiated regulatory limits for W compounds as environmental toxins

in drinking and surface waters in the 1990s (Strigul, 2005). Inclusion of Al and W in future studies may provide a more complete answer re the darter.

Passive samplers

Although chlorinated compounds, including several PAHs, were occasionally identified in the site waters, the levels were low, below USEPA ambient water quality criteria for fresh water aquatic systems (US EPA, 1986). The East Turkey Creek sites did increase in measurable impact as one progressed downstream. In general the reference sites showed less effect than did the test sites. Regarding the sporadic appearance of estrone at one reference site (LC01), this may have been the effect of sprayfield effluent, run-off or a spurious wildlife event. Finding 17α-ethynylestradiol at a single test site (TC01), the most upstream site and the site closest in proximity to the sprayfields, is most likely a result of inadequately treated municipal waste introduced through the sprayfields.

Fish response

These studies were designed to examine three sites on Turkey Creek that are inhabited by the Okaloosa darter and compare the finding to two sites on Long Creek which were taken as reference sites. The initial expectation was that the two sites on Long Creek would be similar and serve as appropriate reference sites. The microarray data indicated that Site C (Long Creek headwaters) was likely contaminated by materials that target the liver. Because exposure to waters from this site affected gene

transcription outside the range of the other sites, the site was not included in the analysis.

All other sites were included in the evaluation. Among these sites, it appears that Site D (Long Creek , LC01) was most like the control dechlorinated water at the University of Florida. For both the liver and the gonad, only few changes in gene transcription were noted between Site D and the control. While Site D is unlikely pristine, it appeared that contaminants at the very active upstream Site C were either diluted below the level required for a response or were bound up in the sediment over the downstream distance to Site D.

Hierarchical clustering of gene expression changes in the liver suggests that waters from Sites D (Long Creek reference), B (Turkey Creek headwaters) and E (Turkey Creek at sprayfields) were similar to each other. At these sites, the predominant gene expression change that was observed was in metabolism, suggesting that within 48 h of exposure the livers of the FHM have increased their metabolic processes in the presence of their *in situ* environment. However, Site E also exhibited what may have been a limitation for reproductive potential if exposure durations were extended. Furthermore, Site A which was clearly downstream of the sprayfields, showed a markedly different liver response when compared to the control or other sites. Exposure of fathead minnows to the waters from Site A for only 48 hours elicited changes in protein synthesis and metal ion transport.

Analysis of changes in gene expression in the testis suggests that exposure to Sites D and B are closely linked and Sites E and A are closely linked. The sites most influenced by the sprayfields appeared to be Sites E and A. Among the top biological

processes affected at Sites E and A are cell division and meiosis, immune response, hypoxia, and cell signaling.

Overall, the microarray data indicated that the waters from the downstream East Turkey Creek sites (E and A) contained compounds that induced potentially adverse genomic responses in both FHM livers and testes. Although the microarrays provided ample thought provoking data, and some important general water quality parameters were found to differ among sites, the sampling for causative analytes using metal burdens in fish and passive sampling for organic (SPMD) and polar organic (POCIS) analytes did not expose these agents. This evaluation demonstrated the importance of a multifaceted approach when attempting to reveal and understand complex ecological issues.

Conclusions

Differences in ambient water quality existed between East Turkey Creek and the reference site. Some parameter disparities may indicate a difference in hydrogeology in the creek drainage or external sources of dissolved organic or inorganic substances. The 48 h fish exposure experiments indicate that critical pathways of toxicity were induced in laboratory-exposed fathead minnows, suggesting that these exposures could interfere with reproduction and growth of endogenous fish. Additional evaluation of the sites should be performed to determine more specifically how these processes are affected.

To better understand the ecological significance of these preliminary findings, a longer exposure regimen with surrogate fish showing reproductive outcomes (21 d reproductive test), changes in plasma levels of sex steroid hormones and vitellogenin, and changes in gonad histology would be paramount. Ultimately, it would be ideal to develop a family or genera-specific microarray by which to cross reference the results of these surrogate evaluations to the Okaloosa darter specifically. This will allow for a better species-specific evaluation of the effect that the East Turkey Creek environment, and other municipal effluent influenced waters, may be having on darter populations. To accomplish this, as well as better understand the unexpected fish effects in the headwaters of Long Creek, further evaluation is highly recommended.

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