ASSESSMENT OF THE EFFICACY OF A CONSTRUCTED WETLAND SYSTEM TO REDUCE OR REMOVE WASTEWATER EFFLUENT ESTROGENICITY AND TOXICITY USING BIOMARKERS IN MALE FATHEAD MINNOWS *(Pimephales promelas* RAFINESQUE, 1820).

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Vitellogenin in *Pimephales promelas* was used to assess estrogenicity of a local municipal effluent. Vitellogenin induction in male *P. promelas* increased in frequency and magnitude with increased exposure duration and was greater (=0.05) than controls after 2 and 3 weeks of exposure. The level of vitellogenesis induced by effluent exposure was high compared to similar studies. A spring season evaluation followed. Biomarkers in *P. promelas* were used to assess the efficacy of a treatment wetland to remove toxicity and estrogenicity in final treated wastewater effluent. Comparisons were made with an effluent dominated stream and laboratory controls. Vitellogenin, GSIs (gonado-somatic indices), HSIs (hepato-somatic indices) and secondary sexual characteristics were biomarkers used in *P. promelas* models to assess aqueous estrogenicity. Biological indicators used to assess general fish health included hematocrit and condition factors. The estrogenic nature of the effluent was screened, concurrent with fish exposure, with GC/MS analysis for target estrogenic compounds including: 17-□□estradiol, estrone, ethynylestradiol, Bisphenol A, nonylphenolic compounds, phthalates, and DDT. Plasma vitellogenin measured in *P. promelas* was significantly elevated ($p<0.0001$) at the inflow site of the wetland and stream sites. GSIs for these exposures were less (□=0.001) at the

wetland inflow site. At wetland sites closest to the inflow, secondary sexual charateristics, tubercle numbers and fat pad thickness, were less (□≡0.0001). Hematocrit and condition factors were less (□≡0.001) at sites closer to the wetland inflow. Seasonal variation was examined by repeating the effluent characterization in summer. Additionally, summer testing included exposure to an effluent dilution series. Fish condition heavily influenced interpretation of the results. Pre-acclimation exposure to spawning stresses may have altered many of the biological markers measured. Results are discussed relative to fish health and pre-exposure environment. Toxicity assessed with *P. promelas* biomarkers was compared with *Ceriodaphnia dubia* and *Vibrio fischeri* toxicty tests on this effluent. Biomarkers of fish health were somewhat less sensitive than *C. dubia* test endpoints, but more sensitive than *V. fisheri*.

Keywords-Vitellogenin, Xenoestrogens, Wastewater effluent, *Pimephales promelas*, Constructed Wetland

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CHAPTER 1

ASSESSMENT OF WASTEWATER EFFLUENT ESTROGENICITY FROM THE CITY OF DENTON, TEXAS USING THE VITELLOGENIN BIOMARKER IN MALE FATHEAD MINNOWS *(Pimephales promelas* Rafinesque, 1820).

Abstract

Vitellogenin is a frequently used biomarker in fish models to assess estrogenicity. Male fathead minnows, *Pimephales promelas,* were exposed to undiluted wastewater effluent for 1, 2 or 3 weeks. Vitellogenin induction in male *P. promelas* increased in frequency and magnitude with increased exposure duration. Vitellogenin concentrations were significantly greater (=0.05) than the control after 2 and 3 weeks of exposure, but not after 1 week. The level of vitellogenesis induced by these effluent exposures was high compared to similar exposures. The receiving ecosystem may show similar estrogenic activity because dilution typically found in streams of the southwestern United States is low. Results are discussed relative to those of similar studies of wastewater receiving systems in the United States and United Kingdom. Our results are preliminary and are intended to better define necessary testing parameters to assess the utility of a treatment wetland system for reduction or removal of wastewater effluent estrogenicity.

Keywords-Vitellogenin, Xenoestrogens, Wastewater effluent, *Pimephales promelas*

Introduction

The egg yolk protein precursor, vitellogenin (VTG), has been used as a biological indicator for xenoestrogens with fish models [1-5]. Under natural conditions, only mature female fish produce vitellogenin. Estrogen is secreted from the ovaries of a mature female ready for spawning and flows through the blood stream to the liver. When estrogen reaches the liver, it reacts with cytoplasmic steroid receptors in liver cells that trigger the release of VTG, which then travels back through the bloodstream to the ovary where it is phagocytosed by the ova and is transformed into yolk proteins [6]. However, both male and juvenile fish also secrete VTG from their livers if estrogen is provided exogenously. Circulating plasma concentrations of VTG in males and juvenile fish or elevated VTG levels in mature females is an indication of xenoestrogens in the aquatic environment. Plasma vitellogenin concentrations increase by approximately a million-fold during the egg forming portion of the reproductive cycle. The enormous range of potential vitellogenin concentrations provides a biomarker which is very sensitive to estrogenic exposure in fish [4].

The approach described above has enjoyed noted success and was declared the universal assay of vitellogenin as a biomarker for environmental estrogens [3]. This approach was used to examine elevated hermaphroditism (intersex) occurrences associated with sewage treatment works effluent in the United Kingdom [4]. Caged male trout were placed in 100% effluent and a 300 fold increase in plasma VTG concentration

was observed in 1 week. The increase presumably resulted from an estrogenic chemical or, more likely, from a mixture of chemicals. The response was observed at all fifteen sewage treatment works sites (100% of sites tested where fish survived). Sumpter (1995) stated the effluent from all sewage treatment works tested has been strongly estrogenic to fish. He reported that the sewage treatment works were of different types and received input of varying composition primarily consisting of domestic influent. It was further stated that because all effluents proved estrogenic, the domestic portion of the influent was the most probable source of the estrogenicity in that locality.

Recent reports in the United States have shown less conclusive evidence for estrogenic responses from fathead minnows and goldfish exposed to wastewater effluent constituents [7-8]. These researchers suggest that differences in wastewater treatment or effluent dilution between the United States and the United Kingdom may be the cause of the apparent difference in effluent estrogenicity. However, the amount of VTG per ml male plasma was similar in both U.S. and U.K. effluent studies [8-9]. In the above mentioned research, fish exposure to wastewater outfalls around the Great Lakes area in Michigan resulted in VTG levels in males an order of magnitude higher at some sites compared to the field reference sites. Moreover, the 2 reference sites were somewhat different in the background VTG measured. The source pond where fish were collected was the reference site with higher VTG levels in male fish compared to the second reference site. The VTG level actually decreased during a 3 week exposure to the second reference site after the fish were transplanted from the source pond to the second reference site. This suggests some unexpected sources of estrogenic exposure or high

variability in background VTG levels in male *Pimephales promelas*. Vitellogenin responses in fish exposed to Las Vegas, Nevada wastewater effluent was attributed to anthropogenic sources of estradiol and the synthetic estrogen, ethinyl estradiol (active ingredient in female oral contraceptives). Sumpter and Jobling (1995) assessed the contribution of ethinyl estradiol to wastewater effluents estrogenicity. Widely used as an oral contraceptive, ethinyl estradiol is excreted by women almost exclusively in conjugated forms considered to be biologically inactive. Sumpter and Jobling observed the conjugated forms (ethinylestradiol glucuronide and sulfate) to be inactive as an estrogen in trout.

With reports of the potential risk of exposure to environmental estrogens, the need to determine the presence of estrogenicity and persistence in wastewater treatment effluents becomes evident. Effluent estrogenicity alone does not cause as much concern as would a persistent estrogenicity that may return to a drinking water supply. This potential was examined in rivers and reservoirs in the U.K. where they found reduced estrogenicity downstream from the wastewater outfall and no estrogenicity in reservoirs receiving these waters [4]. However, recent research in the U. K. indicates increased VTG levels in male winter flounder not only in estuaries which received treated sewage effluent, but also along and away from the coastline [10].

Our research was designed to quantify xenoestrogen presence in a U. S. wastewater effluent. It further provided related information on the relationship between exposure duration and estrogenic response.

Our results are from a pilot study designed to better define necessary testing

parameters to assess the utility of a treatment wetland system for reduction or removal of wastewater effluent estrogenicity. The effectiveness of a treatment wetland is a function of retention time, vegetation type, microbial activity and soil capacity to hold and/or degrade effluent constituents [11]. Using various wetland configurations, sewage treatment works have constructed treatment wetlands to reduce wastewater effluent levels of many contaminants including: suspended solids, phosphorus, nitrogen, organic carbon, metals, and bacteria such as fecal coliforms [12]. Treatment wetlands have also successfully removed toxicity attributed to pesticides such as diazinon and chlorpyrifos [13]. It can, therefore, be hypothesized that wetlands potentially reduce or remove estrogenicity observed in fish exposed to treated sewage effluent.

Objectives

1. Provide a quantifiable means of assessing the presence of xenoestrogens in the wastewater effluent.
2. Provide for establishment of exposure duration versus estrogenic response relationship.
3. Improve future decisions on wastewater treatment and management by providing quantitative data on the estrogenicity of the effluent.

The research was designed to test the following hypotheses.

I. Exposure to Denton, Texas final waste water effluent does not influence vitellogenin levels in male *Pimephales promelas*.

II. Vitellogenin levels in male *Pimephales promelas* are not influenced by duration or exposure to 100% Denton, Texas final wastewater effluent.

Materials and Methods

Twenty mature, male fathead minnows, *P. promelas*, were exposed to an undiluted municipal wastewater effluent typical of the southwestern United States. Ten more fish were exposed to control conditions for 3 weeks. *P. promelas* exposed to effluent were caged and placed in a constructed wetland at the inflow of final effluent from the wastewater treatment plant. Subsamples of five fish were taken weekly for 3 weeks. Control exposure was performed in the University of North Texas Aquatic Toxicology Laboratory in a 72L aquarium containing reconstituted hard water (USEPA, 1989).

After each week a five fish subsample was sacrificed by severing the head after blood samples were collected (modification of methods used by Allen *et al.* 1999). Blood samples were taken from control fish at 3 weeks. Samples were collected by severing the caudal peduncle with a razor and collecting blood with a heparinized hematocrit tube. Blood samples in the hematocrit tubes were centrifuged for 3 minutes at 3,000 RPM. After centrifugation, the eluted plasma was removed from the hematocrit tubes with a Hamilton syringe and injected into aprotinin plated 1.5 ml Eppendorf tubes. Plasma samples were immediately placed on dry ice shipped to the University of Florida Protein Chemistry Research Laboratory for plasma vitellogenin quantification within 24 hours of collection.

Eppendorf tubes were plated with a 10 ul diluted working solution of 0.9% NaCl, 0.9% benzyl alcohol with 3 mg/ml aprotinin (10X solution). The working solution was

prepared by diluting 10 ul of the 3 mg/ml solution to 1 ml with distilled water. Eppendorf tubes containing 10 ul of working solution were then vortexed to coat the tube and then allowed to dry. Eppendorf tubes were refrigerated after drying until the plasma was added.

An additional 600 ul of plasma was collected from 25-30 *P. promelas* exposed in the laboratory to a daily renewed solution of 1,000 ng/L estradiol with triethylene glycol (TEG) as the carrier. Plasma was collected five days after treatment and used to provide a source of purified vitellogenin for standards and control comparisons.

Collected plasma was quantified for vitellogenin using a sandwiched enzyme linked immunosorbant assay (ELISA) as decribed by Denslow *et al.* (1999). The sandwiched ELISA requires plating monoclonal antibodies to carp vitellogenin on microwell plates to which sample vitellogenin binds. Following the initial sample monoclonal antibody binding, polyclonal antibodies and enzyme linked detection bodies are exposed to the sample (Nancy Denslow, personal communication). Competitive binding between sample vitellogenin and enzyme linked detection bodies determines the sample vitellogenin content [14].

The minimum detection limit for the preliminary assay was 5,000 ng/ml plasma vitellogenin. Concentrations below that limit were reported as non-detectable (ND). In statistical analysis of these data, NDs were referred to as censored observations, specifically left-censored in this case. The statistical analysis assumed that the data followed a log normal distribution and were left censored at 5,000 ng/L vitellogenin. The analysis is based on a likelihood ratio test using methods described in Meeker and Duke

(1981) and implemented by the S-Plus [16] function, censorReg.

Results

Analysis of fish plasma showed elevated vitellogenin (VTG) concentrations relative to control samples (Table 1). Plasma VTG concentrations increased with increased duration of exposure with mean concentrations of 6,800, 20,000 and 25,400 ng/ml VTG measured for 1, 2 and 3 weeks of exposure respectively. Control plasma from fish kept in the University of North Texas Aquatic Toxicology Laboratory under static conditions in reconstituted hard water showed no measurable VTG levels. Several plasma samples from the 1 and 2 week exposures to whole effluent also showed no measurable VTG levels. There may have been some background levels of VTG in control fish plasma and plasma samples for 1 and 2 weeks of exposure that were not detected by the ELISA analysis performed.

Statistical analysis assumed the data to be in a log normal distribution and left censored at 5,000 ng/L vitellogenin. Plasma vitellogenin concentrations in laboratory exposed control fish were not different from fish exposed for 1 week (Bonferroni-adjusted P-value, 0.17), However, control fish had statistically less plasma VTG (= 0.05) than fish exposed for 2 weeks (Bonferroni-adjusted P-value, 0.033) and 3 weeks (Bonferroni-adjusted P-value, 0.001).

Table 1 : Measured plasma VTG concentrations (ng/ml) in individual *Pimephales promelas* plasma samples after exposure to City of Denton final whole wastewater effluent August 1998.

1 Week	2 Weeks	3 Weeks	RHW Control
15,000	ND	55,000	ND
19,000	21,000	40,000	ND
ND	48,000	22,000	ND
ND	31,000	3,000	ND
ND	ND	7,000	ND
Mean 6,800 CV% 138.5	Mean 20,000 CV% 103.3	Mean 25,400 CV% 86.7	Mean ND CV% ND

ND = Not detectable at a detection limit of 3,000 ng/ml.

CV% = Coefficient of variation as percent.

Discussion

P. promelas blood plasma increased in VTG concentration after exposure to final wastewater effluent. Increases in plasma VTG concentration to levels less than 5,000 ng/ml were not detected with the sensitivity of this initial quantification assay. However, it is possible that both background and exposure samples possessed VTG concentrations below detection. As more sensitive assays were used to measure VTG levels, degradation of plasma proteins from repeated freeze-thaw process did not allow a more accurate VTG quantification (personal communication, Marjorie Chow, University of Florida Protein Chemistry Research Laboratory).

Plasma vitellogenin concentrations in laboratory exposed control fish were not less than that in fish exposed for 1 week, but were statistically less than fish exposed for 2 weeks and highly significantly less than fish exposed for 3 weeks. The results presented suggest that vitellogenin as a biomarker measured in male *P. promelas* to assess the effluent estrogenicty is different in occurrence and strength with increasing exposure time (Figure 1). As detection limits for vitellogenin in male fatheads minnows are decreased the dose response relation may be better understood.

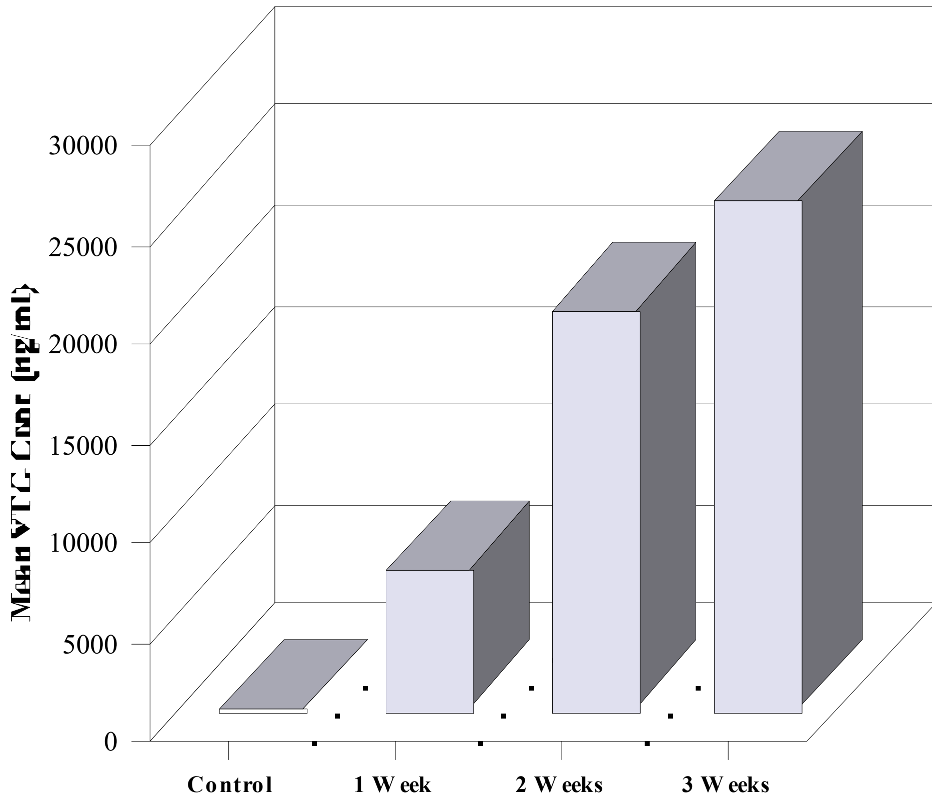

Figure 1 : Plasma vitellogenin concentrations in male fathead minnows (*P. promelas*) exposed to City of Denton (COD) final wastewater effluent (100%), August 1998.

VTG concentrations measured in plasma samples were similar to those found in other wastewater effluent evaluations in Europe and the United States. (Figure 2). For example, Harries, *et al*. (1997) exposed male rainbow trout *(Oncorhynchus mykiss*) in cages upstream and downstream of outfalls for 3 weeks. Plasma VTG concentrations were measured before and after exposure. Plasma VTG concentrations ranged from 1 to 10 ng/ml prior to exposure and increased to 1,000 to 50,000 ng/ml after exposure. The highest VTG concentrations were measured in fish exposed near the outfall and decreased downstream.

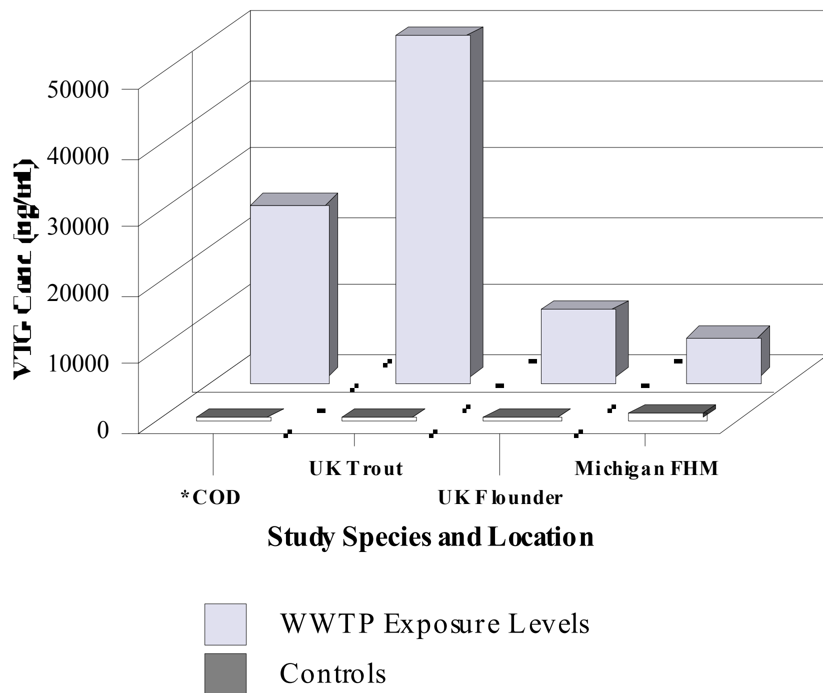

Figure 2 : Vitellogenin levels in male fish exposed to final wastewater effluents in the United Sates and United Kingdom (*COD=City of Denton).

Allen, *et al*. (1999) reported increased VTG levels in male flounder (*Platichthys flesus*) in estuaries that receive wastewater effluent in Europe. Plasma VTG concentrations at reference sites (assumed to have little or no wastewater effluent influx) ranged from five to fifty ng/ml. However, sites that received wastewater effluent from known sources showed significant differences (= 0.05) in plasma VTG ranging from 1,000 to 10 '10^6 ng/ml. They also reported elevated VTG levels in male flounder caught in the coastal waters adjacent to these estuaries.

Another study performed by Nichols, *et al*. (1999) examined rivers in central Michigan that receive wastewater treatment plant effluent. Caged fathead minnows (*P. promelas*) were placed in effluent-receiving rivers for 3 weeks. Background samples were provided from 2 reference sites, and male fish had mean plasma VTG concentrations 190 and 783 ng/ml. These levels were 1 to 2 orders of magnitude higher than male background levels measured in species used for the previous 2 studies [9-10] possibly reflecting higher background VTG concentrations in *P. promelas*. Mean VTG concentrations were 1,089, 3,240 and 5,859 ng/ml in fish caught at the reference stations but exposed for 3 weeks at different sites.

In our study, background levels (as measured by controls) of VTG were below detection limits (5,000 ng/ml). The temporal exposures did show high variability (Table 1, Coefficient of variation range 86.7 - 138.5% of the mean) and some lower levels of VTG were likely below detection. However, the high levels measured indicate induction of vitellogenesis in male fish. Naturally, this process is reserved to mature female fish under the influence of estrogen secreted from the ovaries. When male fish are induced to

produce high levels of VTG, it is generally in response to factors outside the fish.

In this pilot study, VTG concentration had a large variance because of differences in the occurrence and level of induction of vitellogenesis and low sample size. Despite that, it is evident that statistically significant increases in plasma VTG in male fish did occur. The response in the male fathead minnows was an order of magnitude greater in wastewater effluent in this study than in the study performed in Michigan and well within the range of responses found in Europe. In Michigan, fish were caged at sites downstream from wastewater treatment facilities in receiving rivers and effluent was diluted well below 100%, possibly causing the response differences. The implication of such exposures has been discussed in the context of numerous wildlife losses and impairments [17-24, 2]. The impact on humans, although heavily debated, has led to legislation mandating development of screening and testing protocols which are in the process of being implemented (Food Quality Protection Act of 1996 and Reauthorization of the Safe Drinking Water Act of 1996). The status of water reclamation in many areas of the western United States may require greater attention be paid to evidence of increased estrogenic exposures because the dilution factor is often much less than in other areas evaluated such as Michigan and Europe.

Conclusions

Research presented here was designed to determine presence or absence of xenoestrogens in a wastewater treatment plant effluent in the southwestern U.S. using vitellogenin induction in male *P. promelas* as a biomarker. The results show estrogenic activity in final wastewater effluent to be similar to that found by studies in Europe and somewhat higher than previously measured in the United States. The relationship between exposure duration and estrogenic response was also examined and showed an increased response with increased time of exposure. These quantitative data provide rationale for supporting research on the possible ecological consequences associated with chemicals that alter normal hormonal activity.

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CHAPTER 2

ASSESSMENT OF THE EFFICACY OF A CONSTRUCTED WETLAND SYSTEM TO REDUCE OR REMOVE WASTEWATER EFFLUENT ESTROGENICITY AND TOXICITY USING BIOMARKERS IN FATHEAD MINNOWS (*Pimephales promelas* RAFINESQUE, 1820)

Abstract

Biomarkers in *Pimephales promelas* were used to assess the efficacy of a treatment wetland to remove toxicity and estrogenicity in final treated wastewater effluent compared to an effluent dominated stream and laboratory controls. Vitellogenin, GSIs (gonado-somatic indices), HSIs (hepato-somatic indices) and secondary sexual characteristics were used in *P. promelas* models to assess aqueous estrogenicity. Biological indicators used to assess overall fish health included hematocrit and condition factors. The estrogenic nature of final sewage treatment works effluent was screened, concurrent with a 3 week fish exposure, with GC/MS analysis for target estrogenic compounds including: 17-□□estradiol, ethynylestradiol, Bisphenol A, nonylphenolic compounds, phthalates, and DDT. Plasma vitellogenin was measured in *P. promelas* exposed at treatment wetland sites, stream sites and in controls after 3 weeks of exposure. Vitellogenin levels were found to be significantly elevated ($p<0.0001$) at the inflow site of the wetland and stream sites. GSIs for these exposures were significantly less (□=0.001) at the wetland inflow site than controls. At wetland sites closest to the inflow,

secondary sexual charateristics, tubercle numbers and fat pad thickness, were less (□⊟0.0001) than controls. However, HSIs and density of male breeding stripes were not significantly different from controls despite elevated HSIs at the inflow wetland site. Fish health was assessed via hematocrit and condition factors and both were less (□⊟0.001) at wetland sites closer to the wetland inflow than in control fish or fish further downstream in the wetland.

Keywords- Vitellogenin, Xenoestrogens, Wastewater effluent, *Pimephales promelas*

Introduction

Reports of the potential wildlife risk from exposure to environmental estrogens emphasizes the need to better understand both estrogenic presence and persistence in treated wastewater effluents [1-9]. In addition to wildlife exposure, human exposure should also be examined [10] especially in situations when estrogenic effluents may return to a drinking water supply. This potential was examined in rivers and reservoirs in the U.K. where reduced estrogenicity downstream from the wastewater outfall and no estrogenicity in reservoirs receiving these waters was found [8]. However, recent research in the U. K. indicates increased vitellogenin (VTG) levels, an estrogen induced protein, in male winter flounder not only in estuaries which received treated sewage effluent, but also along and away from the coastline [11].

The egg yolk protein precursor, VTG, has been used as a biological indicator for

xenoestrogens with fish models [8-9, 12-15]. Under natural conditions, only mature female fish produce high levels of vitellogenin protein. Estrogen is secreted from the ovaries of a mature female ready for spawning and flows through the blood stream to the liver. When estrogen reaches the liver, it reacts with steroid receptors in hepatocytes that trigger the release of VTG, which then travels back through the bloodstream to the ovary where it is taken up into the ovum by receptor mediated endocytosis and is transformed into yolk proteins [16]. However, both male and juvenile fish also secrete high levels of VTG from their livers if estrogen is provided exogenously. High circulating plasma concentrations of VTG in males and juvenile fish is an indication of xenoestrogens in the aquatic environment. Plasma vitellogenin concentrations increase by approximately a million-fold during the egg forming portion of the reproductive cycle. The enormous range of potential vitellogenin concentrations provides a biomarker which is very sensitive to estrogenic exposure in fish [8].

Plasma vitellogenin concentrations were used to examine the estrogenicity of systems where elevated hermaphroditism (intersex) occurrences were associated with sewage treatment works effluent in the United Kingdom [8,17]. Caged male trout were placed in 100% effluent and a 300 fold increase in plasma VTG concentration was observed in 1 week. The increase presumably resulted from an estrogenic chemical or, more likely, from a mixture of chemicals. The response was observed at all fifteen sewage treatment works sites (100% of sites tested where fish survived). Sumpter (1995) noted the effluent from all sewage treatment works tested was strongly estrogenic to fish. He reported that the sewage treatment works were of different types and received input of

varying composition primarily consisting of domestic influent. It was further stated that because all effluents proved estrogenic, the domestic portion of the influent was the most probable source of the estrogenicity in that locality.

Recent reports in the United States have shown less conclusive evidence for estrogenic responses from fish exposed to wastewater effluent constituents [18-20]. Despite inherent differences in naturally inducible vitellogenin levels among species, it has been suggested that differences in wastewater treatment or effluent dilution between the United States and the United Kingdom may be the cause of the apparent difference in effluent estrogenicity.

Results presented here utilized biomarkers in fish to assess the utility of a treatment wetland system for reduction or removal of wastewater effluent estrogenicity and toxicity. The effectiveness of a treatment wetland is a function of retention time, vegetation type, microbial activity and soil capacity to hold and/or degrade effluent constituents [21]. Using various wetland configurations, sewage treatment works have constructed treatment wetlands to reduce wastewater effluent levels of many contaminants including: suspended solids, phosphorus, nitrogen, organic carbon, metals, and bacteria such as fecal coliforms [22]. Treatment wetlands have also successfully removed toxicity attributed to pesticides such as diazinon and chlorpyrifos [23]. The possibility that treatment wetlands reduce or remove estrogenicity and toxicity as observed in fish exposed to final treated sewage effluent was examined.

Materials and Methods

Exposure Scenario

Prior to exposure, adult male fathead minnows (*P. promelas*) were kept in 72 L aquaria with flow through activated carbon dechlorinated tap water for a period of 6 weeks. Light was available for 16 hours per day and the temperature was kept relatively constant at 23-25 C. Fish were fed frozen brine shrimp twice daily. After the acclimation period fish were divided among exposures.

Twenty-one mature, male fathead minnows were exposed to both 100% final treated wastewater effluent sites (Figure 1) and control conditions for 3 weeks. The fish exposed to effluent were held in 3 replicate cages and placed in a constructed treatment wetland at four sites. Seven fish were place in each large minnow trap with the entrances sealed. Individual cages were aerated with airstones to increase dissolved oxygen. Sites were located in wetland channels divided by land berms to direct flow linearly through the wetland. Site 1 was located at the inflow of final effluent. Sites 2 and 3 were distributed downstream the wetland between the inflow and outflow. Site 4 was located at the outflow of the wetland after the effluent had passed through all four wetland cells. The control exposures were performed in the University of North Texas Aquatic Toxicology Laboratory in 3 twenty-gallon aquaria containing activated carbon dechlorinated tap water for the same duration as the effluent exposures. Seven male fathead minnows were exposed at sites within the stream that received the effluent. The sites included a low flow stream area upstream of the effluent outfall (-50 m), the outfall

(0 m), four downstream sites including a low water dam (631 m), a riffle area (1,762 m), confluence of the stream and reservoir (5,500 m) and a site in the receiving reservoir at the drinking water intake. Exposures were performed after the last freeze in early spring in north-central Texas.

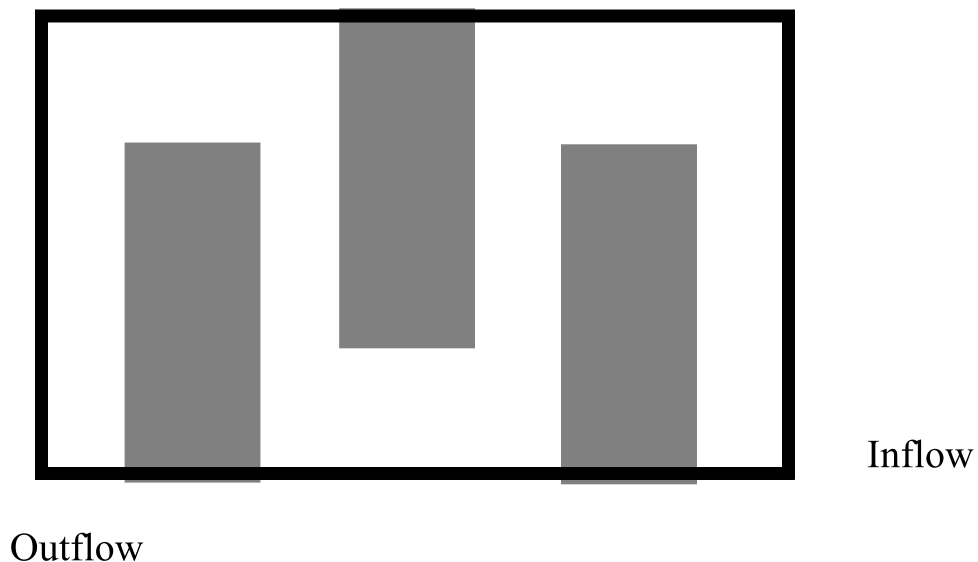

Figure 1 : Wetland schematic showing exposure sites in water channels separated by 3 land berms.

Fish Endpoints

After exposure, total length (cm), weight (g), testes weight (g), liver weight (g), hematocrit (% packed cells) and secondary sexual characteristics including number of tubercles, fatpad thickness and stripe density were recorded. Blood plasma was isolated and vitellogenin quantified at the University of Florida Biomarkers/Protein Chemistry

Core Facility as described below. Condition factor (K; weight $\times 10^5$/length^3)[24] and hematocrit (packed blood cell column height/total blood column height ×100) were calculated for individual fish to assess general fish health. Gonadal-somatic index (GSI; testes weight/total body weight ×100) and hepatic-somatic index (HSI; liver weight/total body weight ×100) were calculated to assess the physiological effects of estrogenic exposure. Secondary male sexual characteristics were recorded and included tubercle number, fatpad thickness and stripe density. Fatpad thickness and stripe density were recorded semi-quantitatively using a rating system of 0=none, 1=moderate, and 2=strong display.

Vitellogenin Quantification

After exposure, blood samples were collected and fish were sacrificed (modification of methods used by Allen *et al.* 1999). Blood samples were taken by severing the caudal peduncle with a razor and collecting blood with a heparinized hematocrit tube. Blood samples in hematocrit tubes were centrifuged at 3,000 RPM in a hematocrit centrifuge for 3 minutes. After centrifugation, the eluted plasma was removed from the hematocrit tubes with a Hamilton syringe and injected into aprotinin plated 1.5 ml eppendorf tubes. The plasma samples were immediately placed on dry ice and prepared for shipment to the University of Florida Protein Chemistry Research Laboratory for plasma vitellogenin quantification.

Eppendorf tubes were plated with a 10 ul diluted working solution of 0.9% NaCl, 0.9% benzyl alcohol with 3 mg/ml aprotinin (10X solution). The working solution was

prepared by diluting 10 ul of the 3 mg/ml solution to 1 ml with distilled water. The Eppendorf tubes containing 10 ul of the working solution were then vortexed to coat the tube and then allowed to dry. The Eppendorf tubes were refrigerated after drying until the plasma was added.

Collected plasma was quantified for vitellogenin using direct enzyme linked immunosorbant assays (ELISA) as decribed by Denslow *et al.* (1999). The monoclonal antibody 2D3 used for this assay was made specifically against carp VTG, however, it crossreacts with fathead minnow VTG. The direct ELISA required plating a series of VTG standards along with unknown sample VTG on microwell plates. Following the initial anti-VTG monoclonal antibody 2D3 binding, biotinylated goat anti-mouse IgG polyclonal antibody and strepavidin conjugated alkaline phosphatase were used consecutively. The bound enzyme then converted the substrate mixture of p-nitro blue tetrazolium/5-bromo-4-chloro-indoyl phosphate into a color product that absorbs light at 405nm. By comparing the unknown absorbance unit against the standard curve with the known VTG concentration, VTG concentration of exposed plasma was calculated [25].

To obtain concentrations within the standard curve, a series of sample dilutions were performed. The samples were first diluted from 1 to 100 and subjected to direct ELISA with a detection limit of 0.5 ug/ml. Samples with higher concentrations than the standard curve range were subjected to further dilutions of 1:10,000, 1:100,000 or 1:1,000,000 and direct ELISA was performed again. Those samples with lower concentrations were repeated with a competitive ELISA at a dilution of 1:20 and 1:50. Competition ELISA is currently more sensitive than the direct ELISA with a minimum

detection limit of 0.2 ug/ml of plasma (personal communication Marjorie Chow, University of Florida Protein Chemistry Research Laboratory).

Wetland Characterization

Wetland characteristics were measured including flow (m/min), nominal residence time [(t-1)=average storage volume (V)/total inflow rate (Q1)], vegetation types and relative cover at time of exposure, and channel length, width and depth. Flow was estimated by eleven replicate measures of travel time of cloth dye (royal blue) through a metered 2 inch PVC half-pipe submerged within the vegetation of the first channel at the left, right and center of the channel. Retention time of the wetland was estimated via inflow volume measures relative to the average storage volume and corrected for effluent displacement [26]. Depth was measured at four locations in each channel. Each depth measurement included a measure on each side of the channel and one central depth measure at each of the four areas per channel. Width was measured at the four locations in each channel where depth was measured. Vegetation types were identified and recorded via digital images taken during the exposure and relative coverage was estimated based on visual inspection and using digital images of the plant types in each channel.

Chemistry

General water chemistry measurements were taken periodically at each site during the fish exposure including: pH (Orion 230A pH Meter), dissolved oxygen (mg/L;

YSI Model 51A Oxygen Meter), temperature (°C; YSI Model 85 Oxygen, Conductivity, Salinity and Temperature Meter), alkalinity (mg $CaCO_3$/L; Potentiometric Titration with 0.1 N H_2SO_4 to a pH of 4.5), hardness (mg $CaCO_3$/L; Colorimetric Titratrion with 0.02 N Ethylenediamine tetracetic acid (EDTA) and calmagite) and specific conductance (umho/cm at 25°C; YSI Model 33 Conductivity Meter).

The nature of the estrogenic effluent components was assessed during the fish exposure with GC/MS analysis of the effluent to quantify levels of known estrogens, estradiol and the synthetic ethynylestradiol, and suspected estrogen mimics such as nonylphenol, di-2-ethylhexylphthalate (DEHP), bisphenol A, dichloro-diphenyl-trichoroethane (DDT). The analytical technique was similar to EPA methods 625 and 8270 (applicable to wastewater) but extends lower detection limits to concentrations more typical of EPA s method 525 (applicable to drinking water). Four samples were taken from each site representing 4 distinct days.

Samples were received in the laboratory the morning collected. Extractions began immediately. Five hundred ml samples were acidified with sulfuric acid to a pH of 2-3 and pulled by a vacuum through a 200 mg Waters HLB solid phase extraction cartridge. Cartridges were conditioned with 6 ml MTBE (methyl-tert-butyl-ether) followed by 6 ml of methanol followed by 6 ml deionized water prior to extraction. Extraction time varied from 2-6 hours depending on suspended solid content of the water. Some samples required 2 cartridges to complete the 500 ml sample volume. In an effort to reduce problems associated with suspended materials, cartridges used for the final 3 sampling dates were modified by the addition of approximately 1 cm of deactivated fused silica

wool to the head of the cartridge. After extraction, the cartridges were rinsed with 6 ml of 5% methanol in water, then analytes were eluted with 8 ml of MTBE. Water was removed from the eluate with a pipette and remaining MTBE was dried through a sodium sulfate column (10mm X 50mm) and blown down under nitrogen to a final extraction volume of approximately 100ul. Surrogate standards and matrix spike standards were added to the water samples prior to extraction. Internal standards were added to the final extract prior to analysis via GC/MS.

Final quantitative instrumental analysis was with Hewlett Packard (HP) 5890 Series II GC, HP 5970 MS and HP Enviroquant quantitative software. Analytes of interest were compared to a five-point internal standard calibration curve with a concentration range from 5-100 ppm (extract concentration) which corresponds to a 1-20 ppb concentration range in the original sample (concentration factor = 500 ml original sample volume/0.1 ml(100ul) final extract volume = 5,000).This compares to an estimated quantitation limit (EQL) of 10 ppb for similar analytes using EPA 8270. Matrix spikes were added at the EQL of 10 ppb and surrogate spikes were added at 20 ppb.

Specific analytical conditions were: Column=DB-5.625, 30 m X 0.25 mm ID with 0.25 micron thickness; Injector temperature= 260°C; oven temperature program=40°C for 5 minutes, 10°C/minute ramp rate, final temperature=300°C held for 14 minutes; MS=electron impact ionization, scanned from 35-500 amu; Injection Volume=2-4 ul.

Results are reported as ug/L in the original sample except for nonylphenols. The family of congeners reported as nonylphenols was represented by five peaks and the average of all five values was used to estimate total nonylphenol concentration for a

given sample. All values reported have been validated by matching retention time and mass spectral characteristics of authenic standards under the same conditions. Values less than 1 ppb were below the lowest standard and below the EQL and should be considered more approximate than values above 1 ppb. Values reported as 0" were non-detectable or less than the EQL of 1 ppb.

Statistics

All analyses were carried out using SAS Version 7 (SAS Institute Inc). Statistically significant differences were accepted at =0.05. Data were analyzed with parametric General Linear Procedures when the assumption of normality and homogeneity were met. The Student Newman Kruels (SNK) or Tukey multiple range test was used when differences were found. When parametric assumptions were not met, the non-parametric Kruskal-Wallis analysis was used with Dunnett s and ranked Tukey non-parametric multiple range test when differences were found. Data are presented as means plus and minus 1 standard error of the mean (SE) or median with the intraquartile range (75% and 50%).

Results

Fish Endpoints

Survival during exposure was 100% for wetland and control fish. Fish health was assessed via condition factor calculations (Figure 2) and hematocrit values (Figure 3).

The condition factor (K) of fish exposed to effluent at wetland sites 1 and 2 was significantly lower (Kruskal-Wallis; $p<0.0001$) than condition factors of dechlorinated tap water exposed control fish (Dunnett s MRT). Hematocrit values were also significantly less (GLM; $p=0.0008$) at wetland sites 1 and 2 than controls and wetland sites 3 and 4 (SNK). Together these data indicate a decrease in toxicity with increased distance in the wetland. A negative trend was observed between vitellogenin concentration and condition among wetland sites. A positive trend was seen between condition and hematocrit.

Mortality of 3 fish at the upstream site (-50m) was observed after 3 weeks of exposure. Two stream sites were lost, riffle site (1,762 m) and the confluence site (5,500 m), during a large rainfall event resulting in cage losses in the heavy flow. Condition of fish exposed upstream of the effluent outfall were less than laboratory control fish (Kruskal-Wallis; $p=0.027$ with Dunnett s MRT), however, the effluent dominated stream sites and reservoir were not less than the laboratory controls. Hematocrit values for the stream sites were not different.

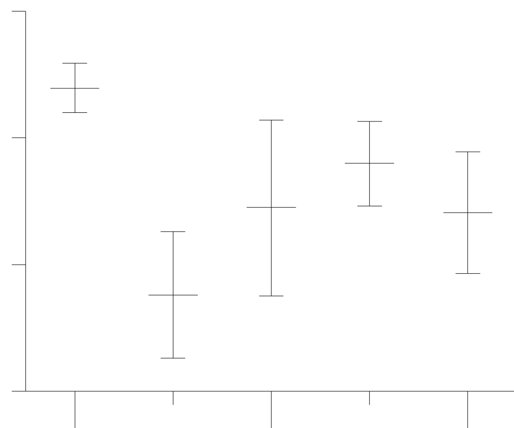

Figure 2: Fish condition factor (K) mean ± 1 standard error with Dunnett s non-parametric multiple range test differences (*).

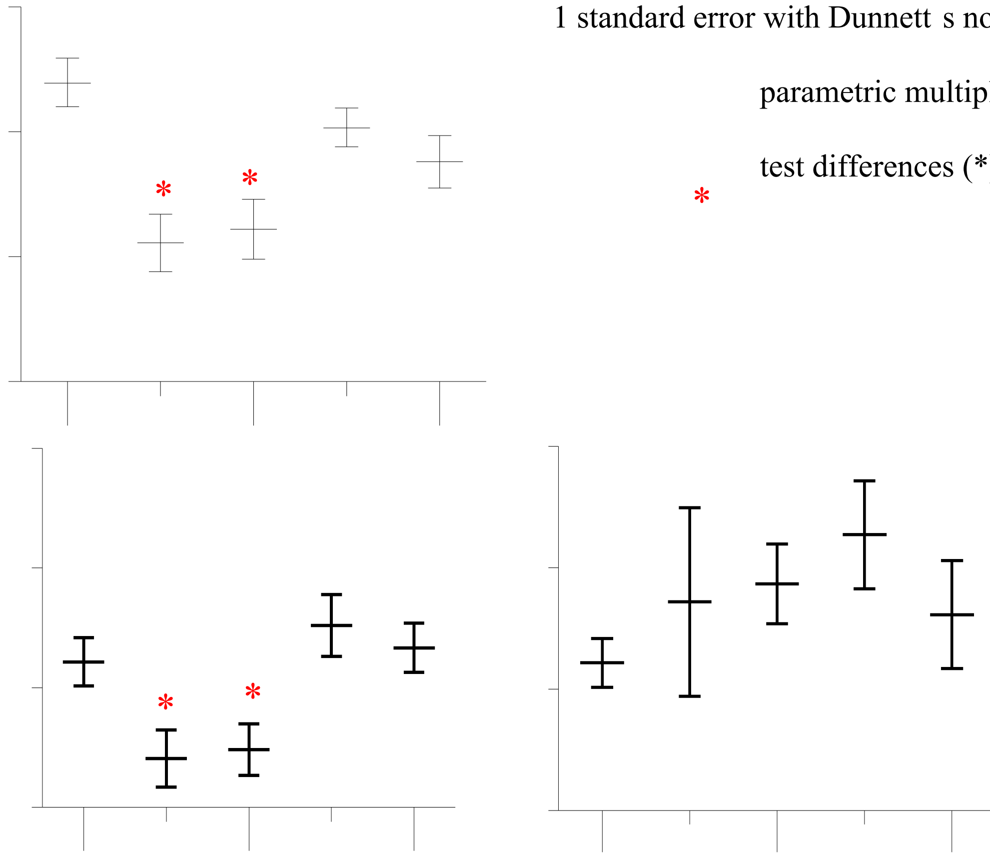

Figure 3: Fish hematocrit value mean ± 1 standard error with Students-Newman-Keuls multiple range test differences (*)for wetland sites.

The lowest GSI (Figure 4) and highest HSI values (Figure 5) were also found in fish exposed at site 1 before any wetland treatment. GSIs were significantly less than controls at site 1 but not at other wetland sites (Kruskal-Wallis; p=0.0087 with Dunnett s MRT). No significant differerences were found for HSI values among wetland sites,

however, site 1 was nearly different from other sites (Kruskal-Wallis; p=0.0584). A negative trend was observed between vitellogenin concentration and GSI. A positive trend was seen between VTG with HSI. GSI and HSI demonstrated an apparent inverse trend. No significant differences or trends were found among stream and reservoir sites and laboratory controls for GSI or HSI

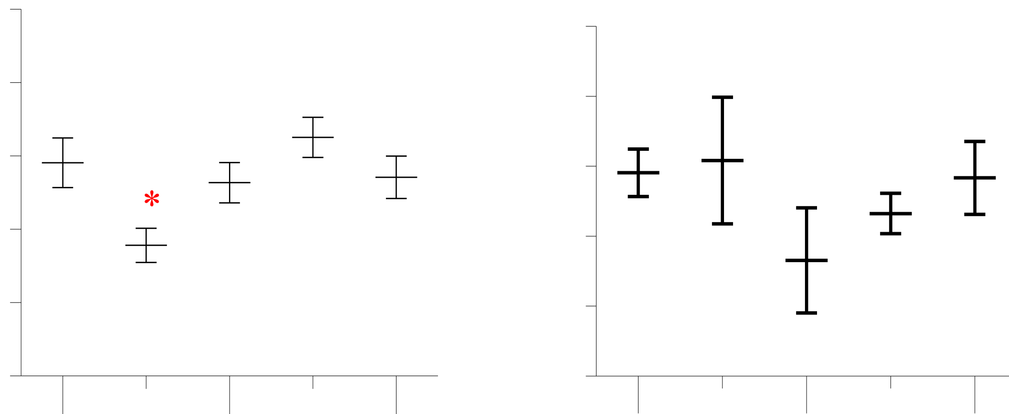

Figure 4: Fish Gonadosomatic Index (GSI) mean $\pm$ 1 standard error with Dunnett s non-parametric multiple range test differences (*).

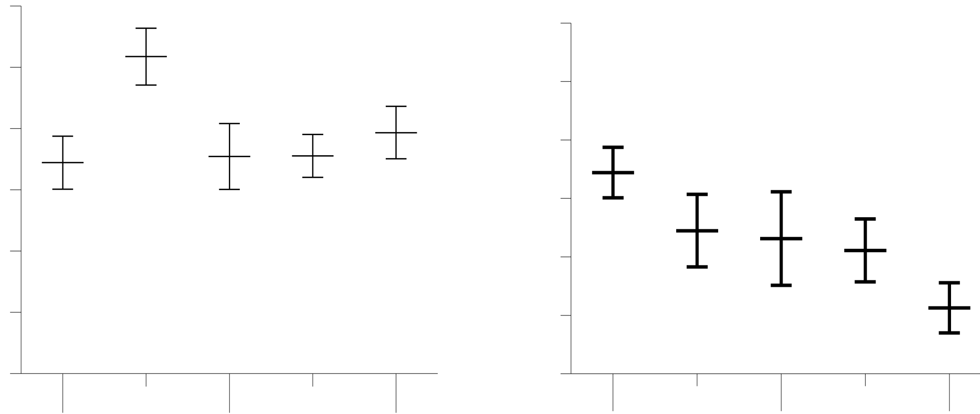

Figure 5 : Fish Hepatosomatic Index (HSI) mean ± 1 standard error.

Secondary male sexual characteristics were different among wetland sites (Figure 6A-C). Tubercle number was markedly less at site 1 and significantly less at site 2 and 4 than in controls (Kruskal-Wallis; p<0.0001 with Dunnett s MRT). Observed frequencies of thickened fatpads were significantly different than expected even frequencies (Likelihood ratio; p<0.0001) with the lowest frequency observed at site1. Observed fatpad thickness was different than expected among the stream and reservoir sites with the low water dam site (0.6 km downstream) being approximately half that of other sites (Likelihood ratio; p=0.0344) .No differences in expected and observed frequencies of stripe density were found among wetland sites or among stream and reservoir sites and controls. Stripe displays were low with all exposures.

A negative trend was apparent between VTG concentration and number of tubercles for wetland sites and stream/reservoir sites. A similar negative trend was

present between VTG concentration and fatpad thickness for both wetland sites and stream/reservoir sites. No trend was evident between stripe density and VTG concentration.

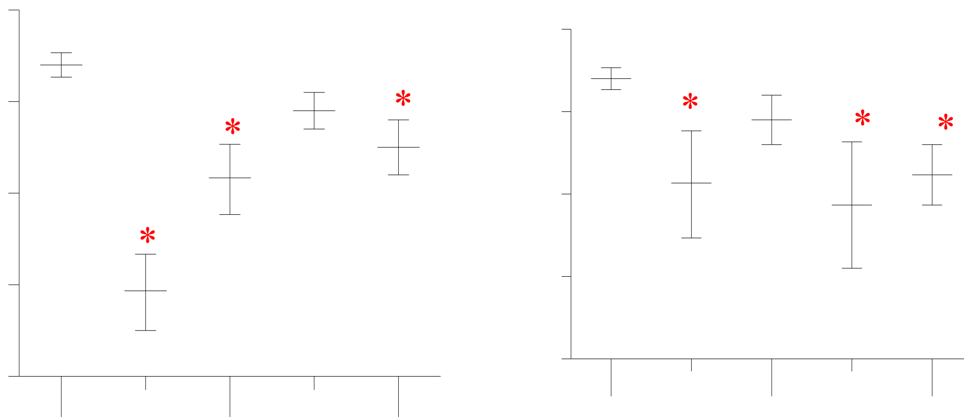

Figure 6A: Tubercle number mean ± 1 standard error with Dunnett s non-parametric multiple range test differences (*).

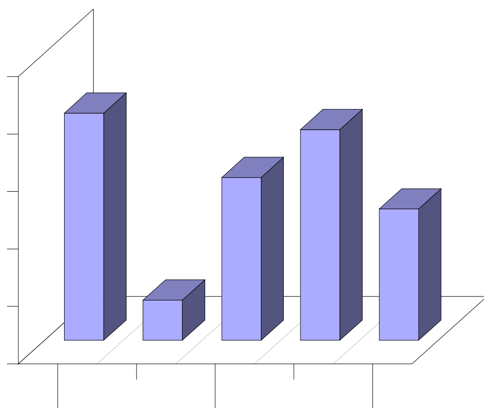

Figure 6B : Fatpad thickness mean.

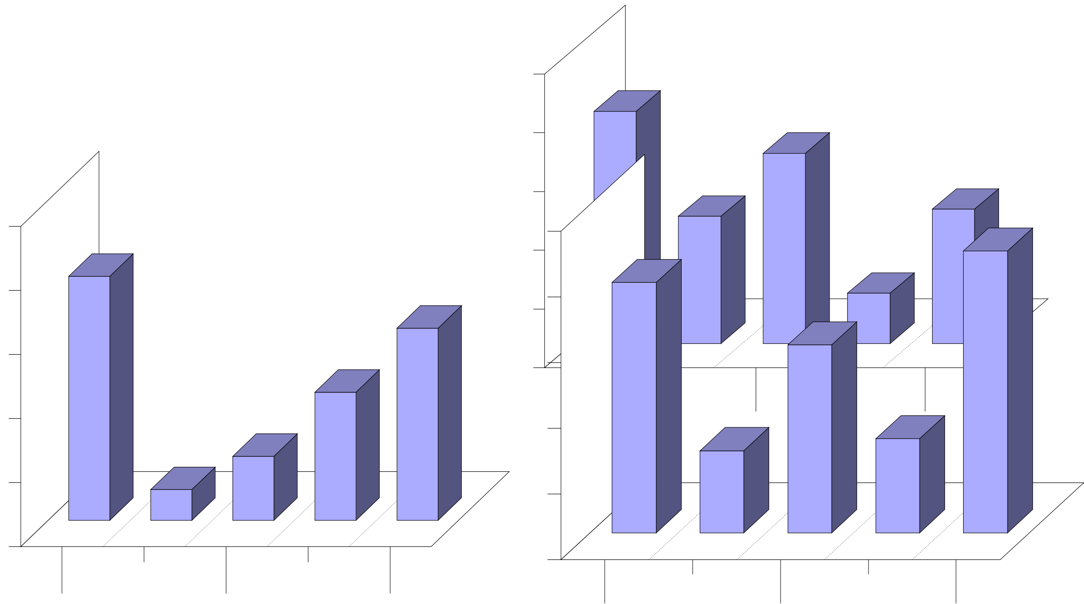

Figure 6C: Stripe density mean.

Vitellogenin Quantification

Vitellogenin levels were found to be highly statistically different among wetland exposure sites (Kruskal-Wallis $p<0.0001$). Fish exposed at site 1 had significantly higher plasma vitellogenin concentrations (mean 55,031 ug/ml, SE 7,204) than measured in controls (mean 0.276 ug/ml, SE 0.155). High concentrations at site 1 were followed by a rapid decline at sites 2, 3 and 4 with corresponding vitellogenin concentrations of 1.0 (SE 0.865), 0.038 (SE 0.038) and 0.028 (SE 0.017) ug/ml respectively (Figure 7). Similar elevations in plasma vitellogein were found in fish exposed to the treatment plant outfall

and low water dam site (631 m) downstream (Kruskal-Wallis; $p<0.0001$). Vitellogenin levels at these sites were significantly higher than laboratory controls, an upstream site (-50 m) and the reservoir which receives this stream. Sites located further downstream (1,762 m and 5,500 m) were lost during a large runoff event.

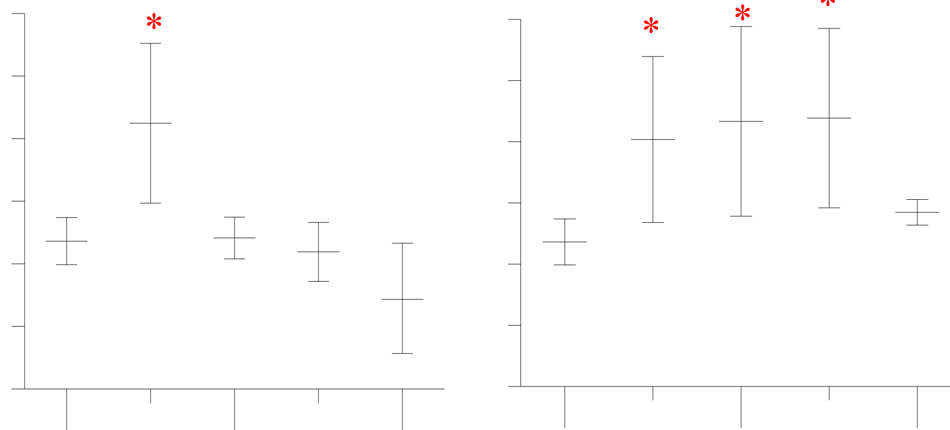

Figure 7 : Vitellogenin levels (log VTG ug/ml) in male fish; mean $\pm$ 1 standard error with Dunnett s non-parametric multiple range test differences (*).

Wetland

Flow was estimated for the first wetland channel only. The deeper channels in other areas of the wetland did not allow for sufficient unidirectional flow to be measured with the techniques attempted. Retention time was thought to be a better indication of

effluent exposure than actual travel time in each channel and wetland as a whole. Flow estimated for channel 1 was 2.9 m/min (median, Q3 = 3 m/min, Q1=2 m/min). The wetland average storage volume derived from 40 length and width measures and 65 depth measures was 80,646 gallons. The volume for channel 1 alone was 10,355 gallons. The average inflow rate was 784 gallons/hour (2,968 L/hour). Nominal residence (retention) time was estimated to be 4.3 days for the wetland and 0.6 days for channel 1. Correcting for actual displacement of effluent as per Sprague (1969) provided a 90% renewal of effluent in the whole wetland at 10 days and a 99% replacement at 20 days. Channel 1 was estimated to be 90% exchanged at 1.4 days and 99% exchanged at 2.6 days.

Macrophyte type varied with wetland channel (Figure 8). Channel 1 possessed the greatest diversity of plants including *Pontederia* (pickerelweed), *Ludwigia*, and *Lemna* (duckweed) with some *Scirpus* (bullrush) sprouting near the end of the exposure. Channels 2, 3 and 4 were heavily dominated by *Typha* (cattails) with *Lemna* covering open areas. The 2,660 ft^2 (140' long X 19' wide) area of channel 1 was 70% inhabited by *Pontederia*, 25% *Ludwigia* and 5% open with actual vegetative coverages of 27% and 19.5% respectively within the areas. Open areas were covered with a layer of *Lemna*. Channel 2 (3,105 ft^2, 135' long X 23' wide) was 75% inhabited with *Typha* with 95% cover within that area and dense duckweed covering the remainder. Channel 3 (3,494 ft^2,137' long X 26' wide) was 70% inhabited with *Typha* at the same cover as channel 2 with *Lemna* covering the open areas. Channel 4 (3,192 ft^2,137' long X 23' wide) was 50% inhabited with *Typha* at a cover of 50% within the inhabited area with *Lemna* over the remaining area.

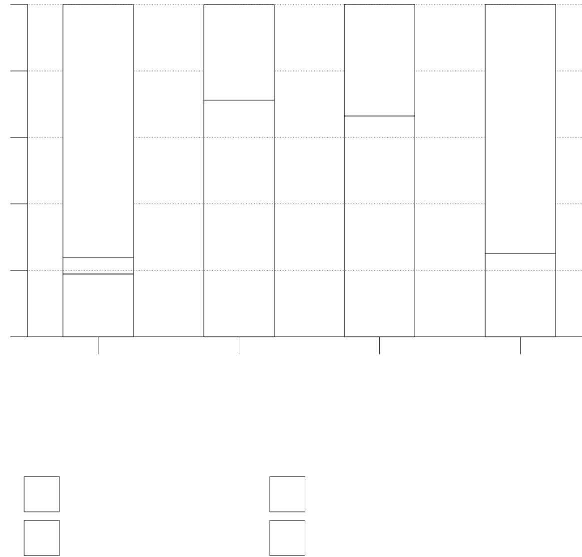

Figure 8: Wetland percent coverage by channel in March 2000.

Chemistry

The wetland was characterized after last freeze during weekly fluctuating cool (~1.7°C) and warm (~27°C) air temperatures. General water chemistry measures were recorded during exposure (Table 1). Aqueous temperatures varied from 10°C to 26°C in the wetland but were relatively constant in the laboratory at 22°C. Site 1 and control were significantly warmer than other wetland sites (Kruskal-Wallis $p<0.0001$; ranked Tukey MRT). Measurements of pH were not different among sites and were approximately 7. Differences in dissolved oxygen (~8 mg/L) readings were found between the laboratory control and wetland sites (Kruskal-Wallis $p=0.0013$; Ranked Tukey MRT). However,

oxygen concentrations at wetland sites were kept around 6 to 7 with aeration within the fish cages. Specific conductance (umho/cm at 25°C) was higher in water samples taken from all wetland sites (~800) than in control (~450) samples (Kruskal-Wallis p0.0010; Ranked Tukey MRT). Differences in alkalinity (mg $CaCO_3$/L) were found between site 4 and control which was somewhat lower than all wetland sites. (Kruskal-Wallis p<0.0373; Ranked Tukey MRT). No differences in hardness (mg $CaCO_3$/L) were found among sites.

Table 1: General water chemistry measurements at wetland sites during March 2000. Significant differences shown with letter designations from ranked Tukey.

Quartiles	Site01	Site02	Site03	Site04	Control
Temperature (°C)					
Q3 (75%)	20	17	16	17	22
Median (50%)	20 B	16 C	14 C	15 C	22 A
Q1 (25%)	19	14	13	13	21
Sample size	15	15	15	15	15
pH					
Q3 (75%)	7.54	7.48	7.42	7.46	7.81
Median (50%)	7.42	7.37	7.33	7.35	7.65
Q1 (25%)	7.17	7.15	6.96	7.12	7.40
Sample size	6	6	6	6	6

Table 1 continued: General water chemistry measurements at wetland sites during March 2000. Significant differences shown with letter designations from ranked Tukey.

Quartiles	Site01	Site02	Site03	Site04	Control
Dissolved Oxygen (mg/L)					
Q3 (75%)	7.6	7.6	7.2	7.2	8.2
Median (50%)	7.2 B	7.1 B	6.8 B	6.8 B	8.0 A
Q1 (25%)	6.2	6.4	6.2	5.9	7.8
Sample size	7	7	7	7	7
Specific Conductance (umho/cm at 25°C)					
Q3 (75%)	820	800	800	800	460
Median (50%)	800 A	800 A	780 A	730 A	450 B
Q1 (25%)	730	730	710	720	450
Sample size	7	7	7	7	7
Alkalinity (mg $CaCO_3$/L)					
Q3 (75%)	130	130	140	165	120
Median (50%)	125 AB	121 AB	124 AB	147 A	101 B
Q1 (25%)	110	116	121	135	100
Sample size	6	6	6	6	6
Hardness (mg $CaCO_3$/L)					
Q3 (75%)	165	170	170	170	180
Median (50%)	156	168	168	169	149
Q1 (25%)	129	164	160	160	130
Sample size	6	6	6	6	6

Measured concentrations of known and suspected environmental estrogens were highly variable throughout the exposure period (Figure 9A-D). Estradiol, DDT and Bisphenol-A were not detected in any samples analyzed. DEHP was detected at concentrations below 1ug/L (range 0.1-0.3 ug/L) in some samples from each date. Ethynylestradiol was found in samples from 3 of the four sampling dates at concentrations ranging from 0.2-0.9 ug/L. Nonylphenol was the most frequently detected analyte and was consistently detected in all five congeners analyzed. The average of these five peaks is provided as a conservative estimate of total nonylphenols in the sample. By this method total nonylphenol concentrations ranged from 1.0-2.2 ug/L.

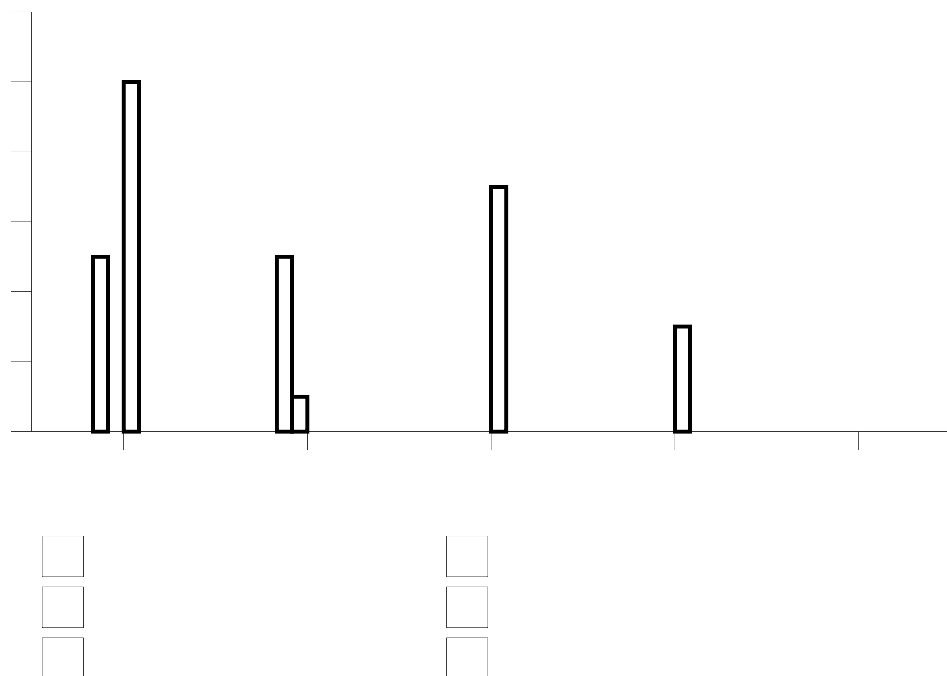

Figure 9A: Estrogenic chemicals in the March 22, 2000 sample.

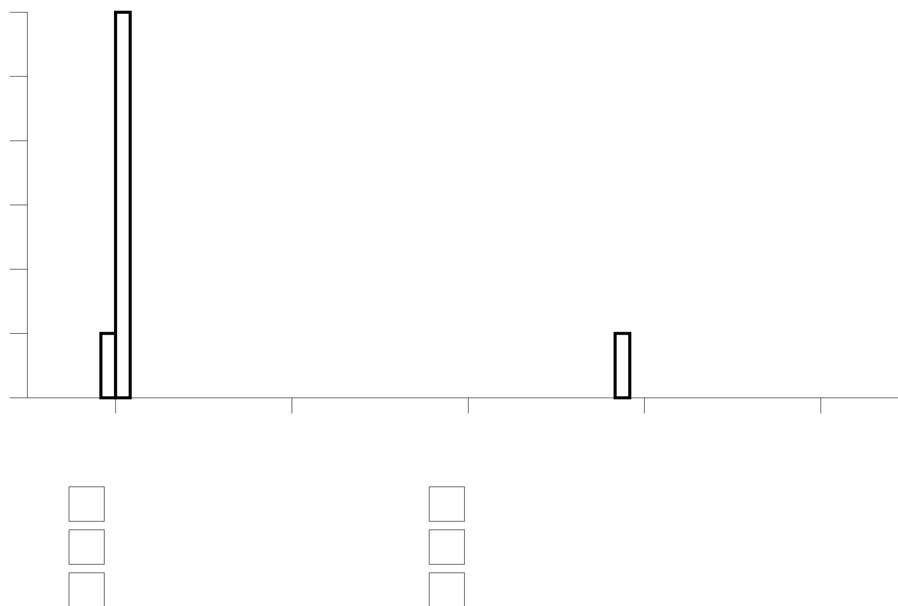

Figure 9B: Estrogenic chemicals in the March 27, 2000 sample.

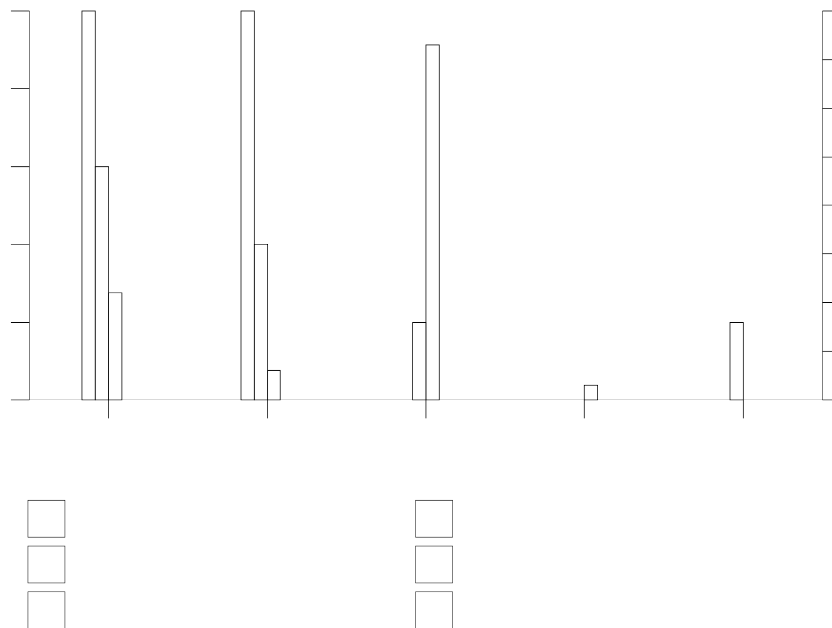

Figure 9C: Estrogenic chemicals in the March 29, 2000 sample. Note change of Y axis scale.

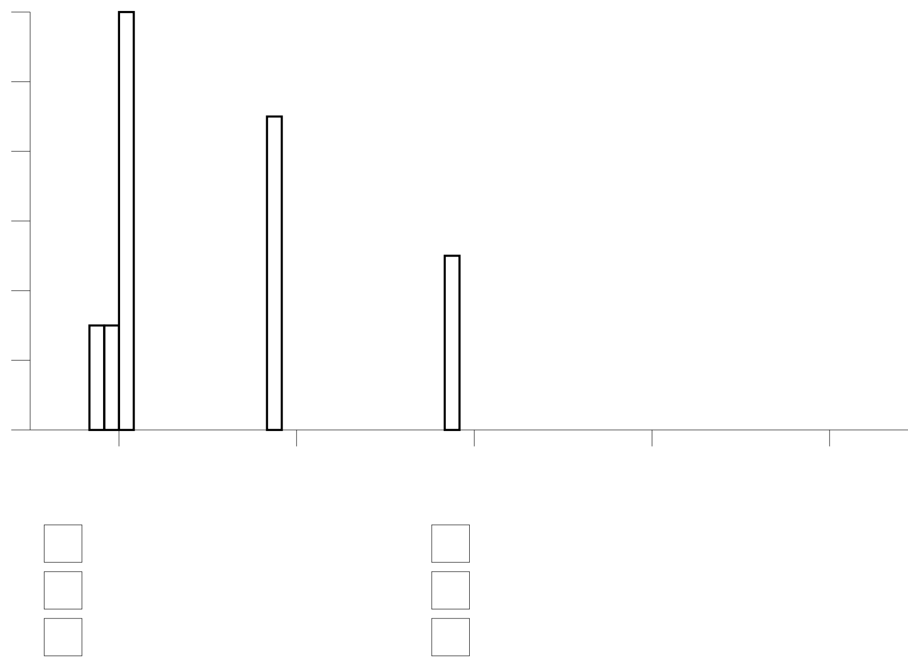

Figure 9D: Estrogenic chemicals in the April 4, 2000 sample.

Discussion

Fish Endpoints

The health of fish was assessed via condition factors and hematocrit values. There was 100% survival of control fish and fish caged at wetland sites. Good agreement existed between these 2 indicators at all wetland sites and in controls. Both showed reduced levels, indicating lessor health, at wetland sites 1 and 2. The nature of the toxicant or conditions causing these reductions in unknown. Similar responses to effluent from each site during this time period was observed in evaluations using different test methods and organisms (see Chapter 4). Fish placed at stream and reservoir sites showed variable results possibly reflecting the smaller sample size.

The levels of nonylphenols measured were below the reported toxic levels for aquatic species [27-28]. However, previous studies of this effluent have found organophosphate pesticides to be a seasonally toxic constituent [23]. Additionally, the presence of ethynylestradiol was detected at concentrations above those which have been

shown to increase plasma vitellogenin to concentrations as high as 10-100 mg/L, a level shown to cause physiological stress via kidney and liver damage and necrosis [29]. Condition factor was negatively correlated with increasing plasma vitellogenin concentrations in this study. The lessor condition factors and hematocrit values recorded for site 1 fish may be a result of estrogenic stimulation of excessive vitellogenesis. Significant associations were found at 36% covariance for condition factor and GSI (Spearman Correlation; r_s= 0.362; $p<0.0001$) and hematocrit and GSI at 43% covariance (Spearman Correlation; r_s= 0.425; $p<0.0001$) indicating that increased effluent estrogenicity is associated with decreases in fish health in wastewater effluent. DEHP levels measured were consistently below 0.5 ug/L and not expected to have been active as a toxicant. However, toxicity contributions of individual chemicals in a complex exposure is not well understood.

GSIs showed a similar trend to the health indicators. The lowest GSI values and highest HSIs were consistently found at wetland site 1, however, no differences were observed in fish at stream sites. As a measure of estrogenic exposure the GSI and HSI would appear inversely related [17]. A negative trend was observed between VTG concentration and GSI as was a positive trend of VTG with HSI. Consequently GSI and HSI appeared inversely related as would be expected. Some changes in GSI and HSI may have been less evident due to variation in these factors related to fish size and stage of maturity.

Estrogenic exposure results in testicular inhibition and consequently lower GSIs [30]. This same type of exposure has been associated with an increase in liver weight via

liver hypertrophy of the estrogen sensitive tissue resulting in higher HSIs [11,31]. Fish exposed to site 1 showed reduced GSIs that was complimented by increased HSIs indicating probable estrogenic effects from the effluent. However, non-estrogenic toxicity of the effluent may have been involved in altering the GSI - HSI relationship through toxic effects at the testis and/or hepatocyte level.

The secondary male sexual characteristics of tubercle number, fatpad thickness and stripe density were also different among wetland sites showing a trend similar to condition, hematocrit and GSI values. This trend was not evident at stream and reservoir sites. Fish exposed at wetland site 1 consistently showed lower values for secondary sexual characteristics indicating lessor health or masculinity. The statistically lower turbercle numbers found at sites 2 and 4 were much less pronounced (difference of 2.3 and 1.2 turbercles, respectively) compared to fish at site 1 (6.0 fewer). Number of male breeding tubercles and fatpad thickness showed an inverse trend with the estrogenic biomarker, VTG concentration, indicating possible demasculinization by estrogenic effluent constituents. However, the cause of these apparent trends, as well as those seen for GSI, HSI and health indicators, may have been toxic in nature caused by some effluent parameter or parameters not measured. The secondary sexual characters, fatpad thickness and stripe density, were semi-quantitative in nature and may have been biased by the limited rating system, however, they were performed consistently and show a pattern similar to those data collected by quantitative methods.

Vitellogenin Quantification

Measured vitellogenin concentrations were markedly high for the wetland inflow site (Site 1), the effluent outfall in the stream, and 631 m downstream at the low water dam. Concentrations of plasma vitellogenin at these sites were 55,031 to 144,263 ug/ml, levels 3 to 4 orders of magnitude higher than previously measured in the area (see Chapter 1) and even higher compared to other U.S. studies [18-20]. However, the levels were comparable to those observed in similar studies including 100% final wastewater effluent exposures in the U.K. [8, 11,17,33]. High vitellogenin induction indicates a high level of estrogenic exposure, in this case environmental exposure. Laboratory research has provided that induction of these levels of vitellogenesis in male flounder was accomplished after a 3 week exposure to 10 ng/L ethynylestradiol [11]. Fathead minnows have been induced to 20,000 ug/ml vitellogenin 2 days after injection with 5 ppm of 17 B-estradiol [34]. The level of induction is often highly variable, as seen in this study, and has been attributed to possible causes including composition of effluent, route of exposure, exposure time, water temperature, species, age and sex of fish [35]. However, of the above mentioned variables, only temperature was different among wetland sites. Other variables measured that were different among sites were dissolved oxygen (higher in controls), specific conductance and alkalinity (both lower in controls). The number of known and suspected estrogens measured varied greatly but were most frequent at site 1.

Naturally low induction of vitellogenesis (to levels less than 0.5 ug/L) occurs in male fish [36]. Once induced to high levels vitellogenesis, the clearance of VTG from fish plasma can take up to 5 months after the estrogenic cue is removed [37]. The

ecological significance of estrogenic exposure in not certain but is being investigated [33,38-39]. Intersexuallity has been associated with vitellogenin induction in wild roach *(Rutilus rutilus*) populations [9] as has disrupted natural sexual cycles [40]. Traditionally reported effects of prolonged synthesis of vitellogenin include metabolic stress leading to kidney and liver damage and necrosis [29], misallocation of physiological building blocks, and calcium losses from the skeleton and scales [41]. These stresses on organisms may increase susceptibility to disease and possibly death [11]. However, recent research found no relationship between an estrogenic effluent inducing elevated VTG and other physiological changes in male fish [33] which is contrary to observations made in this study of north-central Texas final wastewater effluent.

Wetland

The wetland was characterized during fish exposures after last freeze in early spring. The emergent and floating vegetation was growing rapidly over the 3 week period. The first wetland channel, having highest diversity but lowest percent coverage, decreased effluent estrogenicity in less than 140 linear feet indicated by the reduced VTG levels at site 2, 3 and 4 and the corresponding changes in GSI, HSI and secondary sexual characteristics. The wetland reductions were noteworthy when compared to stream sites where VTG levels did not decrease at distances over 630 meters downstream from the effluent outfall. However, the effluent reached that site ~4.4 hours after discharge versus the 13.2 hour estimated average retention time for the distance between wetland site 1 and 2 (90% exchanged at 1.4 days, 99% exchanged at 2.6 days). River estrogenicity

decreases were examined with trout and found at distances from 0.5 to 5.0 km [17] indicating a comparatively persistent estrogenicity in the study area resulting from final treated wastewater effluent. Evaluation of further distances in the stream were not possible after cage losses. However, levels in the reservoir were low relative to the measured creek sites.

Other forms of toxicity were apparently removed after travel through both channel 1 and the more dense *Typha* monoculture of channel 2 as reflected by the improved condition factors and hematocrit values for sites 3 and 4. The reduction in both types of effects may have been related to the rapid growth phase of the wetland plants, however, it is likely that microbial degradation associated with aerobic- anaerobic dynamics contributed to the majority of the transformations [42]. Enhancement of the degradation process was apparently sufficient within the first wetland channel to remove estrogenicity and to remove toxicity within the first 2 chanels.

Chemistry

The chemical analyses of known and suspected environmental estrogens were conducted on a limited number of samples. A total of four samples per site were analyzed and provided only a limited view of actual exposure. The analyses did show the consistent presence of nonylphenol congeners, DEHP and ethynylestradiol. The average concentrations of estradiol alone would have been sufficient to induce high VTG levels [11,35] even if exposure was intermittent [43]. However, the presence of ethynylestradiol at sites 2 though 4 did not correspond with elevated VTG levels indicating a probable

highly variable exposure, bioavailability, or biotransformation of the estrogens to conjugated inactive forms. Similar results were seen for the nonylphenol congeners and DEHP measurements strengthening the likelihood of variation in the biological availability of the causative agents.

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CHAPTER 3

SUMMER SEASON EVALUATION OF WASTEWATER EFFLUENT TOXICTY AND ESTROGENICITY AND ASSESSMENT OF THE UTILITY OF A CONSTRUCTED WETLAND SYSTEM TO REDUCE OR REMOVE THOSE PROPERTIES USING BIOMARKERS IN MALE FATHEAD MINNOWS (*Pimephales promelas* Rafinesque, 1820).

Abstract

The utility of a treatment wetland to remove toxicity and estrogenicity in final treated wastewater effluent has been demonstrated for effluent studies performed in spring. However, the variable nature of effluents and wetland treatment may alter the efficacy of this method with season. A final municipal wastewaster effluent was characterized in summer in north-central Texas for comparison with spring study results. Vitellogenin, GSIs (gonado-somatic indices), HSIs (hepato-somatic indices) and secondary sexual characteristics were biomarkers used in *Pimephales promelas* models to assess aqueous estrogenicity. Biological indicators used to assess overall fish health included hematocrit and condition factors. The estrogenic nature of final sewage treatment works effluent was screened, concurrent with a 2 week fish exposure, via GC/MS analysis for target estrogenic compounds including: 17-βestradiol, estrone, ethynylestradiol, Bisphenol A, nonylphenolic compounds, phthalates, and DDT. Fish (*P. promelas*) were exposed to four sites in a treatment wetland receiving final effluent, effluent receiving stream system sites, an effluent dilution series and control conditions. Fish condition heavily influenced interpretation of results. Pre-acclimation exposure to spawning stresses may have been

implicated in altering many of the biological markers measured. Results are discussed relative to fish health and pre-exposure environment.

Keywords-Vitellogenin, Xenoestrogens, Biomarker, Wastewater effluent*, Pimephales promelas*

Introduction

Reports of the potential wildlife risk from exposure to environmental estrogens emphasizes the need to better understand both estrogenic presence and persistence in treated wastewater effluents [1-9]. In addition to wildlife exposure, human exposure should also be examined [10] especially in situations when estrogenic effluents may return to a drinking water supply (Chapter 1 and 2). This potential was examined in rivers and reservoirs in the U.K. where reduced estrogenicity downstream from the wastewater outfall and no estrogenicity in reservoirs receiving these waters was found [8]. However, recent research in the U. K. showed increased vitellogenin (VTG) levels, an estrogen induced protein, in male winter flounder not only in estuaries which received treated sewage effluent, but also along and away from the coastline [11]. Additionally, recent research in north central Texas showed no decrease in VTG induction up to 0.63 km downstream of a wastewater treatment outfall (Chapter 2).

The egg yolk protein precursor, VTG, has been used as a biological indicator for xenoestrogens with fish models [8-9, 12-15]. Under natural conditions, only mature

female fish produce significant vitellogenin protein. Estrogen is secreted from the ovaries of a mature female ready for spawning and flows through the blood stream to the liver. When estrogen reaches the liver, it reacts with cytoplasmic steroid receptors in liver cells that trigger the release of VTG, which then travels back through the bloodstream to the ovary where it is phagocytosed by the ova and is transformed into yolk proteins [16]. However, both male and juvenile fish also secrete VTG from their livers if estrogen is provided exogenously. Circulating plasma concentrations of VTG in males and juvenile fish or elevated VTG levels in mature females is an indication of xenoestrogens in the aquatic environment. Plasma vitellogenin concentrations increase by approximately a million-fold during the egg forming portion of the reproductive cycle. The enormous range of potential vitellogenin concentrations provides a biomarker which is very sensitive to estrogenic exposure in fish [8].

Plasma VTG concentrations were used to examine elevated hermaphroditism (intersex) occurrences associated with sewage treatment works effluent in the United Kingdom [8,17]. Caged male trout were placed in 100% effluent and a 300 fold increase in plasma VTG concentration was observed in 1 week. The increase presumably resulted from an estrogenic chemical or mixture of chemicals. The response was observed at all fifteen sewage treatment works sites (100% of sites tested where fish survived). Sumpter (1995) stated the effluent from all sewage treatment works tested has been strongly estrogenic to fish. He reported that the sewage treatment works were of different types and received input of varying composition primarily consisting of domestic influent. It

was further stated that because all effluents proved estrogenic, the domestic portion of the influent was the most probable source of the estrogenicity in that locality.

Recent reports in the United States have shown less conclusive evidence for estrogenic responses from fish exposed to wastewater effluent constituents [18-20]. It has been suggested that differences in wastewater treatment or effluent dilution between the United States and the United Kingdom may be the cause of the apparent difference in effluent estrogenicity. However, a recent study in U.S. has reported high vitellogenin induction resulting from exposure to final wastewater effluent and an effluent dominated receiving stream (Chapter 2).

The previous chapter demonstrated the utility of a treatment wetland system for reduction and removal of wastewater effluent estrogenicity and toxicity via biomarkers in fish. The principles mechanisms by which wetlands degrade effluent constituents are discussed in Chapter 2. High levels of estrogenicity and chronic toxicity were observed in the effluent studied during the early spring and were effectively removed by wetland treatment. The seasonality of these effluent properties was examined in this chapter. Research included a summer season evaluation of wastewater effluent estrogenicity and toxicity and assessed of the efficacy of a constructed wetland system to reduce or remove those properties using biomarkers in male fathead minnows (*Pimephales promelas* Rafinesque 1820).

Materials and Methods

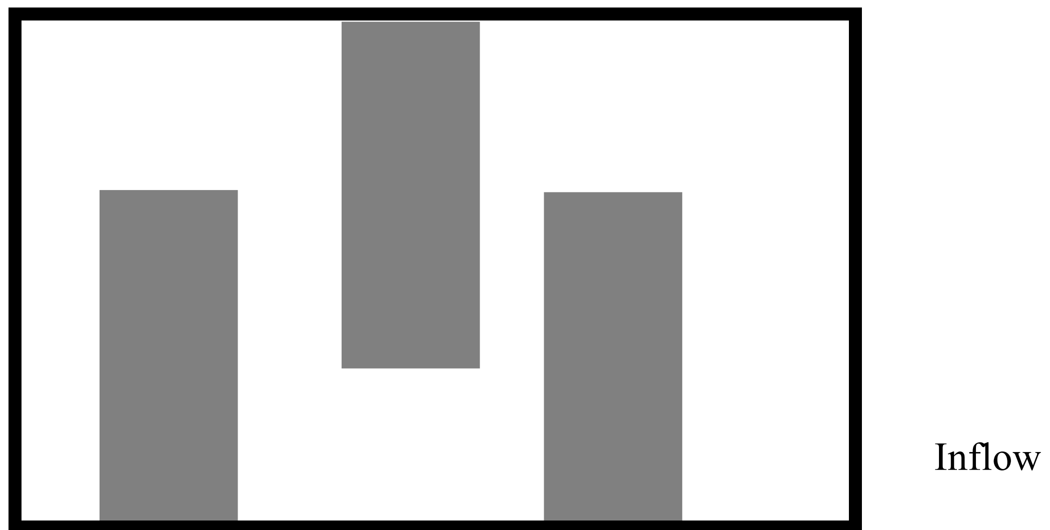
Exposure Scenario

Prior to exposure, adult male fathead minnows (*Pimephales promelas*) were kept in 72L aquaria with flow through activated carbon dechlorinated tap water for a period of 2 weeks. Light was available for 16 hours per day and the temperature was kept at 24-26 C. Fish were fed frozen brine shrimp twice daily. After the acclimation period fish were divided among exposures. Exposures were performed for 2 weeks in summer in north-central Texas with approximate peak wetland macrophyte biomass (June).

Mature, male fathead minnows were exposed to both 100% final treated wastewater effluent sites and control conditions. The fish exposed to effluent were held in 3 replicate aerated cages with 12 fish in each cage and placed in a constructed treatment wetland at 4 sites (Figure 1). Site 1 was located at the inflow of final effluent. Sites 2, 3 and 4 were distributed downstream the wetland in the first 2 wetland channels. Site 2 was located midway down channel 1 of the wetland. Site 3 was located at the end of channel 1 and site 4 at the end of channel 2. No fish were exposed to the end of the wetland at the end of channel 4 (site 4 in March) because of acutely low dissolved oxygen. However, the March study showed the effluent effects were removed by the end of channel 2. The control exposures were performed in the University of North Texas Aquatic Toxicology Laboratory in 3 72 L aquaria containing activated carbon dechlorinated tap water for the same duration as the effluent exposures.

Fifteen male fathead minnows were exposed at sites within the stream system that receives the effluent. The sites included a low flow stream area above the effluent outfall (-50 m), the outfall (0 m), four downstream sites including a low water dam (631 m), riffle area (1,762 m), confluence of the stream and reservoir (5,500 m) and a site in the receiving reservoir at the drinking water intake.

Twenty male fish were exposed to each concentration in a effluent dilution series which included: 100%, 50% and 25% effluent. Dilutions were performed with activated carbon dechlorinated tap water used for control exposures. The effluent dilution exposures were also compared to sulfer dioxide (SO_2) dechlorinated tap water exposures to examine the influence of activated carbon dechlorination on normal tap water chemistry. Tap water was vigorously aerated for 24 hours after the addition of 64 ul of SO_2 saturated deionized water. Static renewals were perfomed daily in 72L aerated aquaria. Exposures were performed at 25°C with a 16:8 light:dark cycle.



Outflow

Figure 1 : Wetland schematic showing exposure sites in water channels separated by 3 land berms.

Fish Endpoints

Length (cm), weight (g), testes weight (g), liver weight (g), hematocrit (% packed cells) and secondary sexual characteristics including number of tubercles, fatpad thickness and stripe density were recorded after exposure. Blood plasma was isolated and vitellogenin quantified at the University of Florida Biomarkers/Protein Chemistry Core Facility as described below. Condition factor (K; weight $\times 10^5$/length^3)[21] and hematocrit (packed blood cell column height/total blood column height $\times$100) were calculated for individual fish to assess fish health. Gonadal-somatic index (GSI; testes weight/total body weight $\times$100) and hepatic-somatic index (HSI; liver weight/total body weight $\times$100) were calculated to assess the physiological effects of estrogenic exposure. Secondary male sexual characteristics were recorded and included tubercle number, fatpad thickness and stripe density. Fatpad thickness and stripe density were recorded semi-quantitatively using a rating system of 0=none, 1=small, 2= moderate, and 3=strong display.

Vitellogenin Quantification

After exposure, blood samples were collected and fish were sacrificed (modification of methods used by Allen *et al.* 1999). Blood samples were taken by severing the caudal peduncle with a razor and collecting blood with a heparinized hematocrit tube. The blood samples in the hematocrit tubes were centrifuged in a hematocrit centrifuge for 3 minutes. After centrifugation, the eluted plasma was removed from the hematocrit tubes with a Hamilton syringe and injected into aprotinin plated 1.5 ml eppendorf tubes. The plasma samples were immediately placed on dry ice and prepared for shipment to the University of Florida Protein Chemistry Research Laboratory for plasma vitellogenin quantification.

Eppendorf tubes were plated with a 10 ul diluted working solution of 0.9% NaCl, 0.9% benzyl alcohol with 3 mg/ml aprotinin (10X solution). The working solution was prepared by diluting 10 ul of the 3 mg/ml solution to 1 ml with distilled water. The Eppendorf tubes containing 10 ul of the working solution were then vortexed to coat the tube and then allowed to dry.
The Eppendorf tubes were refrigerated after drying until the plasma was added (Nancy Denslow, University of Florida).

Collected plasma was quantified for vitellogenin using direct enzyme linked immunosorbant assays (ELISA) as decribed by Denslow *et al.* (1999). The monoclonal antibody 2D3 used for this assay was made specifically against carp VTG, however, it crossreacts with fathead minnow VTG. The direct ELISA required plating a series of VTG standards along with unknown sample VTG on microwell plates. Following the

initial anti-VTG monoclonal antibody 2D3 binding, biotinylated goat anti-mouse IgG polyclonal antibody and strepavidin conjugated alkaline phosphatase were used consecutively. The bound enzyme then converted the substrate mixture of p-nitro blue tetrazolium/5-bromo-4-chloro-indoyl phosphate into a color product that absorbs light at 405nm. By extrapolating the unknown absorbance unit against the standard curve with the known VTG concentration, VTG concentration of exposed plasma was calculated [22].

To obtain concentrations within the standard curve a series of sample dilutions were performed. The samples were first diluted from 1 to 100 and subjected to direct ELISA with a detection limit of 0.5 ug/ml. Samples with higher concentrations than the standard curve range were subjected to further dilutions of 1:10,000, 1:100,000 or 1:1,000,000 and direct ELISA was performed again. Those samples with lower concentrations were repeated with a competitive ELISA at a dilution of 1:20 and 1:50. Competition ELISA is currently more sensitive than the direct ELISA with a minimum detection limit of 0.2 ug/ml of plasma (personal communication Marjorie Chow, University of Florida Protein Chemistry Research Laboratory).

Wetland

Wetland characteristics were measured including flow (m/min), nominal residence time [(t-1)= average storage volume (V)/ total inflow rate (Q1)], vegetation types and relative cover at time of exposure, and channel length, width and depth. Flow was estimated by eleven replicate measures of travel time of cloth dye (royal blue)

through a metered 2 inch PVC half pipe submerged within the vegetation of the first channel at the left, right and center of the channel. Retention time of the wetland was estimated via inflow volume measures relative to the average storage volume and corrected for effluent displacement [23]. Depth was measured at four locations in each channel. Each depth measurement included a measure on each side of the channel and one central depth measure at each of the four areas per channel. Width was measured at the four locations in each channel where depth was measured. Vegetation types were identified and recorded via digital images taken during the exposure and percent coverage was estimated based on visual inspection and using digital images of the plant types in each channel.

Chemistry

General water chemistry measurement were taken periodically at each site including pH (Orion 230A pH Meter), dissolved oxygen (mg/L; YSI Model 51A Oxygen Meter), temperature (°C; YSI Model 85 Oxygen, Conductivity, Salinity and Temperature Meter), alkalinity (mg $CaCO_3$/L; Potentiometric Titration with 0.1 N H_2SO_4 to a pH of 4.5), hardness (mg $CaCO_3$/L; Colorimetric Titratrion with0.02 N Ethylenediamine tetracetic acid (EDTA) and calmagite) and specific conductance (umho/cm at 25°C; YSI Model 33 Conductivity Meter).

The nature of the estrogenic components were assessed via GC/MS analysis of the effluent to quantify levels of known estrogens, estradiol, estrone and the synthetic ethynylestradiol, and suspected estrogen mimics or disruptors including: 5 nonylphenol congeners, di-2-ethylhexylphthalate (DEHP), bisphenol A, dichloro-diphenyl-trichloroethane (DDT) and atrazine. Structural similarities prompted the quantification of related compounds including :dimethylphthalate, di-n-butylphthalate, butylbenzylphthalate, di-n-octylphthalate and phthalic anhydride. The analytical technique was similar to EPA methods 625 and 8270 (applicable to wastewater) but extends lower detection limits to concentrations more typical of EPA s method 525 (applicable to drinking water).

Four composite samples were taken the during the exposure period from each site. Composites samples included five 800 ml aloquats from grab samples taken 5 continuous days. The fifth set of water samples were single day grab samples. The single day samples were used to assess the degradation of natural estrogens in the composites. Samples were placed in 4 L amber bottles at 4°C until the fifth aloquat was collected. Composite samples were received in the chemistry laboratory the morning completed. Single day grab samples were received the morning collected. Extractions began immediately.

Five hundred ml samples were acidified with sulfuric acid to a pH of 2-3 and pulled by a vacuum through a 200 mg Waters HLB solid phase extraction cartridge. Cartridges were conditioned with 6 ml MTBE (methyl-tert-butyl-ether) followed by 6 ml of methanol followed by 6 ml deionized water prior to extraction. Extraction time varied

from 2-6 hours depending on suspended solid content of the water. Some samples required 2 cartridges to complete the 500 ml sample volume. In an effort to reduce problems associated with suspended materials, cartridges used for the final 3 sampling dates were modified by the addition of approximately 1 cm of deactivated fused silica wool to the head of the cartridge. After extraction, the cartridges were rinsed with 6 ml of 5% methanol in water, then analytes were eluted with 8 ml of MTBE. Water was removed from the eluate with a pipette and remaining MTBE was dried through a sodium sulfate column (10mm X 50mm) and blown down under nitrogen to a final extraction volume of approximately 100ul. Surrogate standards and matrix spike standards were added to the water samples prior to extraction. Internal standards were added to the final extract prior to analysis via GC/MS.

Final quantitative instrumental analysis was with Hewlett Packard (HP) 5890 Series II GC, HP 5970 MS and HPO Enviroquant quantitative software. Analytes of interest were compared to a five-point internal standard calibration curve with a concentration range from 5-100 ppm (extract concentration) which corresponds to a 1-20 ppb concentration range in the original sample (concentration factor = 500 ml original sample volume/0.1 ml(100ul) final extract volume = 5,000).This compares to an estimated quantitation limit (EQL) of 10 ppb for similar analytes using EPA 8270. Matrix spikes were added at the EQL of 10 ppb and surrogate spikes were added at 20 ppb.

Specific analytical conditions were: Column=DB-5.625, 30 m X 0.25 mm ID with 0.25 micron thickness; Injector temperature= 260°C; oven temperature program=40°C

for 5 minutes, 10°C/minute ramp rate, final temperature=300°C held for 14 minutes; MS=electron inpact ionization, scanned from 35-500 amu; Injection Volume=2-4 ul.

All results are listed as ppb (ug/L) in the original sample except for the nonylphenols. The family of congeners representing the nonylphenols was represented by five peaks and the average (not sum) of all five values is the best estimate of total nonylphenol concentration for a given sample. All values listed have been validated by matching retention time and mass spectral charateristics of authentic standards analyzed under the same conditions. Values below 1 ppb fell below the lowest standard and below EQL and should be considered more approximate than values above 1 ppb. Values less than the EQL of 1 ppb were reported as non-detectable (ND) .

Statistics

All analyses were carried out using SAS Version 7 (SAS Institute Inc). Statistically significant differences were accepted at =0.05. Data were analyzed with parametric General Linear Procedures when the assumption of normality and homogeneity were met. The Student Newman Kruels (SNK) or Tukey multiple range test was used when differences were found. When parametric assumptions were not met, the non-parametric Kruskal-Wallis analysis was used with Dunnett s multiple range test on ranked data when differences were found. Data are presented as means plus and minus 1 standard error on the mean (SE).

Results

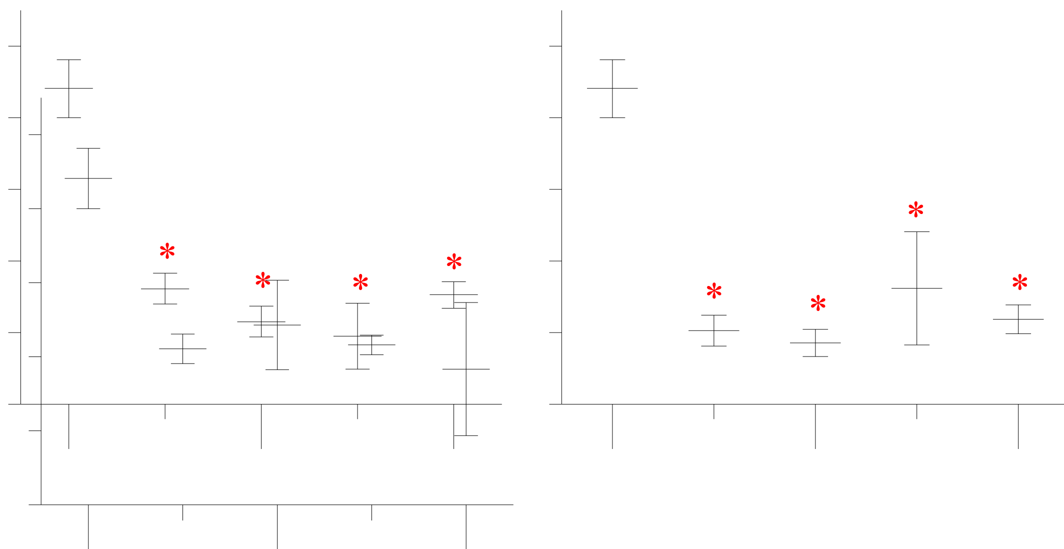
Fish Endpoints

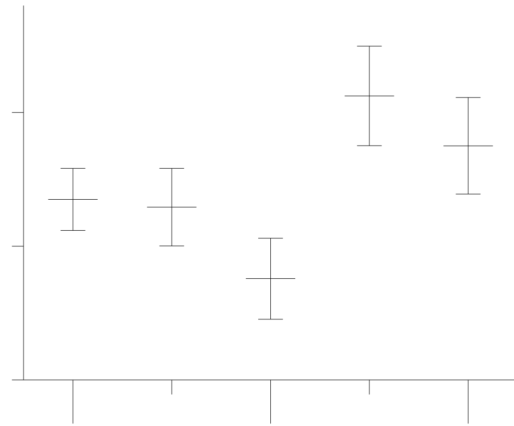
Survivorship during wetland exposures was less than previously observed (Chapter1 and 2) with the control at 92% survival, site 4 at 78%, site 2 at 75%, site 1 at 67% and a low of 56% survival at site 3. Two stream sites were lost to vandalism, the riffle (1,762 m) and confluence sites (5,500 m). The remaining sites suffered high mortality resulting in 67% survival upstream, 87% at the outfall and 47% at the low water dam (631m downstream). Dilution series exposures showed 90%, 30%, 80% and 15% survival for the SO_2 dechlorinated tap water, 25% effluent, 50% effluent and 100% effluent, respectively. Mortality was recorded daily and occurrences were uniform through exposure indicating a steady decline in health and resulting in significant mortality (Fisher s Exact Test, =0.05).

Condition factor (K) and hematocrit values for fish exposed to effluent are shown in Figures 2 and 3. Condition of fish exposed to effluent at wetland sites were all less than controls (Kruskal-Wallis; $p<0.0001$; Dunnett s ranked MRT). Hematocrit values were significantly greater at site 1 than controls (GLM; $p<0.0001$), but were not different from hematocrits of fish exposed to wetland sites 2, 3 or 4 (SNK). The decrease in condition was driven by significant loss of weight over the 2 week exposure period (Kruskal-Wallis; $p<0.0001$; Dunnett s ranked MRT) and the corresponding increase in hemotacrit may have been driven by that reduction in body volume. Nevertheless, the

poor survivorship and relatively ambiguous results may negate their interpretation relative to effluent exposure except to illustrate the importance of pre-exposure condition.

Condition of fish exposed to stream sites were also significantly less than controls (Kruskal-Wallis; p<0.0001; Dunnett s ranked MRT). And condition of fish exposed to the effluent dilution series (including the SO_2 dechlorinated tap water) showed the same result with the exception of the 100% effluent exposure which was not different than controls (Kruskal-Wallis; p=0.0003; Dunnett s ranked MRT). No differences between hematocrit values of controls and stream exposed fish were found. Dilution series exposed fished showed only fish exposed to SO_2 dechlorinated tap water had significantly elevated hematocrit values (Kruskal-Wallis; p=0.002; Dunnett s ranked MRT). Similar reductions in weight of effluent exposed fish were observed in all stream and reservoir exposed fish (Kruskal-Wallis; p<0.0001; Dunnett s ranked MRT) and dilution series exposed fish (Kruskal-Wallis; p=0.001; Dunnett s ranked MRT).





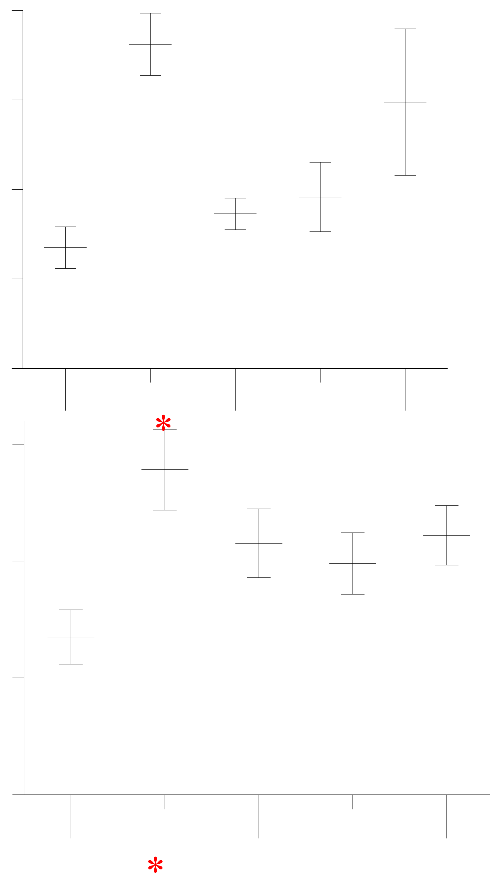


Figure 2: Fish condition factor (K) mean ± 1 standard error with Dunnett s non-parametric multiple range test differences (*).

Figure 3: Fish hematocrit value mean ± 1 standard error with Students-Newman-Keuls parametric (wetland) or Dunnett s non-parametric (stream and dilution series) multiple range test differences (*).

GSI and HSI values for effluent exposures are shown in Figures 4 and 5. No differences in GSI were found for any exposure despite significantly smaller testes weight in wetland (Kruskal-Wallis; p=0.0005) and stream system exposures (Kruskal-Wallis; p=0.0008). Decreases in weight relative to controls negated these declines in testes weight resulting in significant reductions in GSI. However, significant decreases in liver weights for wetland (Kruskal-Wallis; p<0.0001), stream (Kruskal-Wallis; p<0.0001), and dilutions series exposed fish (Kruskal-Wallis; p=0.0007) were sufficient to result in significantly lower HSI values despite lower fish weights. All wetland exposed fish showed significantly lower HSIs than controls (GLM; p<0.0001 with Tukey s MRT). Similarly, all stream exposed fish showed significantly reduced HSIs relative to the control (Kruskal-Wallis; p<0.0001, Dunnett s ranked MRT). However, only the SO_2 dechlorinated tap water and the 25% effluent exposures were different than control HSIs (Kruskal-Wallis; p=0.002, Dunnett s ranked MRT).

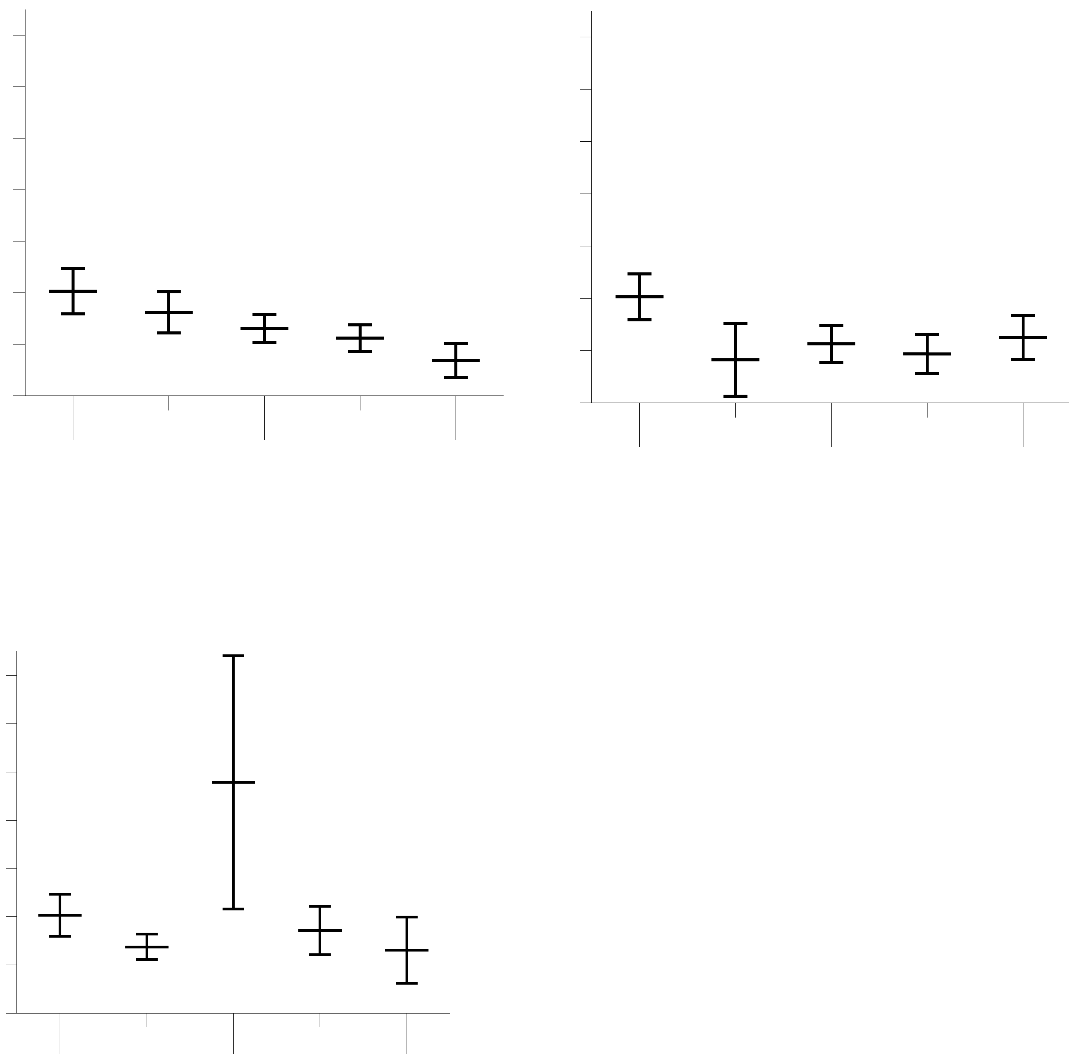


Figure 4: Fish Gonadosomatic Index (GSI) mean ± 1 standard error with Dunnett s non-parametric multiple range test

differences (*).

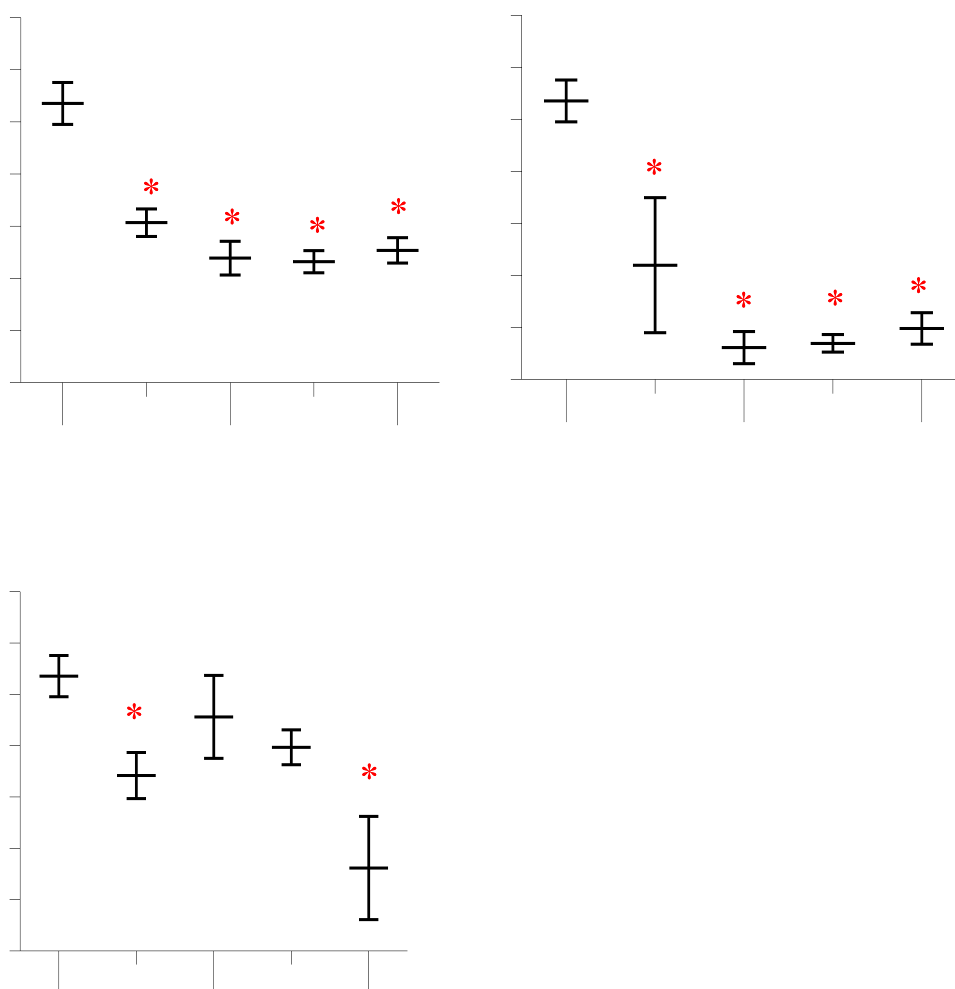


Figure 5: Fish Hepatosomatic Index (HSI) mean $\pm$ 1 standard error with Students-Newman-Keuls parametric (wetland) or Dunnett s non-parametric (stream and dilution

series) multiple range test differences (*).   .

Secondary male sexual characteristics for effluent exposures are shown in Figure 6 A-C. Tubercle number was significantly less in fish at all sites in the wetland (Kruskal-Wallis; p=0.0004 with Dunnett s MRT), stream system (Kruskal-Wallis; p<0.0001 with Dunnett s MRT) and in dilutions series exposures (Kruskal-Wallis; p=0.0004 with Dunnett s MRT). Fatpad densities were similarly reduced in all exposures, however, stripe density was not moderately displayed in the controls or any treatment. The reduction in secondary sexual characteristics is likely related to the poor condition and health of the fish, therefore, separating the effects of exposure from fish condition may not be possible with the available data.

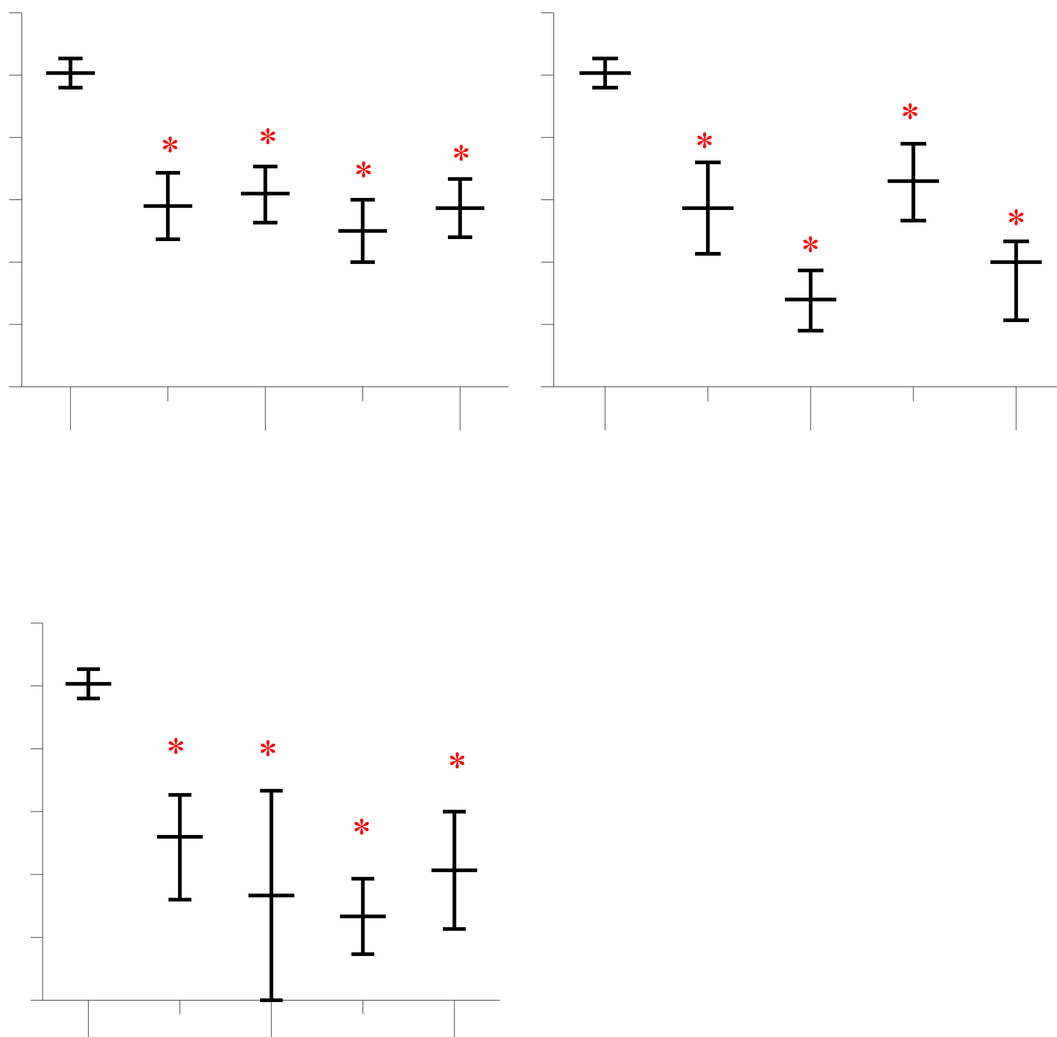
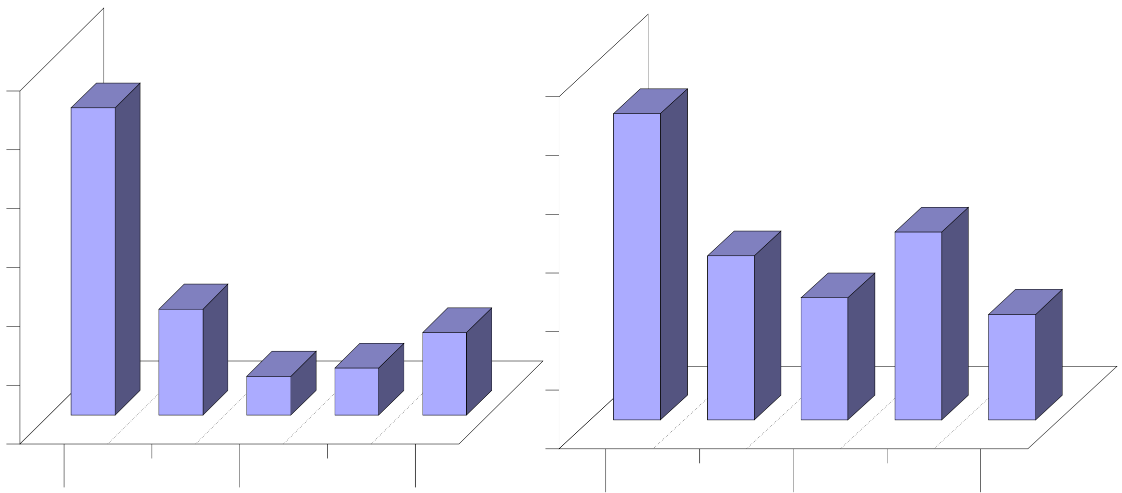
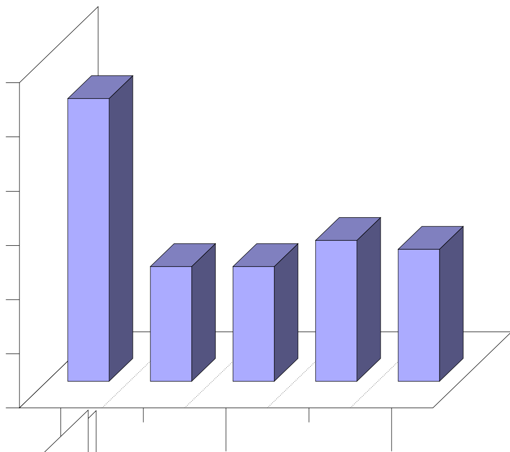


Figure 6A: Tubercle number mean $\pm$ 1 standard error with Dunnett s non-parametric multiple range test differences (*).





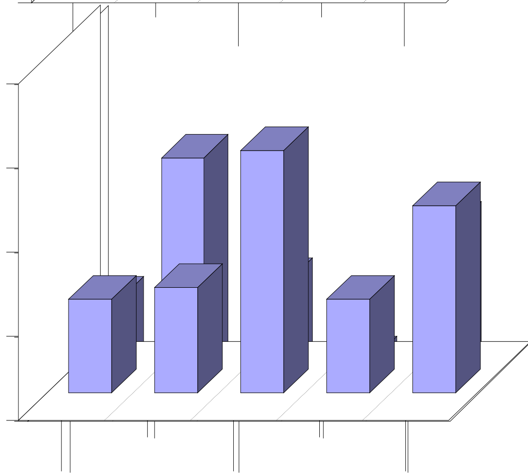


Figure 6B: Fatpad thickness mean.

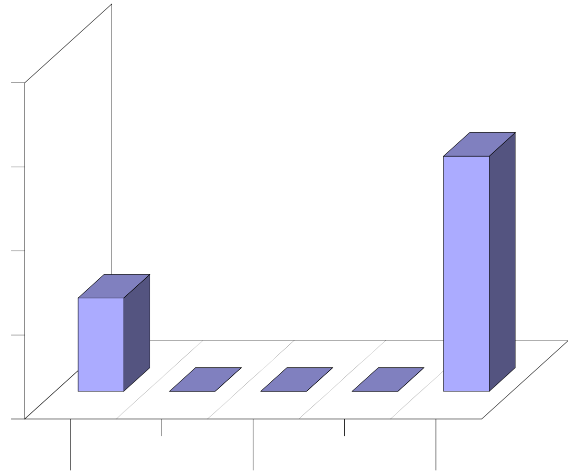


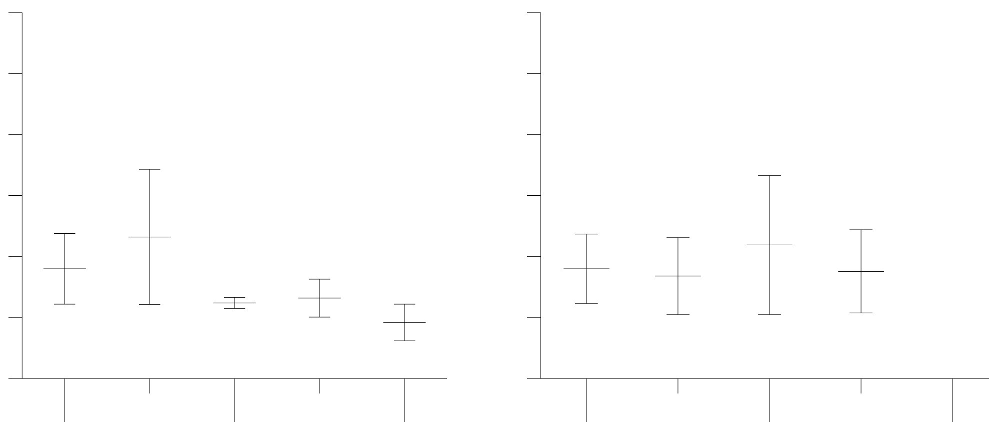
Figure 6C: Stripe density mean

Vitellogenin Quantification

Vitellogenin (VTG) levels were not found to be different for any exposure (Figure 7). Elevated control levels of VTG were seen with 28% of control samples showing measurable concentrations resulting in a mean of 6.3 ug/L plasma VTG (SE 3.8 ug/L). Mean plasma concentration at site 1 was higher at 20.7 ug/L VTG (SE 12.7 ug/L) but was caused by only 17% of fish with measurable levels. Mean plasma VTG was lower at wetland sites 2 through 4, but variable numbers of fish responded (33%, 11%, 17%, respectively).

Similar data were obtained from fish exposed to stream system sites and the dilutions series. A 30% response in fish exposed upstream caused a mean level of 4.8

ug/L VTG (SE 4.3 ug/L), a 20% response at the outfall resulted in a slightly higher mean of 15.4 ug/L VTG (SE 13.8 ug/L), low water dam site fish showed a 20% response with a mean level of 5.7 ug/L VTG (SE 4.8 ug/L) and the reservoir site fish showed no measurable responses. In the dilution series, SO_2 dechlorinated tap water exposed fish showed a 10% response with a mean of 1.9 ug/L VTG (SE 2.0 ug/L), 100% effluent exposure resulted in a 67% fish response with a mean of 139.3 ug/L (SE 132.9 ug/L), 50% effluent exposed fish showed a 20% response with a mean level of 0.5 ug/L VTG (SE 0.4 ug/L) and fish exposed to 25% effluent demonstrated a 17% response with a mean of 0.5 ug/L VTG (SE 0.5 ug/L). However, the higher level in the 100% effluent exposure was strongly influenced by 1 high data point as only 3 fish were available for analysis. Although the percent of fish with measurable levels were similar among experiments, the variability in response was greater than previously observed (Chapter 1-2) and likely was influenced by the environmental conditions to which the fish were exposed prior to acclimation in the laboratory and experimental exposure.



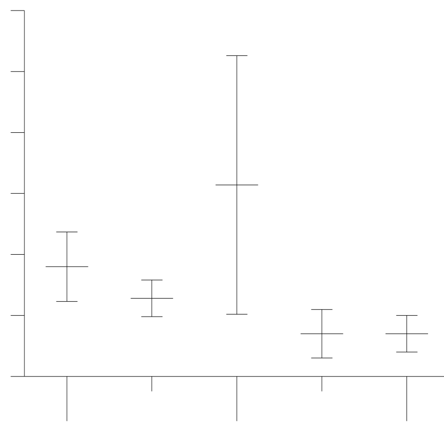


Figure 7: Vitellogenin levels (log VTG ug/ml) in male fish; mean $\pm$ 1 standard error with Dunnett s non-parametric multiple range test differences (*).

Wetland

Characterization of the hydrology of the wetland is discussed in Chapter 2. Emergent macrophyte type varied with wetland channel (Figure 8). Channel 1, which had increased in coverage and diversity since March, again possessed the greatest diversity of plants including *Pontederia* (pickerelweed), *Scirpus* (bullrush), *Sagittaria* (arrowhead), *Ludwigia*, *Ceratophyllum* (coontail) and *Lemna* (duckweed). Channels 2, 3 and 4 were increasingly dominated by *Typha* (cattails), relative to the March sampling period, with *Lemna* and *Ceratophyllum* covering open areas. The area of channel 1 was 40% inhabited by *Pontederia*, 25%

Scirpus, 25% *Sagittaria*, 5% *Ludwigia* and 5% open, but containing dense floating vegetation (*Lemna* and *Ceratophyllum*). Coverages during the June sample were approximately complete constituting an total emergent plant coverage area in channel 1 of 95%. Channel 2 was 90% inhabited with *Typha* with 100% coverage within that area and *Lemna* and *Ceratophyllum* heavily covering the remainder. Channel 3 was 75% inhabited with *Typha* at the same density as channel 2 with *Lemna* and *Ceratophyllum* covering the open areas. Channel 4 was 50% inhabited with *Typha* at a coverage of approximately 100% within the inhabited area and *Lemna* and *Ceratophyllum* in the remaining area.

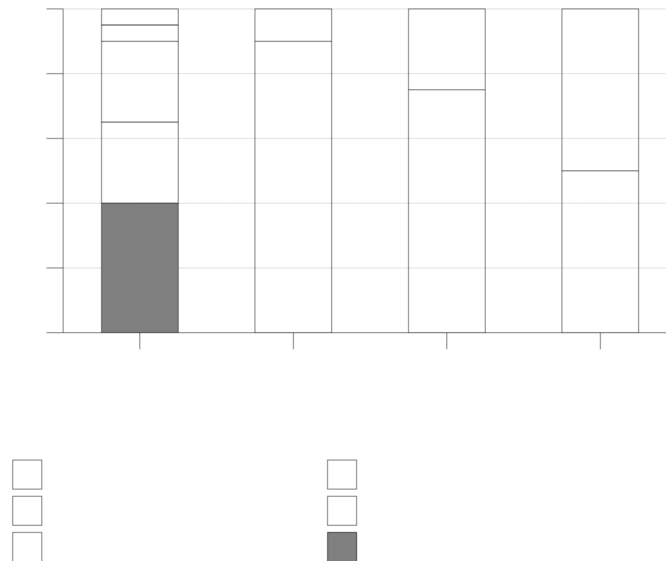


Figure 8: Wetland percent coverage by channel in June, 2000.

Chemistry

The wetland was characterized in June at near peak biomass in warmer temperatures (Table 1). Aqueous temperatures varied from 22.1°C to 27.7°C in the wetland but were relatively constant in the laboratory at 25±1°C. Exposure sites were different in temperature (Kruskal-Wallis p<0.0001) with site 1 (26.4°C) being significantly warmer than the control (25.5°C) and site 4 (24.5°C) significantly cooler (Dunnett s Nonparametric MRT, =0.05). Measurements of pH were lower at wetland sites (7.12 to 7.46) than in the control (7.88) exposures (Kruskal-Wallis p=0.0005, Dunnett s Nonparametric MRT, =0.05) but were all within an acceptable range. Differences in dissolved oxygen readings were found between the laboratory control (~7 mg/L) and wetland sites ranging from 1.6 to 5.6 mg/L (Kruskal-Wallis p<0.0001, Dunnett s Nonparametric MRT, =0.05). However, oxygen concentrations at wetland sites were kept elevated above ambient with aeration within the fish cages. Specific conductance (umho/cm at 25°C) was higher at wetland sites (742 to 787) than control (~470) samples (Kruskal-Wallis p<0.0001; Dunnett s Nonparametric MRT, =0.05). Differences in alkalinity (mg $CaCO_3$/L) were found between site 4 (129) and site 3 (112) and compared to the remaining exposures at site1 (95), 2 (98) and the control (97) exposure (GLM; p<0.0001; Tukey MRT). Hardness (mg $CaCO_3$/L) of samples from wetland sites (151 to 160) were significantly higher than control water (118) hardness (Kruskal-Wallis p=0.0015; Dunnett s Nonparametric MRT, =0.05).

Table 1: General water chemistry measurements at wetland sites during June 2,000.

Significant differences from control shown (S) from Dunnett s ranked MRT.

Quartiles	Site01	Site02	Site03	Site04	Control
Temperature (°C)					
Mean	26.4	26.0	25.2	24.5	25.5
Std. Deviation	0.7	0.8	1.3	0.8	0.5
Significance	S			S	
Sample size	15	15	15	15	15
pH					
Mean	7.2	7.3	7.4	7.5	7.9
Std. Deviation	0.2	0.1	0.2	0.1	0.1
Significance	S	S	S	S	
Sample size	15	15	15	15	15

Dissolved Oxygen (mg/L)					
Mean	5.6	3.9	2.8	1.6	7.1
Std. Deviation	0.6	0.9	2.6	0.5	0.5
Significance	S	S	S	S	
Sample size	15	15	15	15	15
Specific Conductance (umho/cm at 25°C)					
Mean	786.7	786.9	778.4	742.2	469.1
Std. Deviation	28.5	28.2	37.6	170.7	8.3
Significance	S	S	S	S	
Sample size	15	15	15	15	15

Table 1 continued: General water chemistry measurements at wetland sites during June 2,000. Significant differences from control shown (S) from Dunnett s ranked MRT.

Quartiles	Site01	Site02	Site03	Site04	Control
Alkalinity (mg $CaCO_3$/L)					
Mean	95.3	98.6	111.7	129	96.5
Std. Deviation	3.4	4.5	3.7	4.7	4.8
Significance			S	S	
Sample size	15	15	15	15	15
Hardness (mg $CaCO_3$/L)					
Mean	151	156.2	153.0	159.5	117.7
Std. Deviation	6.3	7.0	6.7	6.0	3.2
Significance	S	S	S	S	

Sample size	15	15	15	15	15

Concentrations of known and suspected environmental estrogens were monitored throughout the exposure period (Figure 9A-E). Estradiol, estrone, ethynylestradiol and DDT were not detected in any samples analyzed. Di-2-ethylhexylphthalate (DEHP), dimethylphthalate, di-n-butylphthalate, butylbenzylphthalate and di-n-octylphthalate were detected at concentrations below 1ug/L (range 0.1-0.7 ug/L) in some samples from each date. Phthalic anhydride was consistently measured in samples and ranged from 0.0 to 18.8 ug/L. Nonylphenol was also frequently detected and was consistently found in all five congeners analyzed (mean nonylphenols range, below detection- 4.1 ug/L). The average of the five nonylphenol congeners is provided as a conservative estimate of total nonylphenols in the sample. Bisphenol A was detected infrequently (range, below detection-0.8ug/L). The herbicide atrazine, suspected of interfering with estrogen metabolism and not direct estrogen site binding, was regularly detected at concentrations ranging from below detection to 0.6 ug/L.

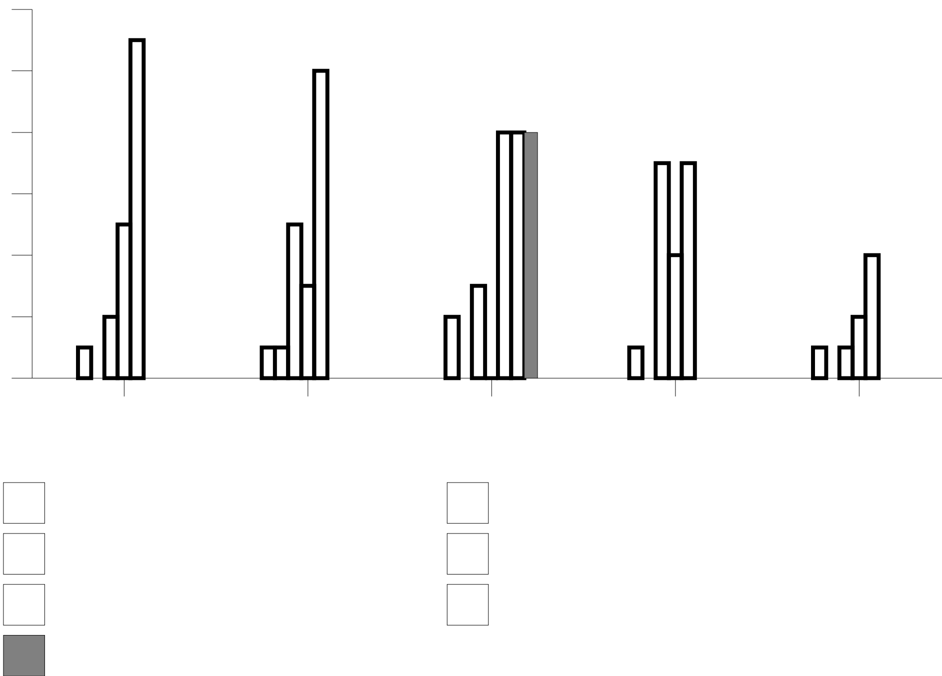


Figure 9A: Estrogenic chemicals in the June 12, 2000 composite samples.

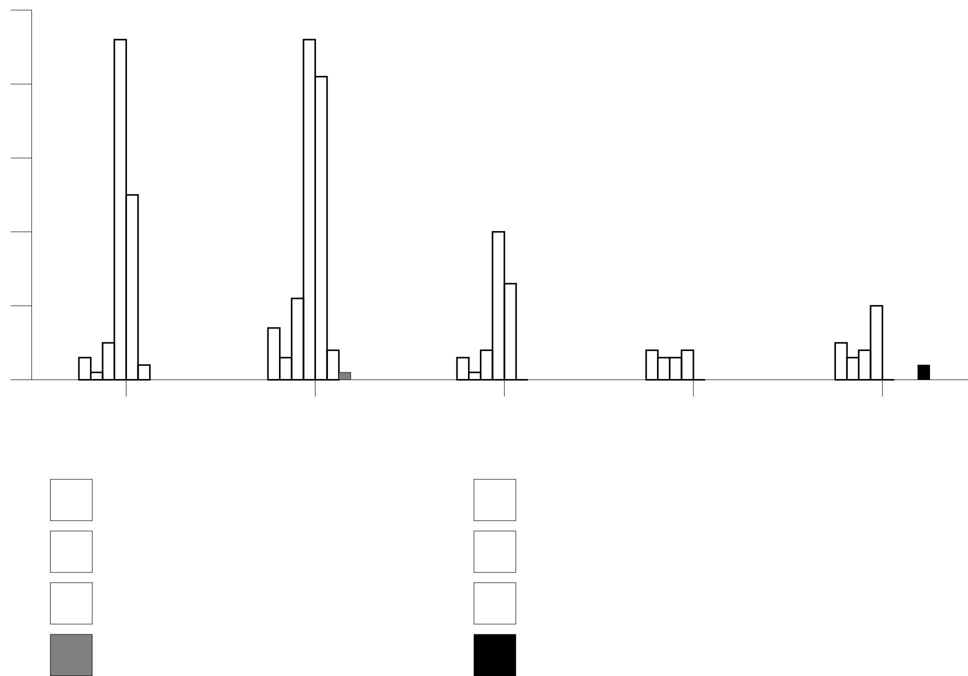


Figure 9B: Estrogenic chemicals in the June 17, 2000 composite samples.

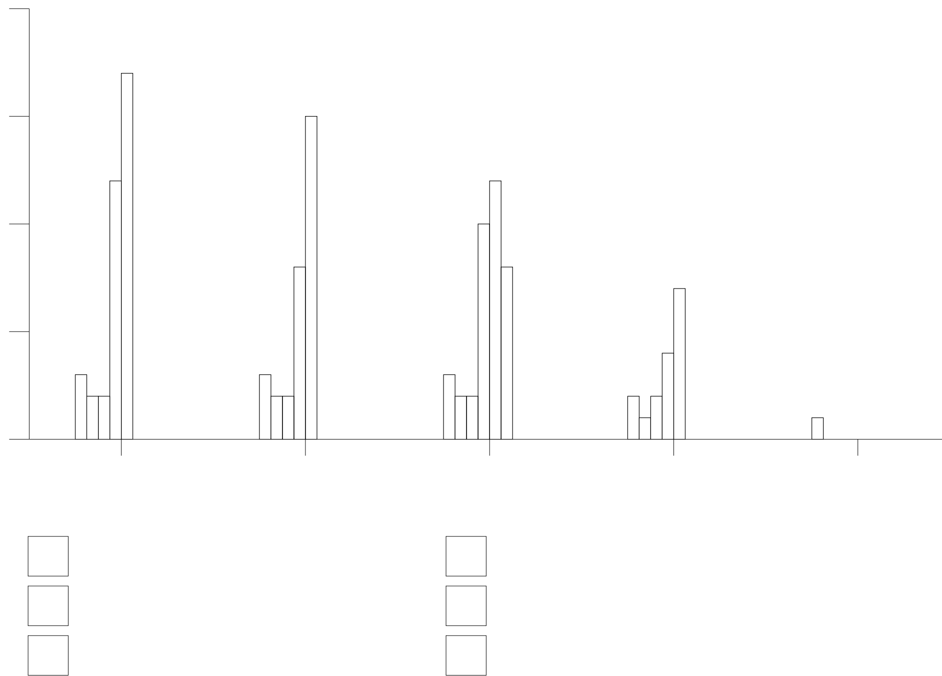


Figure 9C: Estrogenic chemicals in the June 22, 2000 composite samples.

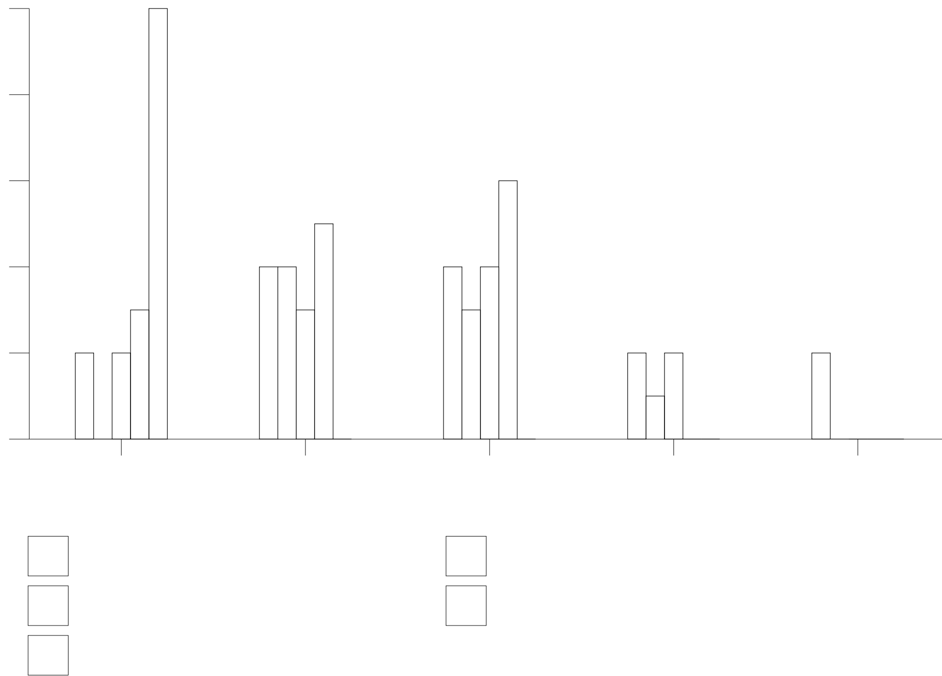


Figure 9D: Estrogenic chemicals in the June 26, 2000 composite samples.

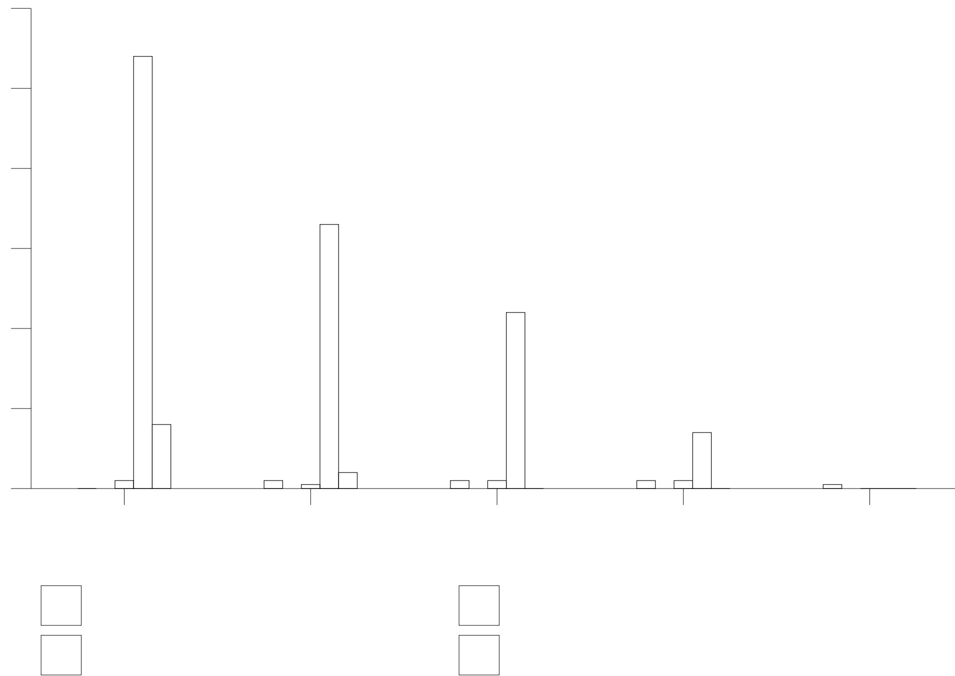


Figure 9E: Estrogenic chemicals in the June 28, 2000 single day samples.

Average analyte concentrations were used to track chemicals through the wetland system. Two distinct trends were evident and were represented by 1) Figure 10A; mostly phthalate derivatives (di-2-ethylhexylphthalate (DEHP), dimethylphthalate, di-n-butylphthalate, butylbenzylphthalate, di-n-octylphthalate and bisphenol A) and 2) Figure 10B; other organic analytes measured (5 nonylphenol congeners, phthalic anhydride, atrazine). In the first trend, an increase in concentration apparently occurred after the inflow of the wetland followed by a subtle decline. In the second trend a steady decrease in concentration was observed with wetland treatment for nonylphenols and phthalic

anhydride, and to a lessor extent atrazine.

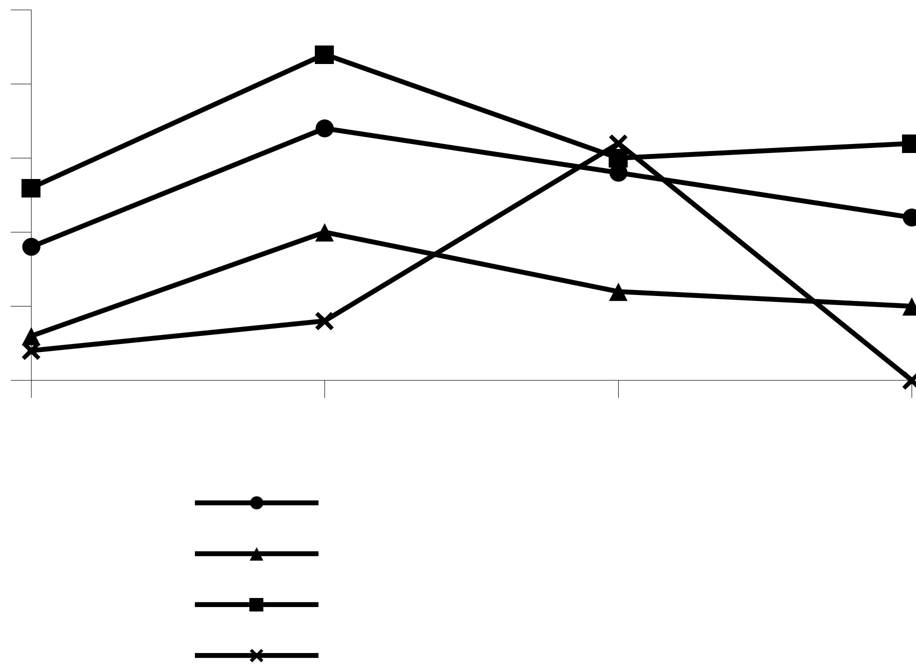


Figure 10A: Estrogenic chemical degradation trend 1 with wetland treatment.

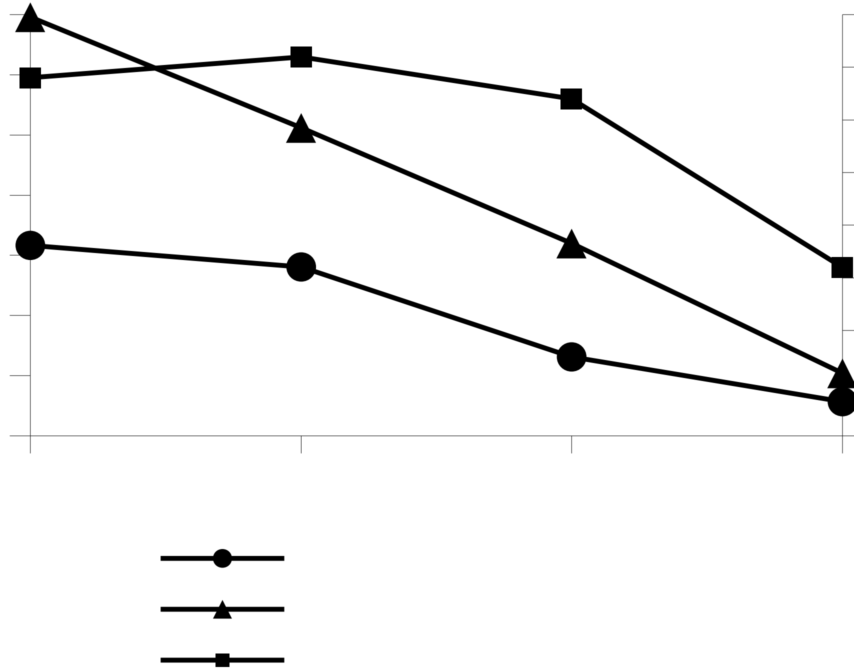


Figure 10B: Estrogenic chemical degradation trend 2 with wetland treatment.

Discussion

Fish Endpoints

Elevated mortality in all tests performed, compared to previous studies (Chapter 1-2), weighed heavily in the interpretation of results. Investigation into the cause of increased mortality led to the conclusion that spawning activity stress led to a weakened state, susceptibility to disease and high mortality. Personnel at the hatchery from which the fish were purchased noted that this is a typical spring and summer season occurrence for male *P. promelas* cultured in ponds (personal communication from supplier, Kertz s Fish Hatchery). The influence of spawning stress had not been previously observed as fish were laboratory raised with controlled spawning exposure (Chapter 1) or purchased during non-spawning periods (Chapter 2). However, four unsuccessful attempts were made from mid to late spring to obtain disease free fish for this study.

The influence of low survivorship was evident in surviving fish condition factors after the additional stress of exposure was endured. The condition of fish, shown to be less in all treatments save one, would likely manifest itself in altered expression of all other parameters measured. Fish condition evaluates the relationship of fish weight to length. Rapid weight losses during the 2 week exposure was indicative of high stress in the exposed animals. Previous fish exposures of this type (Chapter1-2) have not resulted in rapid weight losses over the exposure period. Weight loss was therefor attributed to factors other than nutrition such as spawning stress.

Assessment of other fish endpoints with respect to exposure was thought to be

inappropriate in the presence of poor survivorship and condition and in the absence of toxicity which was measured with other methods during the exposure period (Chapter 4). The baseline health of fish could have directly influenced all other fish endpoints measured. Hematocrit levels appeared higher with most effluent exposures. However, the SO_2 dechlorinated control also showed elevated hematocrit. These apparent hematocrit elevations may have been an artifact of rapid weight loss. Changes in resources allocated to certain physiological functions would also likely be different in unhealthy, stressed fish. It is not likely that fish in poor condition would physiologically allocate significant resources to reproduction and decreases in testes weight would result. Losses in testes weight were masked in GSIs by lower body weight. Loss in liver weight seemed to occur at an accelerated rate with respect to body and testes weight loss. Significantly lower HSIs were observed, but was not attributed to exposure because this occurred without any observable exposure-response trend. The reduction in secondary sexual characteristics was pronounced for tubercle number and fatpad thickness, but not for stripe density because of low occurrence of control stripe displays. Reduction in testes weight would result in overall fewer cells to secrete androgens responsible for expression of secondary sexual characteristics [24].

Vitellogenin Quantification

Concentration of plasma vitellogenin (VTG) can also be influenced by fish condition

because of changes in physiological resource allocation (pers comm. Nancy Denslow, University of Florida Protein Chemistry Research Laboratory). The apparent increases in plasma VTG in fish exposed at wetland site 1, stream outfall and to the 100% dilution series concentration were similar to results from previously conducted 2 week, summer season exposures to this effluent (Chapter 1). However, the expression was highly variable. Measurable concentrations of VTG were found in 17% of fish at wetland site 1, 20% at the outfall and 67% in the 100% effluent dilution series exposure concentration. Measurable levels of VTG were found in 28% of control plasma samples resulting in a mean concentration 1 order of magnitude higher than previously observed (Chapter 1-2). The possible increases in each of the above mentioned exposure groups resulted from a few fish in each group with higher VTG levels, especially in the case of the 100% dilution series treatment in which only 3 fish survived. Previous studies of the estrogenicity of this effluent have shown 60% of fish to have measurable VTG levels after 2 weeks of exposure during this season, 100% after 3 weeks, and no measurable levels in the controls (Chapter 1). Interpretation of VTG induction results in the presence of detracting variables was thought to be inapplicable and circumspect to poor fish condition.

However the presence of elevated VTG occurrences in the control fish did more than increase background levels for comparison, it also demonstrated the pre-acclimation environment of these fish with respect to estrogenic exposure. VTG levels in male fish have been shown to be elevated in the presence of breeding females [25-26]. The elevated control VTG levels (6.3 ug/L) indicate exposure to a source of estrogen.

Considering season and the previous healthy fish received from this provider, the estrogenic source was likely high densities of breeding females. Under natural environmental conditions minimal induction of vitellogenesis (less than 0.5 ug/L) occurs in male fish [27]. However, these pond raised fish were likely kept at densities higher than would naturally occur. Once induced to higher levels vitellogenesis, the clearance of VTG from fish plasma can take up to 5 months after the estrogenic cue is removed [28]. The elevated control VTG levels indicate that these male fish were exposed to spawning related stresses prior to testing.

Wetland

The wetland was characterized during fish exposures in early summer with peak macrophyte biomass. The emergent and floating vegetation had increased greatly since last characterization in March of this same year. The influence of summer season wetland treatment on effluent toxicity and estrogenicity was not ascertained during this portion of the research because of the above mentioned occurrences. However, an effluent toxicity assessment was performed and wetland reduction of toxicity was evaluated in the following chapter.

Chemistry

Differences in general water chemistry between this and previous exposures were higher temperatures and lower dissolved oxygen (DO) levels. Temperature in the wetland was significantly higher at site 1 only (0.9°C warmer). Temperature was not different at any

other wetland site than control temperature. Dilution series exposures were performed at the same temperature as the controls (25°C) but also showed reduced condition factors. Temperature was not thought to have caused undue stress during exposures. DO concentrations were significantly less at all wetland sites. However, aeration within the cages has previously compensated for the lower ambient DO. Comparison with the more controlled laboratory dilution series exposures showed DO levels to be similar between the dilution series (6-8 mg/L) and the controls (6.6-7.6 mg/L) despite uniform reductions in condition factors among exposures in the wetland, stream and to the effluent dilution series in the laboratory. DO was not thought to influence fish condition to the degree observed in this study alone or with the increased temperature at site 1.

Chemical analyses of known and suspected environmental estrogens showed no typical estrogens. The absence of ethynylestradiol, the active ingredient in female oral contraceptives, is noteworthy as it has been consistently detected in this effluent during the spring (Chapter2). Numerous suspected and possible estrogenic chemicals were detected routinely and included 5 nonylphenol congeners, numerous phthalate derivatives, bisphenol A and the endocrine disrupting herbicide, atrazine. However, measured concentrations were lower than have been associated with estrogenic activity [29-30] and potential for further evaluation was negated by poor fish condition.

The fate and transport of measured analytes was examined in the wetland system. Two apparent degradation trends existed, including: 1) phthalate derivatives and bisphenol A and 2) the nonylphenol congeners, atrazine and phthalic anhydride (a phthalate precursor). Measured levels of the first group of chemicals showed increased

concentration after entering the wetland indicating a possible additional source of the chemicals therein or possible degradation from unknown parent compounds. The nonylphenol, atrazine and phthalic anhydride levels demonstrated an apparent trend of decreasing concentration with distance from the inflow of the wetland. A detailed study of fate and transport would be important to understanding actual degradation processes within the wetland system including expanded sampling times, seasons and analytes.

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CHAPTER 4

ASSESSMENT OF TOXICITY REDUCTION IN WASTEWATER EFFLUENT FLOWING THROUGH A TREATMENT WETLAND USING *Pimephales promelas, Ceriodapnia dubia* AND *Vibrio fischeri.*

Abstract

Effluent toxicity is regularly assessed with *Ceriodaphnia dubia* short-term chronic toxicity tests. To assess the ability of a constructed wetland to remove toxicity of a municipal effluent, laboratory *C. dubia, Vibrio fischeri* and bacterial adenosine 5 triphosphate (ATP) tests were performed in the laboratory, and caged *Pimephales promelas* were exposed *in situ*. Final whole effluent was diverted to a constructed wetland and effluent samples were taken daily from four sites, at incremental distances from the inflow, for a 3-week study. Overlapping 7-d *C. dubia* tests, *V. fischeri* assays, and bacterial ATP assays were conducted with samples from each wetland site concurrent with 2 and 3 week fish exposures. Measured endpoints in fish included condition factor and hematocrit. Significant (<0.0001) reductions in survival and fecundity of *C. dubia* were evident at the inflow, but steadily improved with distance from the inflow. Health of exposed fish was lower (□=0.001) during the 3 week exposure at wetland sites closer to the inflow than in controls. *V. fischeri* assays indicated no reduction in effluent toxicity relative to controls. Bacterial ATP measures in wetland samples were higher than controls (<0.0001). A comparison of results among methods, exposure season and wetland sites is provided.

Key Words- *Pimephales promelas, Ceriodaphnia dubia, Vibrio fischeri*, Municipal Effluent, Constructed Wetland

Introduction

Comparative studies of methods for measuring toxicity in municipal and industrial effluents have provided information about the relative strengths of certain tests and variability of effluents [1-4]. Most found organism sensitivities to complex effluents to differ from that derived from laboratory test with single chemicals or simple mixtures. Some have suggested that effluent complexity often preempts the feasibility of chemical identification because of large financial and resource requirements, and that chemical identification is not necessary to assess toxicity [5-6].

Effluent toxicity is regularly assessed using juvenile fathead minnows, *Pimephales promelas*, and the cladoceran, *Ceriodaphnia dubia* [7]. The 7-day *C. dubia* partial life cycle test provides organism survival and fecundity data. Studies comparing results from *Vibrio fischeri* assays (formally MicroTox®) and *C. dubia* tests have shown agreement [2, 8-9]. However, agreement is not always found, particularly when chronic toxicity is shown [1, 10]. Similar variability exist for comparisons between *V. fischeri* and fish toxicity evaluations [11-13].

Recently, condition factor and hematocrit values have been used to assess stress in adult fish exposed to chemicals or effluents implicated in endocrine disruption [14-17].

To date, however, no studies have shown the relationship between these endpoints and the *C. dubia* or *V. fischeri* tests for complex municipal effluents.

Others have shown the utility of treatment wetlands to reduce or remove toxicity of a variety of effluents [5, 18-24], although, none have evaluated toxicity changes during wetland treatment by comparative bioassays. Effectiveness of a treatment wetland is a function of retention time, vegetation type, microbial activity and soil capacity to hold and/or degrade effluent constituents [25]. Using various wetland configurations, sewage treatment facilities have constructed treatment wetlands to reduce levels of many contaminants in effluent [26]. Treatment wetlands have also successfully removed toxicity attributed to pesticides such as diazinon and chlorpyrifos [19].

The objective of this research was to utilize biomarkers in male *P. promelas*, as well as *C. dubia*, *V. fischeri* and bacterial adenosine 5 triphosphate (ATP) bioassays to assess the efficacy of a constructed wetland system for reducing or removing municipal wastewater effluent toxicity. In addition, a comparison of test and organismal sensitivities was made.

Materials and Methods

Wetland Characterization

In fall of 1992 the City of Denton, Texas constructed a half acre experimental treatment wetland at the Pecan Creek Water Reclamation Facility. The wetland measured

approximately 150 '150 ft. with 3 earthen berms separating it into four channels. Channel depth varied from a few inches near the inflow to 2 ft. at the outflow.

The wetland receives dechlorinated final wastewater effluent and had an estimated maximum volume of 150,000 gallons (~570,000L). It was isolated from groundwater by a layer of clay. The inflow and retention time are adjustable. After construction, aquatic macrophytes were transplanted to the first wetland channel. Three plant species were transplanted including: *Sagittaria sp.*, *Pontederia cordata* and *Scirpus validus*. Other species (*Lemna*, *Ceratophyllum* and *Typha*) quickly invaded the wetland. Mosquitofish, *Gambusia sp.*, were stocked in the wetland to control the mosquito larvae population. A variety of turtles, birds, insects and other organisms inhabit the wetland and were self introduced [19].

The wetland was constructed in the third and final year of a Toxicity Reduction Evaluation (TRE) . The TRE identified the organophosphate diazinon as a probable toxicant in the wastewater effluent. Wetland effluent treatment was evaluated for reduction of diazinon related toxicity [19]. After the diazinon toxicity reduction study, final effluent continued to be diverted to the wetland for approximately eight years prior to this study.

Wetland characteristics measured during this study included: flow (m/min), nominal residence time [(t-1)=average storage volume (V)/total inflow rate (Q1)], vegetation type and relative cover at time of exposure, and channel length, width and

depth. Flow was estimated by eleven replicate measures of travel time of cloth dye (royal blue) through a 1 meter, 2 inch diameter PVC half pipe submerged within the vegetation of the first channel. Flow measures were taken at the left, right and center of the channel. Retention time of the wetland was estimated *via* inflow volume measures relative to the average storage volume and corrected for effluent displacement [27]. Depth was measured at 4 locations at incremental distances down each wetland channel. At each location, depth was measured on each side and in the middle of the wetland channel. Width was measured at the 4 locations in each wetland channel where depth was measured. Emergent and floating macrophytes were identified and recorded *via* digital images taken during the exposure periods. Percent coverage was estimated based on visual inspection and digital images of the plant types in each channel.

Water chemistry measurements taken periodically at each site included: pH (Orion 230A pH Meter), dissolved oxygen (mg/L; YSI Model 51A Oxygen Meter), temperature (°C; YSI Model 85 Oxygen, Conductivity, Salinity and Temperature Meter), alkalinity (mg $CaCO_3$/L; Potentiometric Titration with 0.1 N H_2SO_4 to a pH of 4.5), hardness (mg $CaCO_3$/L; Colorimetric Titratrion with0.02 N Ethylenediamine tetracetic acid (EDTA) and calmagite indicator) and specific conductance (umho/cm at 25°C; YSI Model 33 Conductivity Meter).

Pimephales promelas

Sexually mature, male fathead minnows (*Pimephales promelas*,) were purchased from Kertz s Fish Hatchery (Elverson, PA) at 6 to 8 months of age. Prior to exposure,

fish were acclimated in 72 L aquaria with flow-through activated carbon dechlorinated tap water 2 (June) to 6 (March) weeks. Light was available for 16 hours per day and temperature was maintained at 22-25 C. Fish were fed frozen brine shrimp twice daily. After the acclimation period fish were divided among exposure sites.

Fish were exposed to final treated wastewater effluent sites and control conditions for 2 (June) or 3 (March) weeks. Fish exposed to effluent were held in 3 replicate cages and placed in the constructed wetland at 4 sites located in wetland channels (Figure 1). During the March exposures site 1 was located at the inflow of final treated effluent. Sites 2 and 3 were distributed downstream of the inflow. Site 4 was located at the outflow of the wetland after the effluent had passed through all 4 wetland channels. During June exposures sites were moved upstream shortening the wetland treatment distance (Figure 1). Control exposures were performed in 3 72 L aquaria containing activated carbon dechlorinated tap water for the same duration as the effluent exposures. A 3-week exposure was performed after last freeze in March and a 2-week exposure was performed in June of the same year.

After exposure, fish were taken to the laboratory, sacrificed (modification of methods used by Allen *et al.* 1999) and measurements taken including: length (cm), weight (g), and hematocrit. Condition factor (K; weight '10^5 /length^3)[29] and hematocrit (packed blood cell column height / total blood column height '100) [30] were calculated for each fish to assess fish health.

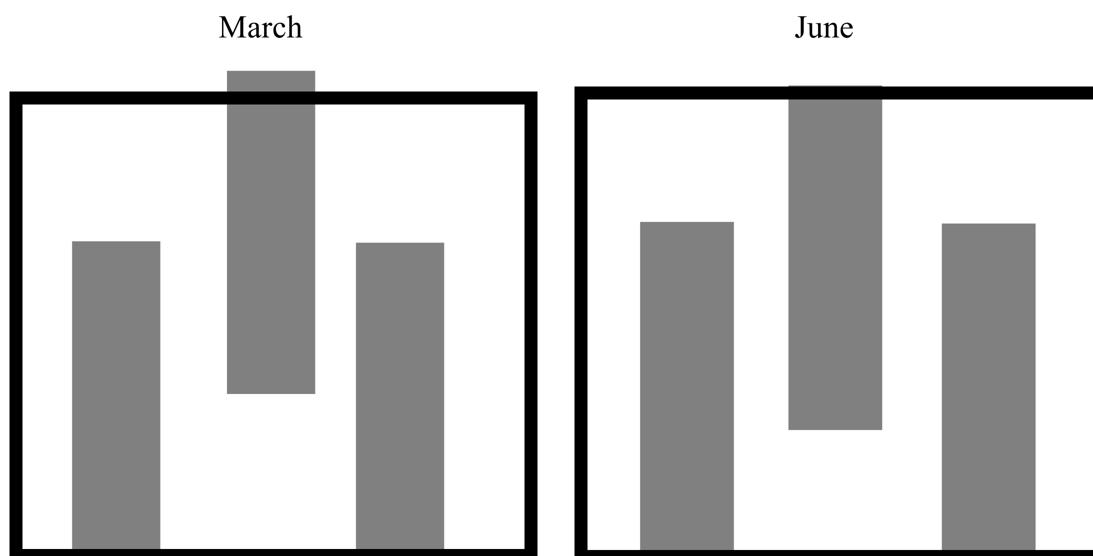


Figure 1 : Wetland schematic showing exposure sites, increasing in distance from the inflow (1), in water channels separated by 3 land berms.

Ceriodaphnia dubia

C. dubia were cultured in standard synthetic reconstituted hard water (RHW)[31] without addition of sodium selenate. RHW also served as control water for all laboratory toxicity tests. RHW was prepared in 50-L batches following procedures outlined by Knight and Waller [32] with the following exceptions: 1) initial water used to prepare RHW was reverse-osmosis deionized water, 2) glass columns were packed with granular activated carbon, and 3) the final solution was not bubbled with CO_2, but vigorously aerated for at least 24 h.

C. dubia were mass cultured as described by Knight and Waller [33] with the following modifications: 1) 500-ml culture jars contained 300 ml RHW, 2) mass cultures were fed 10 ml algae-Cerophyl suspension for the first 4 d, 3) mass cultures were initiated with less than 12-h-old neonates but not necessarily within 4 h of each other, and 4) fluorescent lights were not covered with dark plastic, hence light intensity in the test chamber was approximately 125 lux. *C. dubia* received the same feeding suspension in both mass culture and during 7-d toxicity tests. The final feeding suspension consisted of a mixture of algae and Cerophyl® and was prepared following procedures described by Knight and Waller [33].

C. dubia survival and fecundity were evaluated during the study period. Overlapping toxicity tests were initiated every 2 to 4 days. Five tests were performed in March and six tests in June. 7-d short-term toxicity tests were conducted following general procedures recommended by the U.S. Environmental Protection Agency [7] except the yeast-cerophyl-trout chow (YCT) feeding suspension was replaced by that described above. Constructed wetland effluent samples were obtained daily for test renewals. Samples were collected in 1-L polyethylene cubitainers, brought back to the laboratory, and used when acclimated to 25 C. RHW was used as the control after similar acclimation in 1-L polyethylene cubitainers.

Vibrio fisheri

Vibrio fisheri, a luminescent marine bacterium (formerly MicroTox®), used in effluent toxicity bioassays, was obtained as freeze-dried DeltaToxa™ reagent and held at

15 ºC until used [34]. Wetland samples were stored at 4ºC for less than 72 hours prior to analysis. Assays followed the 82% B-Tox Procedure for low toxicity samples [34]. Briefly, percent light loss or gain was detected following osmotic adjustment of samples and DeltaToxa™. Control (non-toxic ultra pure water) was used as a reagent blank [34]. Assays were performed on 3 samples during March and 13 samples during the June testing period.

Microbial ATP Assay

Wetland bacterial adenosine 5 triphosphate (ATP) was determined using a photoluminescent assay (Sigma Chemical Co.). In this reaction, firefly luciferase catalyzes oxidation of D-luciferin, and light emitted is proportional to the bacterial ATP concentration in the sample. Standard curve and effluent assays followed bioluminescent kit protocols[35]. Photon production for ATP and DeltaToxa™ assays were quantified using a DeltaToxa™ PS1 luminometer. Weekly tests were performed with wetland effluent samples during the June testing period. No bacterial ATP assays were performed in March.

Statistical Analyses

All analyses were carried out using SAS Version 7 (SAS Institute Inc.). Statistically significant differences were accepted at =0.05. Data were analyzed with parametric General Linear Model (GLM) procedures when the assumption of normality and homogeneity were met. The Student-Newman-Kuels (SNK) or Tukey s multiple-

range comparison test was used when differences were found. When parametric assumptions were not met, Kruskal-Wallis analysis was used with Dunnett s or Tukey s (for general water chemistry) multiple-range comparison tests on ranked data when differences were found. Data from each test were analyzed and then pooled, as per Middaugh *et al.* [1], to simulate an exposure duration like that experienced by the fish. Data are presented as means plus and minus 1 standard error.

Results

Wetland Characterization

Flow was estimated for the first wetland channel only. The deeper channels in other areas of the wetland did not have sufficient unidirectional flow to be measured with the techniques attempted. Flow estimated for channel 1 was 2.9 m/min (median, Q3 = 3 m/min, Q1=2 m/min). Retention time was thought to be the best estimate of effluent exposure. The wetland average storage volume derived from 40 length and width measures and 65 depth measures was 80,646 gallons (306,455 L). Volume for channel 1 alone was 10,355 gallons (39,349 L). The average inflow rate was 784 gallons/hour (2,968 L/hour). Average retention time was estimated to be 4.3 days for the wetland and

0.6 days for channel 1. Correcting for actual effluent displacement (Sprague, 1969) provided 90% effluent renewal in the whole wetland at 10 days and 99% replacement over 20 days. Channel 1 was estimated to be 90% exchanged at 1.4 days and 99% exchanged over 2.6 days.

March. Emergent macrophyte type varied with wetland channel (Figure 2). Channel 1 possessed the greatest diversity of plants including *Pontederia* (pickerelweed), *Ludwigia*, and *Lemna* (duckweed) with some *Scirpus* (bullrush) sprouting near the end of the exposure period. Channels 2, 3 and 4 were dominated by *Typha* (cattails) with *Lemna* covering open areas. The 2,660 ft^2 (140' long '19' wide) area of channel 1 was 70% inhabited by *Pontederia*, 25% *Ludwigia* and 5% open with actual vegetative coverages of 27% and 19.5%, respectively within the areas. Open areas were covered with a layer of *Lemna*. Channel 2 (3,105 ft^2, 135' long '23' wide) was 75% inhabited by *Typha* with 95% coverage within that area and *Lemna* densely covering the remainder. Channel 3 (3,494 ft^2,137' long '25.5 wide) was 70% inhabited by *Typha* at the same density as channel 2 with *Lemna* covering the open areas. Channel 4 (3,192 ft^2,137' long '23.3' wide) was 50% inhabited by *Typha* at 50% coverage within the inhabited area and *Lemna* covering the remaining area.

The wetland was characterized after last freeze in early spring, during fluctuating air temperatures from 1.7° to 27°C. Aqueous temperatures varied from 10°C to 26°C in the wetland but were relatively constant in the laboratory dechorinated tap water controls at 23±1°C. Samples from site 1 and controls were significantly warmer than other wetland sites (Kruskal-Wallis p<0.0001; Ranked Tukey s MRT). pH was not different

among sites and was approximately 7. Differences in dissolved oxygen (DO) were found between the laboratory controls (~8 mg/L) and wetland sites (Kruskal-Wallis p=0.0013; Ranked Tukey s MRT). However, oxygen concentrations at wetland sites remained between 6 and 7 mg/L with aeration in the fish cages. Mean specific conductance (umho/cm at 25°C) was higher in samples taken from all wetland sites (~800) than in control (~450) aquaria (Kruskal-Wallis p=0.001; Ranked Tukey s MRT). Differences in alkalinity (mg $CaCO_3$/L) were found between site 4 and controls which were somewhat lower than all wetland sites (Kruskal-Wallis $p<0.0373$; Ranked Tukey s MRT). Hardness was not different (149-169 mg $CaCO_3$/L) between wetland sites and controls.

June. Emergent macrophyte type continued to vary with wetland channel (Figure 2). Channel 1, which had increased in coverage and diversity since March, again possessed the greatest diversity including: *Pontederia*, *Scirpus*, *Sagittaria* (arrowhead), *Ludwigia*, *Ceratophyllum* (coontail) and *Lemna*. Channels 2, 3 and 4 were increasingly dominated by *Typha*, relative to March, with *Lemna* and *Ceratophyllum* covering open areas. The area of channel 1 was 40% inhabited by *Pontederia*, 25% *Scirpus*, 25% *Sagittaria*, 5% *Ludwigia* and 5% open, but containing dense floating vegetation (*Lemna* and *Ceratophyllum*). Coverages during June were approximately complete constituting an emergent plant total area coverage in channel 1 of 95%. Channel 2 was 90% inhabited by *Typha* with 100% coverage within that area and *Lemna* and *Ceratophyllum* covering the remainder. Channel 3 was 75% inhabited by *Typha* at the same density as channel 2 with *Lemna* and *Ceratophyllum* covering the open areas. Channel 4 was again

50% inhabited by *Typha*, but now at a coverage of 100% within the inhabited area and *Lemna* and *Ceratophyllum* covering the remaining area.

The wetland was characterized in June with warmer temperatures at near peak macrophyte biomass. Water temperatures ranged from 22.1°C to 27.7°C in the wetland, but were relatively constant in dechlorinated tap water laboratory controls at 25±1°C. Wetland exposure sites were different in temperature (Kruskal-Wallis $p<0.0001$) with site 1 (26.4°C) being significantly warmer than control aquaria (25.5°C) and site 4 (24.5°C) significantly cooler (Dunnett s nonparametric MRT, =0.05). pH wase lower at wetland (7.12 to 7.46) sites than in control (7.88) exposures (Kruskal-Wallis $p=0.0005$, Dunnett s nonparametric MRT, =0.05) but were all within an acceptable range. DO was different between the laboratory controls (~7 mg/L) and wetland sites which ranged from 1.6 to 5.6 mg/L (Kruskal-Wallis $p<0.0001$, Dunnett s nonparametric MRT, =0.05). However, oxygen concentrations at wetland sites were maintained above ambient by aerating the fish cages. Specific conductance was higher at wetland sites (742 to 787) than in control (~470) samples (Kruskal-Wallis $p<0.0001$; Dunnett s nonparametric MRT, =0.05). Differences in alkalinity were found between site 4 (129) and site 3 (112) and compared to the remaining exposures at site1 (95), 2 (98) and the control (97) aquaria (GLM; $p<0.0001$; Tukey s MRT). Hardness (mg $CaCO_3$/L) of samples from wetland sites (151 to 160) was significantly higher than control water (118) hardness (Kruskal-Wallis $p=0.0015$; Dunnett s nonparametric MRT, =0.05).

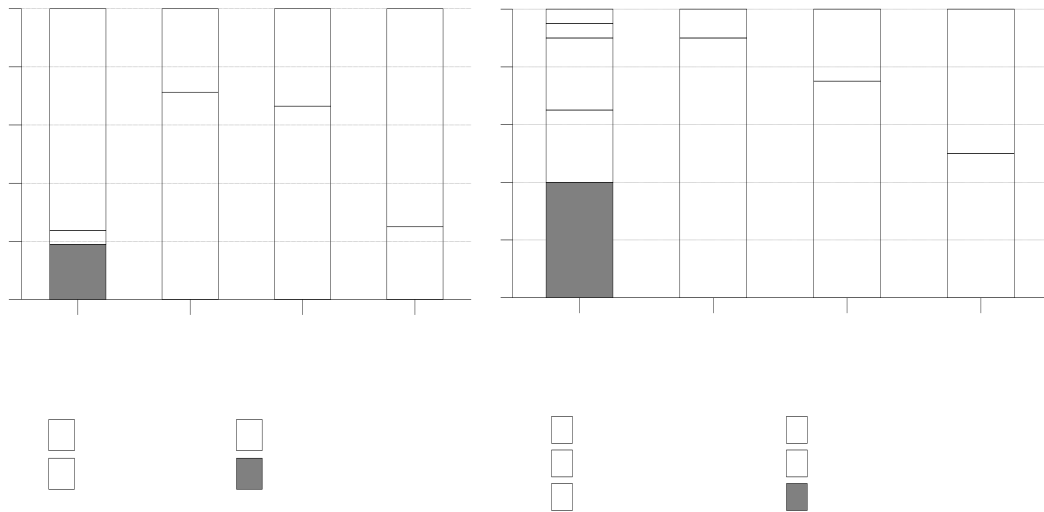
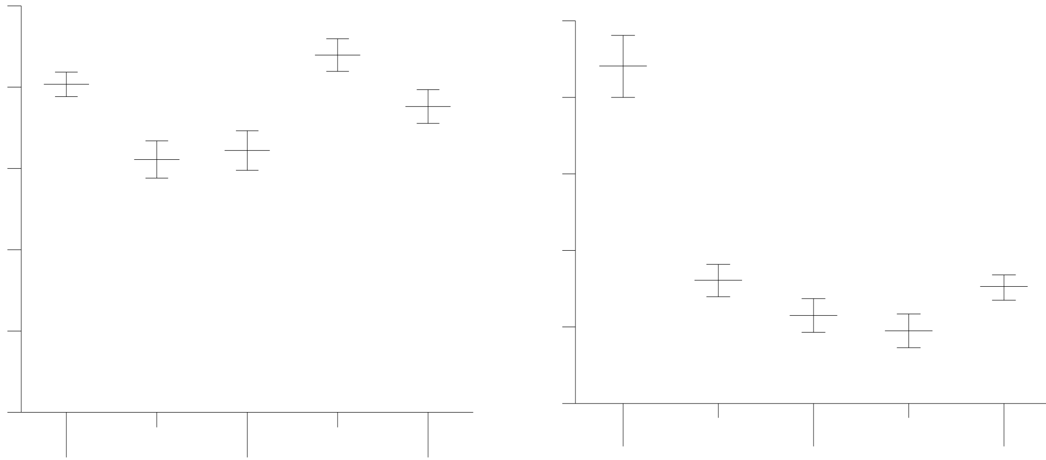


Figure 2: Wetland percent coverage by channel.

P. promelas

March. Surivival during exposure was 100% for effluent exposed and control fish. Fish health was assessed *via* condition factor and hematocrit (Figure 3-4). The condition factor (K) of fish exposed to effluent at wetland sites 1 and 2 was significantly lower (Kruskal-Wallis; $p<0.0001$) than for control fish (Dunnett s MRT). Hematocrit values were also significantly less (GLM; $p=0.0008$) in fish at wetland sites 1 and 2 than in controls and wetland sites 3 and 4 (SNK). A positive trend was apparent between condition factor and hematocrit value.

June. Survivorship during the June exposure period was controls at 92%, site 4 at 78%, site 2 at 75%, site 1 at 67% and a low of 56% survival at site 3. Condition factor



(K) of fish exposed to effluent at wetland sites was less than for controls (Kruskal-Wallis; p<0.0001; Dunnett s MRT). Hematocrit values for site 1 fish were significantly greater than controls (GLM; p<0.0001), but were not different from hematocrit of fish exposed at wetland sites 2, 3 or 4 (SNK).The influence of ancillary stresses preempted evaluation of trends between condition factor and hematocrit.

Figure 3: Fish condition factor (K), mean + 1 standard error with Dunnett s non-parametric multiple range test differences (*).

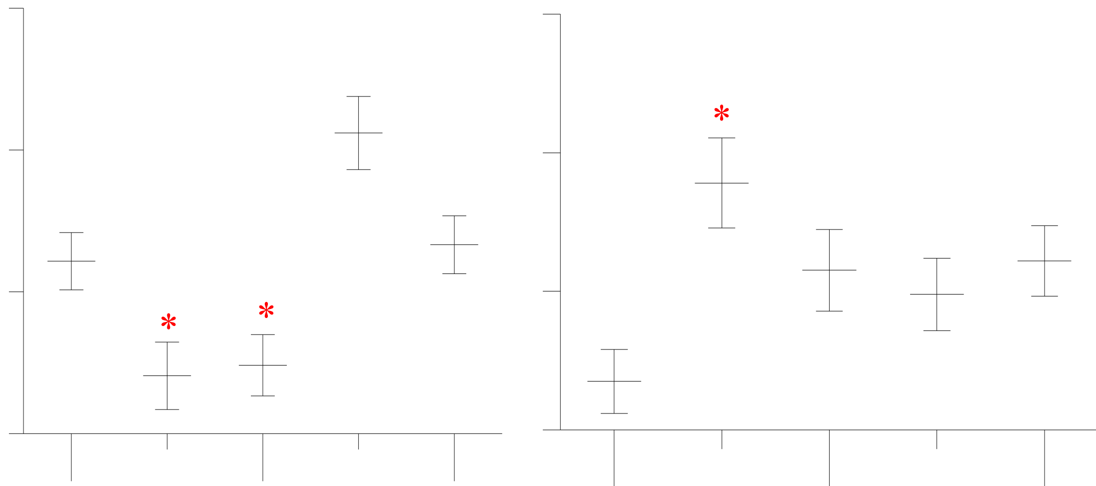


Figure 4 : Fish hematocrit value, mean + 1 standard error with Students-Newman-Keuls multiple range test differences (*) for wetland sites in March and June.

C. dubia

March. Fecundity (mean number of neonates per female) results from *C. dubia* 7-d short-term chronic tests are shown in Figure 5. Survival was significantly less in samples taken from site 1 than controls during the first 2 tests with 80% and 50% mortality, respectively (Fisher s Exact Test, =0.05). However, no other site or any other test showed significant mortality. Tests 1 and 2 showed fecundity in samples from all wetland sites to be significantly less than control fecundity (Kruskal-Wallis, p<0.0001; Dunnett s nonparametric MRT, =0.05). Test 3 and 4 showed significantly lower fecundity in samples from sites 1, 2 and 3 relative to controls (Kruskal-Wallis, p<0.0001; Dunnett s nonparametric MRT, =0.05). Test 5 showed significantly lower fecundity in

samples from wetland sites 2 and 3 only (Kruskal-Wallis, $p<0.0001$; Dunnett s nonparametric MRT, =0.05). Pooling all data to assess effects on survival and fecundity during the 3-week fish exposure, showed a pattern similar to that of the fish results. Pooled data from site 1 showed significant mortality during the 3-week period (Fisher s Exact Test, =0.05). Pooled data also revealed significant reductions in fecundity in samples from sites 2 and 3 (Kruskal-Wallis, $p<0.0001$; Dunnett s nonparametric MRT, =0.05). Finally, pooled data showed no differences between site 4 and control survival or fecundity.

June. Survival was significantly less in samples taken from site 1 (80% mortality) than controls for only the final test (Fisher s Exact Test, =0.05). No other test showed significant mortality during the June testing period. Test 2 showed lower fecundity in samples from site 1 only (Kruskal-Wallis, $p=0.0031$; Dunnett s nonparametric MRT, =0.05). Test 3 showed significantly lower fecundity at site 4 (Kruskal-Wallis, $p=0.0002$; Dunnett s nonparametric MRT, =0.05). Test 6 showed significantly reduced fecundity at sites 2 and 3 (Kruskal-Wallis, $p<0.0001$; Dunnett s nonparametric MRT, =0.05). Conversely, test 4 showed significant increases in fecundity for sites 1, 2 and 3 relative to controls because of lower, but acceptable control reproduction. No significant effects on fecundity or survival were seen in test 1 and 5. Pooled data to assess effects on survival and fecundity throughout the fish exposure period showed no significant differences between wetland samples and controls.

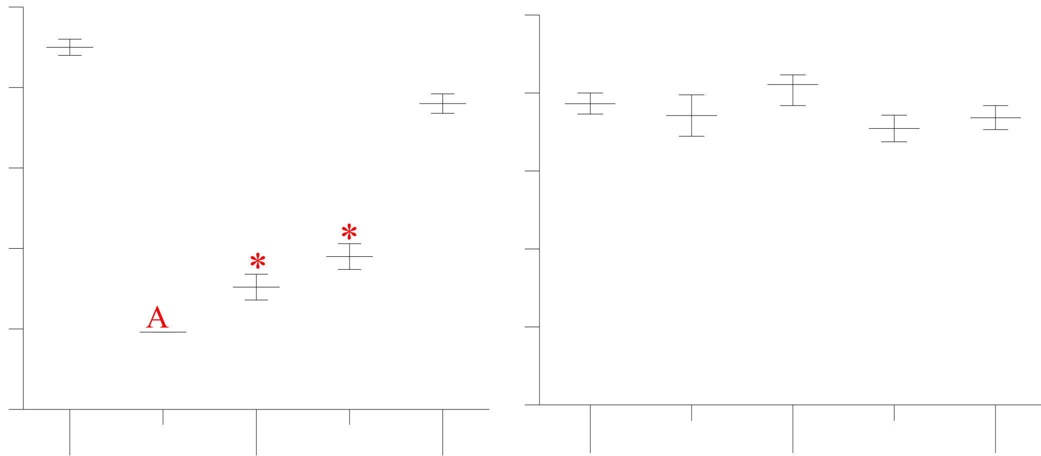


Figure 5 : *Ceriodaphnia dubia* fecundity data for 5 tests, mean + 1 standard error with Dunnett s non-parametric multiple range test differences (* = fecundity, A = Mortality).

Vibrio fischeri

March. Test 1 showed significant elevation in *V. fischeri* luminescence for all wetland sites relative to controls (Kruskal-Wallis, p=0.0131; Dunnett s nonparametric MRT, =0.05). However, tests 2 and 3 showed no difference between wetland samples and controls. Pooled data from the 3 individual tests showed no significant change in luminescence with effluent exposure (Figure 6).

June.One test showed significantly decreased luminescence in a site 1 sample relative to controls (Kruskal-Wallis, p=0.0486; Dunnett s nonparametric MRT, =0.05). However, none of the remaining 12 tests showed significant changes in *V. fischeri*

luminescence with effluent exposure. In addition, pooled data from all 13 tests showed no significant change in luminescence relative to controls (Figure 6).

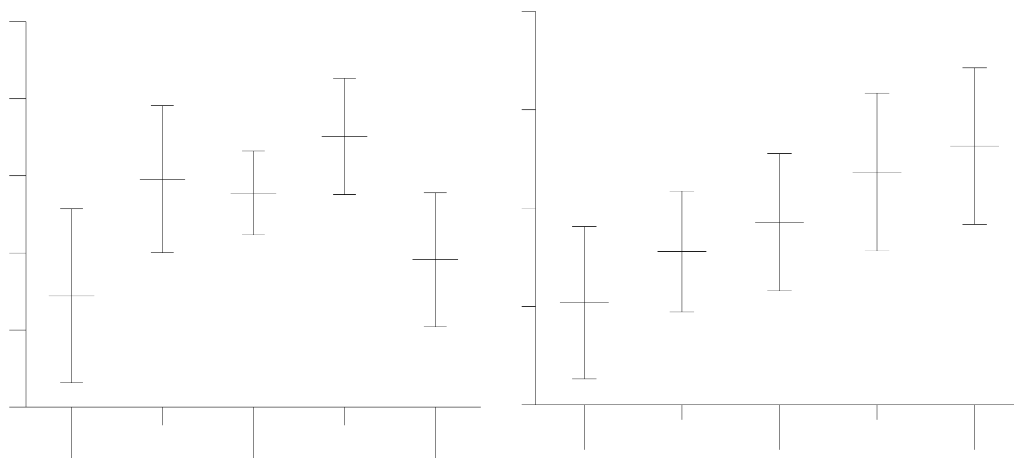


Figure 6 : *Vibrio fischeri* bioluminescence inhibition data, mean $\pm$ 1 standard error with Dunnett s non-parametric multiple range test differences (*).

Microbial ATP Assay

June. Results from wetland bacterial ATP analyses are shown in Figure 7. Bacterial ATP induced photoluminescence at all wetland sites was significantly greater than controls in all 3 assays (Kruskal-Wallis, p=0.0111, 0.0091 and 0.0111, respectively; Dunnett s nonparametric MRT, =0.05). Pooled data showed the same trend of all wetland sites being significantly higher than controls (Kruskal-Wallis, $p<0.0001$, Dunnett s nonparametric MRT, =0.05).

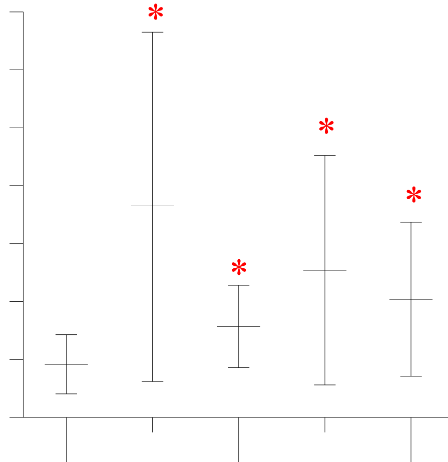


Figure 7: Bacterial ATP photoluminescence data for 3 tests, log mean (umoles ATP '10^{-7} /L) $\pm$ 1 log standard error with Dunnett s non-parametric MRT differences (*).

Discussion

Distinctly different results were observed for the March and June study periods. For this reason, results are discussed by season. A comparison of cumulative results follows in the conclusion.

March

The wetland was characterized during fish exposures after last freeze in early spring. Emergent and floating vegetation grew rapidly over the 3 week exposure period. The first wetland channel had highest diversity of emergent macrophytes, but lowest percent coverage. Toxicity was apparently reduced after travel through both the more diverse channel 1 and the dense *Typha* monoculture of channel 2 as indicated by improved fish condition factors and hematocrit values for sites 3 and 4. However, *C. dubia* seemed more sensitive to toxic effluent constituents. Fecundity was significantly reduced until site 4, which received treatment through 1 to 2 more *Typha* dominated wetland channels.

The toxicity reduction may have been related to the rapid macrophytic growth, however, it is likely that microbial degradation associated with aerobic- anaerobic dynamics contributed to the majority of the transformation [25-26]. Enhancement of toxicant degradation through wetland treatment was apparently sufficient to reduce

effluent toxicity within the first 2 channels (<275 ft, 5,765 ft^2) and to remove toxicity, within the capacity of the tests organisms to monitor, within the entire wetland (549 linear ft, 12,451 ft^2 area).

General water chemistry measures were not thought to have affected exposure results. Temperature was the only factor considerably different among wetland sites, however, the control and site 1 showing the largest difference in effect were different by only 2°C for the *P. promelas* exposures. *C. dubia* and *V. fischeri* tests were conducted at standard testing temperatures. Specific conductance was higher in all wetland sites and dissolved oxygen lower by 1 mg/L. Neither were assumed to be a causative factor in differences found in toxicity testing.

Fish health was assessed *via* condition factor and hematocrit. There was 100% fish survival and good agreement between condition factor and hematocrit. Both showed reduced levels, indicating lower health, at wetland sites 1 and 2. The nature of the toxicants or conditions causing these reductions is unknown. Nonylphenol concentrations measured during this time period (Chapter 2) are below reported toxic levels for aquatic species [36-37]. However, previous studies of this effluent have found organophosphate pesticides to be a seasonally toxic constituent [19]. Additionally, the presence of ethynylestradiol was detected (Chapter 2) at concentrations above those which have been shown to increase plasma vitellogenin to concentrations as high as 10-100 mg/L, a level shown to cause physiological stress *via* kidney and liver damage and necrosis in rainbow trout [38].

C. dubia acute mortality was observed only at site 1 within the first week of testing. Absence of acute toxicity in samples from site 2 demonstrated the wetlands ability to reduce acute toxicity. Furthermore, reduction in chronic toxicity was also shown by site 4. The variable nature of the wetland influent was demonstrated test by test at site 1 by the progression from acute to chronic toxicity followed by no adverse effects, and then back to chronic toxicity over the 3 week study period. Seasonally elevated organophosphate residues have resulted in toxicity to *C. dubia* in the study area [19], although none were detected in monthly grab samples taken by the municipality for organophosphate monitoring. Cumulative impact shown on *C. dubia* over the study period indicates that the toxicants of action are not likely estrogenic as no reports have shown estrogens to be toxic to cladocerans at the measured levels [39-40].

An increase in luminescence of *V. fischeri* in the first assay is not thought to be associated with a toxic effect as toxicity usually manifests itself in decreased luminescence [2]. No other test or the pooled data indicated a toxic response by *V. fischeri*. It is possible that toxic agents actively affecting *C. dubia* and *P. promelas* were of a type ineffective to *V. fischeri* luminescence, such as undetetected organophosphate pesticides [2].

June

Wetland macrophyte total coverage increased considerably from March to June. Total summed coverage of channel 1 in March was only 24%, but rose to 95% in June. Total wetland coverage increased from 47% in March to 78% coverage in June. This

lesser increase may reflect the influence of the early-emerging cattail growth in March. The influence of the wetland on toxicity abatement was less clear during June, however, some *C. dubia* tests did show toxicity at site 1 that was not observed at other wetland sites. Overall, lower toxicity was observed in wetland samples during June. The properties of the wetland which act in toxicity reduction were likely still actively present in June, but were less discernable from the toxicity test results.

Differences in general water chemistry was not thought to have affected results. Providing aeration in cages raised DO sufficiently to sustain fish. This was assumed to be the case as fish exposed at site 1, with highest measured DO concentrations and cage aeration, suffered similar impacts as those at sites with lower measured DO. Temperature differed by ~2°C on average among all exposures and at most sites by less than 1°C. Differences in conductivity have not previously influenced results noticeably and the small differences in pH, alkalinity and hardness were not thought to contribute to observed fish effects. Additionally, no effects in *C. dubia*, *V. fischeri*, or bacterial ATP measures (run at standard testing temperatures) were attributed to general water chemistry differences.

Fish survivorship during the 2-week exposure in June was considerably lower than in the 3-week March exposure. Fish used in the March exposure were purchased pre-spawning season as young adult males and were raised to a larger size (5-7 cm) in the laboratory. June exposed fish were purchased in late spring during the natural spawning season as adult, breeding males. The investment of males in spawning seemed to diminish their ability to withstand ancillary challenges in the form of infection and

disease to which they appeared less susceptible in pre-spawning phase. The rapid body weight loss caused lower condition factors. Control fish not exposed to the same environmental stresses as the effluent exposed fish did not suffer high mortality or rapid weight losses and resultant decreased condition factor. The decrease in condition factor was caused by rapid weight loss over the 2 week exposure period and the corresponding increase in hemotacrit of some fish may have been driven by that reduction in body volume.

Higher temperatures and lower dissolved oxygen were evident compared to the March exposure period and may have contributed to the onset of these fish losses and difficulties. Nevertheless, with fewer observations of toxicity in *C. dubia* tests, together with a lack of measurable estrogens (Chapter 3), these results may not relate to effluent toxicity as much as the presence of other environmental stresses. Poor survivorship and somewhat ambiguous results may negate their interpretation relative to effluent exposure except to illustrate the importance of pre-exposure condition. Data provide a strong rationale for obtaining test organisms during the least stressful life phase possible to ensure healthy baseline standards with which to compare exposure results.

There was less acute or chronic toxicity observed in *C. dubia* tests in June. Acute mortality was observed in a sample from site 1, however, a sample taken from site 2 (70 ft. downstream) the same day was not acutely toxic. Chronic toxicity was observed most often in samples from site 1 and only once in samples from sites 2, 3 and 4, indicating a reduction in chronic toxicity with wetland treatment. Pooled data showed no difference in

survivorship or fecundity during the 3-week testing period possibly denoting a disproportionate fish response with respect to March results.

Toxicity was not observed in the cumulative data from 13 *V. fischeri* assays performed during the June testing period. One test showed significantly decreased luminescence in a site 1 sample. However, this decrease in *V. fischeri* luminescence did not correspond with acute or chronic toxicity for *C. dubia* exposed to the same sample. Conversely, the acute toxicity observed in the final *C. dubia* test was not evident in the corresponding *V. fischeri* assay. Differences in response may be related to the nature of toxic agents as discussed above.

Higher microbial activity, as measured by a bacterial ATP photoluminescent assays, was found in wetland samples relative to the control. This illustrated higher microbial activity in wetland samples and was consistent over the 3-week study period. Bacterial ATP levels were consistently found an order of magnitude higher in samples from site 1 than any other site. This may reflect a higher abundance of organic nutrients present at the wetland inflow. These nutrients may be consumed and degraded with distance preempting effects at sites downstream. Dissolved oxygen concentrations were also consistently higher at the inflow which may have influenced the composition bacterial populations assessed with this method Lower microbial ATP levels were found at sites further downstream as DO rapidly decreased with distance in the absence of light. The bacterial ATP photoluminescent assays provide insight into the aerobic portion of the microbial population which are likely actively involved in the treatment process, but provide no direct relationship to toxicity.

Conclusions

March sampling revealed both acute and chronic toxicity. *C. dubia* was the more sensitive organism used to monitor this effluent during wetland treatment and was the only organism to show acute and chronic toxicity at sites further downstream in the constructed wetland. Adult male *P. promelas* also indicated chronic effluent toxicity through condition factors and hematocrit values, but effects were diminished earlier in wetland treatment. However, the toxic effluent constituents failed to elicit a response from *V. fisheri*. Results of this testing period show a similar organismal response relationship to results of studies which have compared these methods using different exposures [1, 10-13].

June sampling indicated less toxicity stemming from exposure to the test effluent. *C. dubia* again was more sensitive to toxicants when present and both acute and chronic toxicity was observed. Fish data reflected a pre-acclimation influence of spawning activity and showed no relationship to effluent exposure. Additionally, no toxicity was measured with *V. fisheri* bioluminescence assays despite acute and chronic toxicity measured with *C. dubia*. Microbial ATP assays revealed higher levels at all wetland effluent sites and highest levels at the effluent inflow. Although not thought to directly

measure toxicity, microbial ATP data may show an inverse relationship between microbial populations and toxicity with an expanded testing regime.

Overall, *C. dubia* short-term chronic test proved most sensitive and consistent for effluent toxicity testing. Fish results showed the physiological indicators condition factor and hematocrit to be slightly less sensitive to effluent toxicity and to depend heavily on pre-exposure condition. *V. fisheri* results indicated that the use of other methods may be necessitated in conjunction with this assay when monitoring complex municipal effluents for toxicity.

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SUMMARY

Chapter 1

A pilot study was conducted in north central Texas to assess the occurrence of estrogenic endocrine disruption associated with effluent dominated aquatic systems. The physiological biomarker vitellogenin (VTG) was utilized in the *Pimephales promelas* fish model. Naturally, high VTG levels are found only in mature, female fish under the influence of estrogen secreted from their ovaries. When male fish are induced to secrete high VTG levels, it is in response to estrogenic stimuli in the exposure environment. Consequently, the VTG biomarker in fish has been widely used to evaluate aqueous estrogenic exposure.

Results showed increases in VTG concentration with municipal effluent exposure. VTG levels were not significantly increased in fish exposed for 1 week, but were significantly higher in fish exposed for 2 weeks and 3 weeks. Results indicated that the test effluent possessed estrogenic properties. VTG concentrations detected in pilot study samples were similar to those found in other wastewater effluent evaluations in the U.K. and U.S. The estrogenic response was an order of magnitude greater in this study than in previous U.S. studies, but well within the range of responses found in the U.K..

The implication of such exposures has been discussed in the context of wildlife losses and difficulties. The impact on humans, although heavily debated, has led to legislation mandating development of screening and testing protocols which are in the process of being implemented (Food Quality Protection Act of 1996 and Reauthorization

of the Safe Drinking Water Act of 1996). The status of water reclamation in many areas of the southwestern United States may require greater attention to evidence of increased estrogenic exposure because effluent dilution is often much less. Therefore, further research on the implications of environmental estrogen exposure was warranted and assessed in following chapters.

Chapter 2

A spring season evaluation of municipal effluent toxicity and estrogenicity was conducted in Denton, Texas. The study was designed to assess these properties relative to distance from the outfall for an effluent flooded treatment wetland and effluent dominated stream system. The health of fish was assessed via condition factors and hematocrit values to estimate effluent toxicity among sites. There was good agreement between these 2 indicators. Both showed reduced levels, indicating lessor health, at wetland sites closer to the inflow of effluent. The nature of the toxicant or conditions causing these reductions in unknown. Measured chemical analyte concentrations would not account for observed fish toxicity unless acting indirectly (e.g. excessive estrogenic stimulus). Similar responses to effluent was observed using different test methods and organisms (Chapter 4). Fish exposed at stream and reservoir sites showed variable results possibly reflecting the smaller sample size.

Gonadosomatic indices (GSIs) showed a similar trend to health indicators. The lowest GSI values and highest hepatosomatic indices (HSIs) were consistently found at the wetland inflow site, however, no differences were observed in fish at stream sites.

Estrogenic exposure results in testicular inhibition and consequently lower GSIs . This same type of exposure has been associated with increased liver weight, via liver hypertrophy of estrogen sensitive tissue, resulting in higher HSIs. Fish exposed to the wetland inflow showed reduced GSIs that were complimented by increased HSIs indicating probable estrogenicity of the effluent.

The secondary male sexual characteristics tubercle number, fatpad thickness and stripe density were also different among wetland sites showing a trend similar to condition, hematocrit and GSI values. This relationship was not seen at stream and reservoir sites. Fish exposed to the wetland inflow consistently showed lower values for secondary sexual characteristics, indicating lessor health or masculinity. Number of male breeding tubercles and fatpad thickness were inversely correlated with the estrogenic biomarker, VTG concentration, indicating possible demasculinization by estrogenic effluent constituents. However, the cause of these apparent trends, as well as those seen for GSI, HSI and health indicators, may have been toxic in nature and caused by some effluent parameter or parameters not measured. The secondary sexual characters fatpad thickness and stripe density were semi-quantitative in nature and may have been biased by the limited rating system, however, they were performed consistently and show a pattern similar to quantitative data collected.

Measured vitellogenin concentrations were markedly high for the wetland inflow site, effluent outfall in the stream, and 631 m downstream. Concentrations of plasma vitellogenin at these sites were 3 to 4 orders of magnitude higher than previously measured in the area (Chapter 1) and even higher compared to other U.S. studies.

However, the levels were comparable to those observed in similar studies including 100% final wastewater effluent exposures in the U.K. High vitellogenin induction indicates environmental estrogen exposure. Laboratory research has demonstrated induction of high vitellogenin levels with exposure to estrogens at concentrations similar to those measured in this effluent. The level of induction is often highly variable, as seen in this study, and has been attributed to possible causes including: composition of effluent, route of exposure, exposure time, water temperature, species, age and sex of fish.

The wetland was characterized during fish exposures after last freeze in early spring. The emergent and floating vegetation was growing rapidly over the 3 week period. The first wetland channel, having highest diversity but lowest percent coverage, decreased effluent estrogenicity in less than 140 linear feet. This assessment was based on reduced VTG levels in fish further downstream and corresponding changes in GSI, HSI and secondary sexual characteristics. The wetland reductions were noteworthy when compared to stream sites, where VTG levels did not decrease at distances over 630 meters downstream from the effluent outfall. However, effluent reached that site ~4.4 hours after discharge versus the 13.2 hour estimated travel time to reach the first unaffected wetland site. Evaluation of further distances in the stream were not possible after cage losses. However, levels in the reservoir were low relative to creek sites and not significantly higher than controls.

Other forms of toxicity were apparently removed after travel through both channel 1 and the more dense *Typha* monoculture of channel 2 as reflected by improved

condition factors and hematocrit values. The reduction in both types of effects may have been related to the rapid growth phase of the wetland, however, it is likely that microbial degradation associated with aerobic-anaerobic dynamics contributed to the majority of the transformations. Enhancement of the degradation process was apparently sufficient within the first wetland channel to remove estrogenicity and to remove toxicity, as measured by fish biomarkers, within the first 2 channels.

Chapter 3

The exposure scenario performed in early spring was repeated in June of the same year to assess the seasonality of effluent properties and wetland treatment. A dilution series exposure was added for comparison with wetland and stream exposures. Elevated mortality in all tests performed, compared to previous studies (Chapter 1-2), weighed heavily in the interpretation of results. Investigation into the cause of increased mortality led to the conclusion that spawning activity stress led to a weakened state, susceptibility to disease and high mortality. Personnel at the hatchery from which the fish were purchased noted that this is a typical spring and summer season occurrence for male *P. promelas* cultured in ponds. The influence of spawning stress had not been previously observed as fish were laboratory raised with controlled spawning exposure (Chapter 1) or purchased during non-spawning periods (Chapter 2). Four unsuccessful attempts were made from mid to late spring to obtain disease free fish for this study.

The influence of low survivorship was evident in surviving fish condition factors after the additional stress of exposure was endured. The condition of fish, shown to be less in all treatments save one, would likely manifest itself in altered expression of all other parameters measured. Fish condition evaluates the relationship of fish weight to length. Rapid weight losses during exposure was indicative of high stress in exposed animals. Previous fish exposures of this type (Chapter1-2) have not resulted in rapid weight losses. Weight loss was therefor attributed to factors other than nutrition, such as spawning stress.

Assessment of other fish endpoints with respect to exposure was thought to be inappropriate in the presence of poor survivorship and condition, and lower toxicity measured with other methods during the exposure period (Chapter 4). The baseline health of fish could have directly influenced all other fish endpoints measured. Hematocrit levels appeared higher with most effluent exposures. However, the SO_2 dechlorinated controls also showed elevated hematocrit. These apparent hematocrit elevations may have been an artifact of rapid weight loss. Changes in resources allocated to certain physiological functions would also likely be different in unhealthy, stressed fish. It is not likely that fish in poor condition would physiologically allocate significant resources to reproduction and decreases in testes weight would result. Losses in testes weight were masked in GSIs by lower body weight. Loss in liver weight seemed to occur at an accelerated rate with respect to body and testes weight loss. Significantly lower HSIs were observed, but was not attributed to exposure because this occurred without any observable exposure-response trend. The reduction in secondary sexual characteristics

was pronounced for tubercle number and fatpad thickness, but not for stripe density. Reduction in testes weight would result in overall fewer cells to secrete androgens responsible for expression of secondary sexual characteristics.

Concentration of plasma VTG can also be influenced by fish condition because of changes in physiological resource allocation. The apparent increases in plasma VTG in fish exposed at wetland site 1, stream outfall and to the 100% dilution series concentration were similar to previous results. However, expression was highly variable. Measurable concentrations of VTG were found in relatively few fish from each exposure group. Measurable levels of VTG were also found in controls with a mean concentration 1 order of magnitude higher than previously observed (Chapter 1-2). Interpretation of VTG induction results in the presence of detracting variables was thought to be inapplicable and circumspect to poor fish condition.

Presence of elevated VTG in the control fish did more than increase background levels for comparison, it also demonstrated the pre-acclimation environment of these fish with respect to estrogenic exposure. VTG levels in male fish have been shown to be elevated in the presence of breeding females. The elevated control VTG levels (6.3 ug/L) indicate exposure to a source of estrogen. Considering season and the previous healthy fish received from this provider, the estrogenic source was likely high densities of breeding females. Under natural environmental conditions minimal induction of vitellogenesis (less than 0.5 ug/L) occurs in male fish. However, these pond raised fish were likely kept at densities higher than would naturally occur. Once induced to higher levels vitellogenesis, the clearance of VTG from fish plasma can take up to 5 months

after the estrogenic cue is removed. The elevated control VTG levels indicate that these male fish were exposed to spawning related stresses prior to testing.

The wetland was characterized during fish exposures in early summer with peak macrophyte biomass. The emergent and floating vegetation had increased greatly since last characterization in March of this same year. The influence of summer season wetland treatment on effluent toxicity and estrogenicity was not ascertained during this portion of the research because of the above mentioned occurrences. However, an effluent toxicity assessment was performed and wetland reduction of toxicity was evaluated in the following chapter.

Chemical analyses of known and suspected environmental estrogens showed no typical estrogens. The absence of ethynylestradiol, the active ingredient in female oral contraceptives, is noteworthy as it has been consistently detected in this effluent during spring (Chapter 2). Numerous suspected and possible estrogenic chemicals were detected routinely. However, measured concentrations were lower than have been associated with estrogenic activity and potential for further evaluation was negated by poor fish condition.

Chapter 4

Effluent toxicity assessed with *P. promelas* biomarkers during March and June study periods was compared to more traditional effluent toxicity evaluation methods, *Ceriodaphnia dubia* and *Vibrio fisheri*. March testing revealed showed both acute and chronic toxicity. *C. dubia* was the more sensitive organism used to monitor this effluent during wetland treatment, and was the only organism to show acute and chronic toxicity

at sites further downstream in the constructed wetland. Adult male *P. promelas* also showed chronic effluent toxicity through decreases in condition and hematocrit, but effects were diminished earlier in wetland treatment. Toxic effluent constituents failed to elicit a response from *V. fisheri*. Results of this testing period show a similar organismal sensitivity relationship to studies which have compared these methods using different exposures.

June sampling indicated less toxicity stemming from exposure to the test effluent. *C. dubia* again was more sensitive to effluent toxicants. Both acute and chronic toxicity was observed. Fish data reflected pre-acclimation influence of spawning activity and showed no relationship to effluent exposure. Additionally, no toxicity was measured with *V. fisheri* bioluminescence assays despite acute and chronic toxicity measured with *C. dubia*. Microbial ATP assays revealed higher levels at all wetland effluent sites and highest levels at the effluent inflow. Although not thought to directly measure toxicity, microbial ATP data may show an inverse relationship between microbial populations and toxicity with an expanded testing regime.

Overall, *C. dubia* short-term chronic tests proved most sensitive and consistent for effluent toxicity testing. Fish results showed the physiological indicators, condition and hematocrit, to be slightly less sensitive to effluent toxicity and to depend heavily on pre-exposure condition. *V. fisheri* results indicated that the use of other methods may be necessitated in conjunction with this assay when monitoring complex municipal effluents for toxicity.

Ecological Implications

Naturally low induction of vitellogenesis occurs in male fish. Once induced to high levels vitellogenesis, the clearance of VTG from fish plasma can take up to 5 months after the estrogenic cue is removed. The ecological significance of estrogenic exposure in not certain but is being investigated. Intersexuallity has been associated with vitellogenin induction in wild roach *(Rutilus rutilus*) populations as has disrupted natural sexual cycles. Traditionally reported effects of prolonged synthesis of VTG include metabolic stress leading to kidney and liver damage and necrosis, misallocation of physiological building blocks, and calcium losses from the skeleton and scales. These stresses on organisms may increase susceptibility to disease and possibly death. However, recent research found no relationship between an estrogenic effluent inducing elevated VTG and other physiological changes in male fish, contrary to observations made in this study of north-central Texas final wastewater effluent.

Physiological indicators of fish health and endocrine disruption showed ecological impacts to be likely. The deceases in condition and hematocrit, although not specifically connected to estrogenic exposure, would have adverse effects on reproductive success. Competition, from unexposed males in better condition, would plausibly lesson successful mating by exposed males. Moreover, reduction in testes weight, evident in lower GSIs, would certainly reduce the potential for reproductive success. Reduced testes would also mean fewer androgen secreting cells and reduction in secondary sexual characteristics used to attract females. Results of this study demonstrate

numerous factors, resulting from exposure to an estrogenic effluent, that would result in adverse reproductive challenges and likely ecological effects.

Estrogenic exposure results for mature male fish may indicate a far worse problem. The levels of hormonal exposure necessary to induce changes in adult fish are often higher than necessary to affect juvenile fish. Sex ratios have been shown to be dramatically shifted with steroid exposure during critical developmental stages. The effects of estrogenicity on adult fish physiology suggest that far more changes may be occurring in developing fish. Wide shifts from the natural sex ratio of a species can also have dramatic ecological effect, particularly if keystone species are involved.

RECOMMENDATIONS

The study effluent was found to be toxic and estrogenic to *Pimephales promelas*. The level of estrogenicity measured with the VTG biomarker varied with season, fish condition and wetland treatment. Estrogenic responses in fish during this research were higher than previously reported in the U.S.. The determination of high effluent estrogenicity and apparent seasonality provides rationale for more research.

The seasonality of estrogenic effluent components needs further study. Research should include biological and chemical measures of estrogenicity. A more expansive

chemical analysis of estrogenic chemicals should include weekly or monthly effluent characterization. This would provide insight into the influence of local, annual population change on estrogenic effluent components. Biological measures of estrogenicity would also be necessary to determine if the chemically measured estrogenic compounds are biologically available. Less labor intensive means could be used for monthly evaluations. Transgenic yeast containing estrogen receptors could be used for monthly screening with quarterly fish exposure assessments. This scenario would provide a more complete understanding of changes in the study effluent over the year. It may also reveal the most appropriate time to assess ecological changes in the effluent dominated system.

High VTG levels in male fish in spring coincided with decreases in testes weight, condition, secondary sexual characteristics and hematocrit. Decreases in these factors may detract from spawning success. Breeding *P. promelas* studies could be conducted during or after exposure. Breeding pair exposure tests conducted in the laboratory have shown changes in reproductive success with weak xenoestrogen exposures. Such experiments with this effluent may demonstrate the influence of effluent exposure on fecundity and offspring sex ratio. Histopathological measures of estrogen sensitive tissues, such as the ovaries and testes, should be conducted with such exposures to examine occurrence of internal changes in breeding fish and offspring. This would allow assessment of more subtle, longer-term ecological effects.

Preliminary evaluations of transgenerational effects could include *P. promelas* egg exposures. Exposing eggs would demonstrate the influence of estrogenic effluents on the more hormonally sensitive developing fish. Estrogenic exposure below effect levels

in adult fish have shown wide shifts in sex ratio of offspring when eggs are exposed. Although widely used as a aquacultural tool, the ecological implications of unintentional sex ratio shifts in aquatic environments should be evaluated. Another approach to evaluating transgenerational influence of estrogenic effluent could utilize live-bearing *Gambusia affinis*. Adult exposure during internal egg development may provide insight into effluent driven sex ratio changes. Live-bearing egg exposure would be different from direct aqueous egg exposure. However, *G. affinis* models would have the benefit of shorter time to reproductive maturity and lower laboratory rearing space requirements.

The persistence of high levels of estrogenicity in effluent dominated systems needs to be better understood. Two attempts were made to evaluate the receiving system for this effluent. However, distances greater than 0.6 km were not assessed due to repeated cage losses. The distance that effluent remains strongly estrogenic could be valuable in calculating ecological risks of such exposure. In addition to fully characterizing this receiving stream and reservoir with more successful sites, an assessment of the watershed should be conducted. A larger scale study would likely elucidate additional sources of estrogenicity. Ideally, the entire watershed would be surveyed with bioassessment in all major tributaries. Watershed segments with high estrogenicity should be further studied with more strategic sites within that subwatershed. The discovery of strong estrogenicity in a small subset of this watershed should mandate research into the extent of this unfamiliar contamination.

With all recommended experiments on effluent estrogenicity, the need to examine toxicity exists. Municipal effluents are characteristically complex and dynamic and have

been shown to contain both estrogenic and toxic constituents. Any evaluation of effluent estrogenicity could be misinterpreted if toxicity in not assessed concurrently. The influence of each parameter is likely not independent in it s ecological effect.

The chemical evaluation of estrogenic analytes would be incomplete without fate and transport studies of these chemicals. The biological availability of effluent estrogens apparently changed with wetland treatment. Determination of the wetland treatment driven transformations undergone by these chemicals may provide insight into possible wastewater treatment procedures to minimize their influence on aquatic systems.

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