ECOTOXICOLOGICAL INVESTIGATIONS IN EFFLUENT-DOMINATED STREAM MESOCOSMS

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The University of North Texas Stream Research Facility (UNTSRF) was designed to examine contaminant impacts on effluent-dominated stream ecosystems. Stream mesocosms, fed municipal effluent from the City of Denton, TX, Pecan Creek Water Reclamation Plant (PCWRP), were treated with 0, 15 or 140 µg/L cadmium for a 10-day study in August 2000. Laboratory toxicity test and stream macroinvertebrate responses indicated that cadmium bioavailability was reduced by constituents of effluent-dominated streams. The Biotic Ligand Model (BLM) for Cd was used to predict a 48 hour Cd EC_{50} for *Ceriodaphnia dubia* of 280 µg/L in these effluent-dominated streams. This value is higher that an EC_{50} of 38.3 µg/L Cd and a 7-day reproduction effect level of 3.3 µg/L Cd generated for *C. dubia* in reconstituted laboratory hard water. These results support use of a cadmium BLM for establishing site-specific acute water quality criteria in effluent-dominated streams. Although not affected by 15 µg/L treatments, organisms accumulated Cd in 15 µg/L treated streams. Hence, over longer exposure periods, Cd accumulation may increase and a no effect level may be lower than the observed 10-day no effect level of 15 µg/L.

A toxicity identification evaluation procedure was utilized with *in vitro* and *in vivo* bioassays to identify estrogenic compounds in PCWRP effluent, previously identified to seasonally induce vitellogenin (VTG) in male fathead minnows. Steroids, nonylphenol ethoxylate metabolites, and other unidentified compounds were identified as

causative effluent estrogens. These findings suggest that *in vivo* VTG bioassays should be used to confirm *in vitro* Yeast Estrogen Screening assay activity when effluents are fractionated or screened for estrogenicity. A subsequent 90-day cadmium study was initiated to assess long-term effluent and cadmium effects on fish endocrine function. Juvenile fathead minnows were placed in UNTSRF pool sections of replicate streams treated with 0, 5, 20 or 80 µg/L Cd. Male VTG was induced at each treatment level, indicating that PCWRP effluent was estrogenic during fall 2001. 20 and 80 µg/L Cd treatments reduced male circulating estradiol levels and critical swimming performance. Future studies are needed to assess impacts of environmental estrogen exposure on fish calcium metabolism and vertebral integrity.

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OVERVIEW

The University of North Texas Stream Research Facility (UNTSRF) was designed to examine contaminant impacts on and the ecology of effluent-dominated stream ecosystems [1; Chapter 1]. Following design and construction of this system, the facility streams, fed treated wastewater from the City of Denton, TX, Pecan Creek Water Reclamation Plant, were initially tested with 0, 25 or 250 µg/L cadmium for a 10-day study in August 2000 [2]. Laboratory whole effluent toxicity tests were conducted with stream water concurrent with this stream experiment. Laboratory and instream responses indicated that cadmium bioavailability and toxicity were reduced by constituents of effluent-dominated streams. The Biotic Ligand Model, presently used to evaluate site-specific water quality parameter effects on copper and silver toxicity, was utilized to predict a 48 hour Cd EC_{50} for *Ceriodaphnia dubia* of 280 µg/L in these effluent-dominated streams. This value is markedly higher that an EC_{50} of 38.3 µg/L Cd and a 7-day reproduction lowest observed effect level of 3.3 µg/L Cd generated for *C. dubia* in reconstituted laboratory hard water. Results from Chapter 2 [2] support use of the Biotic Ligand Model for establishing site-specific acute water quality criteria in effluent dominated streams. However, periphyton, snail and fathead minnow tissues accumulated Cd in 25 µg/L treated streams that did not affect laboratory *C. dubia* or *Pimephales promelas* toxicity assays. Therefore, longer term Cd exposure in these streams may lead to accumulation in organisms and ultimately lower an observed 10-day no effect level of 15 µg/L.

During the initial UNTSRF study in 2000, adult male fathead minnows were exposed for 12 days to examine effects of cadmium and municipal effluent on vitellogenesis [3; Chapter 3]. Vitellogenin (VTG) induction was not observed in male fish exposed to any stream treatment during this experiment. Previous studies identified that adult male fathead minnow VTG was induced by City of Denton, TX, effluent exposure in March [4], but not June [5], of 2000. Chronologically following this stream study in August 2000, Allen et al. [6] exposed adult male fathead minnows to City of Denton, TX, effluent for 21 days in December 2000. Allen et al. [6] observed VTG induction and detected ethynyl estradiol in effluents during the first week but not the last two study weeks. Results from the Hemming et al. [4], Hemming [5], Brooks [3], and Allen et al. [6] studies indicated that the City of Denton, TX, effluent was seasonally estrogenic and that a correlation may exist between effluent estrogenicity and student enrollment of Texas Woman's University and the University of North Texas, each located in Denton, TX.

To identify the class(es) of compounds responsible for seasonal effluent estrogenicity, a toxicity identification evaluation procedure was applied to the City of Denton's effluent in May 2001 [7; Chapter 4). Effluent was sampled during and following university semester break and extracted using solid phase extraction disks. Each extraction disk was sequentially eluted with methanol, methylene chloride and hexane to fractionate potential estrogens or xenoestrogens based on polarity. Estrogenic activity of effluent solvent fractions was evaluated using an *in vitro* yeast estrogen screening assay (YES assay). Fractions identified as estrogenic by the YES assay were subsequently tested using an adult male Japanese medaka *in vivo* VTG assay to confirm

estrogenicity. City of Denton, TX, effluent was identified as only estrogenic during school session by *in vitro* YES and *in vivo* fish VTG bioassays. These results support the hypothesis that City of Denton, TX, effluent estrogenicity is influenced by population demographics when two university enrollment numbers change. Whereas estrogenic activity was recognized in only the methanol eluate fraction by the YES assay, VTG induction was observed in male medaka exposed for 7-days to methanol and methylene chloride fractions. Estrogen steroids and nonylphenol ethoxylate surfactant degradation products were identified as causative effluent estrogens; however, other unidentified compounds were responsible for male VTG induction following exposure to methylene chloride effluent fractions. These findings suggest that when municipal effluents are fractionated or screened for estrogenicity, *in vivo* bioassays should be used to confirm *in vitro* YES activity.

A 90-day cadmium study was initiated with UNTSRF streams in September 2001 to assess longer term estrogenic effluent and cadmium effects on fish endocrine function [3; Chapter 3). Because several studies [3-7; Chapter 4] supported the hypothesis that seasonal effluent estrogenicity may be influenced by university semester sessions, this experiment was designed to begin and end during the fall 2001 semester, when Denton, TX, effluent was hypothesized to be estrogenic. Juvenile fathead minnows were placed in stream pool sections of replicate streams treated with 0, 5, 20 or 80 µg/L Cd. Fish of the same age were concurrently reared in the laboratory. On day 90, relative to these laboratory reared organisms, male and female gonadosomatic indices were decreased and hematocrits increased by all treatments. Treatment effects were also observed on fish vitellogenesis, steriodogenesis, and hepatic estrogen receptor content. Male hepatic VTG

content was increased in each treatment level, indicating that the City of Denton, TX, effluent was estrogenic during fall 2001 university semester. Conversely, female hepatic VTG and ER content were reduced in each stream compared to laboratory fish. Circulating testosterone levels in female fish were significantly increased in all streams. Male circulating testosterone levels and female plasma estradiol levels were decreased at each treatment level. In addition, 20 and 80 µg/L Cd treatments reduced male circulating estradiol levels and swimming performance. Decreases in plasma estradiol levels is known to alter calcium metabolism and, ultimately, lead to osteoporosis in vertebrates. Impaired swimming performance may have resulted from calcium losses and reduced integrity of caudal vertebrae, previously shown to be affected by cadmium exposure. This research indicates that future studies are needed to assess impacts of estrogen exposure on fish calcium metabolism, vertebral bone strength and swimming performance.

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CHAPTER 1

INTRODUCTION AND DESIGN CONSIDERATIONS FOR THE UNIVERSITY OF NORTH TEXAS STREAM RESEARCH FACILITY

Introduction

In the arid southwestern United States [1-3] and other parts of the world [4, 5] flow of select stream systems is dominated by municipal and/or industrial effluent discharges. For example, Dorn [6] reported that approximately 23% of permitted effluent dischargers in the United States receive less than 10-fold instream dilution. Considering the relationship between a burgeoning global human population and its dependence on water resources, water quantity, particularly in arid regions, is considered the limiting factor to growth and an essential economic commodity [7]. It follows then that in regions currently experiencing or imminently facing water scarcity, maintenance of water quality concurrent with management of water quantity will remain critical.

Numerous investigators studied stream ecosystem responses to water quality changes; however, contaminant insults on effluent-dominated streams have received considerably less attention [8]. Mesocosms have allowed researchers to simulate natural conditions and experimentally manipulate treatments without impacting ecosystems [9-13]. Mesocosm studies have also been used to provide information on contaminant fate and effects for ecological risk assessments [14, 15]. Stream mesocosms are used “to bridge the gap” between laboratory ecotoxicity tests and field bioassessments [16]. If

uncertainty associated with the environmental safety of effluents and contaminants is reduced using lotic mesocosm bioassessments, low uncertainty factors (e.g. approximately 1) may be applied during ecological risk characterizations [17].

Many stream mesocosm studies have focused on benthic macroinvertebrate responses to treatment manipulation [13]. Benthic macroinvertebrates are often ideal for use in bioassessments employing stream mesocosms, primarily because invertebrates integrate exposure to contaminants at a relatively localized level and serve as site-specific indicators of environmental quality [18]. However, information about impacts of contaminant pulses on biota of effluent-dominated systems is lacking [19]. Here, lotic mesocosms are suggested as valuable tools for more direct evaluations of effluent-dominated ecosystem responses to contaminants [20].

Most stream mesocosm studies evaluated single chemicals at multiple concentrations with or without treatment level replication [13]. Regression designs are common and suggested for use in risk assessment when experimental units are scarce [17, 21]. Despite problems associated with pseudo-replication [22], lack of replication has been justified because within unit variability due to treatments can be "substantially more important" than between unit variability [17]. Whereas lack of replication may be sufficient for chemical fate studies, replication is required for univariate techniques such as ANOVA [23] and should be included in the experimental design of chemical effect assessments.

Design of the University of North Texas Stream Research Facility

To examine contaminant impacts on effluent-dominated streams, the University of North Texas Stream Research Facility (UNTSRF) was constructed at the City of Denton, TX,

Pecan Creek Water Reclamation Plant (PCWRP) during the summer and fall of 1999. The design approach to these mesocosms included several considerations: scale, experimental unit number, recirculating vs. once through flow, site location, and water source. UNTSRF streams were designed, in a cost efficient manner, to maximize experimental design flexibility and replication (N = 12), and allow for investigations of multiple levels of biological organization.

Previous investigators contemplated the relationship between mesocosm scale and biological complexity. Hoffman [24] reported that small scale (<1m) laboratory streams are advantageous for study of periphytic community responses because hydrological variation often associated with larger systems is negated. However, these small laboratory systems lack ecological relevance to natural systems and only allow for investigation of microbial responses to contaminants [24]. Larger stream mesocosms are advantageous because they can support multitrophic, multidisciplinary studies that may be of greater ecological relevance [25]. A common problem associated with using large outdoor streams (>50m long) for ecological or ecotoxicological study is longitudinal effects [25]. Longitudinal gradients introduce substantial variability within experimental units such that interpretation of biological responses to treatment manipulation is often confounded by hydrological differences between stream mesocosms [26]. Most experimental stream studies tend to fall between the extreme scale ranges of those described by Hoffman [24] and reviewed by Swift et al. [25]. For example, of 152 total stream mesocosm studies reviewed by Belanger [17], 55% were conducted in stream facilities less than 10 m in length. Further, Belanger [17] found that increasing model system size or length did not correspond to an increase in taxa richness or Shannon-Weiner diversity.

Another disadvantage of large outdoor stream mesocosms is the cost associated with initial construction and routine maintenance of such a facility [25]. The number of experimental units utilized in mesocosm studies is often limited by sample collection and analysis cost considerations. A series of 12 streams were constructed at UNTSRF to maximize the flexibility of future study experimental designs. UNTSRF streams were designed with once through flow; that is, water entering a model stream does not recirculate. Once through was selected over recirculating flow [27] because most ecotoxicological mesocosm studies attempt to maintain constant exposure concentrations, which are easier to maintain with this flow regime [21].

The UNTSRF was constructed on City of Denton, TX, property at PCWRP for several reasons. First, even if only environmentally realistic concentrations of contaminants were added to UNTSRF streams, water leaving stream pool sections is considered an effluent and subject to National Pollutant Discharge Elimination System permitting. Similar to Crossland et al.'s [28] rationale, location of UNTSRF at the City of Denton, TX, PCWRP allowed effluent water leaving stream pools to be treated by PCWRP prior to environmental discharge. Second, locating the facility at PCWRP provided an ample supply of effluent source water for stream flows. In addition, electricity and potable water were provided to UNTSRF by the City of Denton, TX, from existing infrastructure. UNTSRF was constructed approximately 30 m from Pecan Creek that receives effluent from PCRWP. Such a relatively close location to an effluent receiving system facilitated aerial colonization of UNTSRF streams by resident insects of Pecan Creek. Invertebrate taxa in UNTSRF streams are similar to those in a 1994 study of Pecan Creek macroinvertebrates by Wise [29] (Table 1).

Table 1. Benthic macroinvertebrate taxa in Pecan Creek and in UNTSRF riffle sections.

Pecan Creek Taxa 1994 (Wise 1995)	UNTSRF Taxa 2000
Ancylidae	
Baetidae	Baetidae
Caenidae	Caenidae
Chironominae	Chironominae
Coenagrionidae	Coenagrionidae
Empididae	
Heptageniidae	
Hydracarina	Hydracarina
Hydrophilidae	Hydrophilidae
Hydroptilidae	Hydroptilidae
Libellulidae	Libellulidae
Nematoda	Oligochaeta
Oligochaeta	Orthocladiinae
Orthocladiinae	Physidae
Physidae	Pyralidae
Polycentropidae	Simulidae
Tanypodinae	Tanypodinae

UNTSRF streams are conceptually similar to those described by Crossland et al. [28] and Rodgers et al. [16]. A concrete pad was initially constructed to provide a solid base for stream construction; cinderblocks were used to support and maintain slopes for stream riffles. A canopy was suspended approximately 2 m above streams to approximate shading provided by riparian vegetation of a first order stream. A photometer was used to quantify canopy shading at forty percent, a value equal to that employed by Rodgers et al. [16] to shade similar outdoor stream mesocosms.

Dechlorinated, final treated effluent from PCWRP is pumped into two 55,000 l water storage reservoirs and gravity fed to stream mesocosms (Table 2, Figure 1). Dispersal of water to streams is controlled by ball valves which allow for individual stream flow calibration. Chemical treatments are pumped from Nalgene® carboys with high precision peristaltic pumps and delivered to streams by peristaltic tubing (SciLog, Inc.). Water and chemical treatments converge in 160 l mixing chambers (Figures 2, 3). Although previous studies [16] successfully relied on turbulent mixing of inflowing water to homogenize chemical treatments, plexiglass baffles were designed and placed in UNTSRF mixing boxes to further facilitate mixing of chemical and inflowing effluent. Water from mixing chambers flows through two 2.5 m long x 0.6 m wide riffle sections (Figures 2, 3). Mixing boxes and riffle sections were constructed of marine plywood coated with non-toxic marine epoxy paint [16]. Rodgers et al.'s [16] facility succumbed to wood rotting after several years of study (BW Brooks, personal observation). Therefore, prior to paint application, fiberglass was applied to UNTSRF stream riffles and mixing boxes to increase water retention and stream longevity.

UNTSRF riffle sections were designed with different slopes to allow for design

flexibility of future studies. Upstream riffles were designed with a 1:20 slope following Rodgers et al. [16] (Figure 2, Table 1). Downstream riffles were designed with half the

Table 2: Physical characteristics of UNTSRF reservoirs, mixing boxes, riffles, and pools.

Storage Reservoir Volume (2 total)	55,000 L
Mixing Box Volume	160 L
Flow Volume	28 L min^{-1}
Upstream Riffle	
Slope	1/20
Substrate	Gravel
Area	1.5 m^2
Downstream Riffle	
Slope	0.5/20
Substrate	Gravel
Area	1.5 m^2
Pool Volume	568 L

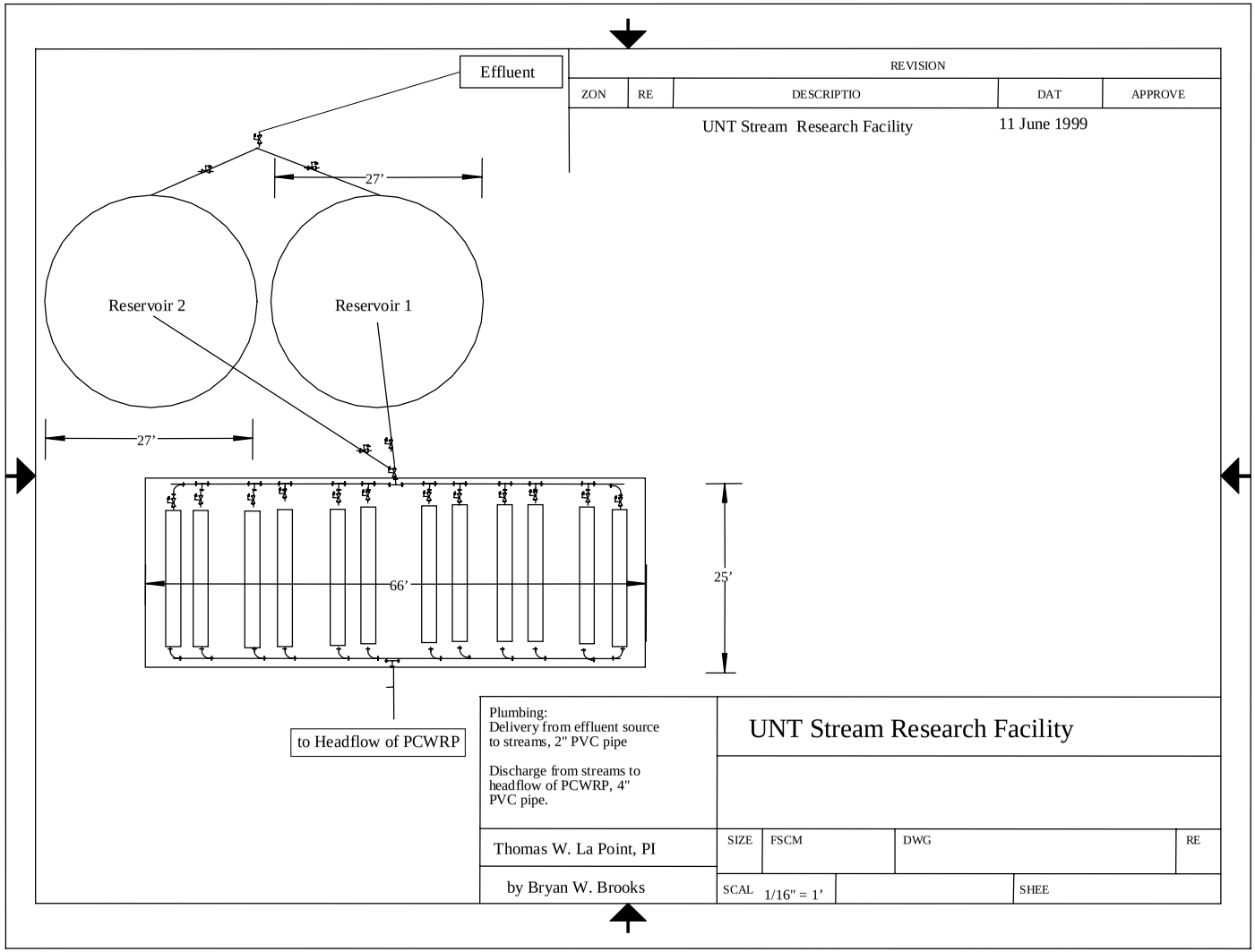

Figure 1: Aerial schematic of the University of North Texas Stream Research Facility .

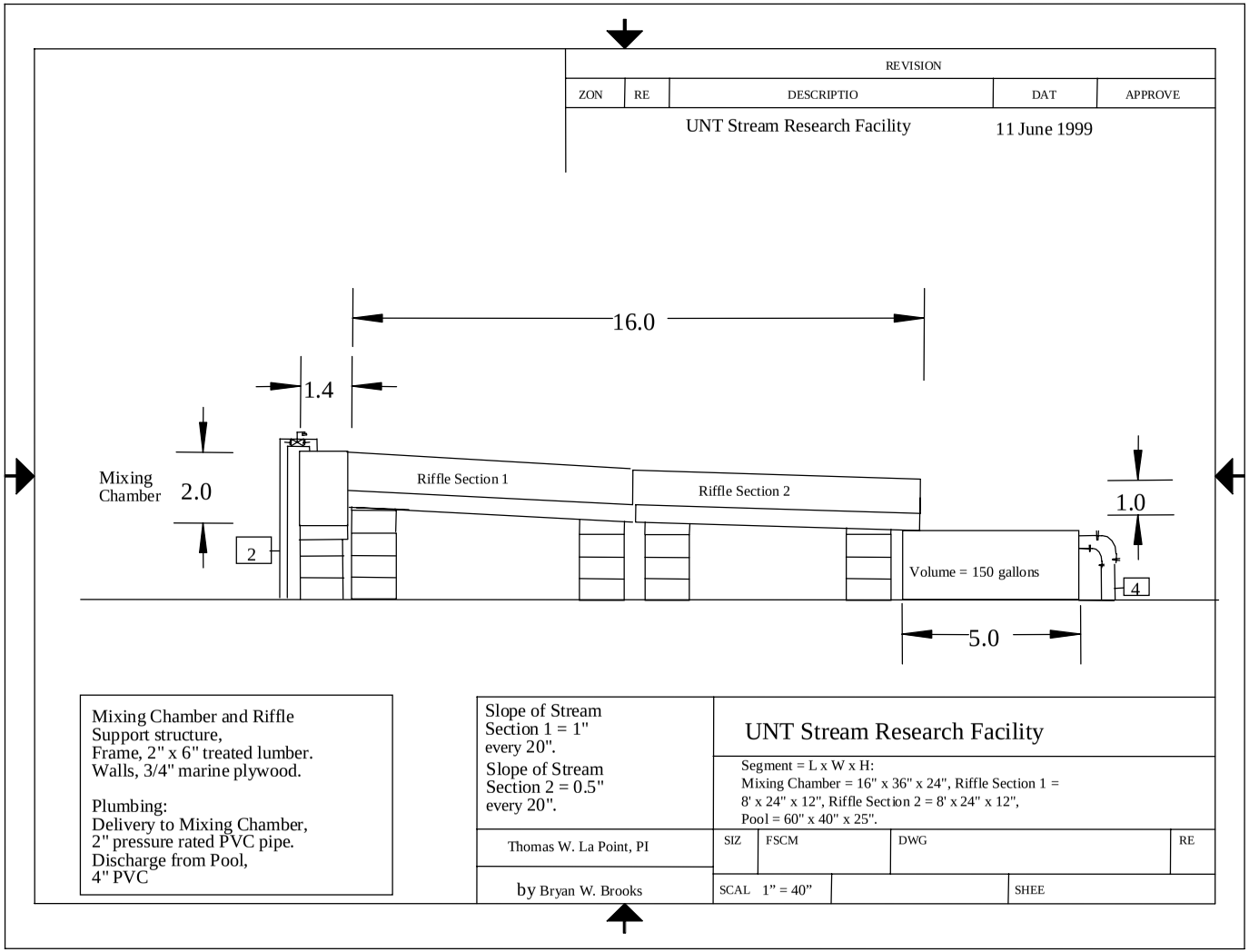

Figure 2: Lateral schematic of a University of North Texas Stream Research Facility lotic mesocosm.

Figure 3: Photograph of the University of North Texas Stream Research Facility.

upstream slope (or, 0.5:20) to allow for study of slower flows and potentially different substrates (Figure 2). Clean gravel substrates (diameter approx. 1.5 cm) were added to riffles at a depth of 5 cm. Plexiglass outflow shoots were thermally molded to downstream riffle sections and provided transfer of water from riffles to 568 l polyethylene pools. Pool sections were included in the stream design to allow for investigation of *in situ* fish population responses to contaminants. Discharge from experimental stream pools are pumped to wastewater inflow and treated by PCWRP.

The UNTSRF was initially utilized for a 12-day experimental study in August 2000 (Chapter 2, Chapter 3). A longer term (90-day) study was performed from September to December 2001 (Chapter 3). This 90-day study was performed during an annual period in which the City of Denton, TX, was previously identified as estrogenic (Chapter 4).

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CHAPTER 2

LABORATORY AND FIELD RESPONSES TO CADMIUM EXPOSURE IN EFFLUENT-DOMINATED STREAM MESOCOSMS

Abstract

Whole effluent toxicity tests measure individual responses, but do not predict natural population or community responses. Whereas southwestern U.S. streams are often dominated by municipal effluents, potential hazard of metals has not been experimentally evaluated in effluent dominated stream systems. Stream mesocosms were used to assess cadmium impacts in streams and to determine relevance to regulatory criteria of standard laboratory tests. My objectives were to verify exposure predictions; to assess cadmium effects on multiple levels of biological organization; and to link laboratory bioassays to system responses after chemical exposure. City of Denton, TX, effluent served as source water for outdoor streams that were colonized by invertebrates from a nearby stream. Because of inherent temporal effluent variability, source water was characterized chemically (standard parameters hourly; alkalinity, hardness, TOC, and metals at 0600, 1400, 2200) and biologically (*C. dubia* bioassays for 0600, 1400, and 2200 samples). Cadmium was added to replicate units at 0, 25 and 250 µg/L during a 10-day study period. Stream mesocosms were sampled on days 0 and 10 for macroinvertebrates, periphyton, and ecosystem metabolism. Concurrent laboratory *C. dubia* and *P. promelas* bioassays were performed with water from each stream. Compared to untreated streams, population, community, and system response variables

were affected by 250 µg/L Cd, but not by 25 µg/L Cd treatments. Laboratory and field results indicate that municipal effluent constituents alter metal bioavailability. Current water quality criteria for metals based on hardness alone do not account for such constituents. My findings support use of the Biotic Ligand Model for establishing site-specific water quality criteria in effluent dominated streams. Although effluent dominated streams reduced Cd bioavailability and acute toxicity, longer term exposure to similar Cd concentrations may increase accumulation in benthic macoinvertebrates and ultimately lower the 10-day no effect level of approximately 15 µg/L observed in this study.
Keywords- cadmium, lotic mesocosms, lab-to-field, Biotic Ligand Model

Introduction

Aquatic organisms are often exposed to complex mixtures of environmental stressors [1, 2]. Whole effluent toxicity (WET) tests are used to assess effects of such mixtures found in industrial and municipal effluents. Under the National Pollutant Discharge Elimination System program, freshwater discharger compliance to permits is limited to standard laboratory testing with *Selanastrum capricornutum*, *Ceriodaphnia dubia* and *Pimephales promelas* bioassays [3]. WET tests may not be protective of all species, are not designed to measure natural population and community responses and do not address issues of contaminant bioaccumulation [4].

Whereas WET test predictions of instream impairment have been extensively investigated [5, 6], adverse effects on aquatic biota may occasionally result [7]. Interactions among municipal effluents and upstream contaminants can reduce agreement

between WET test and field assessment results for a given discharge. Effluent and upstream contaminant mixtures may have cumulative effects on aquatic biota [4]. Further, water chemistry upstream from discharge sites often differs from laboratory dilution water used in standard WET tests [8]. In some circumstances, sites downstream from effluent discharges may exhibit greater biological integrity, as determined by biotic indices, than upstream sites if effluent constituents (e.g. total/dissolved organic carbon) reduce bioavailability of upstream contaminants and serve in a protective fashion, independent of dilution [9]. Responding to such site-specific differences in water quality, USEPA implemented water effect ratios (WER) which are often used for metal contaminants [10]. WER's are calculated as a ratio of LC_{50}'s generated from side-by-side tests using site and laboratory dilution water and have provided an interim approach for determining influence of site-specific constituents on metal bioavailability [11].

The Biotic Ligand Model (BLM) simulates site-specific water quality influences on metal bioavailability [12]. Advancing the "hardness alone" correction used in developing metal water quality criteria and WERs, the BLM further characterizes metal-ligand interactions in aqueous matrices including estimates of binding coefficients interactions with organic matter [12]. Based on user-defined, site-specific water chemistry parameters, the BLM predicts metal speciation, metal-gill accumulation (LA_{50}) and acute toxicity (EC_{50}) to standard toxicity test organisms [12]. An LA_{50} is defined as metal accumulation on the biotic ligand (e.g. metal-gill) necessary to produce a 50% lethal response. Versions of the BLM are presently available for copper and silver; however, BLMs for other metals, including cadmium, zinc, and nickel, are at various stages of development and review by USEPA (R Santore, HydroQual, pers. comm.).

Clements et al. [13] identified that copper toxicity to stream invertebrates was greater in laboratory waters than in field streams. Such differences were attributed to higher field dissolved organic carbon and total suspended solids. Water chemistry differences between laboratory and site-specific waters may be pronounced in effluent dominated streams. Municipal effluents often dominate rivers in the southwestern United States; the Trinity River south of Dallas/Ft. Worth, TX, is often greater than 90% return flow from wastewater treatment plants [14]. In addition, Dorn [15] reported that approximately 23% of permitted discharges receive less than 10-fold dilution. In these scenarios, lotic mesocosm bioassessments are suggested to evaluate effects of wastewater discharges [4]. Because mesocosms allow for treatment manipulation without impacting natural ecosystems [16, 17], stream mesocosms are used to confirm predictions from single-species toxicity tests and evaluate the response of multiple species to stressors [18]. For example, previous investigators have utilized lotic mesocosms to evaluate cadmium effects on streams [19-23]; however, metal effects on effluent dominated systems have not been experimentally tested. Further, application of the BLM to cadmium exposure in effluent dominated streams has not been performed.

Because cadmium concentrations have been detected as high as 90 µg/L in north Texas streams [24], a 10-day cadmium study was conducted in effluent dominated lotic mesocosms at the University of North Texas Stream Research Facility. The objectives of this study were three-fold: 1. assess cadmium effects on periphyton, benthic invertebrates, and ecosystem metabolism of effluent dominated streams, 2. assess laboratory bioassay responses to stream cadmium treatments, and 3. assess water

chemistry parameters that affect metal bioavailability for Biotic Ligand Model estimation of cadmium acute toxicity to *C. dubia*.

Materials and Methods

Experimental Design

Six stream mesocosms, described in Chapter 1, were selected for study at the University of North Texas Stream Research Facility (UNTSRF). Approximately 1 cm diameter pea gravel was added to stream riffle sections to a total substrate depth of 6 cm. Stream substrate samplers and 10 cm x 10 cm unglazed tiles were introduced to riffle sections for collection of invertebrate and periphytic community measures, respectively. Stream substrate samplers were constructed from 7.7 cm diameter PVC pipe and 20 mm x 20 mm polyethylene screen (Figure 1). Stream flows were calibrated to 29 L/min and maintained for a 90-day pre-study period. This study was initially designed to proceed for 30 days; however, a stream system malfunction terminated the study at 12 days. Replicate streams were nominally dosed with 0, 25, or 250 µg/L Cd using peristaltic pumps from 25 August 2000 (Day 0) to 6 September 2000 (Day 12). Treatment levels were chosen to bracket highest reported Cd exposure levels in north Texas streams (90 ug/L; USEPA STORET Database) *and* to experimentally test responses of a previously untested research facility to contaminant insults.

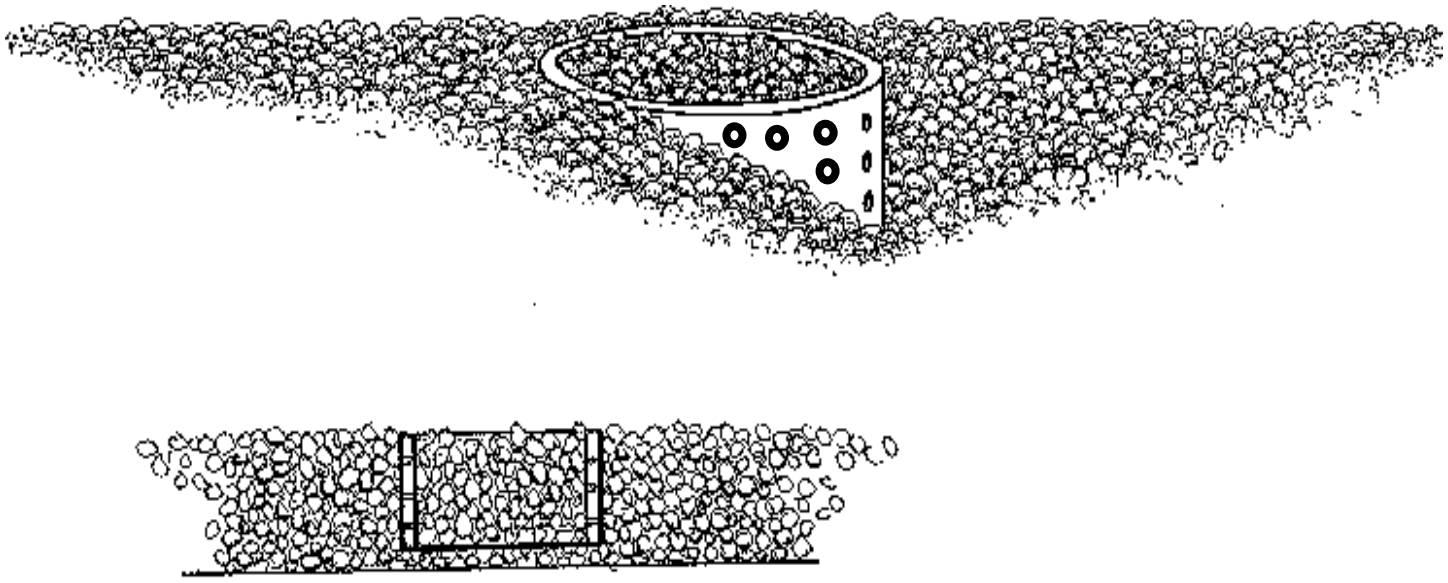

Figure 1: Schematic of stream riffle substrate samplers (courtesy of Peg La Point).

Chemical and Biological Characterization of Source Effluent

Because of inherent temporal effluent variability, source water for UNTSRF streams, the City of Denton, TX effluent, was characterized chemically and biologically during this study. Chemical measures included hourly readings of pH, temperature (^{o}C), specific conductance (µS/cm), dissolved oxygen (mg/L), total dissolved solids (TDS) (mg/L) and turbidity (NTU) with a Hydrolab multiprobe datasonde. In addition, effluent was collected on study days -2, 2, 5, 9 and 12 at 0600, 1400, and 2200 for alkalinity, hardness, total organic carbon, and total and dissolved cadmium and copper analysis. Sample times were chosen at these intervals because the City of Denton, TX, Pecan Creek Water Reclamation Plant has a residence time of approximately eight hours under conditions of normal inflow. These sample times were intended to roughly characterize potential temporal changes in effluent water quality parameters known to affect metal bioavailability and other metal effluent concentrations. Potentiometric alkalinity and colorimetric hardness measurements followed standard methods [25]. Samples for total organic carbon were preserved by acidification to a pH of <2 with H_2SO_4 and refrigerated until analysis with a ThermoGlas TOC analyzer (Franklin, MA). Metal samples were preserved by acidification to a pH of <2 with HNO_3, refrigerated until analysis, and quantified with a Perkin-Elmer Model 2380 flame or furnace atomic absorbtion spectrophotometer, as appropriate [25]. Dissolved metal samples were filtered with a 0.45 um glass fiber filters prior to acidification. Similarly, verification of nominal treatment levels on three study days followed these methods.

Samples were also collected at 0600, 1400, and 2200 and transported to the laboratory on ice for testing of potential effluent toxicity. A seven-day *C. dubia* WET

test was performed on daily samples collected at each sampling time and generally followed methods outlined by USEPA [26]. Deviations from these methods were minimal and are detailed in Hemming et al. [27]. *C. dubia* WET tests were initiated on study day 3.

Stream Sample Collection

Streams were evaluated for treatment effects using laboratory bioassay and stream responses. Downstream riffle outflows were sampled daily at 0600 and transported to the laboratory on ice for toxicity tests. Seven-day, static renewal *P. promelas* and *C. dubia* laboratory toxicity tests were initiated on day 3 and conducted following standard methods [27]. In addition, 48-hour acute and 7 day laboratory *C. dubia* toxicity tests were performed in reconstituted hard water to evaluate cadmium effects on survival and reproduction [27]. Alkalinity and hardness measures of reconstituted hard water and verification of nominal toxicity test treatment levels followed the above-described methods.

Triplicate substrate samplers were collected from stream riffle sections on study days 0 and 10. Unglazed tiles were also sampled on days 0 and 10 for invertebrates and periphytic biomass. Periphyton communities were scraped from tiles, filtered by 0.45 um glass fiber filters, and analyzed for periphytic biomass (AFDW) following standard methods [25]. Invertebrates were sorted from samples and preserved in 70% ethanol. Macroinvertebrates were identified to family or genus [28], except for individuals of the Dipteran family Chironomidae, which were identified to subfamily. Whole system production and respiration was determined for each stream on study days 0 and 10 using

a single curve method [29, 30]. Diel dissolved oxygen concentrations were measured every 4 hours in downstream pool sections. Generation and graphical analysis of dissolved oxygen concentration changes followed methods of Brooks et al. [31]. Briefly, dissolved oxygen concentrations were converted to rate of change and plotted for each period and stream using graphical software (Delta Graph, Version 4.0). Areas under curves estimating gross nighttime ecosystem respiration, gross daytime ecosystem respiration, and gross primary production [30] were digitally integrated as pixel numbers using NIH Image (Version 1.0). Pixel number was subsequently converted to O_2 rate of change ($g\ m^{-3}\ hr^{-1}$).

Biotic Ligand Model

The BLM for cadmium was used with effluent source water chemistry parameters to estimate 48 hr cadmium toxicity to *C. dubia* survival. Because the cadmium BLM, developed by HydroQual, Inc., was under review by USEPA and not available for public use at the time of this study, water quality and laboratory toxicity data was provided to Hydroqual, Inc. for model estimations (KB Wu, HydroQual, pers. comm.). Required water chemisty data for BLM simulations included dissolved cadmium, organic carbon, pH, temperature, percent humic acid, alkalinity, calcium, magnesium, sodium, potassium, chloride, sulfates and sulfides. Calcium, magnesium, sodium, potassium, chloride and sulfate levels were quantified in effluent source waters during this study by the City of Denton, TX, municipal laboratory according to standard methods [25]. Percent of dissolved organic carbon as humic acid and sulfides were not determined in effluent source waters; however, standard default values of 10% humic acid and 0.01 mg/L

sulfides were used for biotic ligand modeling (R Santore and KB Wu, HydroQual, pers. comm.).

Cd Accumulation

Mature male fathead minnows were acquired from Kurtz Fish Hatchery, Elverson, PA, and held in dechlorinated tap water in the laboratory for two weeks prior to stream study initiation. Fish were caged in stream pool sections on day –2 to acclimate. Organisms were fed Tetramin® flake fish food daily during the study period. Fish were removed from streams, transported to the laboratory and gills removed on day 10. Gill tissues from three fish held in the laboratory during the study period were collected for untreated control purposes. Excised gills from three fish per stream were rinsed with dechlorinated tap water, weighed and stored at –80^{o}C. Tissues were dried at 80^{o}C for 24 hours, and then digested with 5 mL trace metal grade nitric acid. Digests were filtered through 0.45 um glass fiber filters, brought to a 25 mL volume with Milli-Q water, and Cd measured with a Perkin-Elmer Model 2380 flame or graphite furnace atomic absorbtion spectrophotometer [32]. Snail and periphyton communities were sampled on study days 0 and 10 for Cd accumulation. Only one dead snail was observed and collected on day 10 in high treatment streams. Samples were transferred to the laboratory on ice, rinsed with EDTA to remove Cd not incorporated in tissues [33], and were subsequently frozen at -20^{o}C until further analysis. Digestion of snail and periphytic tissues followed those used for gill samples; however, Cd was measured in these tissues with a Varian SpectrAA Model 600 graphite furnace atomic absorbtion spectrophotometer. Triplicate analytical measures were performed on each sample and

tissue Cd concentrations were verified with a Cd reference material (Tort-2, National Research Council, Canada).

Statistical Analyses

Analyses of treatment effects were performed by two-way ANOVA (time and Cd factors) using SPSS (Version 10.05). Proportional data and benthic macroinvertebrate abundance were arc sine (square root (y)) and log (x+1) transformed, respectively [34]. Statistical power increases as replication of experimental units increases. However, cost associated with experimental unit replication often precludes appropriate replication, based on *a priori* power analysis, in mesocosm studies. Because two replicate streams per treatment level were utilized in this study, statistical significance of instream response variables was determined at $\alpha = 0.10$ to reduce the chance of type II errors.

Results

Effluent Characterization and Treatment Level Verification

Source effluent water quality, characterized by 0600, 1400 and 2200 grab samples, did not exhibit dramatic fluctuations between sample time points (Table 1). Dissolved oxygen concentrations followed typical diel variation with mean values of 4.5, 7.3, and 4.2 mg/L at 0600, 1400 and 2200, respectively. Effluent total Cu concentrations increased to a mean of 19.3 µg/l in 1400 samples compared to 17.5 µg/L at 0600 and 17.7 µg/L at 2200. Dissolved Cu concentrations were slightly elevated at 1400 compared to

Table 1: Mean water quality characteristics (±SD) of UNTSRF (City of Denton, TX effluent) at 0600, 1400, and 2200.

Parameter	0600	1400	2200
pH	6.90 (±0.10)	6.90 (±0.09)	6.88 (±0.09)
Temperature (^{o}C)	29.3 (±0.18)	31.3 (±0.12)	30.8 (±0.10)
Dissolved Oxygen (mg/L)	4.5 (±0.29)	7.3 (±0.43)	4.2 (±0.23)
Alkalinity (mg/L, $CaCO_3$)	64 (±8.22)	69 (±8.94)	69 (±12.45)
Hardness (mg/L, $CaCO_3$)	145.6 (±11.52)	140 (±5.66)	142.4 (±9.21)
Total Organic Carbon (mg/L)	7.62 (±1.05)	7.80 (±2.05)	7.31 (±2.20)
Total Dissolved Solids (mg/L)	0.5751 (±0.0061)	0.5779 (±0.0018)	0.5765 (±0.0024)
Specific Conductance (µS/cm)	898.4 (±9.7)	903.0 (±2.8)	900.6 (±3.9)
Turbidity (NTU)	9.17 (±3.20)	12.2 (±5.45)	9.43 (±3.86)
Cu (µg/L) Total	17.5 (±1.3)	19.3 (±3.1)	17.7 (±2.6)
Dissolved	16.5 (±1.0)	17.3 (±3.3)	16.7 (±0.6)

0600 and 2200 samples (Table 1).

C. dubia survival and reproduction were not adversely affected by source effluents sampled at 0600, 1400, and 2200 on study days 3 through 10. Instead, *C. dubia* fecundity (neonates female^{-1}) was higher in temporal effluent samples than in laboratory reconstituted hard water controls. Mean fecundity of reconstituted hard water control, 0600, 1400 and 2000 organisms were 22.3 (±1.34), 31.7 (±4.03), 31.6 (±4.97) and 29.9 (±2.99), respectively.

Cadmium treatment levels were verified at lower than nominally dosed values. Average total and dissolved Cd concentrations (±SD) in streams 2 and 10, treated nominally with the low dose of 25 µg/L, were 17 µg/L (±1.4) and 14.5 µg/L (±0.7), and 13 µg/L (±1.4) and 12 µg/L (±2.8), respectively. Average total Cd concentrations in the high dose, nominal 250 µg/L treatments, streams 6 and 8, were 146.5 µg/L (±17.7) and 141 µg/L (±21.2), respectively. Dissolved Cd was detected at an average of 131 µg/L (±2.8) in stream 6 and 120 µg/L (±39.6) in stream 8.

Laboratory Toxicity Test Responses

Based on analytically verified concentrations, a Cd EC_{50} value of 38.3 µg/L (95% C.I.: 30.5 - 46.1) was determined for a 48-hour laboratory *C. dubia* toxicity test performed in reconstituted hard water. Also based on measured concentrations, 7-day no observed Cd effect concentration (NOEC) and lowest observed Cd effect concentration (LOEC) for *C. dubia* reproduction were determined to be 1.1 and 3.4 µg/L, respectively. Alkalinity and hardness of reconstituted hard water were 110 mg/L ($CaCO_3$) and 170 mg/L ($CaCO_3$), respectively. In toxicity tests performed with stream waters, 0 and 25

µg/L Cd treatment levels did not affect *C. dubia* survival. However, in high treatment level streams (250 µg/L nominal Cd), 100% *C. dubia* mortality was observed at 120 hours and 96 hours for streams 6 and 8, respectively (Table 2). LT_{50}'s, the time required to produce 50% mortality [35], were calculated for *C. dubia* at 71 hours in stream 6 and 51 hours in stream 8 using Probit analysis [36]. *C. dubia* reproduction was not reduced by 0 or 25 ug/L treatments. Average neonates produced per female were 30.8, 30.4, 21.4 and 28.5 for streams 4, 12, 2, and 10, respectively (Table 2). Laboratory reconstituted hard water control organisms produced an average of 22.3 neonates per female (Table 2). Similar to *C. dubia* treatment responses, *P. promelas* survival and growth were not affected by 0 and 25 µg/L treatments, but were significantly affected by 250 µg/L treatments (Table 2).

Biotic Ligand Model

The BLM was performed for data from low and high Cd treated streams. Three steps were followed during biotic ligand modeling. First, biotic ligand modeling was performed to calculate an acute LA_{50} of 7.53 nmol/wet weight (g) for 48-hr cadmium toxicity to *C. dubia*. Second, the BLM was used to predict 48-hr LA_{50}'s for *C. dubia* exposed in laboratory toxicity tests to water from streams 2, 10, 6 and 8 at 3.698, 3.562, 7.05 and 7.159 nmol/wet weight (g), respectively. Third, using the BLM, a 48-hr LC_{50} value of 280 ug/L Cd was estimated for *C. dubia*, based on an LA_{50} of 7.53 nmol/wet weight (g) and effluent source water chemistry (Table 1) plus two estimated model variables (% humic acid and sulfides).

Table 2: Seven day, laboratory toxicity test responses to stream Cd treatments. RHW = laboratory reconstituted hard water. ND = not determined. SRF = stream research facility mesocosm.

		Treatment Level						
			0 µg/L Cd		25 µg/L Cd		250 µg/L Cd	
Organism	Response	RHW	SRF 4	SRF 12	SRF 2	SRF 10	SRF 6	SRF 8
C. dubia	Survival (%)	100	100	100	100	100	0	0
C. dubia	Fecundity (neonate #)	22.3	30.8	30.4	21.6	28.5	0	0
P. promelas	Survival (%)	90	93.3	90	86.7	96.7	6.7	3.3
P. promelas	Growth (mg)	0.26	0.27	0.30	0.29	0.24	ND	ND

Macroinvertebrate Responses

Benthic macroinvertebrate abundances were unaffected by treatment structure. Average invertebrate abundance per substrate sampler was 448 (±98.5) organisms or 2.9 organisms cm^{-3}. Richness, quantified as taxa number, and taxa diversity were low in all streams and also not significantly affected by treatments (data not shown). Taxa richness was typically less than 10 per substrate sampler; Shannon-Wiener diversity (log $base_2$) was less than 2 in all samples examined. However, statistically significant macroinvertebrate population changes were observed in response to Cd treatments. The most dominant stream riffle taxa in substrate samples and on tiles were snails, *Physa* sp., and Chironomidae. On study day 0 *Physa* sp. and Chironomidae comprised 53.7% and 23.9%, and 68% and 17% of total macroinvertebrate abundance in substrate samplers and on tiles, respectively. The only significant macroinvertebrate responses to treatments included a reduction of *Physa* sp. population abundance (Time x Cd, $p = 0.03$; Figure 2) and an increase in Chironomidae abundance in 250 µg/L Cd treated streams on day 10 (Time x Cd, $p = 0.007$; Figure 3). In addition to abundance measures in substrate samplers and on tiles, *Physa* sp. abundances were determined by counting snails on tiles in each stream on study days 0, 5 and 10. On day 0, average *Physa* sp. abundance on tiles was 0.59 (±0.12) organisms cm^{-2}. On study days 5 and 10, *Physa* sp. abundance was significantly reduced in 250 µg/L treatments to an average of 0.12 (±0.09) cm^{-2} and 0.02 (±0.019) cm^{-2}, respectively. *Physa* sp. abundance was not affected by 25 µug/L treatments (Figure 4).

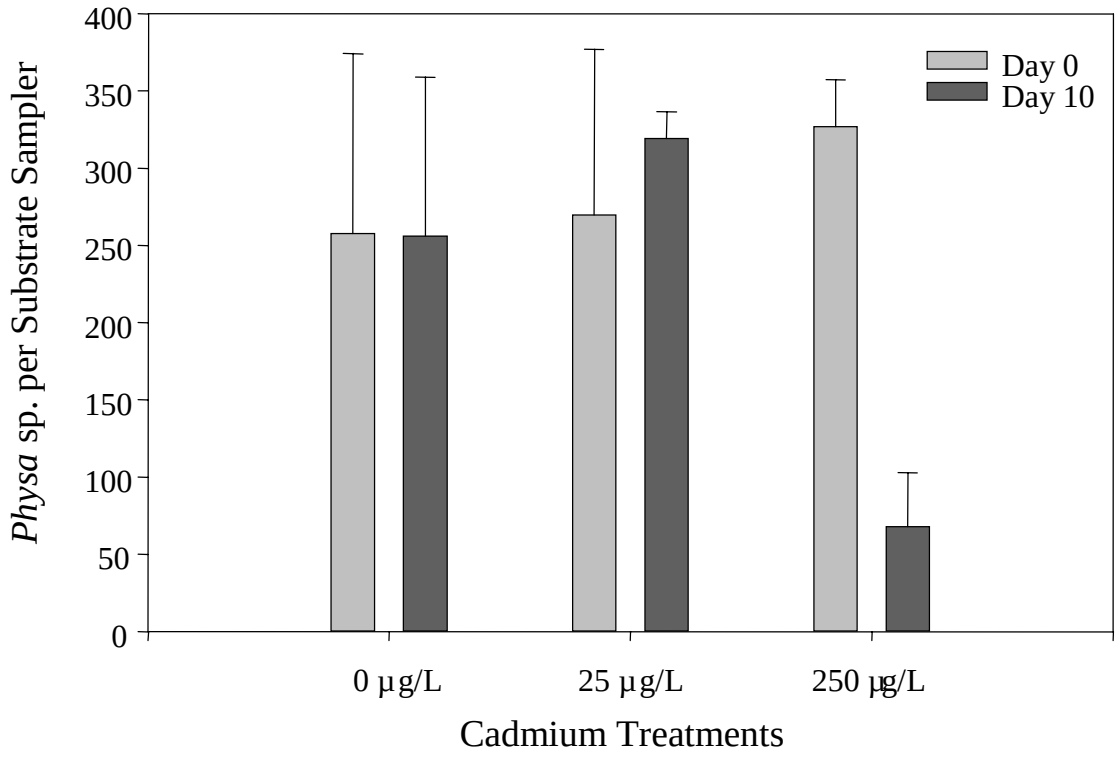

Figure 2: Effects of cadmium on *Physa* sp. abundance (±SD) in stream riffle substrate samplers (Cd, p=0.04; Cd x Time, p=0.03).

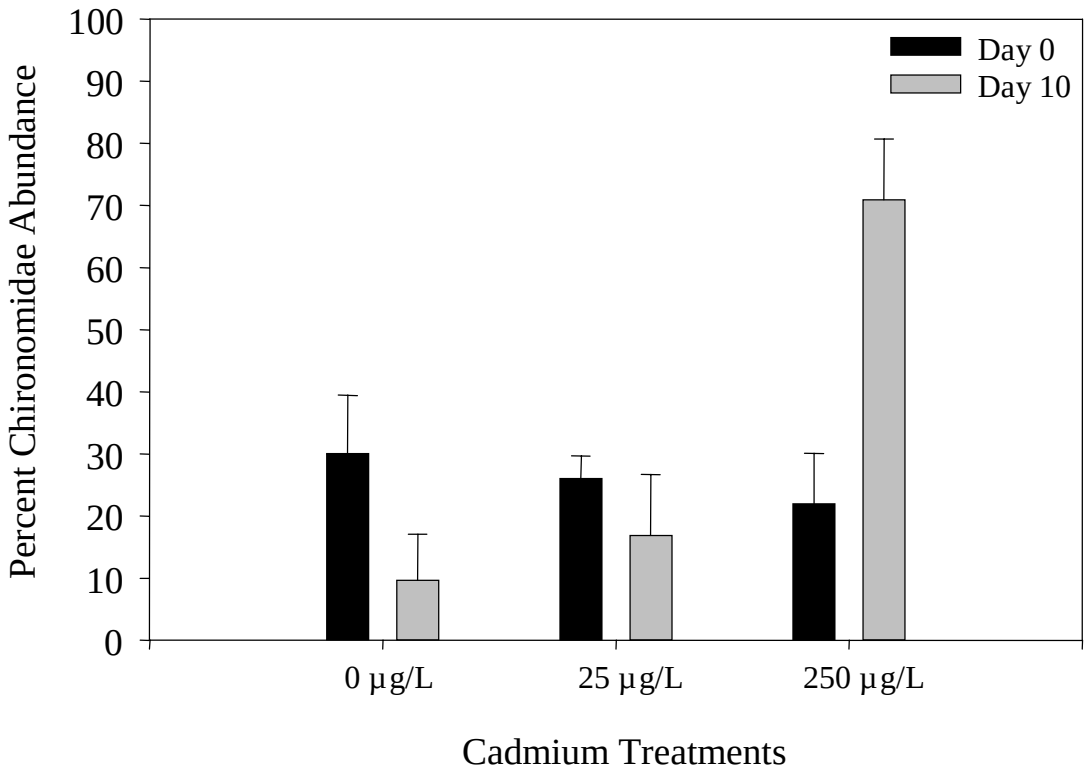

Figure 3: Effects of cadmium on Chironomidae percent abundance (±SD) in stream riffle substrate samplers (abundance, Cd, p=0.07; Cd x Time, p=0.007).

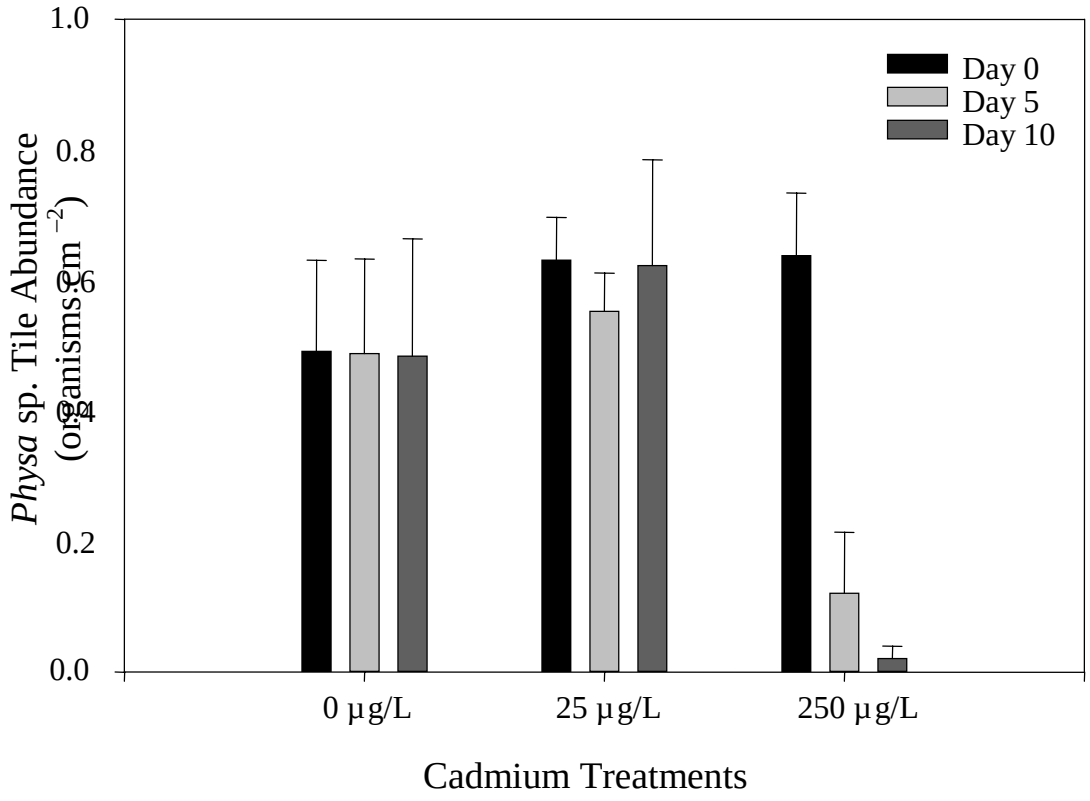

Figure 4: Effects of cadmium on *Physa* sp. abundance (±SD) on stream tile samplers.

Pyralidae lepidopterans were identified in substrate samples and also collected from tiles used for periphyton analysis. Pyralidae abundance was greater on tiles than in substrate samplers, perhaps because unglazed tiles provided a better substrate than riffle pea gravel for case building or better grazing on periphyton. Although no statistically significant differences were observed from tile measures (Time x Cd; $p = 0.38$), Pyralidae organisms were not observed on tiles from 250 µg/L treatments (Figure 5). Rather, empty cases were observed on high treatment tiles.

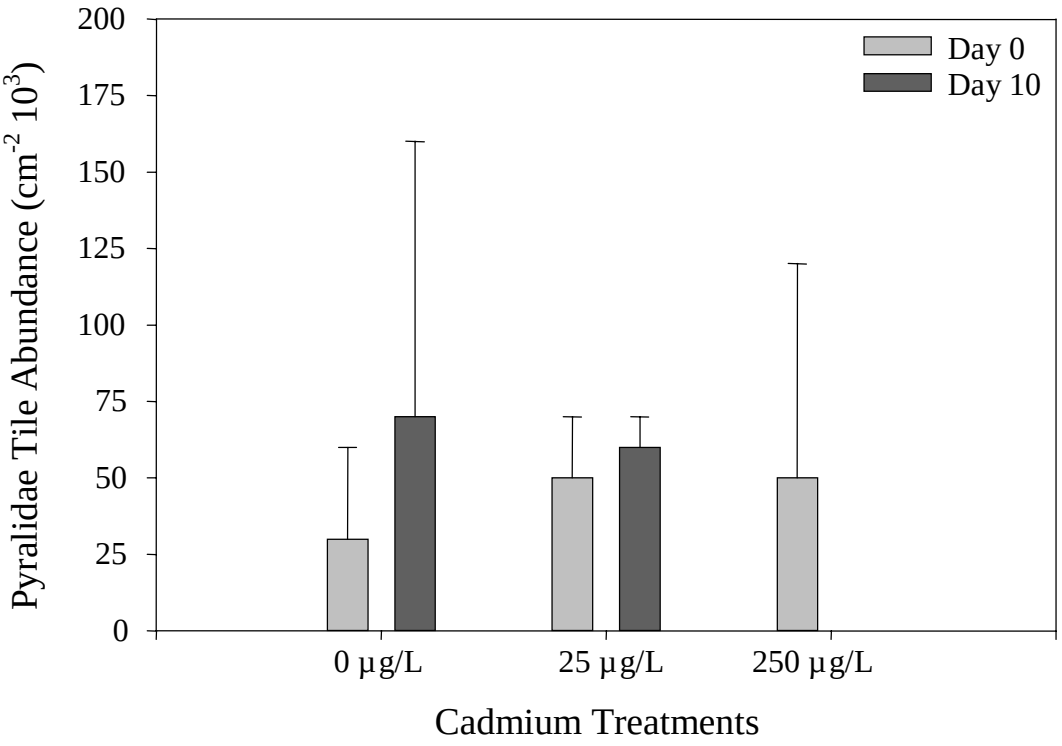

Figure 5: Effects of cadmium on Pyralidae abundance (±SD) on stream tile samplers (Time x Cd; p = 0.38).

Periphyton and Ecosystem Metabolism Responses

Periphyton biomass (AFDW) was unaffected by 0 and 25 µg/L treatment levels; however, 250 µg/L Cd treatments significantly increased periphyton growth (Cd, p = 0.004; Cd x Time, p=0.004; Figure 6). The large increase in periphytic biomass was not accompanied by a change in ecosystem metabolism. Ecosystem gross (Figure 7A) and net (Figure 7B) primary productivity, determined from diel dissolved oxygen dynamics, were not affected by Cd treatments (α=0.10).

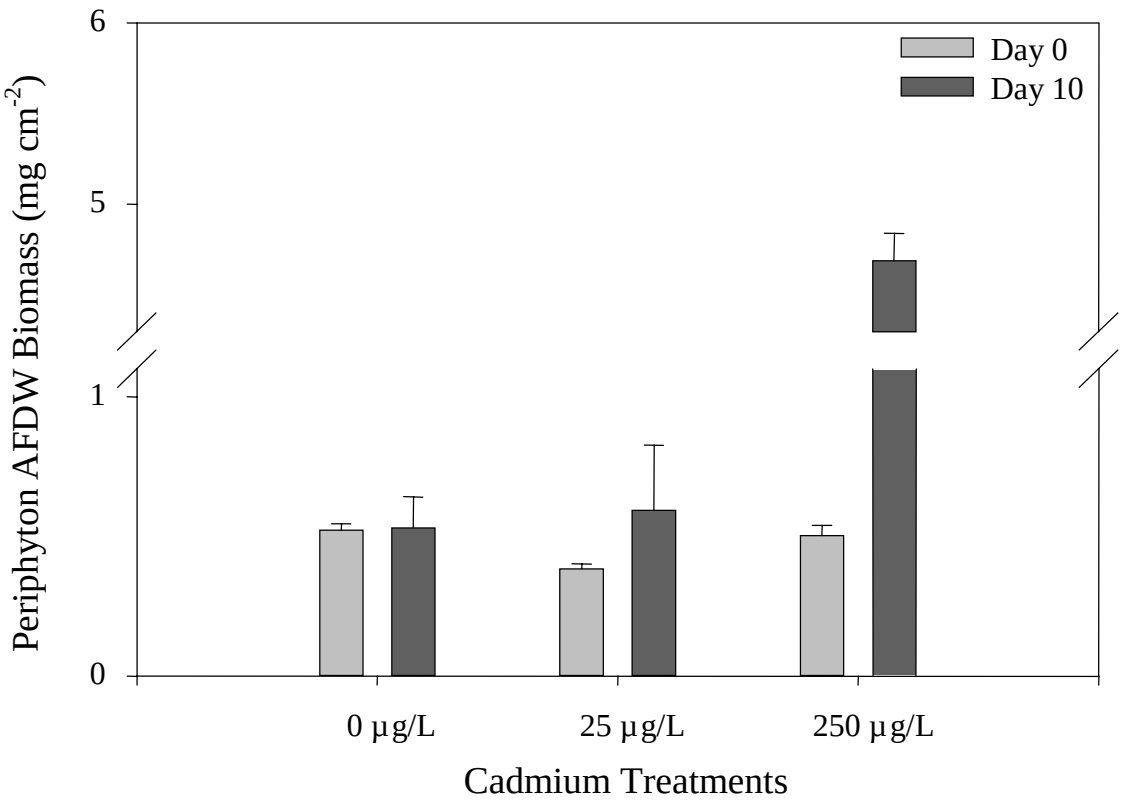

Figure 6: Effects of cadmium treatments on periphytic biomass (AFDW) (±SD) from stream tile samplers (Cd, p=0.004; Cd x Time, p=0.004).

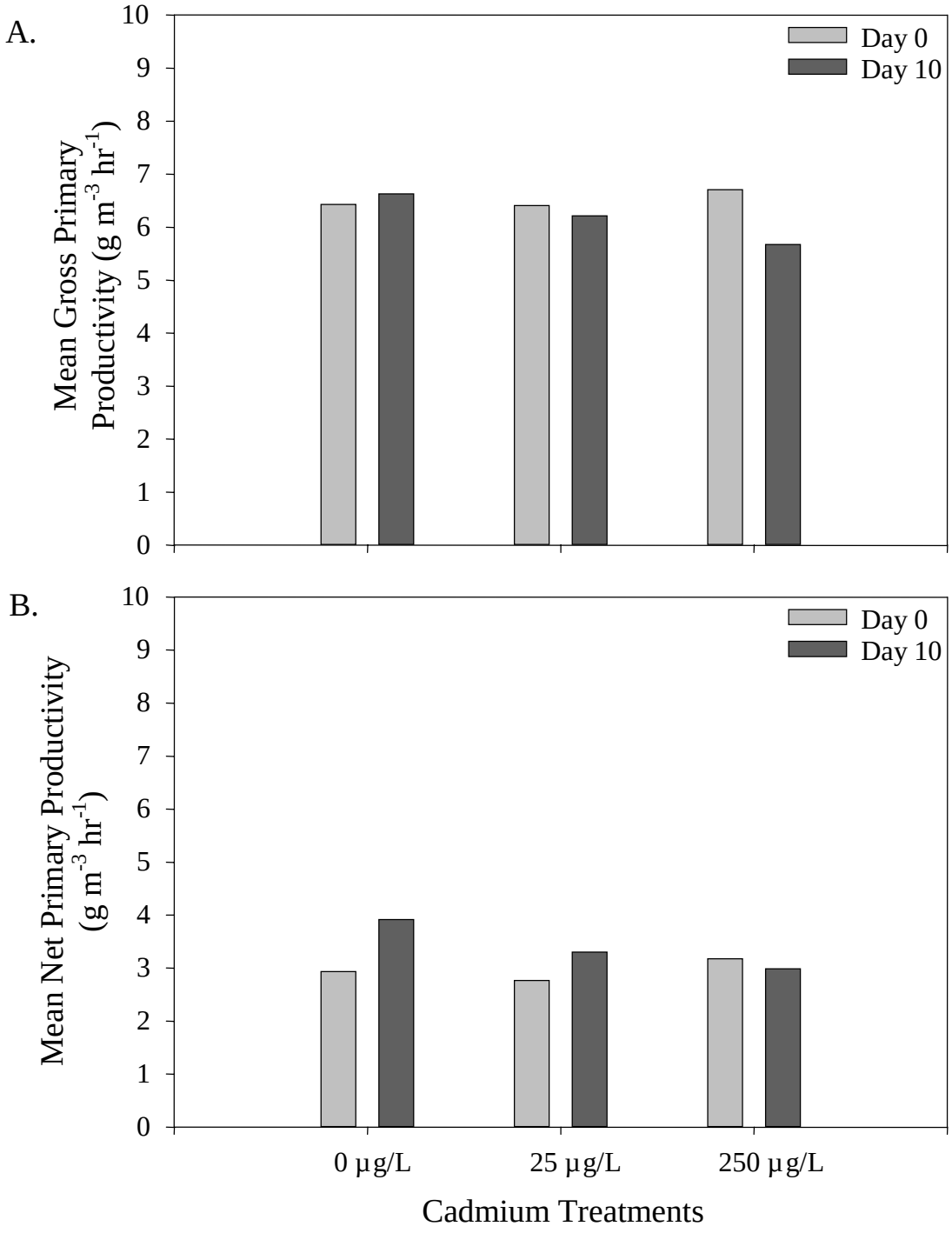

Figure 7: A. Effects of cadmium on mean gross primary productivity and, B. mean net primary productivity of streams (α=0.10).

Cd Accumulation

Adult fathead minnow gill Cd accumulation in 250 µg/L treated streams ranged from 62.3 to 879 µg/g (Table 3). Cd accumulation was detected in three of six fish from 25 µg/L streams (Table 3). Cd was not detected in gill tissues from laboratory fish or untreated streams (Table 3). Instrument detection limit was 1 µg/L for fish gills and 0.5 µg/L for snail and periphyton analysis. Day 0 periphyton and *Physa* sp. Cd tissue concentrations were determined at 5.95 µg/g and 1.59 µg/g, respectively (Table 3). By day 10, periphyton and *Physa* sp. accumulated Cd in 25 ug/L and 250 µg/L treatments (Table 3). Average Cd accumulation in 25 µg/L treatments was 422.5 µg/g for periphyton and 288 µg/g for *Physa* sp. on study day 10 (Table 3). In high dosed streams, periphyton (2 replicates) accumulated Cd to an average of 2860.8 µg/g by day 10 (Table 3). One dead *Physa* sp. collected from stream 8 accumulated 1485.7 µg/g Cd; no organisms were found on stream 6 tiles on day 10 (Table 3).

Table 3: Cadmium accumulation (µg/g, dry weight) in fathead minnow gills, and *Physa* sp. and periphyton tissues collected from streams on study day 0 and 10. NM = not measured, NS = no sample. *Physa* sp. and periphyton values are based on one sample from each stream.

Treatment	Stream	Tissue	Day 0	Day 10
Lab	Lab	Fish Gill	NM	<3.3, <3.5, <1.5
0	4	Fish Gill	NM	<3.1, <1.6, <1.3
0	12	Fish Gill	NM	<1.4, <1.6, <1.5
25	2	Fish Gill	NM	3.04, 2.82, <12.3
25	10	Fish Gill	NM	3.9, <1.9, <3.9
250	6	Fish Gill	NM	62.3, 475.3
250	8	Fish Gill	NM	369.7, 413.4, 879
0	4	*Physa*	0.22	3.46
0	12	*Physa*	0.39	1.48
25	2	*Physa*	2.82	315
25	10	*Physa*	1.42	261.07
250	6	*Physa*	2.25	NS
250	8	*Physa*	2.45	1485.71
0	4	Periphyton	1.47	27.35
0	12	Periphyton	9.10	6.94
25	2	Periphyton	6.46	240.83
25	10	Periphyton	6.50	604.17
250	6	Periphyton	10.78	3725
250	8	Periphyton	1.40	1796.67

Discussion

Laboratory Toxicity Tests

Reported cadmium effects on laboratory toxicity test organisms in effluent dominated streams are lacking. However, cadmium effects on freshwater organisms were reviewed in a recently updated ambient water quality criterion document [37]. In this study, a 7 day LOEC of 3.4 µg/L Cd was observed for *C. dubia* fecundity in reconstituted hard water (170 mg/L $CaCO_3$). Zuiderveen and Birge [38] reported a 7-day *C. dubia* reproduction LOEC of 1.4 ug/L for a lower water hardness of 100 mg/L ($CaCO_3$). Winner [39] reported a 10-day *C. dubia* reproduction NOEC of 0.5 µg/L at a hardness of 90 mg/L ($CaCO_3$). Closer to the average 1400 effluent hardness value of 140 mg/L ($CaCO_3$) measured in this study, a 7-day reproduction effect was observed at 8 µg/L Cd in water hardness of 169 mg/L ($CaCO_3$) by Masters et al. [40]. Effects of 250 µg/L nominal Cd treatments on *P. promelas* survival (Table 2) are generally consistent with findings of Birge et al. [41] who reported a lower 8-day LC50 of 76 µg/L at a water hardness of 101 mg/L ($CaCO_3$).

If cadmium toxicity to *C. dubia* in this study was dependent on hardness alone, average dissolved Cd concentrations of 14.5 and 12 µg/L in low treated streams would be expected to have significantly reduced *C. dubia* reproduction. Rather, no adverse effects on *C. dubia* survival or reproduction were observed in low cadmium treatments (Table 2). Parameters other than water hardness are known to influence metal bioavailability and toxicity [42, 43]. For example, total organic carbon (TOC) is known to form complexes with metals [44, 45] and decrease metal bioavailability and toxicity to aquatic organisms [46-49]. TOC levels are often higher in effluents than upstream dilution waters [50]. The average TOC concentration of municipal effluent source waters used in this study was 7.58 mg/L

(Table 1). These relatively high levels of organic matter likely formed complexes with cadmium and subsequently reduced Cd bioavailability to *C. dubia* in low treatment levels.

To further examine this observation, BLM procedures were performed that included organic carbon in model simulations of Cd speciation and biotic ligand accumulation. Based on effluent water chemistry and default model parameter inputs for % humic acid and sulfides, biotic ligand modeling predicted a 48-hour Cd *C. dubia* EC_{50} of 280 µg/L and an LA_{50} of 7.58 nmol/g (wet weight). This EC_{50} is dramatically higher than the EC_{50} of 38.3 µg/L estimated from a 48-hour toxicity test with laboratory water. In low and high stream treatments, Cd accumulation values lower than this LA_{50} were estimated with the BLM and 50% *C. dubia* mortality was not observed at 48-hrs. Therefore, BLM predictions of 48-hr Cd toxicity to *C. dubia* in this effluent dominated stream system are consistent with observed *C. dubia* laboratory toxicity test responses following exposure to stream treatments. It is interesting to note that constituents of municipal effluents affecting metal bioavailability were not only present at high enough levels to decrease acute cadmium toxicity to *C. dubia*, but that these constituents apparently complexed other waterborne metals in source water effluents. For example, average effluent dissolved copper concentration was 16.8 µg/L (Table 1).

Stream Responses

Overall, macroinvertebrate communities were not affected by low (25 µg/L) Cd treatments. However, high Cd treatment significantly reduced *Physa* sp. abundance (Figures 2 and 4). Effects of high Cd treatments on *Physa* sp. abundance are consistent with previous studies. For example, at a water hardness of 200 mg/L ($CaCO_3$), an acute

Cd LC_{50} was reported at 100.2 µg/L for juvenile *Physa gyrina* [51]. Spehar et al. [52] reported a lower 28-day Cd LC_{50} of 10.4 µg/L for *Physa integra* tested at a water hardness of 58 mg/L ($CaCO_3$). Sub-lethal effects on snail populations were also reported in the range of exposure of high Cd treated streams. Using pond water with a hardness of 250 mg/L ($CaCO_3$) as testing media, Cheung and Lam [53] observed significant affects on *Physa acuta* hatchability by 130 µg/L Cd treatments. Cd exposure of 200 µg/L and 150 µg/L significantly reduced resource consumption and absorbtion rates, respectively, of the freshwater snail *Radix plicatulus* [54].

Previous investigators identified that snail populations may indirectly reduce early instar chironomidae abundance by grazing periphytic communities [55, 56]. In this study, *Physa* sp. composed greater than 50% of benthic macroinvertebrate community abundance in all streams on day 0. On day 10, high Cd treatment (250 µg/L) resulted in a shift of community composition as chironomidae abundance replaced *Physa* sp. abundance (Figures 2 and 3). Such a decrease in grazer-scraper abundance significantly increased periphytic biomass in 250 µg/L streams on study day 10 (Figure 6). Pearson and Crossland [57] observed a similar response of increased primary productivity when lindane treatment decreased macroinvertebrate grazer abundance in artificial streams. Chironomid abundance was significantly increased on day 10 in high Cd treated streams (Figure 3). This response may have resulted from metal resistance and/or resource availability. Some chironomids may be resistant to metal pollution relative to other aquatic invertebrates [23, 58] and chironomid abundances are often positively correlated with periphytic biomass [59]. Therefore, high Cd treatment indirectly increased

chironomid abundance by increasing periphytic biomass and directly reducing *Physa* sp. abundance and, subsequently, resource competition.

An increase in periphytic biomass did not affect ecosystem metabolism, previously suggested as a sensitive measure of environmental stress [19, 60]. In high Cd treatments, primary production and respiration were not affected by Cd treatments in this study (Figures 7A and 7B). Rather, average gross primary productivity was lowest in high Cd treatments (Figure 7A), perhaps resulting from impaired periphytic photosynthesis. Hill et al. [61] found that cadmium treatment of 100 µg/L affected photosynthesis of periphytic communities. Potential shifts of periphytic community structure could have resulted from high Cd treatments that reduced snail grazers. Calow [62] and Patrick [63] identified that composition of periphyton communities may be altered by herbivory and metal exposure, respectively. For example, Genter et al. [64] found snail and zinc treatment to alter periphytic AFDW biomass and community structure. Munoz et al. [65] hypothesized that snail herbivory increases exposure of periphytic communities by disrupting external mucilage, which affords some protection from contaminants. AFDW biomass was not significantly increased in low Cd treated streams in this study; therefore, absence of an approximately 15 µg/L dissolved Cd treatment effect on periphytic biomass does not support Munoz et al.'s hypothesis. Genter et al. [66] found zinc treatment of 50 ug/L to reduce diatom abundance and increase dominance of green algae in periphytic communities. Honig and Buikema, Jr. [67] observed a similar shift from diatom to green algae dominated periphyton following treatment with an artificial refinery mixture that contained 250 µg/L chromium. Increased biomass but not primary productivity may have resulted if tolerant, but less

productive, green algae contributed to AFDW biomass measures. However, taxonomic examination of periphytic communities was not performed in this study.

Although macroinvertebrates and periphytic biomass were not affected by low Cd treatment, fathead minnows, periphyton and snails accumulated Cd in low treatments on day 10 relative to untreated streams (Table 3). In addition to providing a food resource for invertebrate grazers, periphyton serve as a sink for metal contamination in streams [68]. In low dosed streams, periphyton Cd accumulation increased from 6.46 and 6.50 ug/g in streams 2 and 10 on day 0, respectively, to 240.83 and 604.17 µg/g on day 10, respectively (Table 3). Periphytic Cd accumulation in high treated streams was an order of magnitude higher than low dosed streams (Table 3). Gomot [69] found that growth of the snail *Helix aspersa* was slowed by dietary exposure of 100 µg/g Cd and completely inhibited by 800 µg/g Cd. In low dosed streams, higher Cd accumulation was also observed in snail tissue relative to untreated streams (Table 3). *Physa* sp. accumulated 315 and 261 µg/g Cd in streams 2 and 10 by day 10, respectively. Cd accumulation was determined for one dead *Physa* sp. in stream 8, a high treated stream, at over 1400 ug/g. A critical body residue approach has not been applied to *Physa* sp. to evaluate the relationship between Cd accumulation and effect; however, Carlson et al. [70] reported that Cd tissue residues of 125 µg/g resulted in partial mortality of the snail *Helisoma* sp.

Conclusions

Constituents of municipal effluents affected cadmium bioavailability and toxicity to aquatic organisms. Low cadmium treatments, analytically verified at approximately

15 µg/L, to effluent dominated lotic mesocosms did not significantly impact laboratory toxicity test organisms, benthic macroinvertebrate communities, periphyton biomass or ecosystem metabolism. The Cd Biotic Ligand Model estimations were consistent with observed *C. dubia* laboratory toxicity test responses. Although snail abundance and periphytic biomass were unaffected by low Cd treatments, *Physa* sp. and periphyton accumulated Cd in this treatment level at two orders of magnitude higher concentration than untreated streams. These findings indicate that although effluent dominated streams reduced Cd bioavailability and acute toxicity, longer term exposure to similar Cd concentrations may increase accumulation in benthic macoinvertebrates and ultimately lower the no effect level of approximately 15 µg/L observed in this study.

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CHAPTER 3

RESPONSE OF FATHEAD MINNOWS TO 12-DAY AND 90-DAY CADMIUM EXPOSURE IN EFFLUENT-DOMINATED STREAMS

Abstract

An increasing body of evidence indicates that endogenous and exogenous estrogens are present in many municipal effluents, including that of the City of Denton, TX, at levels high enough to elicit biological consequences. Although cadmium has also been reported to affect endocrine function in fish and has been identified as a non-point source contaminant in several north Texas streams, interaction effects of municipal effluents and cadmium exposure on fish have not been explored. To test the hypothesis that cadmium exposure affects fish endocrine function, short-term and longer-term cadmium and municipal effluent studies were performed with fathead minnows (*Pimephales promelas*). Over a 12-day period, replicate experimental streams received municipal effluent as source water and were nominally dosed with 0, 25 or 250 µg/L Cd. VTG induction was not observed in adult male fish at any treatment level and indicated that cadmium and a municipal effluent did not induce vitellogenesis in male fathead minnows during the 12-day study period. A longer-term, 90-day study was performed with juvenile fathead minnows exposed to 0, 5, 20, 40 or 80 µg/L Cd in effluent-dominated streams. Male and female fathead minnow vitellogenesis and steroidogenesis were affected by stream treatments. In addition, swimming performance of male fathead minnows was impaired by 20 and 80 µg/L Cd treatments. Such reduced swimming performance may have resulted from plasma estradiol supression and subsequent

bone calcium losses and reduced integrity of caudal vertebrae. Future studies are needed to assess impacts of estrogen and xenoestrogen exposure on fish calcium metabolism, vertebral bone strength and swimming performance.

Keywords- vitellogenin, municipal effluent, cadmium, *Pimephales promelas*

Introduction

Recent investigations report that the synthetic estrogen, ethinyl estradiol (EE2), and endogenous estradiol (E2) and estrone, significantly contribute to the estrogenic activity of select wastewater treatment plant effluents [1-4]. These estrogenic compounds are used extensively in oral contraceptive formulations, hormone replacement therapy, and as growth enhancement agents in livestock production. Concerns regarding potential environmental exposure to pharmaceutical estrogens arise because of their greater potency relative to other well-studied environmental estrogens, such as alkylphenol ethoxylates, chlorinated compounds, and pesticides [5-8]. There is little information regarding the environmental fate of steroids in aquatic systems because it is often assumed that environmental concentrations are lower than those required to impact aquatic life. For example, an environmental assessment, a simplistic ecological risk assessment, with standard toxicity test organisms is currently required by the U.S. Food and Drug Administration for pharmaceuticals only if predicted environmental concentrations are greater than 1 μg/L [9]. Water quality criteria do not exist for steroid or non-steroid pharmaceuticals [10].

Effluent exposure to ng/L levels of EE2 and E2 is known to affect aquatic life [1, 3]. Investigations in Sweden, the United Kingdom, Canada and Germany quantified maximum

EE2 levels in municipal effluents at 4.5, 7, 15 and 42 ng/L, respectively [3, 11]. EE2 concentrations were measured at 25 ng/L in the City of Denton, TX municipal effluent in December 2000 [12]. In addition, Hemming et al. [4] reported EE2 concentrations ranging from 300 to 500 ng/L in Denton, TX, effluent in March 2000. These values are an order of magnitude higher than other EE2 municipal effluent concentrations [13].

Exposure to steroid hormones and environmental estrogens has a number of potential consequences for aquatic wildlife, the most severe of which is complete sex reversal [14, 15]. In addition, environmental estrogen exposure has impaired gonadal development, resulting in intersexed fish and reduced fertility [16, 17]. As little as 2 ng/L of EE2 has been shown to induce vitellogenin and inhibit testicular growth in male rainbow trout [8]. Steroid hormones have feedback mechanisms at the level of the pituitary and hypothalamus, but primarily act at target tissues to stimulate and maintain the reproductive tract and regulate gametogenesis in the gonads [18]. Sex hormone effects in target tissues are receptor-mediated events; depending on hormone levels and the number of hormone receptors present. Androgen and estrogen receptors are relatively conserved among vertebrate species [19, 20]. In oviparous animals, the liver is considered a target tissue for estrogens as hepatocytes produce vitellogenin (VTG) in response to stimulation [21]. VTG is the glycophospolipoprotein precursor of egg yolk, which provides nutrition for the developing embryo. E2 may regulate testicular androgen production and, thus, expression of secondary sexual characteristics and successful reproduction [16]. Androgens and estrogens also have effects in peripheral tissues such as the developing bone, muscles, skin, and fat deposits, which maintain the sexual phenotype [22]. Further, Sumpter and Jobling [23] suggested that long term exposure to estrogens in wastewater effluent may result in calcium loss from fish skeletons and scales.

Although cadmium bioavailability may be reduced in effluent-dominated streams (Chapter 2), cadmium exposure may have consequences for vertebrate endocrine function. Cadmium bioaccumulation, primarily in liver, kidney and reproductive tissues, may lead to nephropathy and hepatic, ovarian and testicular necrosis [24]. In addition to exerting toxicity at the organ level, cadmium was found to disrupt steroidogenesis [25-27]. Specifically, Sangalang and O'Halloran [25] observed *in vivo* testicular necrosis and decreased *in vitro* testosterone production in rainbow trout exposed to 25 µg/L and 10 µg/L Cd, respectively. Low ug/L cadmium exposure also activated human estrogen receptors and stimulated human MCF-7 breast cancer cell proliferation [28, 29]. Laboratory studies of fish endocrine responses to cadmium exposure have varied with study species. Cadmium exposure of 1.1 mg/L inhibited rainbow trout estrogen receptors in an *in vitro* recombinant yeast assay [30]. *In vivo* cadmium exposure impaired vitellogenesis of winter flounder [31] and rainbow trout [32]. For example, Haux et al. [32] found that four week treatment of 100 µg/L Cd decreased female rainbow trout plasma VTG levels. However, Thomas [33] reported cadmium stimulation of female Atlantic croaker estrogen receptor and vitellogenesis following one month exposure to 1 mg/L Cd. Foran et al. [34] observed a similar hepatic VTG increase in male Japanese medaka exposed to 1 µg/L Cd for two weeks.

A number of environmental contaminants are known to particularly affect the integrity of caudal vertebrae in fish [35-38]; cadmium exposure also compromises calcium content and integrity of fish vertebrae. Such decreased skeletal calcium content may result from disruption of fish calcium metabolism [39] and, subsequently, impaired swimming performance may occur [40]. For example, Muramoto [41] found that 100

day exposure to10 µg/L Cd reduced vertebral calcium content and increased vertebral deformities of carp, *Cyprinus carpio*. Similarly, Bengtsson et al. [42] observed vertebral abnormalities and altered swimming performance of the minnow *Phoxinus phoxinus* following 70 day exposure to 33.7 µg/L Cd.

Whereas municipal effluent estrogenicity has been assessed with male fish VTG induction [1, 2, 4], potential interaction effects of cadmium and estrogenic municipal effluents on fish populations have not been investigated. Therefore, an experimental test of cadmium and municipal effluent effects was performed using a model stream facility that utilized municipal effluent as a water source. The primary objective of this study was to evaluate short term cadmium effects on adult male fathead minnow (*Pimephales promelas*) vitellogenesis and longer term effects on fathead minnow endocrine function and swimming performance in municipal effluent dominated streams.

Materials and Methods

Experimental Design

Two studies were performed using lotic mesocosms at the University of North Texas Stream Research Facility (UNTSRF). In study 1, replicate streams were nominally dosed with 0, 25, or 250 µg/L Cd using peristaltic pumps from 25 August 2000 (Day 0) to 6 September 2000 (Day 12). In study 2, replicate streams were nominally treated with 0, 5, 20, or 80 µg/L Cd from 21 September 2001 (Day 0) to 19 December 2001 (Day 90).

Study 1

Six-to eight-month-old, sexually mature male fathead minnows were acquired from Kurtz Fish Hatchery, Elverson, PA, and held in the laboratory for two weeks prior to stream study initiation. Fish were transported from the laboratory and eight organisms were caged in each stream pool section on study day –2 to acclimate. During the study period, organisms were fed Tetramin® flake fish food and fish mortality was monitored daily. Effluent water quality and Cd treatment level verification were monitored throughout the study period and methods are summarized in Chapter 2.

Study 1 was originally designed to last 14 days; however, a stream system malfunction terminated the study following 12 days of exposure. On day 12, organisms were removed from streams and transported to the laboratory. Organisms were weighed and length measured prior to euthanization by decapitation. Condition factor (K), a general measure of fish health [43], was calculated as weight * 10^5/length^3. Liver and testes were excised, weighed and immediately placed on dry ice prior to storage at –80^{o}C. Hepatosomatic and gonadosomatic indices were calculated as tissue weight/somatic weight * 100. During the study period, eight organisms were held in the laboratory. On day 12, these untreated organisms were also euthanized and endpoints collected according to the above procedures.

Liver VTG levels were determined following previously reported methods [44-46]. Briefly, whole livers were homogenized and centrifuged at 10,000 x g for 30 minutes at 4^{o}C. Protein content of whole liver homogenates was measured using bovine serum albumin standards (Sigma Chemical), a Bio-Rad Protein Assay protein dye and a microplate reader. VTG was determined by SDS-Page and Western blotting with a

monoclonal anti-VTG antibody developed against carp that cross reacts with fathead minnow (Cayman Chemical Co.) [47]. After the nitrocellulose image of each gel was developed, gels were scanned and subsequently analyzed using Scion Image software. VTG bands (170 kDa and 130 kDa) were quantified as percent optical density relative to positive controls. Positive control livers used for this study were collected from fathead minnows exposed to 1 µg/L estradiol for 7d.

Study 2

Fathead minnow eggs were collected from permanent breeding cultures at the University of North Texas Aquatic Toxicology Laboratory. Eggs were hatched and juvenile fish held for two weeks in the laboratory. During the two-week period, juveniles were fed Artemia nauplii *ad libitum* twice daily. Two-week old fathead minnows were transported to the UNTSRF and 100 juveniles were placed in each stream pool section on 19 September 2001 (day -2) to acclimate. Fish were not fed during the study period. From 21 September 2001 (day 0) to 19 December 2001 (day 90) replicate streams were dosed with 0, 5, 20 or 80 ug/L Cd using high precision peristaltic pumps. Each stream was sampled weekly for metals (Cd, Cu, Zn, Ni, Ag, Cr, Pb) and alkalinity and hardness. Metals were quantified by graphite furnace atomic absorbtion spectrophotometry according to methods described in Chapter 2. Source effluent water chemistry including pH, dissolved oxygen, temperature and specific conductance was measured hourly using a Hydrolab datasonde multiprobe.

On study day 90, fish were removed from each stream, enumerated and transported to the laboratory in ambient stream water. Eight fish from each stream were

sub-sampled to evaluate critical swimming performance. Swimming performance was evaluated using a 2 L Brett [48] type swim tunnel filled with dechlorinated tap water and followed previously reported methods [49, 50]. The swim tunnel was composed of 75 mm diameter, 450 mm long acrylic tube with 1 mm mesh flow filters at each end to provided rectilinear flow. Water was delivered to the tunnel at a maximum speed of 65 cm sec^{-1} by a ½ hp Teel centrifugal pump; tunnel water speed was quantified with a Marsh-McBurney flowmeter. Each fish was introduced to the tunnel and allowed to acclimate undisturbed for 5 min. An initial 10 cm sec^{-1} flow rate was produced and fish were swum for 1 min swimming trials. An incremental increase of 10 cm sec^{-1} flow rate was performed every minute until fatigue. Fatigue occurred when the fish stopped swimming, was caught against the rear mesh screen and did not resume swimming when gently prodded [48-50]. Critical swimming speed was calculated as: $U_{crit} = u_1 + (t_1/t_2 \times u_2)$; where u_1 = the highest swimming speed (cm sec^{-1}) maintained for 1 min, u_2 = the speed increment (10 cm sec^{-1}), t_1 = the time swum at fatigue speed and t_2 = the time swimming period (1 min) [48]. Critical swimming speed was normalized by individual body length (cm) [48].

Following each individual swimming performance trials, the fish was measured, weighed and euthanized by decapitation. Blood was collected with a heparinized capillary tube after severing the caudal peduncle. Hematocrit was calculated as percent packed cells following centrifugation at 3,000 rpm for 3 minutes. Plasma was removed from each capillary tube with a microsyringe, volume recorded, placed in a microcentifuge tubes and immediately placed on dry ice prior to storage at -80^{o}C. Liver and gonadal tissue were dissected, weighed for calculation of hepatosomatic and

gonadosomatic indices, and liver tissue immediately placed on dry ice until storage at –80^{o}C. Hepatic protein levels and VTG were measured using techniques reported above for study 1. Hepatic estrogen receptor content was determined from whole liver homogenates by SDS-PAGE and Western blotting [34]. A primary antibody for estrogen receptor was developed in mouse by Affinity Bioreagents, Inc. (Stillwater, MN) against the DNA-binding domain of human ER. Developed nitrocellulose images were digitally scanned and analyzed with Scion Image software according to methods described for study 1.

Plasma steroid concentrations were determined with a steroid enzyme immunosorbant assay developed by Munro and Lasley [51] and followed previously reported methods [44-46]. Briefly, plasma was extracted three times with 100 uL ethyl acetate in a 10 mL test tube. During each extraction, samples were vortexed for 5 seconds and subsequently centrifuged at 4,000 rpm for 5 minutes. Supernatant was transferred to a new test tube and samples and estradiol (E2) and testosterone (T) standards brought to dryness under a nitrogen stream. A 96-well, flat-bottomed Falcon Pro-Bind (Fisher, St. Louis MO) microplate was coated with either bicarbonate-buffered dilutions of 1:10,000 anti-E2 or 1:20,000 anti-T antibodies (UC-Davis Clinical Endocrinology Laboratory, C. Munro). Standards and samples were reconstituted in 250 uL phosphate buffered saline solution with BSA. 25 uL reconstituted sample was added in duplicate and four serial dilutions to the 96-well microplate. In addition, a standard dilution of horseradish peroxidase (HRP) conjugated to either E2 or T was added to each microplate well (UC-Davis Clinical Endocrinology Laboratory, C. Munro). The microplate was rinsed and remaining liquid gently tapped out after a 2 hour incubation

period of sample, standard and HRP conjugate. HRP substrate was then added to the microplate and optical density measured at 405 nm with a Tecan® UV/Vis Rainbow microplate reader. Steroid concentrations were determined using a five-point standard curve.

Statistical Analysis

Analyses of treatment effects were performed using SAS (Version 8.0). A one-way ANOVA and SNK for post-hoc comparisons of treatment levels were performed. Statistical significance of fish response variables were determined at $\alpha = 0.05$.

Results

Study 1

Effluent Characterization and Treatment Level Verification

Source effluent water quality, evaluated at 0600, 1400 and 2200, did not exhibit dramatic fluctuations throughout this study (Chapter 2). Cadmium treatment levels were verified at lower than nominally dosed values. Average total and dissolved Cd concentrations (±SD) in streams 2 and 10, treated nominally with 25 µg/L Cd, were 17 µg/L (±1.4) and 14.5 µg/L (±0.7), and 13 µg/L(±1.4) and 12 µg/L (±2.8), respectively. Average total Cd concentrations in nominal 250 µg/L treatments, streams 6 and 8, were 146.5 µg/L (±17.7) and 141 µg/L (±21.2), respectively. Dissolved Cd was detected at an average of 131 µg/L (±2.8) in stream 6 and 120 µg/L (±39.6) in stream 8.

Fish Responses

Fish mortality was observed in stream 6, a 250 µg/L Cd treated stream, as only 2 of 8 organisms survived to day 12. Fish mortality was not observed in other streams. Fish condition was significantly reduced by 25 and 250 µg/L Cd treatments (Figure 1; $\alpha = 0.05$). Hepatosomatic (HSI) and gonadosomatic (GSI) indices were not significantly

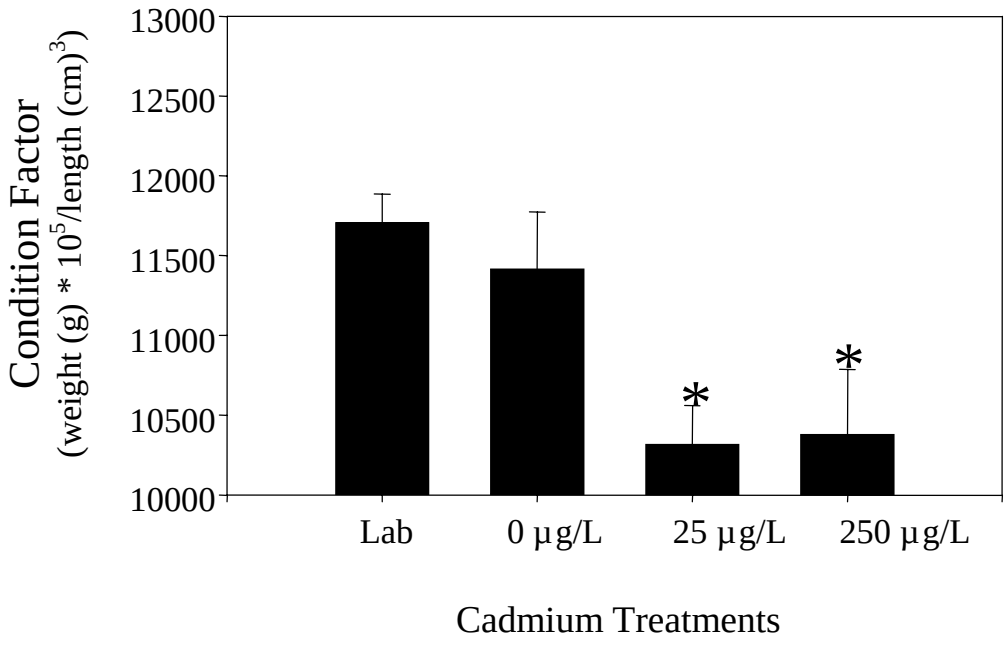

Figure 1: Effects of municipal effluent (0 µg/L) and municipal effluent and cadmium treatments (25 µg/L, 250 µg/L) on condition of adult male fathead minnows (±SD; *: $p<0.05$).

affected by treatment levels (Figure 2; $\alpha = 0.05$). VTG induction was not observed in laboratory control fish or organisms exposed to Cd treatment levels. Although VTG bands were observed for positive control organisms treated with 1 ug/mL estradiol, VTG bands were not observed at the 170 kDa or 130 kDa positions in sample lanes. Therefore, integrated optical density of expected VTG band regions was not quantified with Scion Image software.

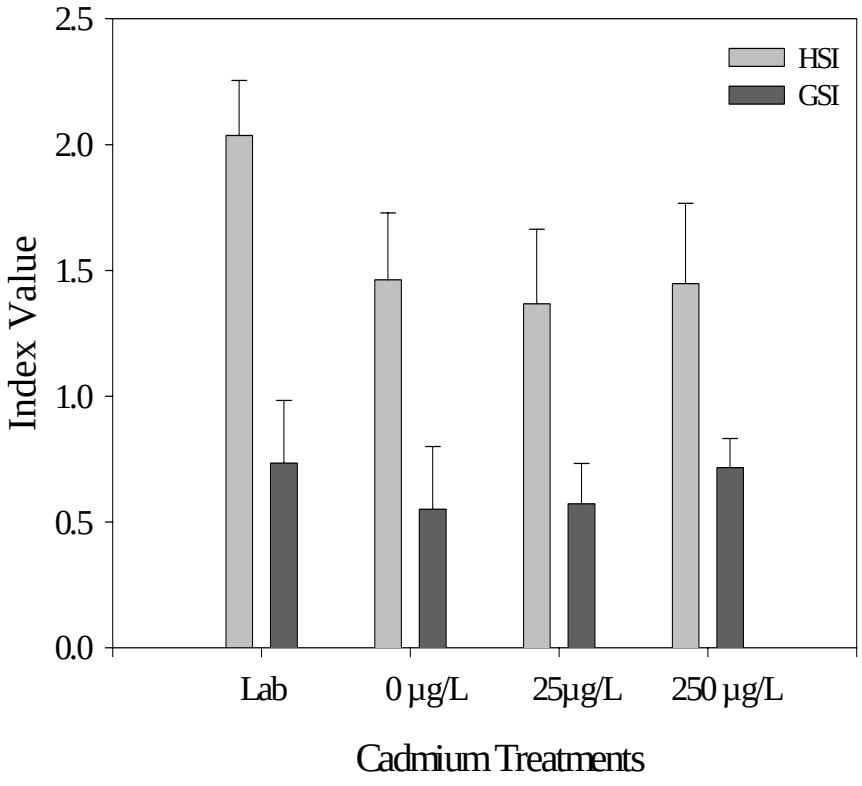

Figure 2: Effects of municipal effluent (0 µg/L) and municipal effluent and cadmium treatments (25 µg/L, 250 µg/L) on hepatosomatic and gonadosomatic indices (±SD) of adult male fathead minnows.

Study 2

Effluent Characterization and Treatment Level Verification

Mean values of municipal effluent source water chemical parameters are provided in Table 1. Based on these parameters, source effluent water quality of study 2 was generally similar to those values measured in study 1 (Chapter 2). Zn, Cu and Ni were

Table 1: Mean water quality parameters (±SD) of source effluent during study 2 (pH, temperature, dissolved oxygen, specific conductance, turbidity, N=2082); alkalinity, hardness, N=16).

Parameter	Effluent Source Water
pH	6.96 (±0.15)
Temperature (^{o}C)	24.5 (±1.64)
Dissolved Oxygen (mg/L)	6.65 (±1.07)
Specific Conductance (us/cm)	715.72 (±28.9)
Turbidity (NTU)	18.96 (±35.7)
Alkalinity (mg/L, $CaCO_3$)	87.7 (±13.3)
Hardness (mg/L, $CaCO_3$)	139.6 (±9.03)

consistently detected in stream water samples (Table 2). Highest average Zn concentrations were detected in streams 2, 7 and 9 at 152.4, 418 and 324.8 ug/L, respectively. These average stream concentrations were markedly higher than other streams and resulted from several high values, as implied from associated standard deviations (Table 2). An explanation for these high Zn concentrations is not clear; high Zn values were not observed between streams 2, 7 or 9 on concurrent sample dates.

Table 2: Mean metal concentrations (±SD) in UNTSRF streams during study 2 (N= 16).

Stream Number	Treatment Level	Metal Concentration (µg/L) Cd	Cu	Zn	Ni
3	0	2.25 (±1.48)	14 (±8.3)	39.1 (±9.04)	13.88 (±13.73)
11	0	3.75 (±1.58)	18.1 (±10.2)	49.1 (±27.7)	9.9 (±12.2)
4	5	6.9 (±4.1)	13.5 (±8.5)	81.1 (±158)	12.7 (±12.1)
12	5	7.5 (±3.2)	18.2 (±8.1)	40.3 (±6.76)	18.5 (±12.4)
2	20	18.9 (±7.5)	14.1 (±7.1)	152.4 (±455.6)	15.6 (±16.5)
7	20	27 (±11.7)	12.9 (±7.3)	418 (±1517)	13.4 (±14.1)
5	80	74.8 (±30.8)	12.6 (±6.7)	46.8 (±31.3)	14.5 (±15.5)
9	80	79.75 (±31.6)	14.7 (±8.3)	324.8 (±1134)	13.9 (±14.2)

Stream Cd treatments were analytically verified at concentrations near nominal treatment levels (Table 2). Specifically, average Cd levels in streams 4 and 12, nominally treated with 5 µg/L Cd, were 6.9 (±4.1) and 7.5 (±3.2) µg/L, respectively. Streams 2 and 7, nominally treated with 20 µg/L Cd, were verified at averages of 18.9 (±7.5) and 27 (±11.7) µg/L, respectively. Nominal 80 µg/L treated streams, streams 5 and 9, were verified at 74.8 (±30.8) and 79.75 (±31.6) µg/L, respectively. Therefore, nominal treatment levels are reported in figures describing fish responses to Cd treatment.

Fish Responses

Fish survival was not significantly affected by Cd treatments; an average of 36.25 (S.D. = ±5.2) organisms was recovered from stream pool sections on day 90. Male ($p=0.04$) and female ($p=0.02$) hematocrit were significantly increased in all streams relative to laboratory reared fish (Figure 3). Although male and female condition and HSI were not significantly affected, GSI was significantly reduced in males and females at all Cd treatment levels (Figure 4). Hepatic VTG was non-significantly induced in male fathead minnows ($p = 0.11$; Figure 5). Conversely, hepatic VTG content was reduced, although also not significantly, in female *P. promelas* ($p = 0.1398$; Figure 5). Hepatic ER content was not significantly affected by treatments; however, it is interesting to note that average ER content was higher in males and lower in females than untreated laboratory fish (Figure 6). Circulating plasma steroid levels were affected by each treatment level. Male testosterone and estradiol levels were reduced in all treatments; most significant reductions in male steriod levels were observed in 20 and 80 µg/L Cd treatment levels (Figure 7). Female estradiol and testosterone were also significantly reduced in 20 and

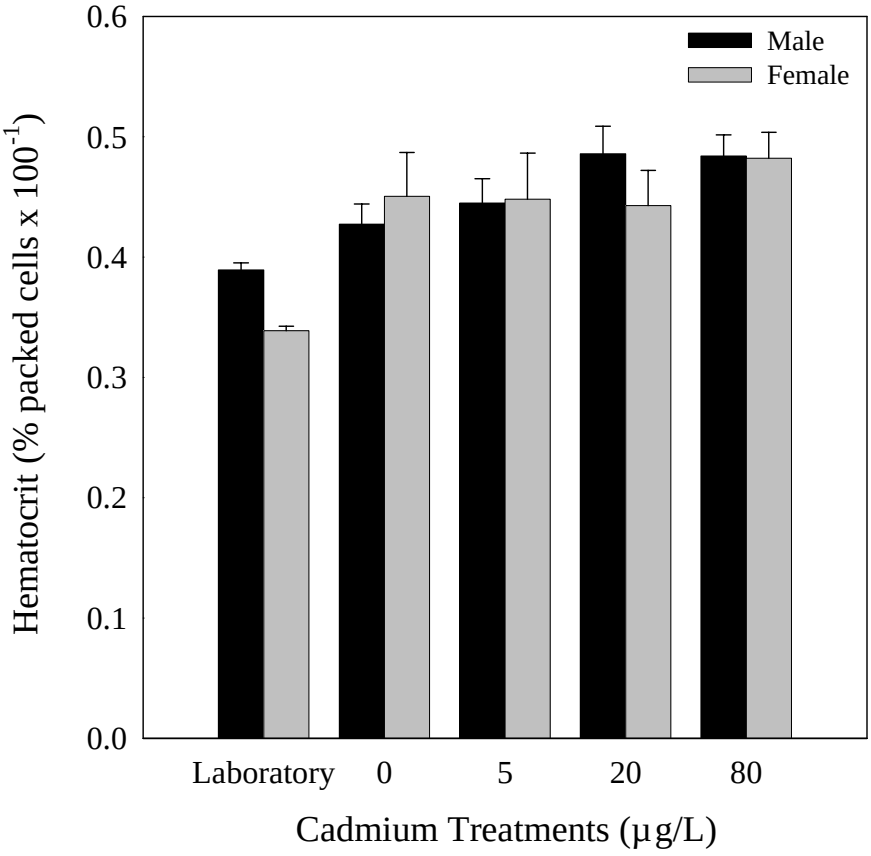

Figure 3: Effects of 0, 5, 20 and 80 µg/L Cd on hematocrit (±SD) of male and female fathead minnows (male p=0.04, female p=0.02).

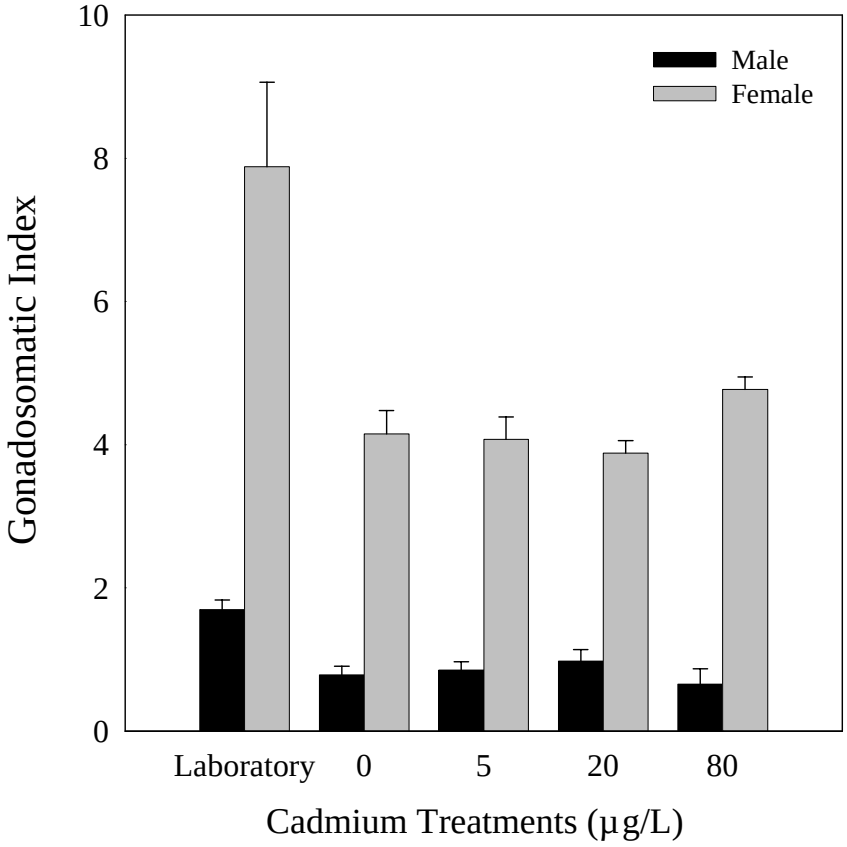

Figure 4: Effects of 0, 5, 20 and 80 µg/L Cd on gonadosomatic indices (±SD) of male and female fathead minnows (male p=0.004, female p=0.014).

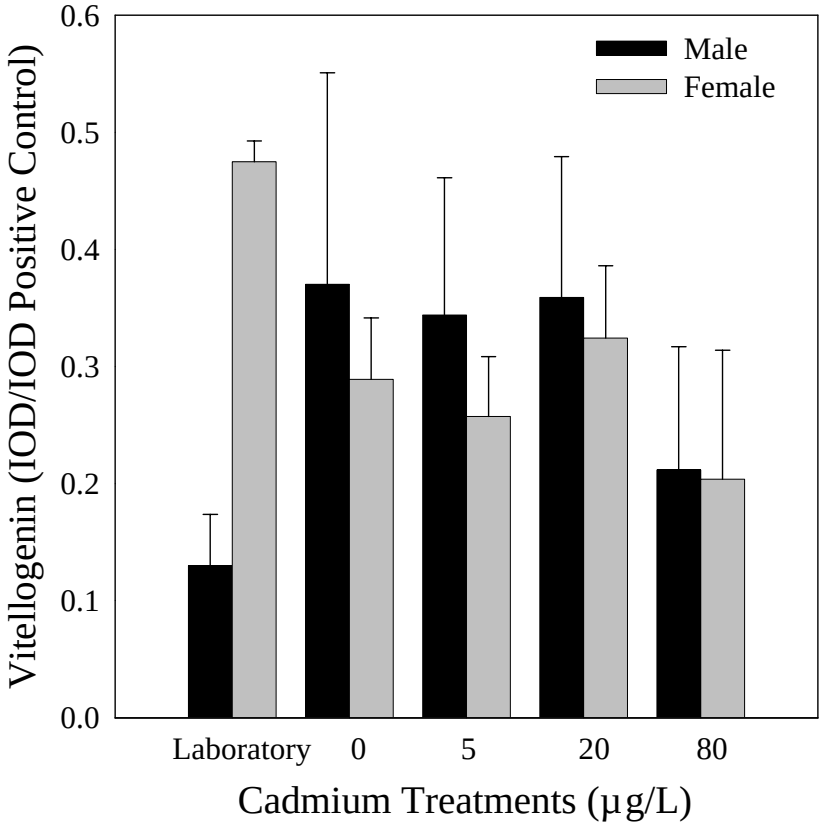

Figure 5: Effects of 0, 5, 20 and 80 µg/L Cd on male and female fathead minnow hepatic vitellogenin (±SD; male p=0.11, female p=0.14).

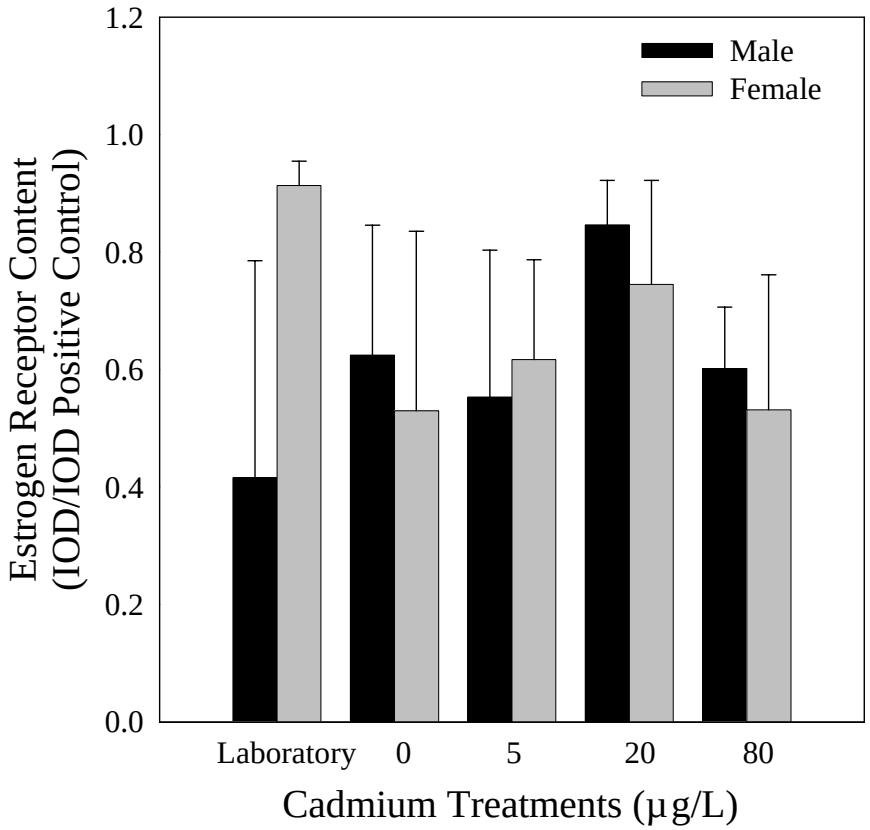

Figure 6: Effects of 0, 5, 20 and 80 µg/L Cd on male (p = 0.49) and female (p = 0.19) fathead minnow hepatic estrogen receptor levels (±SD).

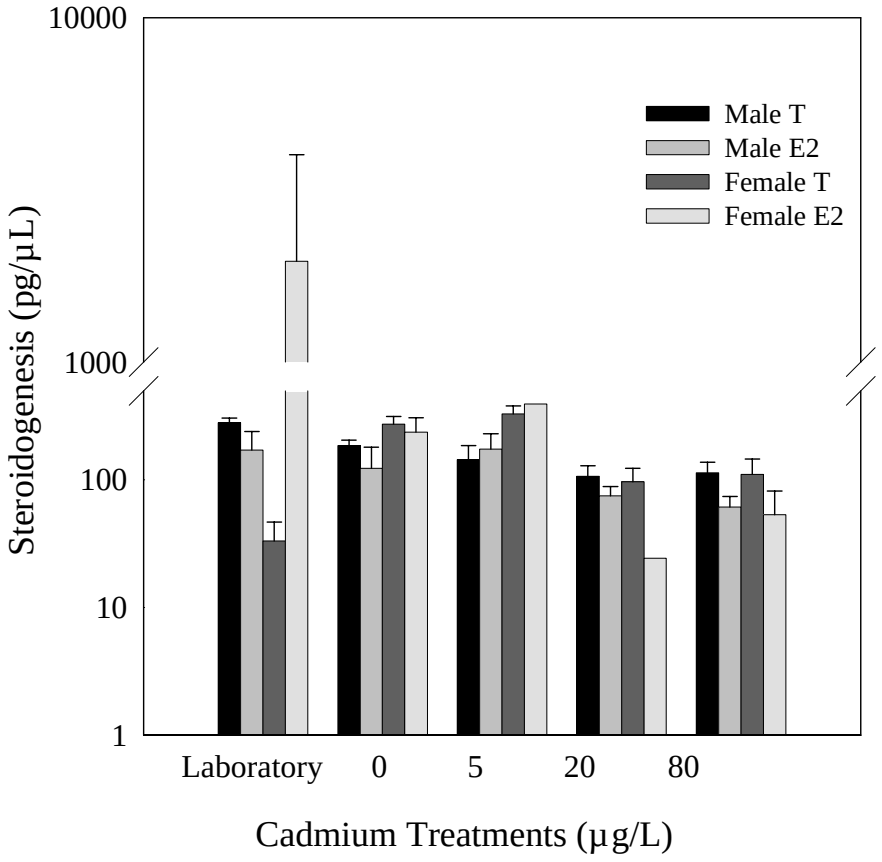

Figure 7: Effects of 0, 5, 20 and 80 µg/L Cd on male and female fathead minnow plasma steroids (±SD; male T, p=0.009; male E2, p=0.008; female T, p=0.072; female E2, p=0.034).

80 µg/L Cd treatments (Figure 7). Male critical swimming performance (body lengths (cm sec^{-1})) was affected by 20 and 80 µg/L Cd treatments (p=0.055, Figure 8). However, female swimming performance was not affected by any treatment level (p=0.3587, Figure 8).

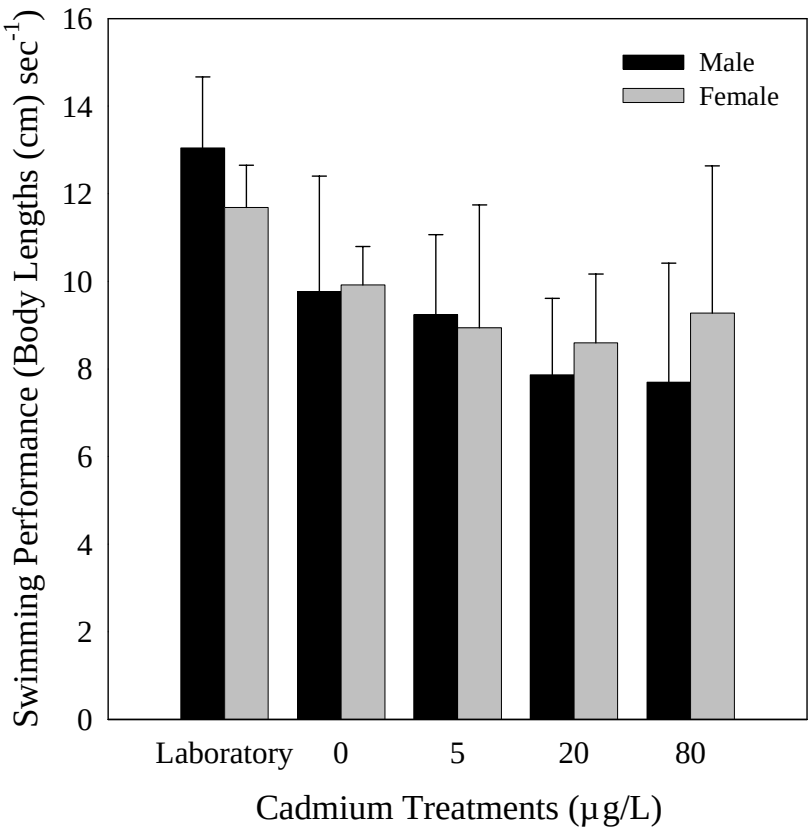

Figure 8: Effects of 0, 5, 20 and 80 µg/L Cd on male and female fathead minnow critical swimming performance (±SD; male p=0.055; female p=0.359).

Discussion

Cadmium or cadmium and effluent effects on fathead minnow endocrine function have not been reported; however, basic endocrinology of fathead minnows was previously investigated [52]. In study 1, vitellogenesis was not significantly induced in adult male fathead minnows following 12-day treatment with a municipal effluent and cadmium. Following a 90-day exposure period in study 2, vitellogenesis was observed in male fathead minnows from each stream mesocosm (Figure 5). Relative to untreated streams, 5, 20, and 80 µg/L Cd + effluent treatments did not induce hepatic VTG content in an additive manner. Rather, the lowest average male and female hepatic VTG contents were detected at the 80 µg/L Cd treatment level (Figure 5). Prior investigations indicated that cadmium induces VTG production in some fish species [33, 34], presumably through activation of the estrogen receptor (ER) [28]. Specifically, Thomas [33] observed Cd treatment of 1 mg/L for 30 days to stimulate Atlantic croaker vitellogenesis and steroidogenesis. However, VTG induction was not observed in other fish species treated with cadmium [31, 32]. Foran et al. [45] observed an upregulation of Japanese medaka hepatic ER following 2-week treatment with 500 ng/L EE2. This EE2 concentration is similar to previously reported City of Denton, TX, effluent EE2 levels ranging from 300 to 500 ng/L [4]. Longer term exposure to this estrogenic municipal effluent (Figure 5) resulted in downregulation of female hepatic ER content, though not significantly, in study 2 relative to laboratory reared organisms ($p=0.19$, Figure 6). Further, compared to fish from untreated streams, 5, 20, and 80 µg/L Cd + effluent treatments did not result in upregulation or downregulation of hepatic ER content following a 90-day study period (Figure 6).

Olsson et al. [53] observed an interesting interaction when juvenile rainbow trout received concurrent interperitoneal (IP) injections of estradiol (E2, 10 mg kg^{-1}) and cadmium (0.2 mg kg^{-1}). Fish that received only E2 IP injections exhibited higher hepato-somatic indices (HSI) and VTG induction relative to controls. Comparable HSI and VTG responses in fish have been observed following exposure to several estrogenic effluents [1, 2], including the city of Denton, TX effluent [4, 12]. However, compared to E2 only, IP treatment cadmium + E2 treatments reduced rainbow trout HSI and vitellogenesis by inhibiting induction of VTG mRNA [53]. If the study 1 effluent was estrogenic, VTG induction and elevated HSI in untreated streams, but not in Cd treatments, may have supported Olsson et al.'s [53] findings that cadmium exposure inhibits VTG synthesis and decreases HSI in male fathead minnows. VTG was not induced and HSI not increased by any stream treatment in study 1 (Figure 2). Although steroid concentrations were not measured in municipal effluent source waters during this experiment, such an observation indicates that the City of Denton's effluent was not sufficiently estrogenic to induce VTG synthesis in study 1. These findings also indicate that cadmium does not induce VTG or affect HSI and GSI in male fathead minnows exposed for 12 days to ~15 ug/L and ~145 µg/L treatments. During a longer 90-day exposure to 80 ug/L Cd in study 2, mean hepatic VTG content was induced to a lesser degree in male fish than those sampled from 5 and 20 µg/L Cd treated streams (Figure 5). Similarly, female hepatic VTG content was lowest among Cd treatments in 80 µg/L streams. This suggests that 80 µg/L Cd treatment may have impaired vitellogenesis in both males and females, perhaps by inhibiting induction of VTG mRNA as suggested by Olsson et al. [53].

Other studies observed VTG induction in male fathead minnows exposed to the City of Denton, TX, effluent [4, 12]. Analysis of effluent steroids identified ethinyl estradiol levels from 300 to 500 ng/L in March 2000 [4] and at 25 ng/L in December 2000 [12], respectively. However, Hemming [54] did not detect EE2 or E2 in the City of Denton, TX, effluent or observe male fathead minnow VTG induction following a two-week effluent exposure during June 2000. Other xenoestrogenic compounds, namely nonyl phenol ethoxylate surfactant degradation products, phlalate plasticizers and the herbicide atrazine, were identified in Denton effluent during March and June 2000 [53]. The 14-day exposure duration of Hemming's study [54] in June 2000 is comparable to the 12-day exposure period of study 1. A 7-day shorter exposure duration in study 1 than those of Hemming et al. [4] and Allen et al. [12] does not account for lack of effluent estrogenicity in study 1 or the Hemming [54] study because male VTG induction was quantified following less than one-week of exposure in December 2000 [12].

Sources of such apparently seasonal estrogenicity in the City of Denton, TX effluent are unknown. Two universities, University of North Texas and Texas Women's University, account for approximately 40% of the Denton, TX population. If these demographics are considered, it may be plausible that absence of effluent estrogenicity in June 2000 [53] and in August 2000 (study 1) and presence of estrogenicity in March and December 2000 [12] and September to December 2001 (study 2) potentially resulted from changes in city population with university sessions. For example, when fathead minnows were exposed to the city of Denton, TX effluent in December 2000, induction of VTG mRNA increased when universities were in session but decreased when classes dismissed for winter break [12]. Tilton et al. [55] previously evaluated seasonal

estrogenicity of two wastewater treatment plant effluents. Although numerous factors including sludge age, retention time and flow rate may influence removal of pharmaceuticals during the WWTP process [56], Tilton et al. [55] evaluated WWTP's that primarily treated high domestic, low industrial areas. One of the WWTP studied by Tilton et al. [55] serviced only a college campus; no relationship was observed between school session and effluent steroid concentrations or estrogenicity.

Clearly, factors that contribute to temporal variation of municipal effluent estrogenicity warrant further investigation. *In vitro* procedures have been successfully utilized to identify causative estrogenic compounds in municipal effluents [57]. Such identification procedures may be useful for screening seasonal effluent estrogenicity and identifying problematic estrogen contaminants in municipal effluent discharges [58]. Chapter 4 discusses an application of estrogen identification procedures, which included an *in vitro* screening assay and an *in vivo* VTG confirmatory bioassay, to the City of Denton, TX effluent before and after university school sessions in May 2001.

Male and female condition of stream fish in study 2 was not significantly different from laboratory fathead minnows of the same age. A previous investigation of fathead minnow responses to estrogenic City of Denton, TX, municipal effluent indicated that fish condition was significantly reduced following a three week exposure period [42]; however, adult male fathead minnows in this previous study were caged *in situ*. In study 2, fathead minnows were exposed to effluent and cadmium treatments uncaged in 568 L pool sections. Longer term fish exposure in UNTSRF pools may have reduced any stress associated with *in situ* caging. Fathead minnows are omnivores that feed on diatoms and

algae [59]. Abundant algal food sources were available in pool sections and possibly contributed to lack of significant treatment effects on fish condition.

In addition to effects of stream treatments on endocrine function endpoints discussed above, other physiological responses were observed during study 2. Male and female GSI were significantly reduced in all streams (Figure 3). Such a reduction in male GSI is similar to previous studies that evaluated laboratory E2 [60, 61] or municipal effluent exposure [2, 4]. Male and female hematocrits were significantly increased in all streams (Figure 3). This increase in hematocrit is converse to that observed by Hemming et al. [43] when adult male fish were exposed to the City of Denton, TX, for 3 weeks. Although the specific mechanism by which fish hematocrit was increased in study 2 is unknown, an increase of hematocrit previously resulted from higher red blood cells needed to compensate for hypoxia [62]. However, hypoxia has not been reported following response to Cd treatment. Rather, Larsson [63] observed a decrease in flounder (*Pleuronectes flesus*) hematocrit and hemoglobin following 5, 50 and 500 µg/L Cd exposure for nine weeks.

Male swimming performance was significantly reduced by 20 and 80 µg/L Cd treatments (Figure 8). Swimming performance is positively related to hematocrit in healthy fish. Poor swimmers, typical of ambush predators, have low hematocrits (e.g. lantern fish: 8%) whereas hematocrits of excellent swimmers are notably greater (e.g. mackerel: 50%) [64]. If male hematocrit would have been decreased by 20 and 80 µg/L Cd treatments, the observed impairment on swimming performance at these treatment levels could have been attributed to decreased hemoglobin and blood anemia, similar to that observed by Larsson [63]. However, reduced swimming performance does not

appear to be related to increased hematocrit, which was significantly increased in all treatment levels relative to laboratory controls. In addition to affecting swimming performance, 20 and 80 µg/L Cd treatments reduced male circulating estradiol levels (Figure 7). Plasma estradiol levels antagonize the action of parathyroid hormone, which controls bone resorbtion [65]. Estradiol is prescribed in hormone replacement therapies for postmenopausal women to reduce bone calcium loss and prevent osteoporosis [65]. Decreases in male plasma estradiol following 20 and 80 ug/L Cd treatment in study 2 may have led to alteration of calcium metabolism, hypocalcemia, and subsequently, reduced bone strength. For example, Larsson [63] observed plasma hypocalcemia in the flounder *Pleuronectes flesus* treated with 5, 50, and 500 µg/L Cd. Further, a 70-day exposure of the minnow *Phoxinus phoxinus* to 33.7 µg/L Cd resulted in vertebral abnormalities and altered swimming performance [42]. Therefore, it is plausible that impaired swimming performance of male fathead minnows treated with 20 and 80 µg/L Cd for 90 days may have resulted from decreased plasma estradiol, bone calcium losses and reduced integrity of caudal vertebrae. This research suggests that future studies are needed to further assess impacts of estrogen and xenoestrogen exposure on fish calcium metabolism, vertebral bone strength and swimming performance.

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CHAPTER 4

ESTROGENICITY IDENTIFICATION EVALUTATION OF A MUNICIPAL EFFLUENT USING *IN VITRO* AND *IN VIVO* BIOASSAYS PROCEDURES

Abstract

Hemming et al. (2001), Allen et al. (2001), Brooks (Chapter 3) and Huggett et al. (2002) identified that the City of Denton, TX effluent periodically induces vitellogenesis in male fish. Two universities, The University of North Texas and Texas Woman's University, compose approximately 40% of the City of Denton, TX, population when enrollment increases during fall and spring semesters. Although numerous compounds are reported to cause estrogenicity in effluents, periodic estrogenicity may be influenced by changes in city population characteristics when university semesters conclude. To test the hypothesis that City of Denton effluent estrogenicity results from demographic changes, an estrogen identification procedure using solid phase extraction and fractionation, and an *in vitro* recombinant yeast screen (YES) and an *in vivo* Japanese medaka (*Orzyias latipes*) vitellogenin bioassay was performed on effluent samples collected prior to and following university semester break in May 2001. Effluent was identified as estrogenic by the YES assay in methanol fractions, the fraction that contained polar steroids, from samples collected during university semester sessions. Vitellogenin induction was also observed in male Japanese medaka exposed to methanol fractions. However, methylene chloride fractions also induced vitellogenesis in male fish. These findings indicate that steroids and surfactants, as well as other unidentified estrogens, may contribute to estrogenicity of the City of Denton,

TX effluent. Further, *in vivo* assays should be used concurrent with *in vitro* YES activity when effluents are screened for estrogenicity.

Keywords- municipal effluent, TIE, YES assay, vitellogenin, *Orzyias latipes*

Introduction

Complex mixtures of chemicals known to modulate fish endocrine function have been identified in municipal effluents [1]. Perhaps the most potent of effluent estrogen contaminants is ethinylestradiol (EE2), a synthetic estrogen pharmaceutical [1]. A major formulation in birth control pills, EE2 is prescribed to over ten million women annually in the United States [2]. In addition, hormone replacement therapies consisting of conjugated equine estrogens may represent significant loadings to the environment. Andrews and Briggs [3] estimated that 5 to 13 million post-menopausal women are prescribed hormone replacement drugs. Conjugated equine estrogens (estrone, and 17α- and 17β-estradiol) are primarily the active estrogenic components of hormone replacement therapeutics [4]. Whereas these endogenous compounds are less potent than EE2, they are likely to be present in effluents at higher levels [5].

Approximately 80% of 17β-estradiol (E2) is excreted in the urine and 7% in feces, demonstrating a large degree of enterohepatic circulation in which the conjugated metabolites are hydrolyzed by gut microflora and resulting E2 is readily reabsorbed [6]. In contrast, 60% of ethinylestradiol is eliminated in the feces with up to 16% existing as unchanged parent compound [7]. Glucuronide and sulfate conjugates constitute the remaining 40% in urine [8]. Previous biotransformation studies indicate that synthetic and

naturally derived estrogens are primarily excreted in the urine and/or feces as a sulfate or glucuronide conjugate [9]. These conjugates are readily hydrolyzed in the gut to release the parent compound, suggesting that similar metabolic events, involving microbial activity, may occur in sewage treatment facilities. In United Kingdom effluents, synthetic and naturally derived estrogens were detected as unconjugated, biologically active compounds [10].

Both endogenous and synthetic estrogens are known to induce vitellogenesis in male fish following binding to and activation of nuclear estrogen receptors [1]. Consequently, *in vivo* male vitellogenin induction is widely used to study contaminant estrogenicity under laboratory and field conditions. *In vitro* assays, including the yeast estrogen screen assay (YES), are also used to evaluate compounds for estrogen activity [11-15] and monitor estrogenic activity of effluents and surface waters [10, 16-18]. In this assay, the human estrogen receptor (ER) and estrogen response elements (ERE) are integrated into the genome of a yeast, *Saccharomyces cerevisae*. When estrogenic compounds activate the estrogen receptor, transcription of a β-galactosidase reporter gene is activated, ultimately leading to a quantifiable colorimetric change [19]. Desbrow et al. [19] suggested that one benefit of the YES assay was its response to biologically active compounds. Other advantages of using *in vitro* assays like YES or MCF-7, a human breast cancer cell screen [20, 21], to screen for estrogenicity have included shorter assay time and lower expense than *in vivo* VTG fish assays.

Previous studies have used a toxicity identification procedure to explore municipal effluent estrogenicity. For example, using the YES assay and chemical analyses, Desbrow et al. [19] identified ethinylestradiol, estrone and estradiol as causative estrogenic compounds in a United Kingdom municipal effluent. Other studies evaluated

the relationship between *in vitro* YES activity and *in vivo* male fish VTG induction. Fawell et al. [18] observed similar sensitivity between YES activity and *in situ* male rainbow trout VTG following exposure to several municipal effluent study sites. In laboratory studies, Metcalfe et al. [16] also observed comparable sensitivity between *in vitro* YES assay and *in vivo* Japanese medaka VTG assay responses to natural and synthetic estrogens.

Because many estrogenic compounds that induce male vitellogenesis have been identified in municipal effluents, it has been difficult to identify causative estrogenic compound(s). For example, *in vivo* data from the City of Denton, TX indicated that during March and December 2000 municipal effluent exposure induced VTG in male fathead minnows [22, 23]. Analytical measures indicated that steroids were present in City of Denton, TX effluent during March and December 2000, but not in June studies [22, 23]. In June and August 2000, this effluent was not estrogenic to male fathead minnows [24, Chapter 3]. However, xenoestrogens including nonylphenols, bisphenol A, phthalates, and atrazine were detected at low ug/L levels during both March and June 2000 study periods [24].

Although effluent estrogenicity may be related to City of Denton, TX population demographic changes with enrollment in two universities (Chapter 3), causative estrogenic contaminants have not been identified. Therefore, the purpose of this study was to identify causative compound(s) potentially responsible for City of Denton, TX effluent estrogenicity. An identification procedure was chosen that included solid phase extraction, effluent fractionation with solvents of different polarities, and subsequent evaluation of solvent fractions for estrogenic activity using an *in vitro* YES assay and a

confirmatory *in vivo* adult male Japanese medaka VTG assay. The primary objectives of this study were: 1. identify causative estrogenic compounds using a modified identification approach from Desbrow et al. [19]; 2. utilize *in vivo* VTG responses to confirm *in vitro* estrogenicity ; 3. determine if population demographic changes corresponded with effluent estrogenicity; and 4. provide information on effluent estrogenicity to guide experimental design of a 90-day study on the effects of cadmium and municipal effluents on fish endocrine function (Chapter 3).

Materials and Methods

Study Site: Pecan Creek Water Reclamation Plant

Final treated effluent was sampled from the City of Denton Pecan Creek Water Reclamation Plant (PCWRP) on 02 May 2001, sample date 1, and 16 May 2001, sample date 2. PCWRP is a conventional activated sludge wastewater treatment plant that operated at a capacity of 15 million gallons per day (MGD) with an average outflow of approximately 12.5 MGD during this study. PCWRP received wastewater from approximately 80,000 residential customers and 150 industries in 2001 (D. Hunter, City of Denton, pers. comm.). Bar screens, vortex grit removal, primary clarification, extended aeration, secondary/final clarification comprised the major PCWRP treatment process sequence. Sludge thickening was accomplished by gravity settling of waste activated sludge, followed by dissolved air floatation. Final effluent received chlorination for disinfection and was subsequently dechlorinated with sulfur dioxide prior

to discharge. Residence time of PCWRP was estimated at eight hours for both study dates (K. Thuesen, City of Denton, pers. comm.); therefore, a sampling time of 1500 was chosen to approximately evaluate PCWRP treatment of early morning sewage inflow. Pecan Creek, the receiving system for PCWRP, is perennially dominated by municipal effluent. Approximately 5500 m downstream from effluent discharge, Pecan Creek flows into Lake Lewisville, an impoundment that supplies water to several municipalities including Dallas, Lewisville and Denton, TX.

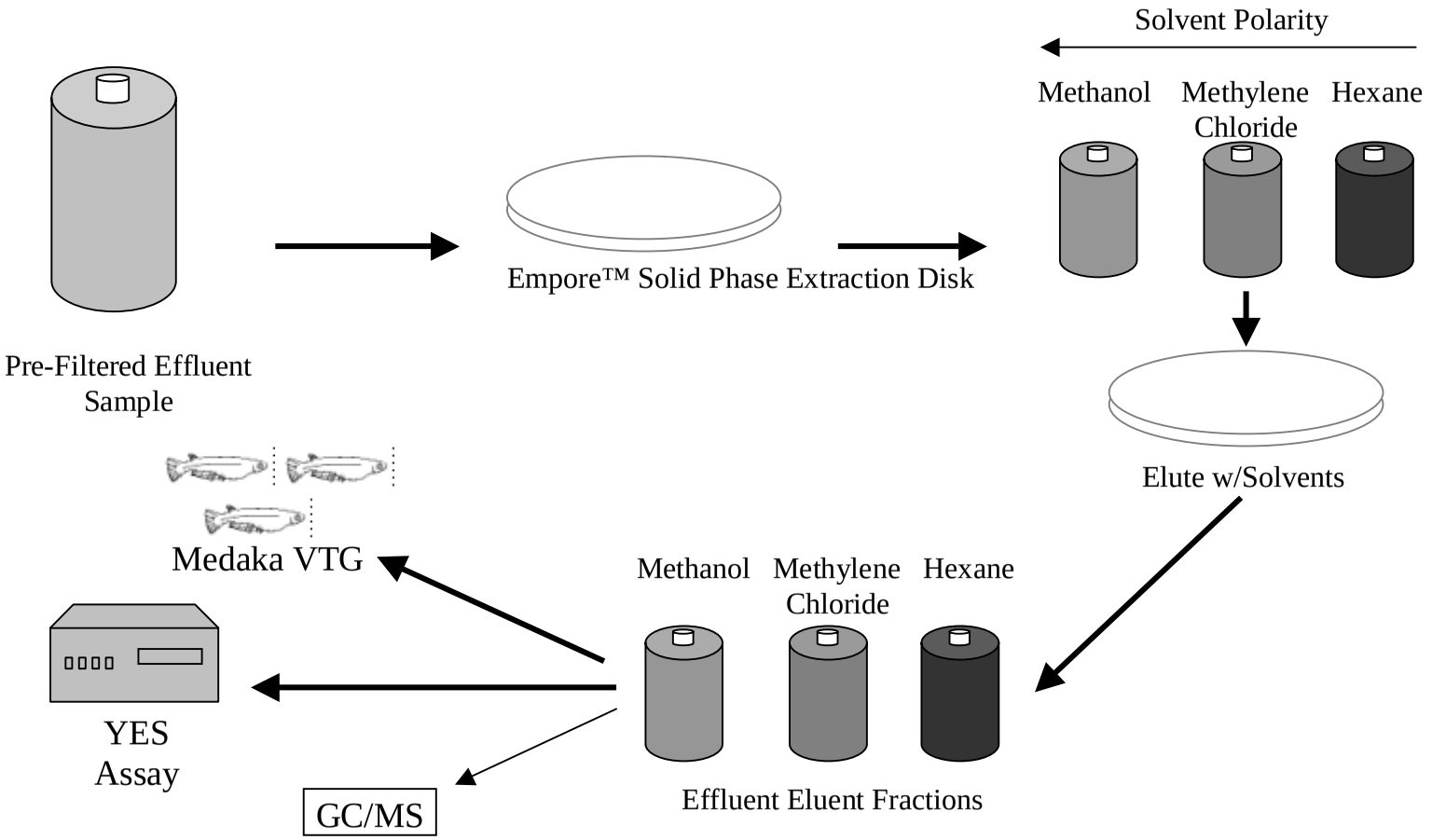
Effluent Sampling Procedure

Effluent samples were collected from PCWRP at 1500 on two sample dates. Sample day 1, 02 May 2001, was selected as a weekday sampling time during the final week of spring university session. Sample day 2, 16 May 2001, was selected as a weekday sample during the week following spring 2001 university session break. Effluent water chemistry, including pH, temperature (^{o}C), specific conductance (µS/cm), dissolved oxygen (mg/L), total dissolved solids (mg/L) and turbidity (NTU), was measured using a Hydrolab multiprobe datasonde. Effluent grab samples were collected with 20 L cubitainers and transported to the laboratory on ice. Potentiometric alkalinity (mg $CaCO_3$/L) and colorimetric hardness (mg $CaCO_3$/L) titrations of effluent subsamples followed standard methods [25]. Less than 24 hours after collection, 1 L effluent sub-samples were prefiltered with 0.7 um Millipore glass fiber filters. Pre-filtered effluent samples were extracted with Empore™ SDB-XC solid phase extraction disks that were sequentially conditioned with ACS grade acetone, ACS grade methanol and Milli-Q water [26]. Extraction disks were stored at -80°C prior to analysis.

YES Assay

Effluents were initially screened for estrogenic activity using the YES assay [12, 19]. To examine the relationship between *in vitro* and *in vivo* responses and to confirm *in vitro* activity, estrogenic effluent samples screened by the YES assay were further evaluated for VTG induction with adult male Japanese medaka (Figure 1). Empore™ SDB-XC extraction disks were sequentially eluted with three solvents of decreasing polarity: methanol, methylene chloride and hexane. Such a solvent elution scheme was previously observed to selectively fractionate a complex mixture of estrogenic compounds based on relative polarity of analytes originally extracted from samples to extraction disks [27]. 30 mL solvent fractions were reduced to dryness a nitrogen stream and reconstituted in 300 μL ethanol. 5 mL growth media was inoculated with either estrogen response element (ERE, a negative control) or estrogen response element and estrogen receptor (ERE+ER) cells. Cells were incubated overnight at 30°C and then diluted to an optical density (OD) of 0.057, determined at 630 nm with a Tecan UV/Vis microplate reader. Each solvent fraction (100 μL) was incubated with 700 μL of ERE+ER cells, ERE cells or ethanol. Following a 24hr incubation at 30°C, 100μL of each cell suspension and 400μL of chromogenic substrate (ONPG) was added to a 96 well microplate. Following microtiter plate incubation at 30°C during color development, OD was determined at 405nm. 17β-estradiol was used for standard curve generation. OD values were corrected for ethanol controls, turbidity and ERE interactions according to Payne et al. (2000) where: OD = ERE+ER ($Test_{540nm}$ - ($Test_{630nm}$ - Con_{630nm}) - Con_{540nm}) - ERE ($Test_{540nm}$ - ($Test_{630nm}$ - Con_{630nm})- Con_{540nm}).

Figure 1: Generalized schematic describing effluent toxicity identification evaluation. Procedures included effluent extraction, elution of disk with solvents of different polarity, evaluation of solvent fraction estrogenicity with *in vitro* yeast estrogen screen and *in vivo* Japanese medaka vitellogenin (VTG) assays and analysis of effluent steroids.



Japanese Medaka (Orzyias latipes) Vitellogenesis

Adult male Japanese medaka were exposed to methanol, methylene chloride and hexane eluents for a 7d static renewal study. Medaka were cultured and tested in Balanced Saline Solution (BSS) [28]. Standard water chemistry of exposure media was evaluated according to standard methods and included pH, temperature, dissolved oxygen, alkalinity, hardness and salinity [25]. Preparation of methanol, methylene chloride and hexane eluent fractions from solid phase extraction disks followed YES assay procedures. Two adult male Japanese medaka were randomly placed in three replicate 800mL exposure chambers per treatment. 40uL aliquots of reconstituted ethanol eluents from sample day 1 were added to exposure chambers prior to daily renewals. On day 7, fish were anaesthetized with MS-222. After the isthmus was incised, blood was collected with a heparinized hematocrit tube. Blood samples from the two fish in each exposure chamber were pooled. Plasma was isolated following centrifugation at 6,000 x g for 10 minutes and stored at –80^{o}C prior to VTG analysis.

Plasma VTG levels were determined following previously reported methods [29-32]. Protein content of plasma was measured using bovine albumin standards (Sigma Chemical) and a Bio-Rad Protein Assay protein dye with a Tecan UV/Vis microplate reader [29-32]. VTG was determined by SDS-Page and Western blotting with a mouse monoclonal antibody developed by Heppell et al. [33] against striped bass (Cayman Chemical Co.). After the nitrocellulose image of each gel was developed, gels were scanned and subsequently analyzed using Scion Image software. VTG bands (170 kDa and 130 kDa) were quantified as percent integrated optical density (IOD) relative to

positive controls. Positive control livers used for this study were collected from Japanese medaka exposed to 1 ug/mL estradiol for 7d.

Quantitation of Effluent Estrogens

Analysis of effluent estrogen steroids was performed for effluent sample days 1 and 2. Analytical procedures followed previously reported methods [26, 34]. Compounds were eluted from solid phase extraction disks with two 15 mL methanol additions. Methanol fractions were combined, mixed with 1 g hot sodium sulfate, and reduced to dryness with nitrogen. Samples were subsequently derivatized with 50 μL of a 1000:2:2 mixture of MSTFA (n-methyl-N-(trimethylsilyl)-trifluoracetamide):TMSI (trimethylsilylimidazole):DTE (dithioerytrol). Derivatized samples were evaporated to dryness by rotary evaporation, reconstituted in 100μL hexane and transferred to GC vials. Samples were analyzed with a Hewlett Packard 6890 series gas chromatograph (GC) coupled to a Hewlett Packard 5973 mass spectrometer. A 0.9 mL gas flow rate and a J&W DB-5MS capillary column (30 m x 250 um x 0.25 um) were utilized. For each GC sample run, a temperature profile of 160°C for 1 min, increased to 290°C at 10°C/min and held at 290°C for 10 min was followed for a 24 min total run time. Samples were analyzed by mass spectroscopy in selective ion monitoring (SIM) mode. Ions 425, 416 and 342 *m/z* were utilized for monitoring EE2, E2 and estrone, respectively. Estrogen quantitation was accomplished with a three-point calibration curve.

Statistical Analysis

Analyses of treatment effects were performed using SPSS (Version 10.05). Treatments effects were evaluated with ANOVA and Dunnett's multiple comparison test. Statistical significance of fish response variables was determined at $\alpha = 0.05$.

Results

Effluent and BSS Water Chemistry

Water quality measures collected from the City of Denton, TX municipal effluent at 1500 were similar on sample days 1 and 2 (Table 1). Standard water chemistry of Balanced Saline Salt solution utilized in Japanese medaka eluent studies was consistent with previous studies [30-32, 35] and is provided in Table 1.

Estrogenic Activity of Effluent Samples

Effluent collected on sample days 1 and 2 were evaluated with the YES assay. Sample day 1 exhibited estrogenic activity, but only in the methanol fraction (Figure 2). Estrogenic responses were observed in Japanese medaka exposed to fractions of effluent collected on sample day 1. Methanol ($p<0.05$) and methylene chloride ($p<0.001$), but not hexane, fractions of sample day 1 effluent significantly induced vitellogenesis in male fish (Figure 3).

Table 1: Water quality characteristics of City of Denton, TX effluent and BSS medaka exposure media. BSS = Balanced Saline Salt solution. Sample day 1 = 02 May 2001, sample day 2 = 16 May 2001. NM = not measured.

Parameter	Sample Day 1	Sample Day 2	BSS
pH	7.25	7.37	6.70
Temperature (^{o}C)	24.72	25.4	25±1
Dissolved Oxygen (mg/L)	7.12	7.01	8.69
Specific Conductance (µS/cm)	880.9	836.1	NM
Salinity (g/L)	NM	NM	1.61
Total Dissolved Solids (mg/L)	0.5643	0.5351	NM
Turbidity (NTU)	1.7	2.1	NM
Hardness (mg/L $CaCO_3$)	164	156	86
Alkalinity (mg/L $CaCO_3$)	80	75	33

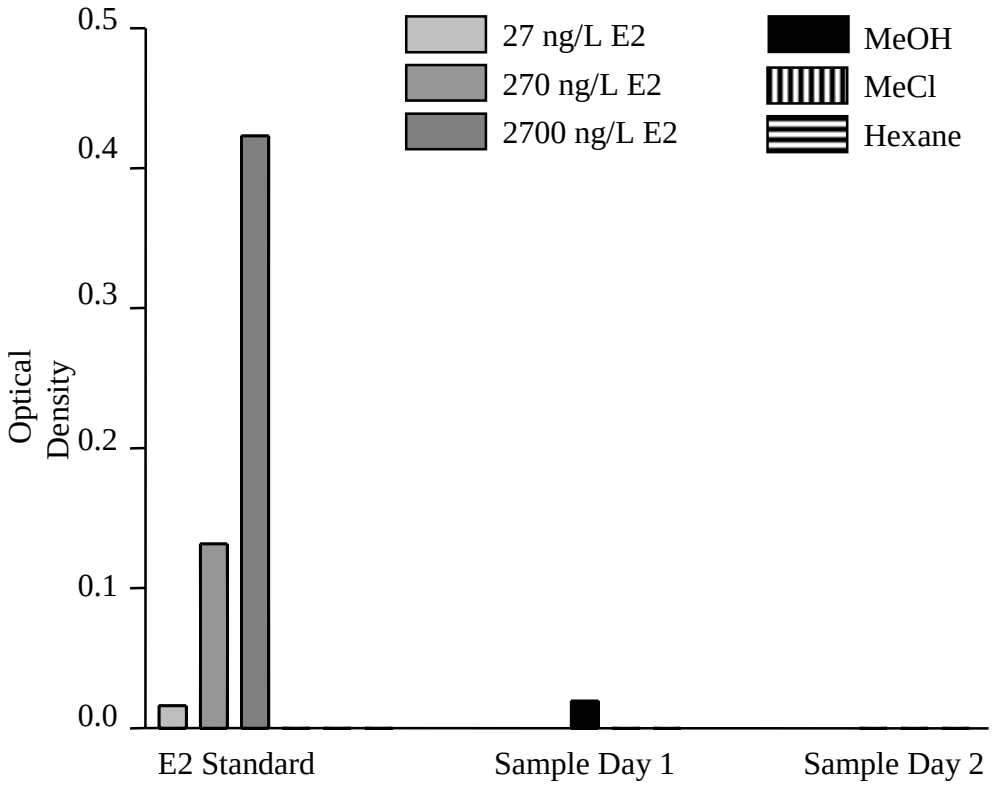


Figure 2: Yeast Estrogen Screen (YES) assay activity for estradiol standards and solvent fractions (methanol, methylene chloride, hexane) extracted from Empore™ SDB-XC solid phase extraction disks. Sample Day 1 = 02 May 2001, Sample Day 2 = 16 May 2001. MeOH = Methanol, MeCl = Methylene Chloride.

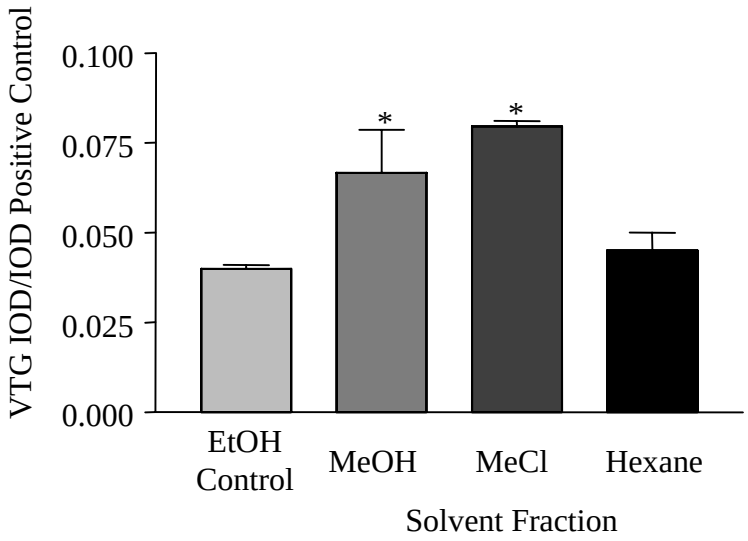


Figure 3. Plasma VTG induction (±SD) in adult male Japanese medaka exposed for 7d to solvent fractions from City of Denton, TX, effluent collected on 02 May 2001. MeOH fraction, $p < 0.05$, N=3; MeCl fraction, $p < 0.001$, N=3.

Analytical Chemistry

EE2, E2, and estrone were quantitated in effluent sampled on study day 1 and 2. Average E2 and estrone concentrations of duplicate sample injections were 1.2 and 6.02 ng/L, and 0.3 and 1.2 ng/L on sample days 1 and 2, respectively. EE2 was not detected in effluent samples from either sample date.

Discussion

Previous investigators observed that the City of Denton, TX effluent was estrogenic during certain periods [22, 23] but not others [24, Chapter 3]. Presence and absence of effluent estrogenicity in these studies appeared to correspond with enrollment of two university student populations [24, Chapter 3]; however, the source(s) of effluent estrogenicity were unknown. Results from this study indicated that the City of Denton, TX effluent was estrogenic to *in vitro* and *in vivo* bioassays on a sample date concurrent with spring university semesters (Figures 2 and 3). Approximately 39% of 80,000 residential City of Denton water utilities customers were students enrolled at University of North Texas (UNT) or Texas Woman's University (TWU) during the 2001 spring semester (UNT: 25,652 [36]; TWU: 5,717 [37]). Interestingly, YES activity was not observed in effluent samples from sample day 2 (Figure 2). This corresponds with an 86% reduction of enrolled students reported between sample day 1 and sample day 2 (UNT: 3,719 (R. Anzaldua, UNT, pers. comm.); TWU: 571 [37]). A reduction of E2 and estrone concentrations was also observed on sample day 2.

Whereas previous studies indicated that the YES assay responds to a large number of endogenous and exogenous estrogens, it exhibits greatest sensitivity to estrogenic steroids. Desbrow et al. [19] reported the YES assay as 1000, 5000 and 50000 times less responsive to bisphenol A, ocylphenol and nonylphenol, respectively. Others observed greater sensitivity of YES activity to steroids relative to other xenoextrogens (Arnold et al. 96). Whereas Routledge and Sumpter [13] and [38] observed EE2 to have higher potency to the YES assay than E2, Metcalfe et al. [16] reported that EE2 and E2 were approximately equipotent. YES responses to xenoestrogen mixtures have been reported

as additive [14]. In this study, only the methanol fraction from sample day 1 was estrogenically active to the YES assay. Because only natural estrogens were chemically evaluated in this sample, an estradiol toxicity equivalent approach was implemented to evaluate relative effluent estrogenicity. A toxic equivalent approach was previously used to compare the response of biologically active TIE fractions in the YES assay to a standard curve of 17β-estradiol, a compound which elicits a similar biological response [39]. This estradiol toxic equivalent approach was used to calculate that the methanol fraction from sample day 1 contained 14 ng/L estradiol equivalents.

Metcalfe et al. [16] successfully utilized *in vitro* YES activity to confirm estrogenic impacts to Japanese medaka. Fawell et al. [18] indicated that *in vitro* YES responses to effluents predicted *in situ* male VTG induction at most sites; however, effluent at one site induced male vitellogenesis without a corresponding increase in YES activity. In this study, *in vitro* YES activity was employed as an initial screening assay and *in vivo* VTG induction in male fish was used as a confirmatory assay. Whereas YES activity was only observed in methanol fractions from sample day 1 in this study, methanol and methylene chloride fractions significantly increased plasma VTG content in Japanese medaka (Figure 3). Snyder et al. [27, 40] identified that the TIE approach used in this study fractionates most polar compounds including EE2, E2 and estrone in methanol fractions, moderately polar compounds including nonylphenol ethoxylate degradation products in methylene chloride fractions, and most nonpolar compounds including PAHs, PCBs and organochlorine pesticides in hexane fractions. Although not as responsive to the YES assay as steroid estrogens, degradation products of the nonylphenol ethoxylate surfactants were consistently observed by Hemming [24] at low

ug/L levels in the City of Denton, TX effluent in March and June 2000. These levels are consistent with other findings [27].

To evaluate which TIE solvent elution contained nonylphenol ethoxylate surfactant metabolites, I performed an additional laboratory extraction and elution study. Triplicate 1 L MilliQ water samples were spiked with 10 ug/L 17B-estradiol (Sigma Chemical, St. Louis MO) and nonylphenol (ChemService, West Chester PA). These volumes were extracted to solid phase with Empore SDB-XC extraction disks. Disks were subsequently eluted with 30 mL methanol, methylene chloride and hexane, evaporated to 1 mL with a nitrogen stream, and estradiol and nonylphenol measured by GC/MS according to methods described in Hemming et al. [23]. Nonylphenol and estradiol were detected and quantitated only the methanol fraction with mean percent recovery of 70.68% and 82.32%, respectively. Increased plasma VTG content in Japanese medaka exposed to methanol and methylene chloride TIE fractions indicate that compounds with polarities similar to steroids and nonylphenol ethoxylate degradation products also contributed to effuent estrogenicity on sample day 1.

A recent report indicated that performance of the YES assay is comparable to other *in vitro* estrogen receptor transcriptional assays and suggested a need for development of testing procedure standards [41]. However, although noted as a valuable research tool, the YES assay was not included in Tier 1 screening assays as recommended by the U.S. Environmental Protection Agency's endocrine disruptor screening and testing advisory committee [42]. Primary questions associated with use of YES activity in estrogen screening studies include limited understanding of contaminant transport across yeast membranes compared to invertebrates or vertebrates and potential

metabolism of compounds by yeast prior to nuclear receptor binding [42]. Metabolism and membrane transport is more understood for the human breast cancer cell assay, MCF-7. MCF-7 has been successfully used in estrogen TIE procedures [21, 39] and may provide a valuable tool for future effluent estrogenicity screening or fractionation TIEs.

This study confirmed previous reports that estrogenicity of the City of Denton, TX effluent, as measured by YES activity and VTG induction in adult male fish, coincides with enrollment numbers at two Denton universities. These findings indicate that E2 and estrone were primarily responsible for effluent estrogenicity; however, nonylphenol ethoxylate degradation products, and other unidentified compounds, may have contributed to estrogenicity of the City of Denton, TX effluent. Results of this study generally concur with findings of Huggett et al. [43] who performed *in vitro* and *in vivo* TIE procedures reported herein on municipal effluents from several New York City wastewater treatment plants. Huggett et al. [43] observed similar discrepancies between *in vitro* and *in vivo* responses to estrogenic effluents from New York. *In vivo* bioassays and other *in vitro* assays should be used concurrent with *in vitro* YES activity when effluents are screened or fractionated for estrogenicity.

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