C I T Y O F B O U L D E R
WATER RESOURCES ADVISORY BOARD
INFORMATION ITEM

MEETING DATE: August 19, 2013

AGENDA TITLE: Information Item - WWTF Activated Sludge Upgrades and Removal of Emerging Contaminants

PRESENTERS:
Jeff Arthur, Director of Public Works for Utilities
Chris Douville, Wastewater Treatment Manager
Bret Linenfelser, Water Quality / Environmental Services Manager

EXECUTIVE SUMMARY

Emerging Contaminants, and Endocrine Disrupting Compounds (EDCs) in particular, are a growing concern within water quality and wastewater treatment. Some of these compounds, such as steroid hormones and pharmaceuticals, have been discovered in various water environments including treated wastewater effluent, and have been shown to cause negative impacts to the reproductive systems of aquatic life. A significant amount of water quality research has been performed on Boulder Creek, with attempts to understand the implications of the wastewater effluent which makes up the majority of the creek flow during most of the year. A long-standing collaboration between the City of Boulder (city), United States Geologic Survey (USGS), and the University of Colorado (CU) has resulted in significant findings related to the impacts of wastewater effluent from the city's 75th Street Wastewater Treatment Facility (WWTF).

Summarized in a 2012 publication from Environmental Science and Technology, research has shown that the endocrine-disrupting effects of the wastewater effluent have been significantly reduced following the WWTF upgrade to activated sludge treatment in 2008. Prior to the upgrades, the WWTF utilized a process technology called trickling filter/solids contact and the documented estrogenic effects to fish in Boulder Creek were very pronounced. After the upgrades went online, repeat studies were conducted where fish were exposed to various amounts of Boulder Creek water and wastewater effluent. Physiological fish data, as well as chemical data from laboratory analysis, confirm that the activated sludge upgrades have significantly reduced the endocrine-disrupting effects of the final effluent.

The city has learned additional information about EDCs in wastewater effluent from participation in other research studies. The purpose of this information item is to highlight the important work with USGS and CU. Highlights from participation in other research efforts will be discussed during the August 19 WRAB Meeting.

This item is being presented as an information item to provide the WRAB with context for future recommendations on capital projects at the WWTF. No formal action by the WRAB is requested at this time.

FISCAL IMPACT

This issue does not have a significant short-term budget impact. Minor amounts of staff time are utilized to participate in ongoing collaboration with research teams, and some minor expenses are experienced as part of "in-kind" contributions affiliated with research efforts. None of the emerging contaminants are on the immediate Federal or State regulatory horizon. Thus, any facility modifications necessary to remove one or more classes of compounds is a long-term issue and will be addressed in the 20-year CIP planning horizon.

COMMUNITY SUSTAINABILITY ASSESSMENTS AND IMPACTS

- Economic: Emerging contaminants in wastewater, once more fully understood and regulated, have the potential to cause significant impacts on capital and operating costs of wastewater treatment. Advanced technologies may be required to affect removal of many of these compounds. The concern is a long-term one that will eventually lead to higher rates for wastewater service.

- Environmental: Research has demonstrated both negative environmental and human impacts from even low quantities of emerging contaminants in waters, such as endocrine disrupting compounds, hormones, and trace organics. It is important to understand the occurrence of these compounds, the concentrations, how they behave, and their fate in wastewater treatment.

- Social: Emerging contaminants are receiving more and more attention from news media, so the public are becoming aware of these concerns, and questions/comments from the public validate this growing awareness. Community education around this issue will continue to grow in importance.

BACKGROUND

The Boulder Creek watershed, and Boulder Creek in particular, has been studied extensively over the years. Much has been learned about the water quality of Boulder Creek, and some of the information reveals environmental and human health implications due to the presence of a host of compounds collectively known as Endocrine Disrupting compounds (EDCs). EDCs have

been shown to disrupt, modify, or otherwise negatively affect an aquatic organisms' growth and reproduction processes. Examples of EDCs are steroid hormones, pharmaceuticals, and nonylphenols. EDCs are a subset of what are referred to as Emerging Contaminants – compounds that are receiving a heightened amount of attention from the scientific and environmental communities, and have the potential to become regulated compounds in the future.

In the 1990s, the city started a broad, collaborative research effort with staff from the USGS and CU to understand water quality in Boulder Creek. One significant effort called "The Millennium Study" documented the occurrence of natural and anthropogenic organic compounds in Boulder Creek, upstream and downstream of the 75th St. WWTF, from samples collected in year 2000. Findings of this study revealed significant concerns with the occurrence and concentration of pollutants in Boulder Creek, downstream of the WWTF. Additional studies in subsequent years revealed more concerning information, including an imbalance male/female fish ratios downstream of the WWTF when compared to upstream, as well as physiological data from fish demonstrating atypical physiology (increased yolk protein consistent with feminization, reduced gonads in male fish) and disruption of the endocrine system. While the city is not the only discharger along Boulder Creek, the 75th St. WWTF is certainly the predominant point-source discharger. For an expanded understanding of the conditions in Boulder Creek and influence of the WWTF effluent, refer to **Attachment A** that contains slides from presentation shared at the 2007 WEFTEC Conference.

From 1990 through late 2007, the city's WWTF utilized the treatment process called trickling filter/solids contact (TF/SC). Rock trickling filters (from the original facility construction) were used to host a microbiological biofilm (fixed-film treatment) and remove BOD, while small aerated reactor tanks downstream of the TFs were used to support a microbiological population mixed in liquid suspension to further remove some BOD and improve settleability of the secondary solids. Downstream of the TF/SC process train, the city also had a single nitrification trickling filter (NTF) to perform ammonia removal (ammonia oxidizing bacteria biofilm on plastic media). Hydraulic and solids residence time within the TF/SC process was low (1 to 2 hours HRT, and 1 day SRT); conditions that are not favorable for EDC removal.

ANALYSIS

The city's 75th St. WWTF was upgraded in year 2008 to include a new microbiological process known as activated sludge, replacing the previous TF/SC process. The key driver for the upgrade was to fully remove ammonia-nitrogen, due to regulatory mandates. The project also included several other significant components, such as a hydraulic and process capacity expansion and partial nitrogen removal capabilities with denitrification. Although it was not a project driver, the upgrade to activated sludge was anticipated to yield improved removal of endocrine disrupting compounds due to results shown in previous research by others. The theoretical reason for the improved removals with activated sludge is due to biodegradability of the compounds when afforded longer exposures to the microbiological population, when compared to other wastewater treatment processes such as TF/SC. Other fate pathways such as

sorption and chemical oxidation also likely occur during treatment from influent to effluent, but the key difference with activated sludge is the long solids retention times (SRTs) which are necessary when a facility fully-nitrifies to remove effluent ammonia.

Prior to the city completing the 75th Street WWTF upgrades, Larry Barber (USGS), Alan Vajda (CU-Boulder, now CU-Denver), and others approached the city with the idea of performing baseline research with the TF/SC treatment process still online, then repeating the research once the activated sludge upgrades were operational to capture a so-called "pre/post" study to understand the effects of a major WWTF infrastructure upgrade on Boulder Creek and endocrine disruption of fish. Highlights of the pre/post study are summarized here. For more detailed information on the study, reference **Attachment B** (white paper, Barber et al. 2012) and **Attachment C** (poster presentation, Vajda et at. 2013).

Study Method

In order to further explore the relationship between the endocrine-activity of the Boulder WWTF effluent and the reproductive disruption observed in downstream white suckers, it was necessary to conduct experiments to expose fish to the WWTF effluent under controlled conditions. A fish exposure mobile (FEM) was designed in a mobile trailer and deployed at the 75th St. WWTF to conduct controlled exposures to fish from effluent and Boulder Creek reference water. The FEM unit is outfitted with stainless steel pumps and holding tanks to store and continuously deliver WWTF effluent and Boulder Creek reference site water. Boulder Creek water is heated to equalize temperature with the WWTF effluent temperature, and a gravity flow system distributes the water to 60 glass aquaria, allowing exposure of fish to WWTF effluent, Boulder Creek reference water, and mixtures of the two.

Adult male fathead minnows (*Pimpehales promelas*) in breeding condition were tested under stimulatory photoperiod and temperature conditions to WWTP effluent, reference water, and a mixture of the two for 28 days. Male fathead minnows are one of the species used by the U.S. Environmental Protection Agency for endocrine disruption studies. Male fathead minnows exhibit quantifiable secondary sexual characters that are stimulated by androgens and antagonized by estrogens. Further, male fathead minnows readily produce the yolk precursor vitellogenin upon exposure to exogenous estrogens. Vitellogenin is normally produced in the livers of mature females in response to endogenous estrogens, and its elevation in males or immature females is a reliable biomarker of exposure to estrogens.

Over the course of the 28-day experiment, changes to the expression of secondary sex characters, changes to gonadal sperm abundance, and concentrations of vitellogenin in the plasma of exposed males were monitored. The occurrence of EDCs including biogenic and pharmaceutical steroidal estrogens and alkylphenolic surfactants was measured. The scope of this investigation extends well beyond the suite of biological analyses typically utilized in most municipal water quality testing laboratories. Further, the integration of biological and chemical analyses utilized in this investigation exceeds the analytical capacity of most individual research laboratories. Such comprehensive studies can only be accomplished through intensive inter-agency and interdisciplinary collaboration.

Results

The baseline condition with TF/SC, sampled in June 2007, was followed with post-upgrades sampling in June 2008 with the new activated sludge system in operation. The study revealed two important data sets: 1) chemical data for pollutants, and 2) physiological fish data. **Table 1** shows a summary of several key chemical parameters and associated final effluent concentrations. Removal efficiencies of most parameters increased, and some parameters were removed to non-detectable concentrations.

Table 1. WWTF Effluent Chemical Parameter Data, Pre and Post WWTF Upgrade

Parameter	Pre-Upgrade Concentration (ug/L)	Post-Upgrade Concentration (ug/L)
Caffeine	1.0	0.031
Bisphenol A	0.008	0.014
4-nonylphenol	0.34	0.21
Triclosan	0.45	0.039
17β-Estradiol	0.013	<0.001
Estrone	0.22	<0.001
∑ NPEO (Total nonlyphenolethoxylate)	6.2	2.3
∑ OPEO (Total octylphenolethoxylate)	2.0	0.031

Note: Pre-upgrade data from 6/20/07, and post-upgraded data from 6/16/08.

Physiological data from the fish exposure studies are summarized in **Table 2** below. The difference in post-upgrade biological fish markers was significant and correlates well with reduced effluent concentrations of estrogenic chemical compounds.

Table 2. Biological Parameter Data from Boulder Creek, Pre and Post WWTF Upgrade

Parameter	Pre-Upgrade Value	Post-Upgrade Value
Minimum Ratio of WWTF Effluent-to Boulder Creek Water with an observed negative impact on fish (trailer experiments)	25%	No effect at 100%
Gonadosomatic Index (gonad mass / body mass * 100)	Fall – 2 Spring – 1	Fall – 6 Spring – 4
Downstream Sex Ratios – M / F / Intersex	20 / 60 / 20	50 / 50 / 0
Plasma Vitellogenin (ng/mL)	Downstream approx. 100 times upstream	Downstream essentially equivalent to upstream

Note: Pre-upgrade data from various dates before August 2007, and post-upgraded data after 2007.

NEXT STEPS

- Utilities staff from the wastewater treatment and water quality workgroups will continue to collaborate with USGS and CU research teams to further understand water quality issues in Boulder Creek and influent of wastewater effluent

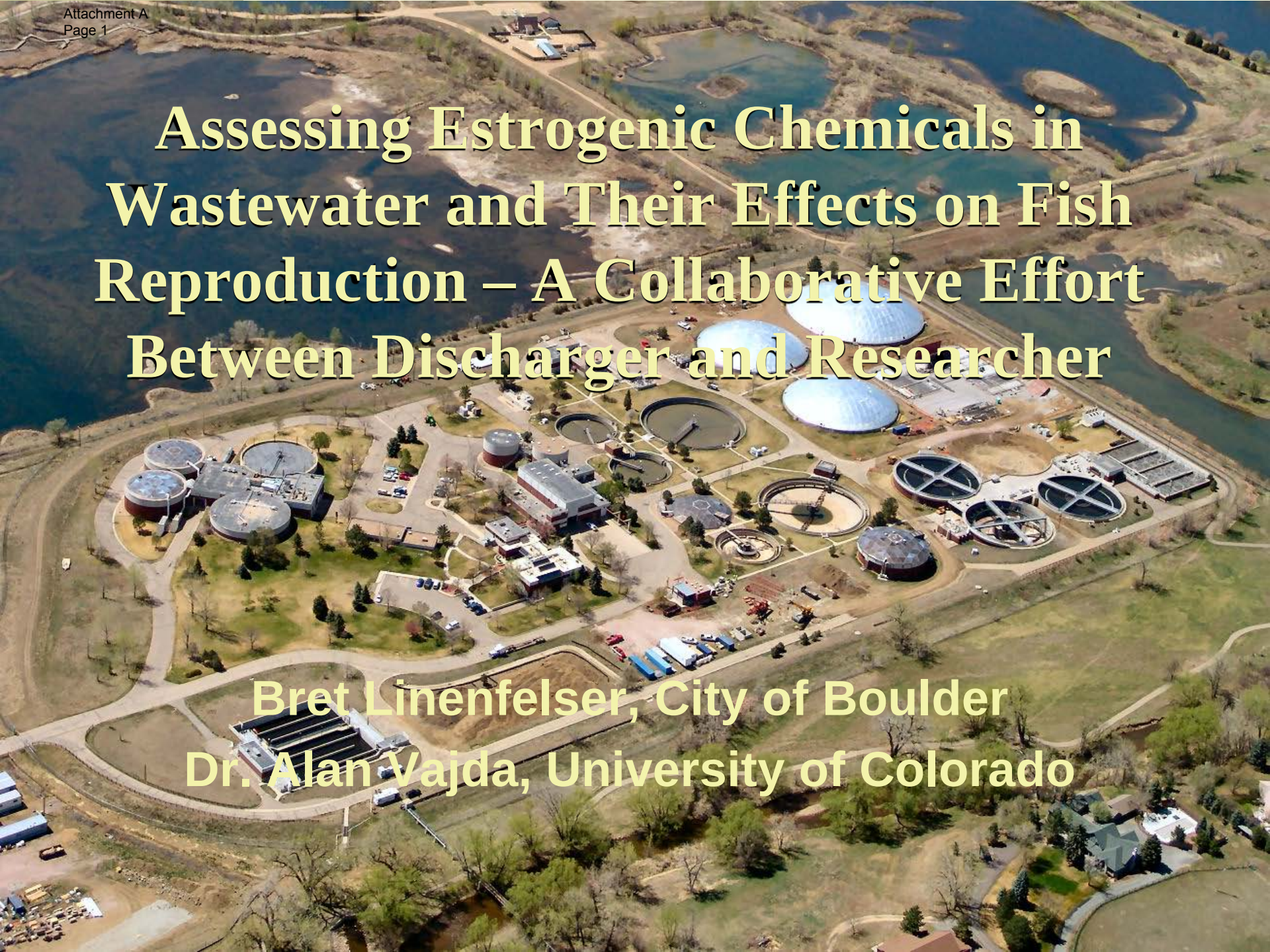
- Utilities staff will continue to evaluate other research opportunities (through organizations such as WERF) to participate in future studies, either as an integral team member or a partner utility
- As new WWTF upgrades are considered for separate drivers such as current regulatory mandates (i.e. nitrogen, phosphorus) or aging infrastructure replacement, impacts on emerging contaminant removal will be considered, when applicable
- Continue to advance public education and outreach efforts to advocate source control of emerging contaminants, such as pharmaceuticals and personal care products.

ATTACHMENTS

A: Slides from Presentation at WEFTEC Conference, "*Assessing Estrogenic Chemicals in Wastewater and Their Effects on Fish Reproduction – A Collaborative Effort Between Discharger and Researcher*". Linenfelser and Vajda 2007.

B: Journal Article from Environmental Science and Technology, "*Fish Endocrine Disruption Responses to a Major Wastewater Treatment Facility Upgrade*". Barber et al. 2012.

C: Poster Presentation shown at various conference venues, "*Infrastructure Investment Improves Ecosystem Health*". Vajda et al. 2013.



Assessing Estrogenic Chemicals in Wastewater and Their Effects on Fish Reproduction – A Collaborative Effort Between Discharger and Researcher

Bret Linenfelser, City of Boulder
Dr. Alan Vajda, University of Colorado

Better Living Through Chemistry

- In the past 70 years, humans have benefited from the production of a large array of chemicals
  - *pesticides, pharmaceuticals, personal care product ingredients, cosmetics and plastics.*

- It is estimated that more than 80,000 different chemicals have been released in to the environment

So What is the Concern?

- Exposure to some chemicals, specifically endocrine disrupting compounds (EDCs), elicits physiological responses in plants and animals, including fish
- Estrogenic EDCs are capable of feminizing fish below WWTPs as well as producing contraceptive-like effects
- Changes in fish have been documented in Colorado streams, including Boulder Creek below the city of Boulder, Colorado, WWTP

What's Been in Your Newspaper?

- **Pharmaceuticals in Waterways Raise Concern**
  - *Effect on Wildlife, Humans Questioned*
- **Male fish becoming female?**
  - *Researchers worry about estrogen and pollutants*
- **Effluent tipping scales on fish gender**
  - *A landmark study has found that wastewater from sewage-treatment plants in Boulder and Denver is causing gender deformities in suckers living downstream.*
- ***Feminization of fish***
  - *The effect of estrogenic compounds and their fate in sewage treatment plants and nature.*
- **OKAY, As if we Don't Have Enough to Worry About Now Comes Intersex Fish and the Potentially Frightening Explanation for Their Condition**

What's Been in Your Newspaper?

- **Excreted Drugs: Something Looks Fishy**
- **Fish sex change investigated**
  - *CU group establishes treatment plant effluent as culprit*
- **Mutant Fish Found in Colorado Rivers**
- **Contracepting the Environment**
  - *Environmentalists Mum on Poisoned Streams*
- **A Fish Named Wayne/Wanda?**

Interagency Collaboration Between the city of Boulder and Researchers

- Few studies have been active, collaborative effort between the discharger and researcher
- Since 2000, Boulder has actively supported collaboration with researchers
- Active participant stems from the benefits of advancing the understanding of observed effects and possible strategies to reduce those effects
- Boulder Creek and the 75th Street WWTP are an important source of information for Boulder and its residents

What are EDCs and What Might be Present in WWTP Effluent in the United States?

- Certain EDCs exhibit estrogenic action
- Natural estrogens are hormones capable of feminizing male vertebrates, inducing reproductive cancers, or blocking reproduction
- Common EDCs entering and leaving a domestic WWTP's are naturally produced by plants and animals with some found in products used by the general public

Wastewater Contaminants

Natural Steroids
Estradiol
Estrone
Estriol

Synthetic Steroids
17α-ethynylestradiol

Detergent Metabolites
4-n-Nonylphenol
4-tert-Octylphenol
Octylphenol,
diethoxy-octylphenol,
monoethoxy-para-Nonylphenol

Pharmaceuticals
Fluoxetine
Clomazapine

Nonprescription Drugs
Caffeine
Cotinine
Methyl salicylate

Antibiotics
Sulfonamides
Tetracyclines

Antioxidiants
BTA

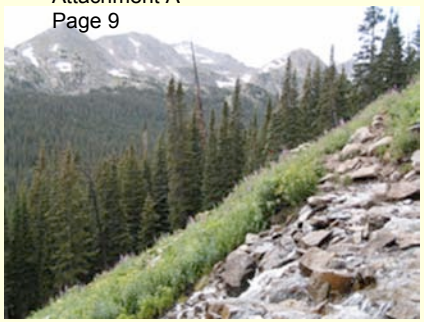
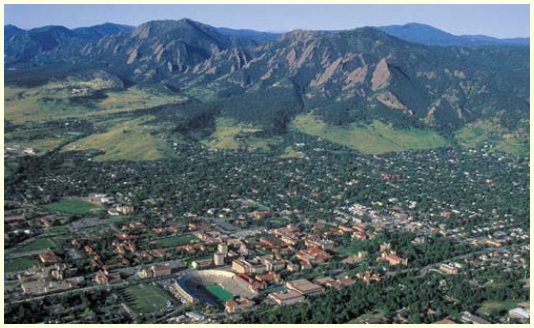
Disinfectants
Triclosan

Insect Repellants
DEET

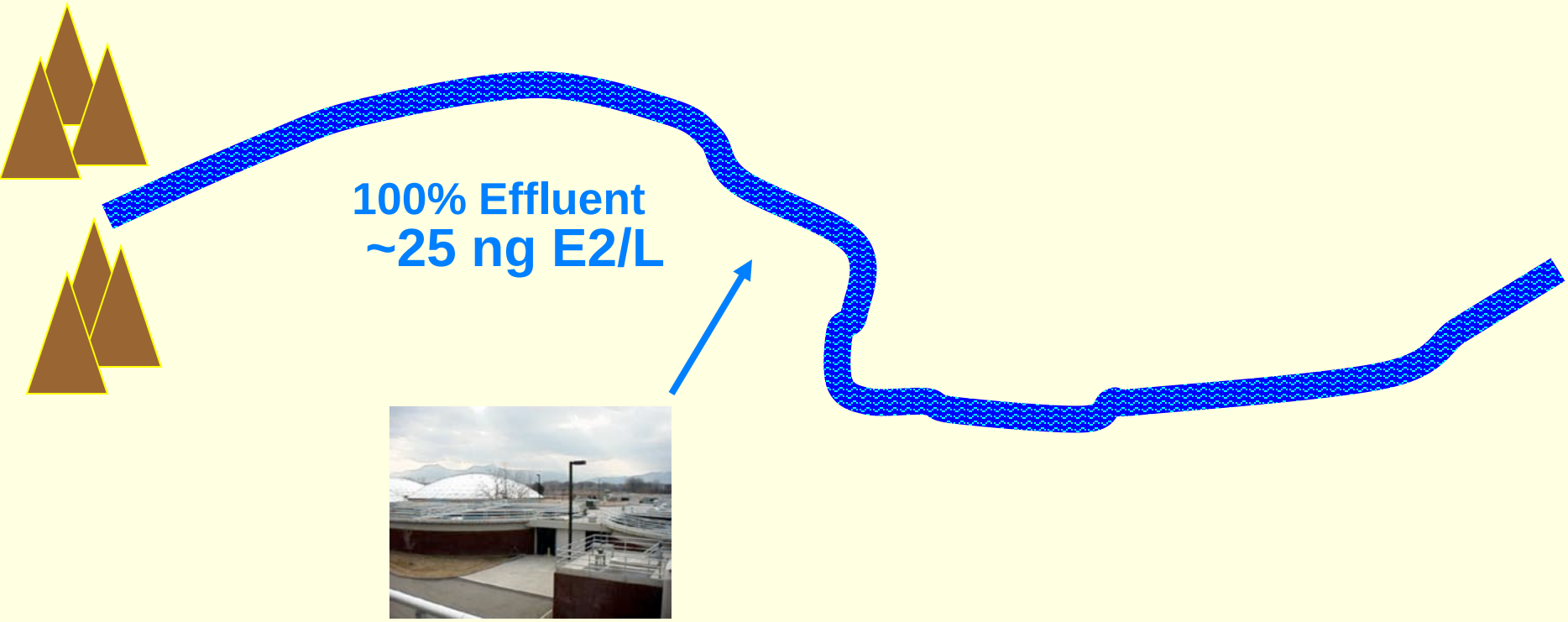
PAHs
Fluoroanthene
Napthalene
Phenanthrene
Pyrene

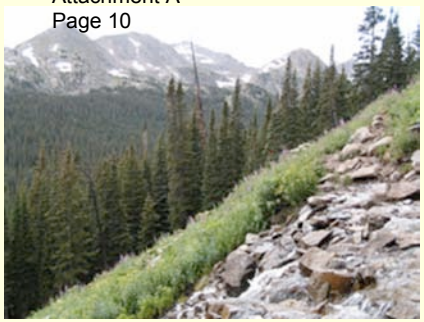
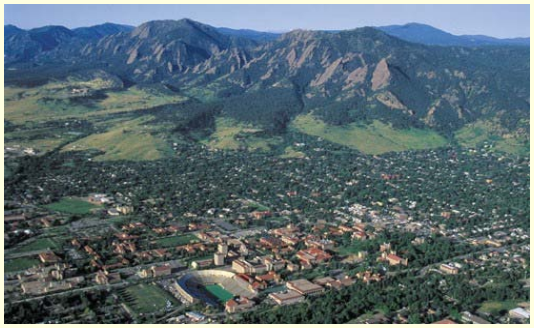
Pesticides
Carbaryl
Chlorpyrifos
Diazinon
Metalachlor

Plasticizers
Bisphenol A

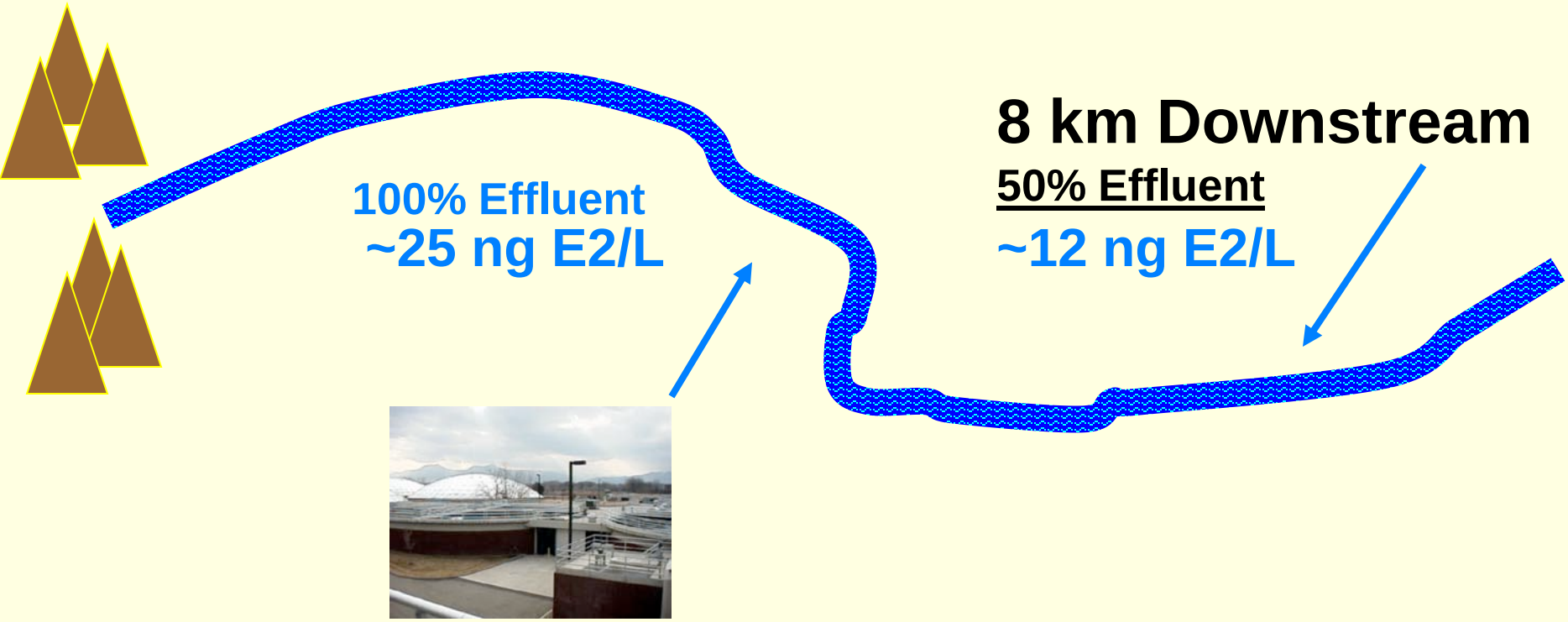



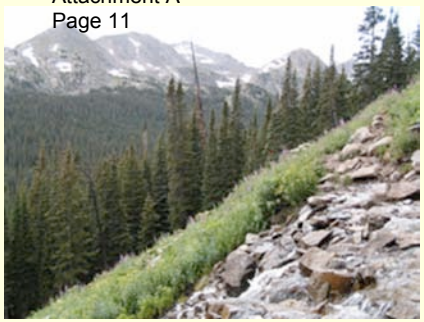
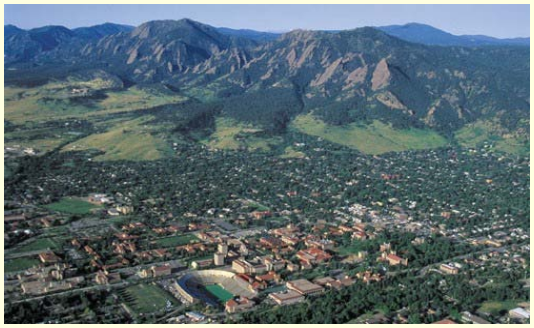
Boulder Creek, Boulder, CO, USA



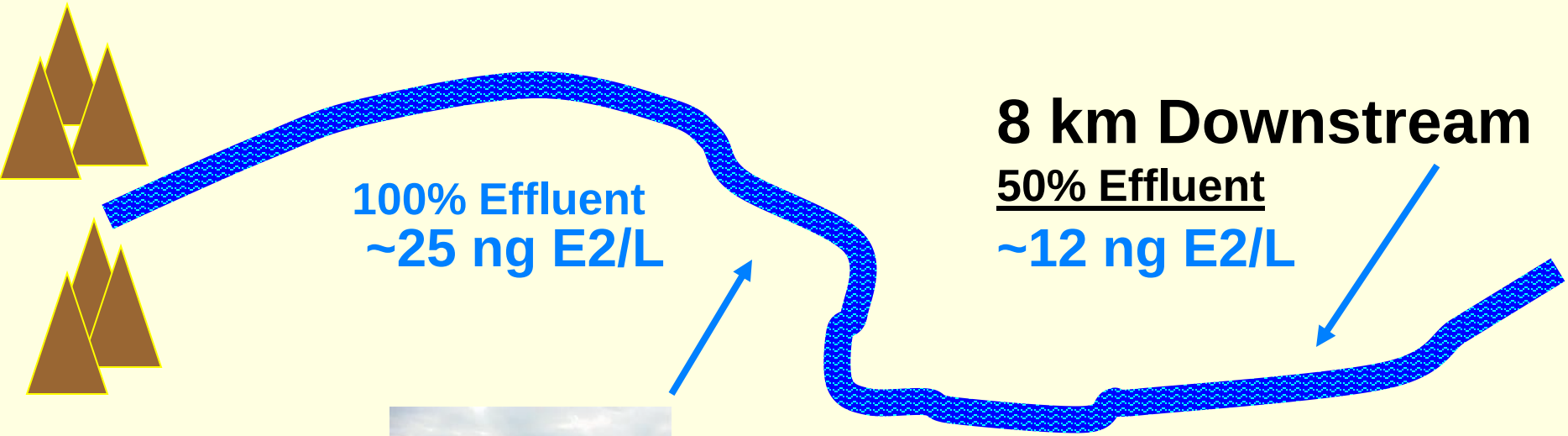



Boulder Creek, Boulder, CO, USA

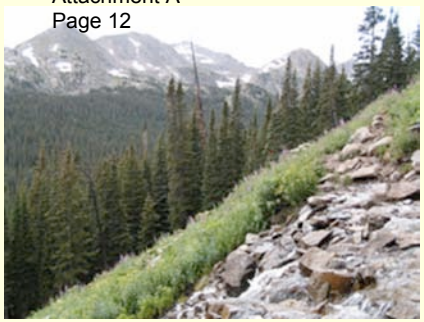
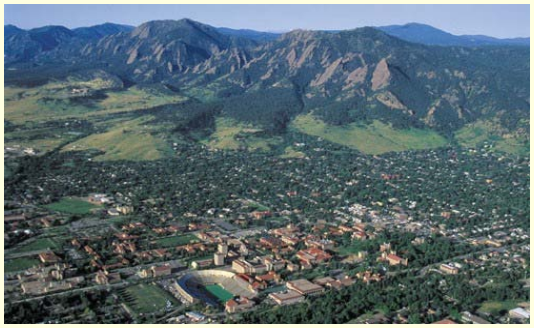


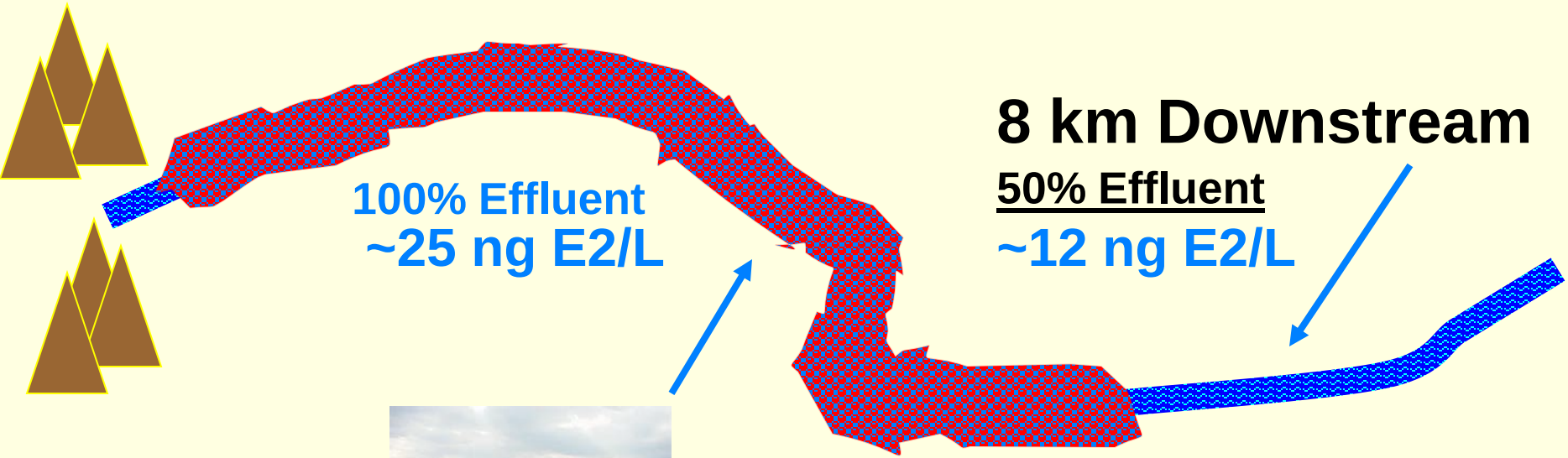
Boulder Creek, Boulder, CO, USA



White Sucker
(*Catostomus commersoni*)

Boulder Creek, Boulder, CO, USA

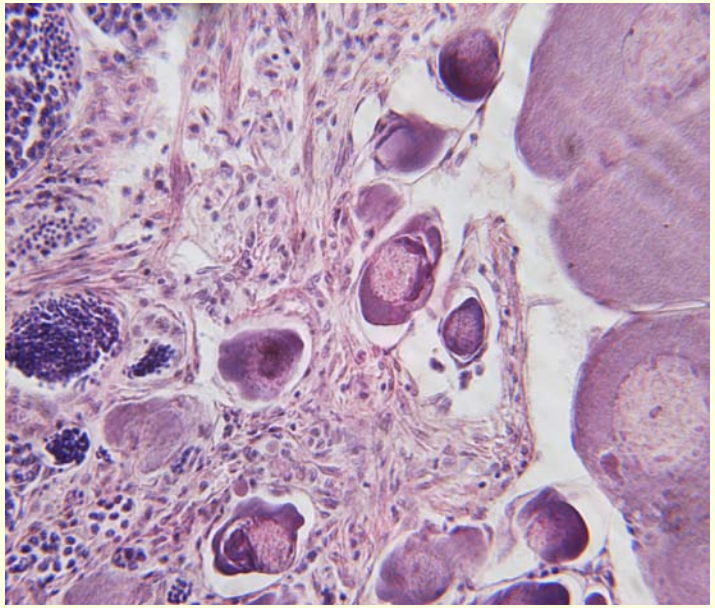


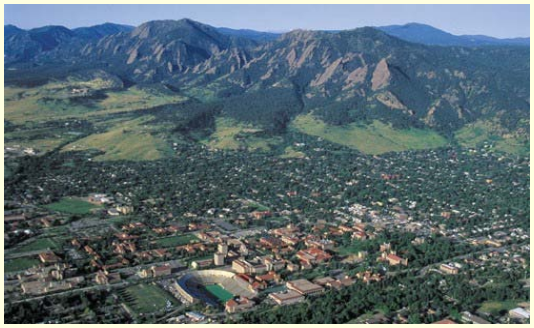
White Sucker
(*Catostomus commersoni*)

Evidence of Reproductive Disruption in White Suckers Below the City of Boulder

(Vajda et al. 2004; Woodling et al. 2006; Vajda et al. 2007)

- Gonadal Intersex (20%)
- Female-biased sex ratios (M:F, 1:5)
- Impaired ovarian development
- Decreased testicular sperm abundance
- Elevated plasma vitellogenin in males





8 km Downstream

<u>50% Effluent</u>
1.2 ng/L E2
0.5 ng/L EE2

Effluent

<u>100% Effluent</u>
2.4ng/L E2
1.2 ng/L EE2

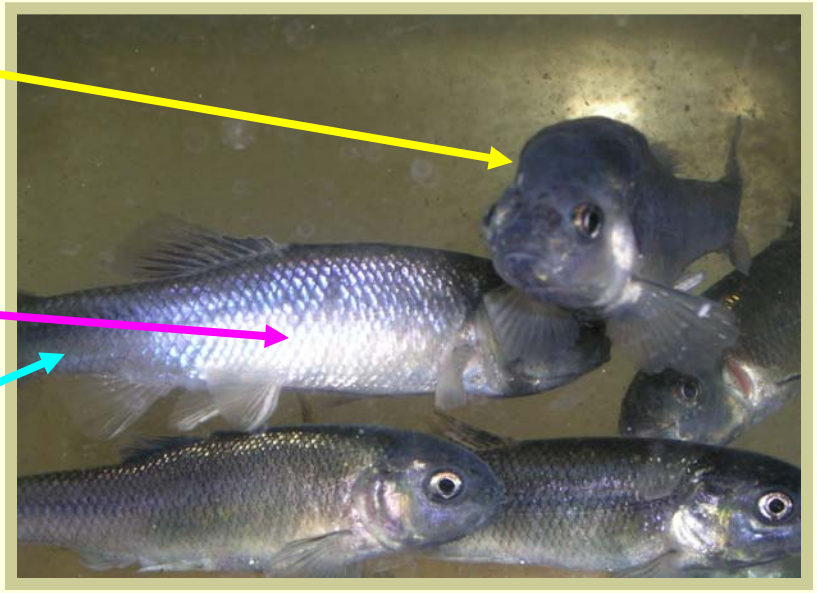
<u>F</u>ish <u>E</u>xposure <u>M</u>obile

EAST
ENCY
T

100% Reference
50/50 Mixture
100% Effluent

Fathead Minnow *(Pimephales promelas)*

1. Secondary sex characters
2. Gonadosomatic index
3. Sperm abundance
4. Plasma vitellogenin



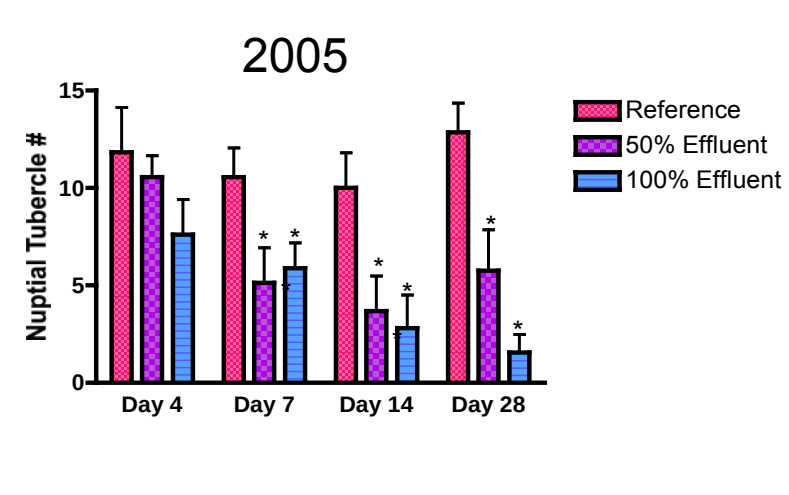
Recrudescent males

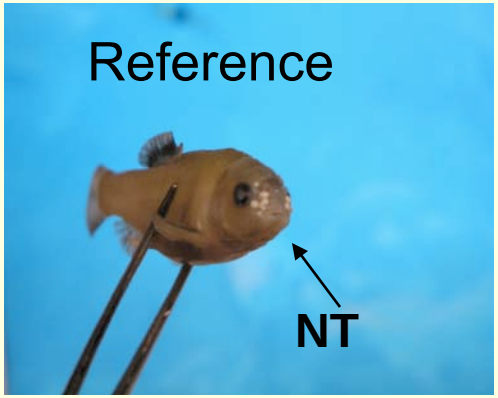
Sampled on days 4, 7, 14, and 28

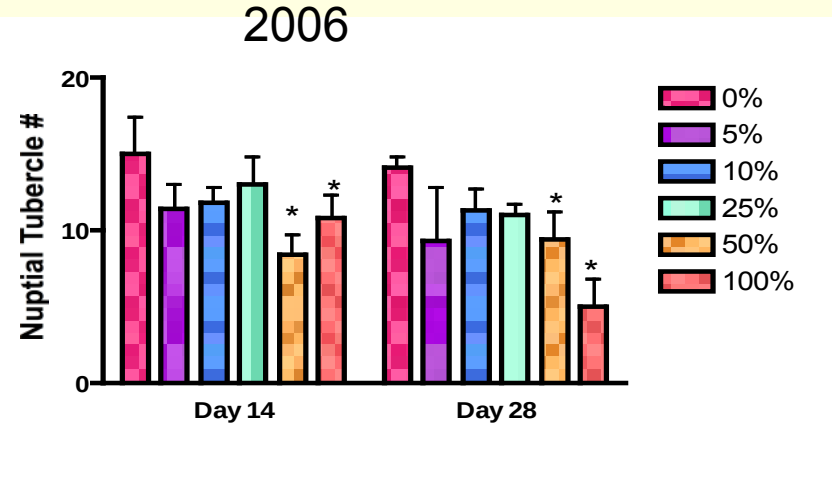
Stimulatory photoperiod (16L:8D) and temperature (21°C)

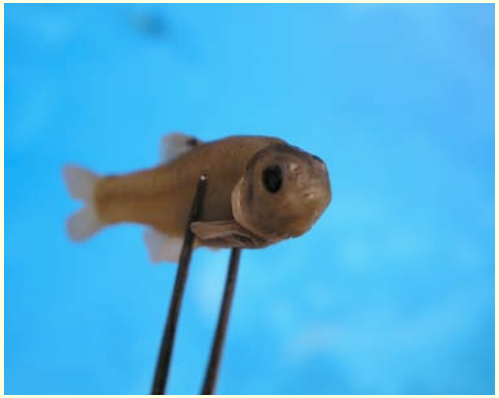
Controlled aeration, feed, and flow

Effluent Reduces Nuptial Tubercle Expression



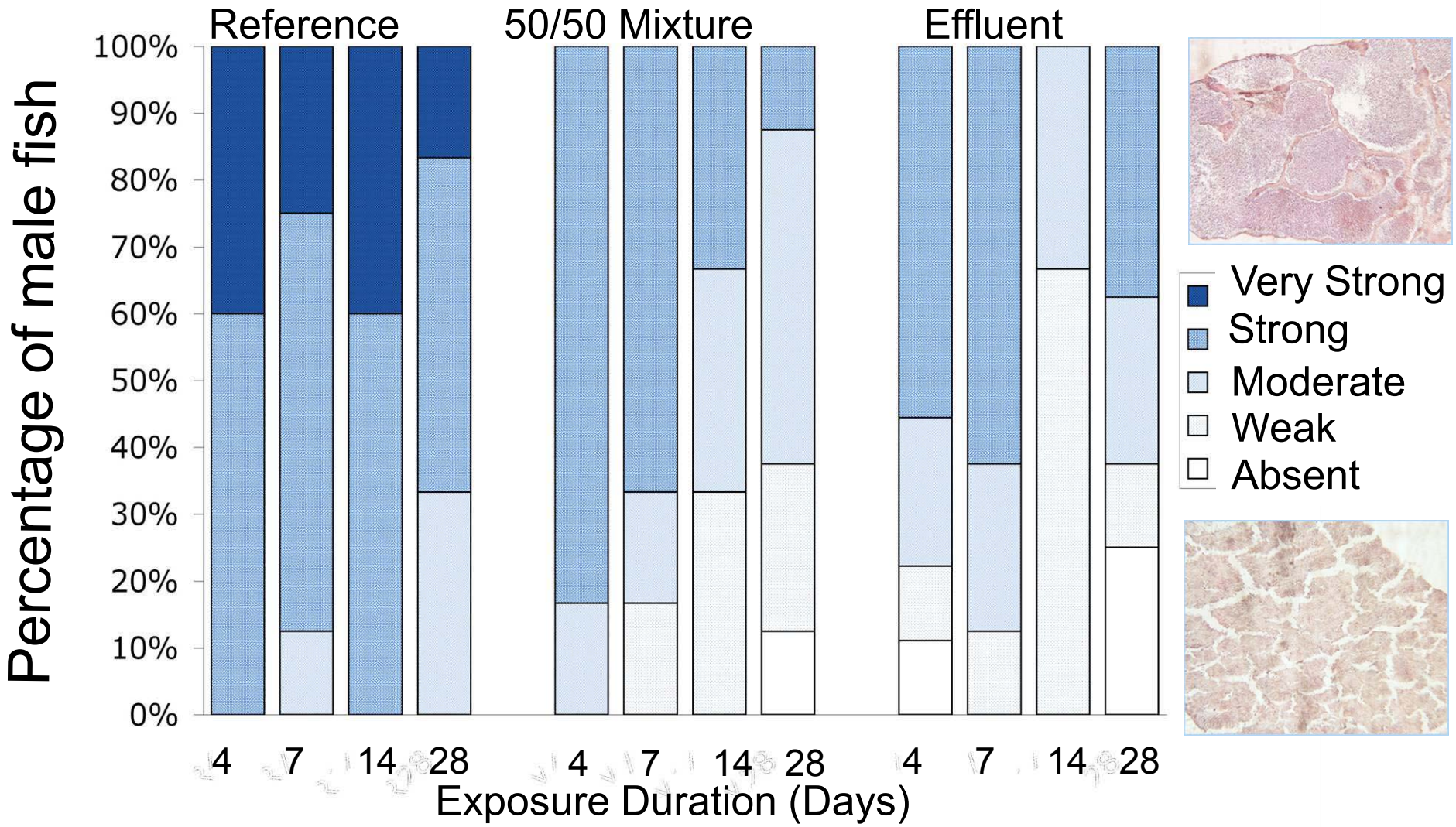






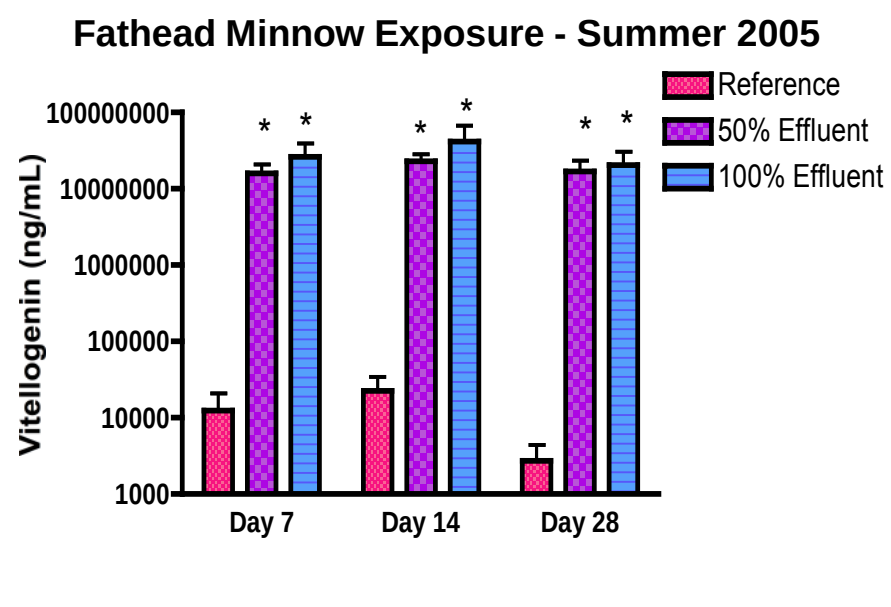
Significant effect of exposure ($P<0.0001$)

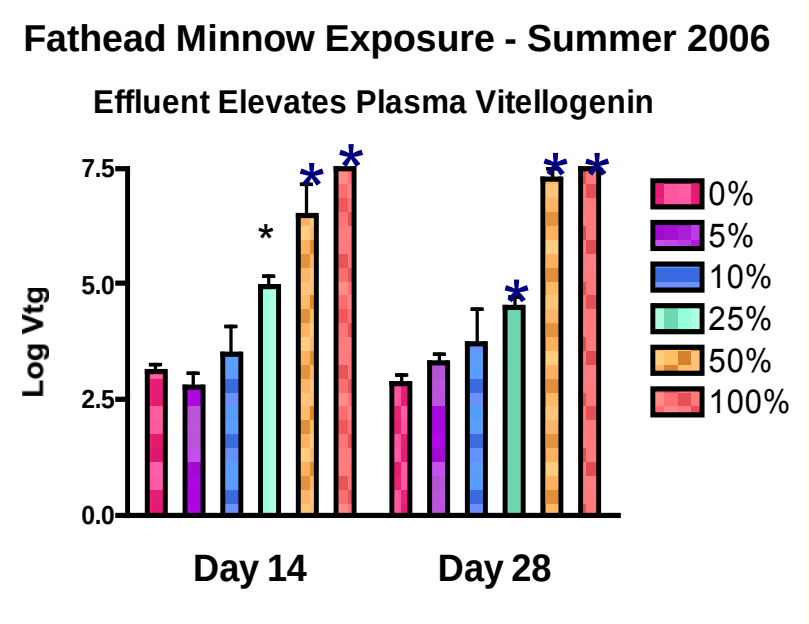
Effluent Decreases Sperm Abundance

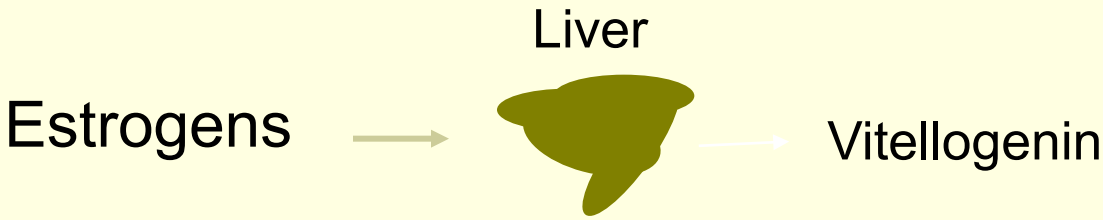


Kruskall-Wallis P<0.001

Effluent Elevates Plasma Vitellogenin







Significant main effect of exposure (P<0.0001)

1. Secondary sex characters: reduced with 50% and 100% effluent by day 7.
2. GSI: reduced with 100% effluent by day 14.
3. Sperm abundance: reduced with 50% and 100% effluent.
4. Plasma vitellogenin: elevated with 50% and 100% by day 7.

Research Summary

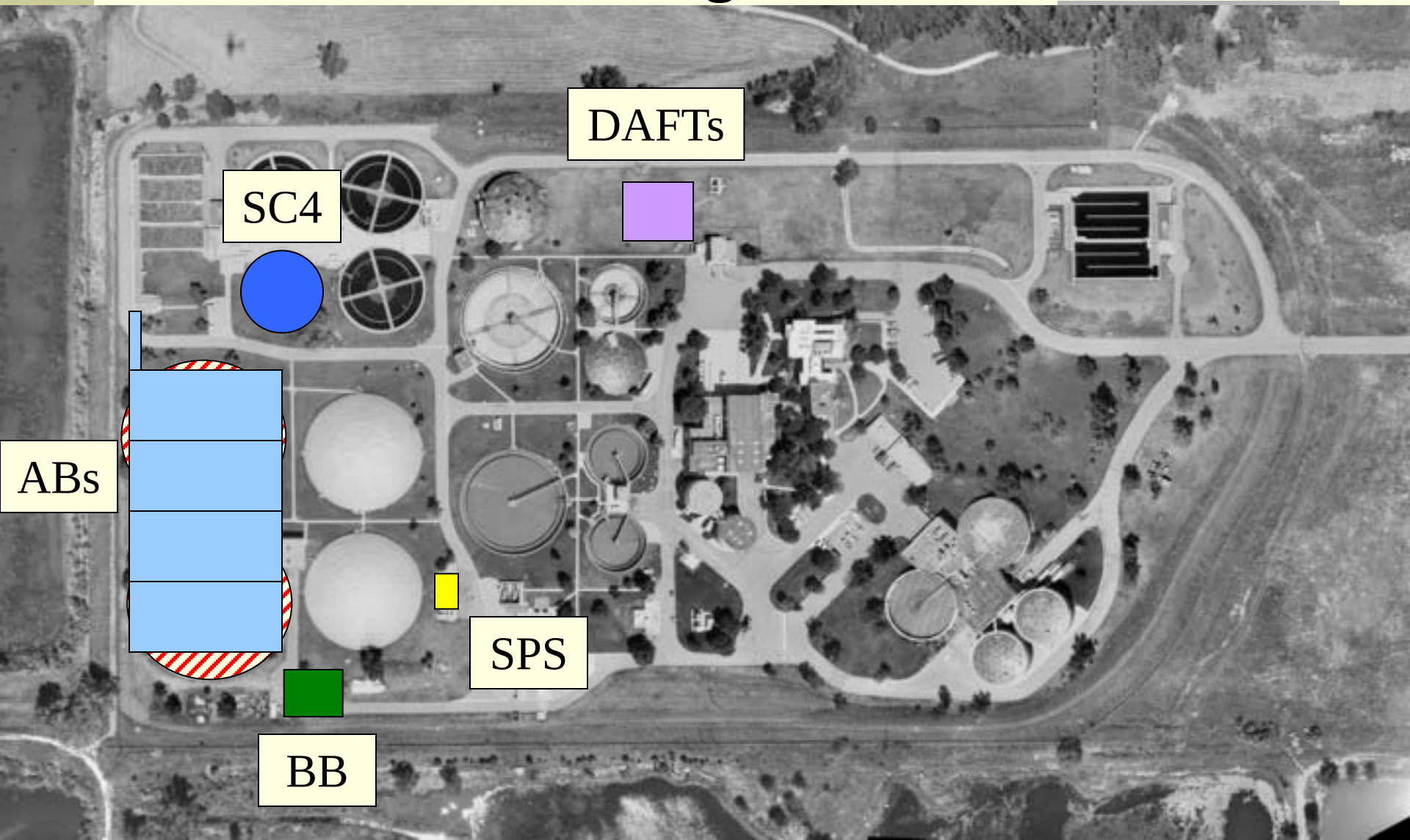
1. City of Boulder WWTP effluent feminized and demasculinized breeding male fathead minnows
2. Reproductive disruption evident within 7 days
3. 50% effluent is equipotent to 100% effluent (for most endpoints)

Evaluation of Treatment Technologies [Existing Process]

- Trickling filter - solids contact secondary process, and nitrifying tricking filter for enhanced ammonia removal

- Sampled at 5 process locations

Evaluation of Treatment Technologies
[New Activated Sludge Process]



Evaluation of Treatment Technologies

- Unique opportunity - evaluate treatment technologies while supporting assumption that the same, or very similar, sources of EDCs are present.
  - Evaluate EDC removal efficiency
  - Conduct tests on fish reproduction and feminization before/after wastewater plant renovation
  - Evaluate impacts of different levels of WWTP treatment on estrogenic endpoints in fish

Collaborative Efforts Have Resulted in Research Advancement at Minimal Expense to the City

Entity	Contribution	Estimated Expense	Benefit to Research
city of Boulder	Site access/security	$15,000 (Staff resources for communications)	Access to WWTP and trailer in secured area
	Power	$20,000 (Installation) $3,000/ year (Electricity)	Run required fish experiments and research trailer operations
	Support for WWTP sample collection	$5,000 (Staff resources)	Treatment process sampling to evaluate contaminant removal
USGS	Sample collection Laboratory analysis	$50,000 $200,000	Development of analytical methods for emerging contaminants with low-level laboratory analysis
University of Colorado	Sample collection/ research Fish aquaria studies	$180,000 $25,000	Development/implementation of flow-through study, characterization of threshold effluent concentrations and histology
Colorado Division of Wildlife	Fish collection	$8,000	Electroshocking

Collaborative Efforts Have Resulted in Research Advancement at Minimal Expense to the City

Entity	Contribution	Estimated Expense	Benefit to Research
city of Boulder			secured
			ts and ns
			evaluate
USGS			nods for w-level
University of Colorado	Sample collection/ research Fish aquaria studies	$150,000 $25,000	Development/implementation of flow-through study, characterization of threshold effluent concentrations and histology
Colorado Division of Wildlife	Fish collection	$8,000	Electroshocking

Contribution Ratio

City/Other

$43,000/$463,000

Public Education and Alternative Disposal Options

Partners for a Clean Environment

- *A partnership of local governments and businesses to encourage*

  and recognize environmental achievements.

WatershED Outreach Program

Take-Back Program

- This is not a cure-all process
  - Prescriptions
  - Other medications
- Comply with laws and regulations
  - Controlled Substances Act of 1972
  - U.S Drug Enforcement Administration

Pharmaceutical Drop-Off Day

Bring your expired, excess and unwanted medications for proper disposal

Saturday, September 8, 2007
8:00 AM to 2:00 PM

Longmont United Hospital
1950 Mountain View Avenue

This is a free event!

Items accepted include:

prescription
and
over-the-counter medications

pills ? liquids ? patches ? inhalers

REMEMBER – Never flush

Conclusions

- It is only a matter of time before emerging contaminants become regulated
- Decision to be an active participant stems from the benefits from advancing the understanding the effects of EDCs and possible strategies to reduce effects
- City's support has resulted in significant advancement in understanding at minimal cost to the city

Conclusions

- Education and outreach on alternative disposal methods can minimize environmental impact
- Boulder wants to leverage existing efforts partner with local groups to avoid overlaps and ensure a consistent message is delivered to the community

Acknowledgements

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  - United States Geological Survey, Boulder
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  - Department of Integrative Physiology, University of Colorado at Boulder

Questions?

Environmental Science & Technology

Article

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Fish Endocrine Disruption Responses to a Major Wastewater Treatment Facility Upgrade

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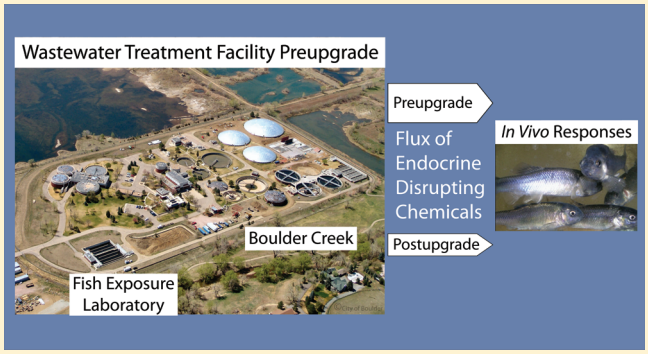
Supporting Information

ABSTRACT: The urban-water cycle modifies natural stream hydrology, and domestic and commercial activities increase the burden of endocrine-disrupting chemicals, such as steroidal hormones and 4-nonylphenol, that can disrupt endocrine system function in aquatic organisms. This paper presents a series of integrated chemical and biological investigations into the occurrence, fate, and effects of endocrine-disrupting chemicals in the City of Boulder Colorado's WWTF and Boulder Creek, the receiving stream. Results are presented showing the effects of a full-scale upgrade of the WWTF (that treats 0.6 m^3 s^{-1} of sewage) from a trickling filter/solids contact process to an activated sludge process on the removal of endocrine-disrupting compounds and other contaminants (including nutrients, boron, bismuth, gadolinium, and ethylenediaminetetraacetic acid) through each major treatment unit. Corresponding impacts of pre- and postupgrade effluent chemistry on fish reproductive end points were evaluated using on-site, continuous-flow experiments, in which male fathead minnows (*Pimephales promelas*) were exposed for 28 days to upstream Boulder Creek water and WWTF effluent under controlled conditions. The upgrade of the WWTF resulted in improved removal efficiency for many endocrine-disrupting chemicals, particularly 17β-estradiol and estrone, and fish exposed to the postupgrade effluent indicated reduction in endocrine disruption relative to preupgrade conditions.



INTRODUCTION

Effluent from municipal wastewater treatment facilities (WWTFs) play an important role in maintaining streamflow in urban aquatic ecosystems and providing water for municipal and agricultural reuse.[1] One of the key differences between WWTF effluents and native streamwater is the complex mixture of anthropogenic organic and inorganic chemicals in effluents,[2−5] which have potential ecological implications such as endocrine disruption in fish.[6,7] The dynamic nature of effluent-impacted streams makes it difficult to establish direct relationships between chemical occurrence and biological effects.[8] A substantial fraction of the domestic and commercial chemical use within cities is collected, treated, and discharged through WWTFs, which provide a final point of contaminant control prior to release to the stream environment. The physicochemical properties of individual contaminants and the level of treatment determine their concentrations in the final WWTF effluent, and it has been shown that activated sludge processes can be effective at removing endocrine-disrupting compounds.[9−12] Although there are opportunities for water-quality improvements by using technological advancements in treatment processes,[13,14] there also are uncertainties in extrapolating laboratory and pilot scale experiment data to potential environmental benefits of full-scale WWTF upgrades on stream ecosystems due to limited "real world" data.

In this integrated study, water-quality sampling and fish-exposure experiments were coordinated with a municipal WWTF operation to provide quantitative data on the effect of different treatment technologies (biological filter replaced with activated sludge) on the chemical composition and fish endocrine disruption effects of the effluent. Such data are essential to validating model predictions of the potential impacts of major WWTF upgrades on the environmental health of the receiving aquatic ecosystem. During 2006 and 2007, the City of Boulder, Colorado implemented an approximately $50,000,000 upgrade of its WWTF from a combined trickling filter/solids contact process to activated sludge treatment.[15] Although the upgrade was designed to enhance nitrogen removal, it also provided the opportunity to evaluate the

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removal of endocrine-disrupting compounds, which has been shown to be enhanced by activated sludge treatment.[9]

The impact of the Boulder WWTF discharge on the hydrology, chemistry, and biology of Boulder Creek has been extensively studied[4,7,16−23] and provide baseline data on preupgrade conditions. Significant reproductive disruption, consistent with exposure to measured levels of estrogenic endocrine-disrupting chemicals, was observed in free-living male and female white suckers (*Catostomus commersoni*) downstream from the WWTF outfall but not at the upstream reference site during studies conducted from 2001 to 2004.[7,18] Reproductive effects included gonadal intersex, female-biased sex-ratios, asynchronous ovarian development in females, reduced sperm abundance in males, and elevated plasma vitellogenin in males. The white sucker was the only fish species consistently found in Boulder Creek at both upstream and downstream sites in sufficient numbers for analysis, and it is unknown whether similar adverse effects occur in other fish species.

As a follow-up to the field studies, a series of controlled 28-day fish exposure experiments were conducted at the Boulder WWTF using an on-site, continuous flow bioassay facility designed to evaluate endocrine disruption.[24,25] Controlled exposure of adult male fathead minnows (*Pimephales promelas*) to the Boulder WWTF effluent in 2005 resulted in reduced secondary sexual characteristics, sperm abundance, and elevated vitellogenin. Fish exposed to the upstream Boulder Creek reference water had no endocrine disruption. Results from the on-site fathead minnow studies were consistent with exposure to estrogenic endocrine-disrupting chemicals, including steroidal estrogens, bisphenol A, and alkylphenols, and parallels the effects observed in free-living white suckers. This evidence of effluent-induced reproductive disruption in multiple fish species suggests that additional native species may be threatened where large volumes of estrogenic wastewater are discharged into relatively small streams. The reproduction disruption observed in Boulder Creek was comparable with intersex in wild fish observed in wastewater impacted streams in the United Kingdom.[6]

This paper describes a multitiered field approach to quantify the effects of a full-scale WWTF upgrade on the levels of endocrine-disrupting compounds in the final effluent and associated biological responses. Results from this comparative study provides integrated hydrological, chemical, and biological data allowing insight into how WWTF effluent impacted stream ecosystems may respond to municipal infrastructure modifications. This is important because a large number of WWTF in the United States are approaching their design life and will need to be upgraded in the future.

■ STUDY DESIGN

Site Description. Evaluation of stream ecosystem changes related to municipal infrastructure modifications and chemical use trends requires long-term data sets acquired in a consistent manner. In 2000, a series of investigations were initiated into the occurrence, fate, and effects of contaminants along Boulder Creek, Colorado, from a snowmelt-driven headwater stream to a WWTF effluent-dominated prairie stream ecosystem.[16] Boulder Creek provides the water supply for the City of Boulder: water is withdrawn in the upper watershed, passes through the urban-water cycle (drinking water treatment, municipal use, and WWTF treatment), and is discharged back into Boulder Creek downstream from the City. Between 2000 and 2010, the population of Boulder increased from 100,200 to 103,600; during the same period the average daily inflow to the WWTF decreased from 0.7 to 0.6 $m^3 s^{-1}$ (15.9 to 13.8 million gallons per day) due to water conservation programs.[15] The WWTF inflow is comprised of 70% domestic wastewater and 30% mixed commercial and industrial wastewater.

This study focuses on the reach of Boulder Creek upstream and downstream from the WWTF outfall. Although Boulder Creek passes through the Boulder urban corridor, the site upstream from the WWTF is relatively unimpacted, and most contaminants were below detection limits. Downstream from the WWTF outfall, flow in Boulder Creek consists of 30 to 80% effluent, and multiple wastewater contaminants have been detected.[4,16,22,23] In the study reach, Boulder Creek is a fifth order pool-and-riffle stream with an average hydraulic gradient of 0.0039. The stream-bed surface consists of gravel and cobbles interspersed with depositional zones containing fine-grained material. Predominant fish habitat is transitional between a high-gradient cold-water and a low-gradient warm-water stream. This increase in stream temperature is reflected in the increased downstream abundance of warm-water-tolerant fish species such as suckers (*Catostomus sp.*), bass (*Micropterus sp.*), and carp (*Cyprinus sp.*) and exclusion of cold-water species such as trout (*Salmo sp.*).

Anthropogenic stressors on stream ecosystems often result in subtle effects that are difficult to measure, and understanding the impacts of dynamic changes associated with urbanization require long-term chemical and biological data and an ecologically relevant reference site. Boulder Creek provides an ideal field site for this study due to (1) long-term data on WWTF effluent and streamwater quality and stream ecology, (2) close proximity of an appropriate reference site with absence of endocrine disruption, (3) streamflow that is effluent dominated and contains a mixture of endocrine-disrupting chemicals, and (4) native fish populations downstream from the WWTF that show evidence of endocrine disruption.

Infrastructure Changes and Chemical Use. During the course of normal WWTF operations there are a number of factors that influence effluent chemistry and can vary over time, including minor short-term disruptions, seasonal differences in treatment efficiency, long-term trends in hydraulic and chemical loading, and major infrastructure changes. A series of experiments were conducted at the Boulder WWTF and in Boulder Creek between 2000 and 2011 to investigate the relationships between the occurrence of endocrine-disrupting compounds in the effluent and streamwater and fish endocrine disruption. These studies captured the full-scale conversion of the WWTF from trickling filter to activated sludge treatment.

During this same period, the nonionic-surfactant derived contaminant 4-nonylphenol was shown to have widespread environmental occurrence and act as an endocrine-disrupting chemical.[2,3,24,26,27] In response to potential biological concerns, the European Union restricted 4-nonylphenol use in many products, and subsequent reductions in effluent concentrations and endocrine disruption were documented.[28] In 2005 the U.S. Environmental Protection Agency (USEPA) established toxicity-based 4-nonylphenol freshwater aquatic life ambient water-quality criteria of 1.7 and 6.6 $\mu g\ L^{-1}$ for chronic and acute exposure, respectively,[29] similar to concentrations (10 $\mu g\ L^{-1}$) shown to cause endocrine disruption in fish.[27,30] Coincident with the published water-quality criteria and potential for increasing regulatory actions were voluntary reductions in 4-

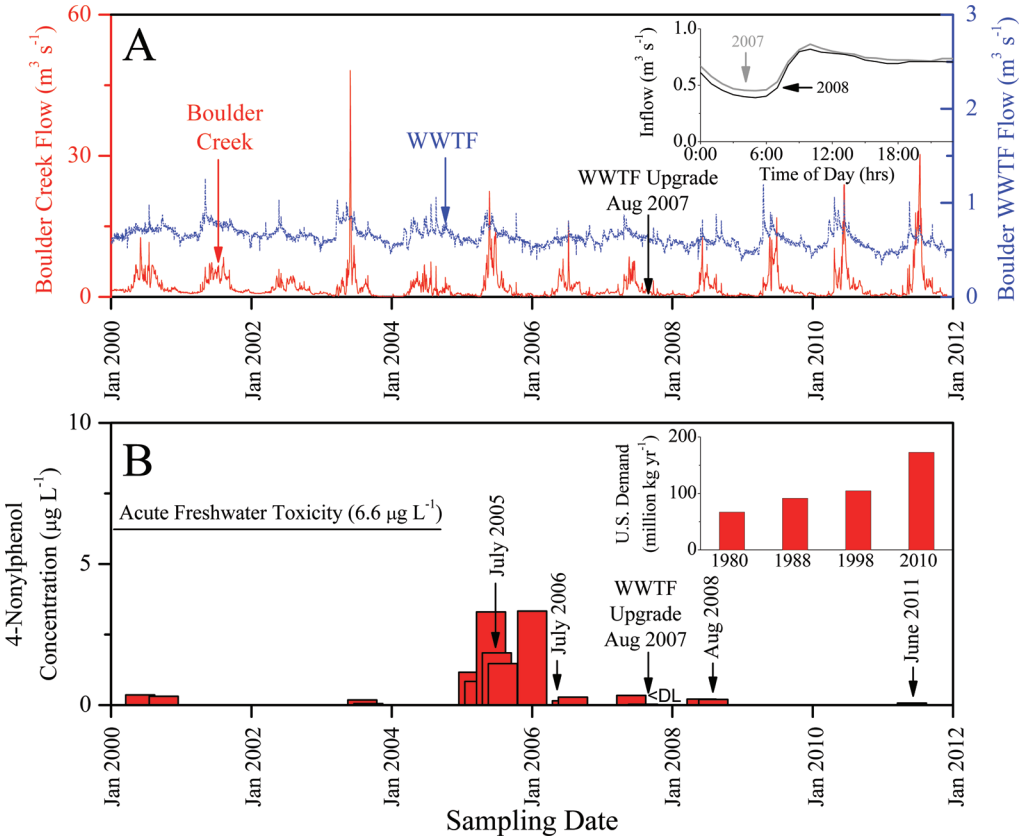

Figure 1. (A) Hydrograph from 2000 to 2011 for Boulder Creek at the 75th Street U.S. Geological Survey stream gage and the Boulder Wastewater Treatment Facility (WWTF). Inset shows inflows on the pre- (June 20, 2007) and postupgrade (June 16, 2008) water quality sampling dates. (B) Concentrations of 4-nonylphenol in the Boulder WWTF effluent between 2000 and 2011. Inset shows U.S. demand for 4-nonylphenol over time.[29,43,44]

nonylphenol based products by U.S. producers and marketers.[31,32]

In addition to large-scale chemical and water use trends, the demographics of the city of Boulder has an annual cycle related to the influx of students to the University of Colorado, which results in abrupt increases and decreases in population of about 25%. It has been established that such rapid demographic changes can have a significant impact on the WWTF chemical loading patterns of specific drugs and chemicals.[33]

Sampling and Chemical Analysis. A long-term series of investigations into the occurrence of endocrine disrupting and other chemicals in Boulder Creek was initiated during 2000[16] and maintained through 2011 (Figure 1). Multiple water quality samplings of the WWTF effluent (as well as upstream and downstream Boulder Creek sampling sites) have been conducted.[16,23] Weekly samplings (for 5 weeks) of the WWTF effluent and upstream Boulder Creek water were conducted during the July 2005, August 2006, August 2008, and June 2011 fathead minnow exposure experiments discussed in the following section. During each sampling event, quality assurance involved field duplicate and duplicate matrix spike samples, field and laboratory distilled water blanks, and spiked distilled water samples.

A challenge in evaluating the fate of contaminants in WWTFs and receiving streams is the temporal variability in flow and chemical loading, which vary over time scales from minutes to decades.[34−36] The variability in chemical composition can affect the estrogenicity of the effluent.[37] An alternative approach to fixed-point, high-frequency sampling strategies[34] is to sample the same approximate parcel of water as it passes through a hydrological system.[22] An internal process water-quality sampling of the WWTF, including the influent and the effluents from the major treatment units, was conducted on June 20, 2007, prior to the conversion from trickling filter to activated sludge treatment. In order to track the same parcel of water, the WWTF inflow on the day of sampling and design volumes of the various treatment processes were used to calculate the hydraulic retention times used to determine sampling intervals. Multiple grab samples were collected to determine contaminant concentrations after each unit process (Figure SI-1 and Table SI-1; influent, primary clarifier effluent, trickling filter effluent, secondary clarifier effluent, and final effluent after chlorination/dechlorination). A follow-up study was conducted on June 16−17, 2008, approximately 10 months after the upgrade was completed, using a similar sampling scheme (influent, primary clarifier effluent, activated sludge influent, activated sludge effluent, secondary clarifier effluent, and final effluent).

Water samples were collected from the outflow of each treatment unit using stainless steel (organics) or/Teflon (inorganics) equipment and were split for subsequent analysis. Trace element samples were filtered through 0.4-μm Nucleopore membranes and acidified with ultrahigh purity nitric acid. Nutrient samples were filtered and chilled. Unfiltered water samples for analysis of organic carbon, acidic organic compounds, neutral organic compounds, and steroidal hormones were collected in precleaned amber glass bottles.

Samples for acidic organic compound analysis were preserved with 1% (v/v) formalin.

The same general analytical procedures were applied over the course of the 10-year investigation, including analysis of water samples from the long-term monitoring, pre- and postupgrade unit process studies, and fish exposure experiments. Nutrients were analyzed using standard methods.[38] Trace elements were analyzed by inductively coupled plasma-mass spectrometry.[39] Acidic organic compounds, including 4-nonylphenolethoxycarboxylic acids (4-NPEC; 1−4 ethyleneoxide, EO, oligomers), ethylenediaminetetraacetic acid (EDTA), and nitrilotriacetic acid (NTA), were analyzed by evaporation, derivatization with acetyl chloride/propanol to form the propyl esters, and gas chromatography/mass spectrometry (GC/MS) analysis.[2] Neutral organic compounds, including 4-nonylphenol and 4-nonylphenolethoxylates (4-NPEO: 1−4 EO oligomers), were isolated by continuous liquid−liquid extraction with methylene chloride followed by GC/MS analysis.[2] Steroidal hormones were isolated from water by octadecylsilica solid phase extraction, derivatized to form the trimethylsilyl ethers, and analyzed by gas chromatography/tandem mass spectrometry.[40]

Biological Analysis. On-site fish exposure experiments using reproductively stimulated 12-month-old adult male fathead minnows were conducted during July 2005 and July 2006,[25] prior to the WWTF upgrade, and during August 2008 and June 2011 following the upgrade. The on-site bioassay system was specially designed to evaluate fish endocrine disruption, and all materials in contact with test solutions and exposed organisms were glass, stainless steel, and Teflon.[24] Male fathead minnows were exposed for 28 days under continuous flow conditions to (1) WWTF effluent pumped from the outfall following chlorination/dechlorination, (2) Boulder Creek water pumped from a site located upstream from the WWTF outfall, and (3) a 50:50 mixture of the two.

For all experiments, 10 randomly collected fish were processed as initial controls. The remaining fish were weighed, measured, and randomly distributed to exposure treatments housed in 10-L aquaria. In every experiment, 5 to 6 fish were placed into each aquarium. All pre- and postupgrade exposure experiments were conducted under similar controlled conditions of flow (200 mL min^{-1}; ∼6 daily volume replacements), temperature (22 °C), lighting (16-h on/8-h off), diet (frozen brine shrimp), and aeration (>85% oxygen saturation). The continuous-flow nature of the exposure experiments incorporated the intrinsic variability in chemical composition of the WWTF effluent and stream.

A variety of biomarkers of fish endocrine disruption were evaluated using methods described elsewhere.[24,25] In this paper, primary sexual characteristics (gonadosomatic index and sperm abundance), secondary sexual characteristics (number and prominence of nuptial tubercles and dorsal fat pad prominence), and plasma vitellogenin induction were used to compare the effects of exposure to the WWTF effluent and the reference streamwater. Proportional, non-normal scores for degree of nuptial tubercle prominence, dorsal fat pad prominence, and sperm abundance were analyzed by Kruskal−Wallis tests. Remaining data were tested for homoscedasticity and analyzed by two-way ANOVA. Concentrations of vitellogenin were log transformed prior to statistical analysis. Significance was accepted at the 5% level. Biological results are expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Long-Term Changes. Boulder Creek is a snowmelt-driven stream (with physical flow modification from diversions and dams), and the annual hydrograph consists of a runoff (May to August) and base-flow (September to April) component (Figure 1A). There are differences in magnitude of flow and timing of peak discharge from year to year. For example, stream flows associated with the multiyear drought leading up to 2002 were among the lowest on record, whereas peak flow following a massive snow storm during the spring of 2003 were among the highest on record. In contrast to the extreme variations in streamflow, discharge from the WWTF was more constant on a decadal, annual, and daily basis. Based on the model of Johnson,[35] the natural variation in Boulder Creek flow from winter to summer between 2000 and 2010 produced differences in minimum and maximum annual flow ranging from 12- to 910-fold, with an average of 230-fold. Assuming a relatively steady state WWTF contaminant load, the natural hydrological variations greatly exceed the variations that result from differences in sewage treatment (∼0.5-fold) and in-stream biodegradation (∼0.1-fold). This seasonal variation in streamflow produces a repeating "roller coaster" exposure of fish to endocrine-disrupting compounds and other WWTF-derived contaminants. In Boulder Creek, the greatest concentrations are predicted to occur during the fall and winter months, a period of reproductive vulnerability for fishes. Because it is not possible to rely on in-stream dilution to minimize ecotoxicolgical impacts of contaminants during periods of low flow, it is important for WWTF, as the last point of control, to achieve the maximum treatment feasible.

Between 2000 and 2010, concentrations of 4-nonylphenol in the Boulder WWTF varied from <0.05 to 5.0 $\mu g\ L^{-1}$ (Figure 1B) and are consistent with results reported for other WWTFs.[8,24,41,42] Although the sample collection was sporadic, concentrations of 4-nonylphenol have remained low in the Boulder WWTF since mid-2006 despite a national trend of increasing 4-nonylphenol consumption.[43,44] The USEPA 4-nonylphenol water-quality criteria[29] provided a basis for action, and the City of Boulder's industrial pretreatment program initiated voluntary efforts to reduce sources of 4-nonylphenol to the WWTF,[15] a possible source of the concentration decrease observed in 2006. Throughout the study, 4-nonylphenol typically comprised <5% of the total 4-nonylphenol pool including the 4-NPEO and 4-NPEC compounds (data not presented).

The fish exposure experiments conducted during July 2005 and August 2006 indicated a decrease in the magnitude of multiple endocrine disruption biomarkers,[25] in general agreement with predictions based on the observed decreases in 4-nonylphenol observed during the same period. Concentrations of other endocrine-disrupting compounds, such as the steroidal estrogens, also varied with time. For example, the biogenic sex hormone 17β-estradiol was consistently detected in the effluent at ∼0.001 $\mu g\ L^{-1}$, whereas the synthetic oral contraceptive 17α-ethinylestradiol was detected only sporadically[25] and may be related to the demographics of the university population (i.e., concentrations decrease when students leave town).

Contaminant Removal under Pre- and Post-Upgrade Conditions. Attenuation of endocrine-disrupting compounds in WWTFs involves physical, chemical, and biological processes that occur at different stages of treatment. For example, sorption and settling occur in the primary clarifier,

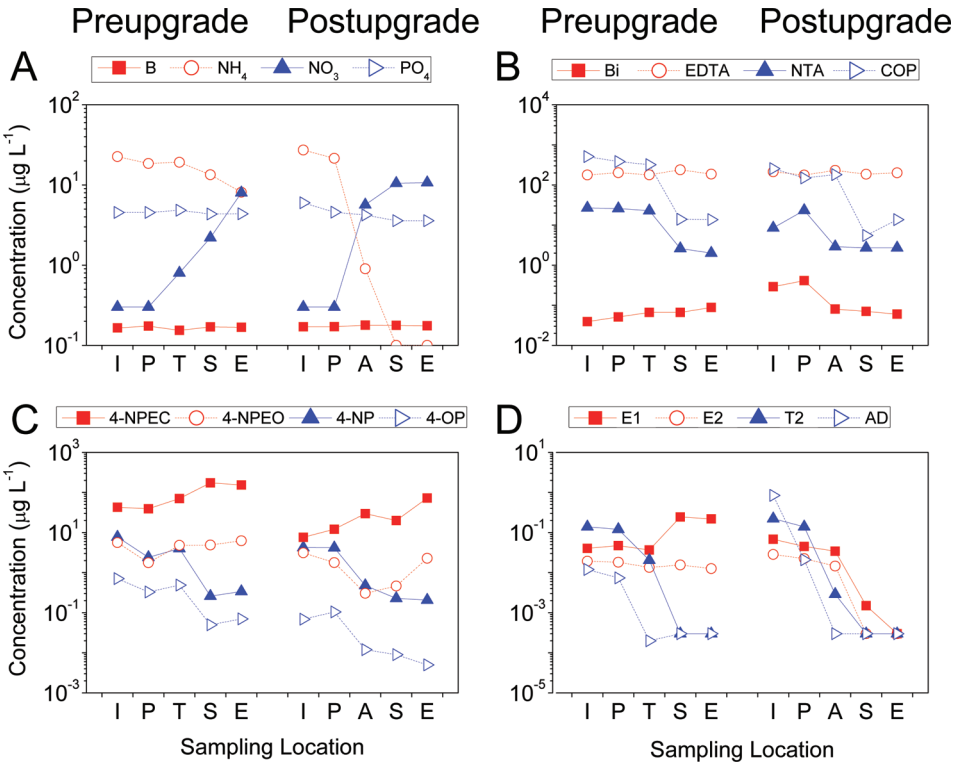

Figure 2. Concentrations of select constituents in the influent and the effluents from the different unit processes at the Boulder Wastewater Treatment Facility (WWTF) under pre- and postupgrade conditions: (A) boron, B, ammonium, NH_4, nitrate, NO_3, and phosphate, PO_4; (B) bismuth, Bi, ethylenediaminetetraacetic acid, EDTA, nitrilotriacetic acid, NTA, and coprostanol, COP; (C) 4-nonylphenolethoxycarboxylic acids, 4-NPEC, 4-nonylphenolethoxylates, 4-NPEO, 4-nonylphenol, 4-NP, and 4-*tert*-octylphenol, 4-OP; and (D) estrone, E1, 17β-estradiol, E2, testosterone, T2, and androstenedione, AD. [I, WWTF influent; P, primary clarifier effluent; T, trickling filter effluent (preupgrade); A, activated sludge effluent (postupgrade); S, secondary clarifier effluent; E, final effluent].

biodegradation and sorption occurs in the trickling filter and activated sludge units, and chemical oxidation occurs in the chlorination stage.[9−12] This study evaluated a single WWTF before and after a full-scale process upgrade and eliminated the uncertainty typically associated with evaluating different treatment technologies using intersite comparisons. In order to compare contaminant removal under pre- and postupgrade conditions, two sampling events were conducted on approximately the same day of the year to replicate flow and environmental conditions (Figure 1A insert). The change from a trickling filter/solids contact process to an activated sludge process resulted in the total treatment system hydraulic retention time increasing from 11 to 25 h and the solids retention time increasing from 4.3 to 10 days (Table SI-1). Tables SI-2 to SI-4 summarize the quality assurance data for the pre- and postupgrade water quality sampling, including results for blanks, duplicates, and matrix spikes.

Boron was used as a conservative wastewater tracer to estimate the relative persistence of co-occurring chemicals.[4,22,45] Concentrations of boron in the WWTF influent were similar during both samplings, and there was little removal during any treatment process under pre- and postupgrade conditions (Figure 2A). The boron results (as well as other conservative constituents such as chloride, lithium, and sodium) suggest that the hydraulic retention time based sampling followed the same approximate parcel of water through the treatment process. The WWTF upgrade was designed to enhance nitrogen removal, and there was a significant (student t test, 2 tailed, $p < 0.05$) reduction in ammonium concentrations and loads and total nitrogen loads following the upgrade (Figure 2A, Figure 3). Nitrate concentrations increased between the influent and final effluent

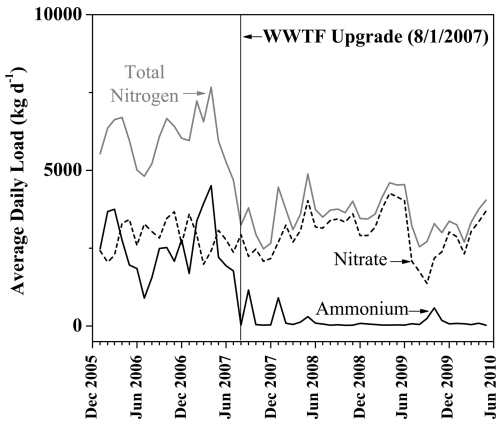

Figure 3. Monthly means of average daily loads of total nitrogen, nitrate, and ammonium in the Boulder Wastewater Treatment Facility (WWTF) effluent prior to and following the August 2007 upgrade.

during both treatment processes, and there was no significant difference between pre- and postupgrade conditions. Phosphate had little removal during preupgrade treatment, and concentrations showed a slight decrease under postupgrade conditions.

The trace element bismuth is widespread in WWTF effluents[4,22,23,46−48] because of its use in over the counter and prescription pharmaceuticals; however, the fate of bismuth in freshwater environments is poorly understood.[49] During the

preupgrade sampling, bismuth concentrations increased from 0.039 μg L^{-1} in the influent to 0.088 μg L^{-1} in the final effluent. During the postupgrade sampling, bismuth concentrations in the influent (0.29 μg L^{-1}) were nearly an order of magnitude greater than preupgrade values and removal during treatment (primarily in the activated sludge process) reduced concentrations to 0.062 μg L^{-1} in the final effluent. The most likely mechanisms for removal of bismuth are oxidation/reduction reactions and formation of insoluble hydroxide and oxide species.[50]

The rare earth element (REE) gadolinium is an important tracer of medically derived contaminants in wastewater due to its use as a contrast agent for magnetic resonance imaging.[17,51,52] Compared to boron concentrations, which remained relatively constant, gadolinium concentrations increased through the treatment process during both samplings. The anthropogenic nature of the gadolinium is shown by the strong anomaly that occurs when the REE data are normalized to their natural abundances[53] as shown in Figure 4. During the preupgrade sampling, gadolinium concentrations (0.024 to 0.12 μg L^{-1}) and anomalies (14 to 69) increased in magnitude through the treatment process, similar to the trend of increasing concentrations observed for bismuth (Figure 2B). In contrast to the boron results, the gadolinium data suggest that slightly different parcels of water were sampled.[34,52] During the postupgrade sampling, the influent (and primary clarifier effluent) gadolinium concentrations were less than one-half the preupgrade concentrations and only had a slight gadolinium anomaly (1.7 compared to 1.5 which is considered to be the natural background). Both the gadolinium concentrations and the magnitude of the anomaly increased in the activated sludge effluent to values similar to the preupgrade samples (0.11 μg L^{-1} and 18, respectively) and persisted through the final effluent. Both the pre- and postupgrade samplings were initiated at ~9 a.m., a time period that reflects the maximum changes in hydraulic and chemical loading to WWTFs and captures the daily cycle of morning urinary excretion of drugs administered the previous day.[34,36,52] Concentrations of extremely specific contaminants such as gadolinium, that are excreted by only a few individuals per 100,000 population, can vary by orders of magnitude over very short time intervals (5–10 min) in WWTF influent as the result of discrete flushing events,[34] whereas contaminants such as boron, that have diverse sources and inputs, have relatively stable concentrations[54] (influent and effluent boron concentrations were 165 and 168 μg L^{-1} in the preupgrade samples and 170 and 175 μg L^{-1} in the postupgrade samples).

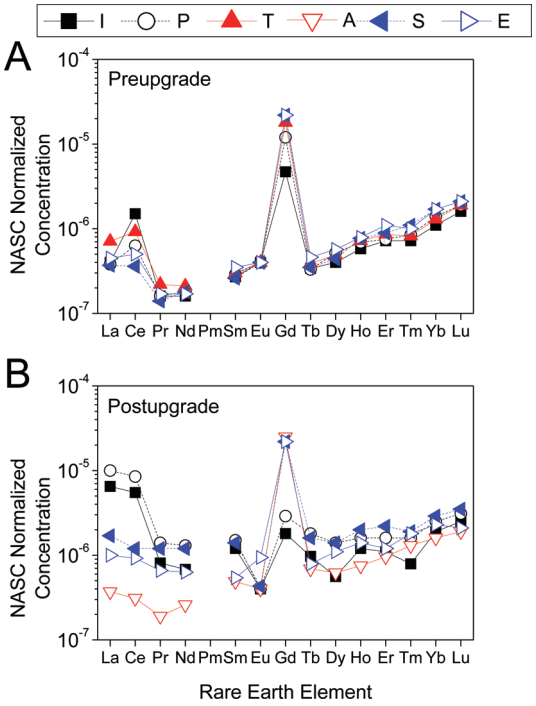

Figure 4. Concentrations of dissolved rare earth elements (REE) normalized to their natural abundance in the North American Shale Composite (NASC)[53] under (A) preupgrade conditions and (B) postupgrade conditions. [I, WWTF influent; P, primary clarifier effluent; T, trickling filter effluent (preupgrade); A, activated sludge effluent (postupgrade); S, secondary clarifier effluent; E, final effluent].

The aminocarboxylic chelating agents EDTA and NTA have widespread occurrence in WWTF effluents[2,55] and can be powerful modifiers of chemical behavior.[56] The biorecalcitrant and nonsorptive compound EDTA had the highest concentrations of any organic contaminant measured in the WWTF effluent (Table SI-5), and behaved in a conservative manner similar to boron under pre- and postupgrade conditions (Figure 2B). In contrast to EDTA, the poorly sorbed but readily biodegradable compound NTA was effectively removed during both samplings. The fecal steroid coprostanol is recalcitrant to biodegradation but readily sorbed to organic-rich particles[57] and was effectively removed under both treatment configurations.

The endocrine-disrupting alkylphenolic compounds showed variable behavior during the pre- and postupgrade samplings (Figure 2C, Table SI-5). The primary source of alkylphenol compounds to WWTF effluents is alkylphenolpolyethoxylate nonionic surfactants used in domestic, commercial, and industrial products that undergo degradation during treatment to form neutral and acidic degradation products.[41,42] During both the pre- and postupgrade samplings, concentrations of 4-nonylphenol decreased during treatment, likely due to multiple processes including sorption, biotransformation, and volatilization. In contrast, concentrations of 4-NPEC increased during WWTF treatment, indicating production from other degradates of the parent surfactant compounds. After an initial reduction in the early treatment stages, 4-NPEO concentrations also increased upon further treatment in the preupgrade sampling (and to a lesser extent postupgrade) due to degradation of other transformation products. Concentrations of 4-*tert*-octylphenol and 4-*tert*-octylphenolethoxylates, which are more estrogenic, were an order of magnitude lower than the 4-nonylphenol compounds but followed a similar trend.

The steroidal sex hormones are potent endocrine-disrupting compounds, and during the preupgrade sampling, 17β-estradiol was only partially removed during treatment (Figure 2D). Estrone concentrations increased, likely due to biologically mediated oxidation of 17β-estradiol.[21,58,59] Concentrations of 17β-estradiol, estrone, and estriol in the preupgrade influent were 0.019, 0.040, and 0.220 μg L^{-1}, respectively, whereas concentrations in the final effluent were 0.013, 0.220, and 0.0024 μg L^{-1}, resulting in removals of 35 and 99% for 17β-estradiol and estriol and a 440% increase for estrone. Under postupgrade conditions, concentrations of 17β-estradiol, estriol, and estrone decreased to below detection limits (0.0005 μg L^{-1}) in the final effluent (>99% removal), consistent with the greater removal efficiency of the activated sludge process.[9,12]

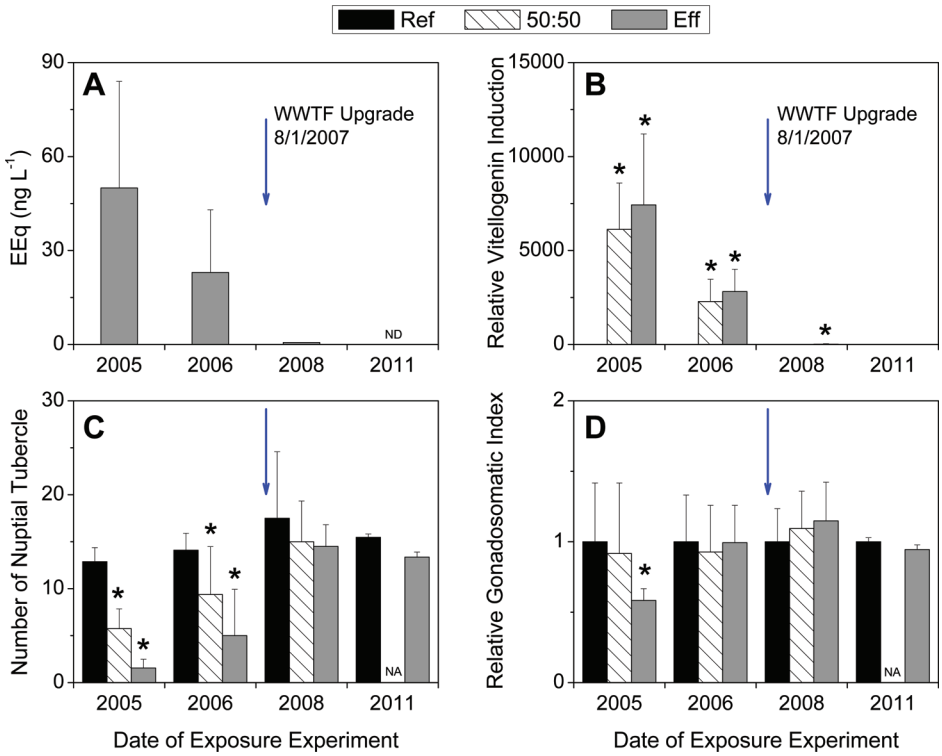

Figure 5. Summary of estrogenic effects in the Boulder WWTF effluent (Eff) prior to the upgrade (2005 and 2006)[25] and following the upgrade (2008 and 2011) compared to upstream Boulder Creek reference water (Ref) and a 50:50 mixture of the two (50:50) using on-site fish exposure experiments. (A) Average WWTF effluent estradiol equivalency quotient (EEq) as a function of exposure experiment based on weekly measurements of multiple endocrine-disrupting chemicals;[7] (B) relative plasma vitellogenin concentrations (normalized to the mean Ref concentration) in adult male fathead minnows exposed to 100% Ref, 50:50 Ref:Eff, and 100% Eff for 28 days; C) nuptial turbercle abundance in adult male fathead minnows exposed to 100% Ref, 50:50 Ref:Eff, and 100% Eff for 28 days: and (D) relative gonadosomatic index normalized to the mean Ref concentration in adult male fathead minnows exposed to 100% Ref, 50:50 Ref:Eff, and 100% Eff for 28 days. Biological results are expressed as mean ± standard error of the mean (SEM). Bars with asterisk indicate a significant difference from the upstream Boulder Creek reference water exposure for the same year (Kruskal–Wallis test, $p < 0.05$). Sample size (n) varies from 7 to 17. ND, not determined.

During both pre- and postupgrade samplings, the androgens testosterone and androstenedione were detected in the influent at similar or greater concentrations than the estrogens but were removed more rapidly during treatment. Similar removals among the treatment processes were observed by Fan et al.,[11] who report that mass flux of estriol was eliminated by 52% in the primary clarifier, the mass of 17β-estradiol showed no change, and the mass of estrone increased by 33%.

Biological Responses to WWTF Modification. The adult male fathead minnow exposure experiments[25] conducted in 2005 and 2006 provided baseline conditions for endocrine disruption against which to compare the impact of the WWTF upgrade (Figure 5). In 2005, endocrine disruption among WWTF effluent-exposed male fish was rapid and severe compared to the upstream reference water; nuptial tubercle number and fat pad prominence (data not shown) were significantly reduced ($p < 0.05$), relative plasma vitellogenin induction was elevated more than 7000-fold ($p < 0.05$), relative gonadosomatic index decreased by 50% ($p < 0.05$), and sperm abundance decreased ($p < 0.05$; data not shown). In 2006, male fish exposed to WWTF effluent showed less severe demasculinization of secondary-sex characteristics, there was no significant effect on gonadosomatic index or sperm abundance, and relative vitellogenin induction was elevated 2800-fold ($p < 0.05$). The primary differences in the effluent chemistry between the 2005 and 2006 exposures were lower concentrations of 4-nonylphenol and 17α-ethinylestradiol (17β-estradiol and estrone remained relatively constant), as reflected in significant ($p < 0.05$) decreases in the effluent estradiol equivalency quotients (EEq) calculated from 17β-estradiol relative potency factors and measured chemical data (see Table SI-5 for calculation details).[7] The decrease in calculated EEq in 2006 was consistent with the decline in severity of endocrine disruption effects.[25]

In the 2008 and 2011 postupgrade exposure experiments, there was a dramatic reduction in the concentrations of most of the endocrine-disrupting compounds in the WWTF effluent relative to preupgrade conditions and a resulting reduction in EEq. Following the upgrade there was no significant effect of 28-day effluent exposure on any measured index of primary or secondary sex characters ($p > 0.05$). Vitellogenin induction in 2008 was elevated only 2-fold ($p < 0.05$) relative to the upstream reference water, and no significant induction was observed in 2011 ($p > 0.05$).

The endocrine disruption biomarkers used in this study were selected to provide consistent data between the multiple fish exposure experiments conducted under pre- and postupgrade conditions and did not specifically assess impacts on behavior, fertility, immune function, or metabolism. However, they can be used in the context of the adverse outcome pathways framework which provides a link between molecular initiating events and effects at a level of biological organization relevant to risk assessment.[60] For example, it has been shown that WWTF effluent can disrupt fish reproductive output in the

absence of secondary sex character inhibition or vitellogenin induction.[61] Likewise, other factors, such as nitrate concentrations, can potentially contribute to fish endocrine disruption.[62] The relatively constant nitrate concentrations in the effluent under pre- and postupgrade conditions (Figure 3), coupled with the postmodification reduction of endocrine disruption, indicate that nitrate was not a major factor contributing to the observed preupgrade endocrine disruption.

Although a number of studies have evaluated the effect of WWTF treatment processes on endocrine disruption using in vitro bioassays, few studies have directly evaluated the effect of treatment on the occurrence of endocrine-disrupting compounds and in vivo biological effects. Filby et al.[61] evaluated the effects of three pilot-scale advanced treatment processes (granular activated carbon, ozonation, and chlorine dioxide) applied to an activated sludge WWTF effluent on the estrogenic reproductive health impacts on fathead minnows and observed that the advanced treatments reduced the concentrations of 17β-estradiol, estrone, and 17α-ethinylestradiol by an additional 53 to 100%. In contrast to the standard WWTF effluent, there was no vitellogenin induction in male fish exposed to the effluent treated with additional chlorination or granular activated carbon; however, ozonation did not eliminate vitellogenin induction. Similar reductions in estrogenic activity following chlorination has been observed in laboratory studies using *in vitro* yeast two-hybrid assays.[10] Gunnarsson et al.[63] compared the effect of six pilot-scale treatment processes on the reduction of endocrine-disrupting compounds and gene expression of endocrine disruption in male rainbow trout and observed that the measured concentrations and gene expression in conventional activated sludge treated effluent was reduced following additional treatment by sand filtration, moving bed biofilm reactor, ozonation, and combinations of the three processes. Although these pilot-scale studies indicate that reduction in adverse endocrine-disrupting effects can be achieved by using advanced treatment technologies, it is important to validate the results under full-scale operational conditions. Likewise, it is important to better understand the effects of less advanced (and costly and energy intensive) technology upgrades such as conversion from biological filter plants, and biological filter plants with additional tertiary treatment, to activated sludge plants.[64]

Ecosystem Implications. The WWTF upgrade was designed to enhance the removal of ammonium nitrogen which can be toxic to fish and other aquatic life. The lower ammonium concentrations in the postupgrade effluent (Figure 3) likely will have a positive effect on the stream ecosystem due to reduced toxic effects. The conversion from trickling filter/solids contact to activated sludge treatment also enhanced the removal of select endocrine-disrupting chemicals, such as 17β-estradiol, estrone, and 4-nonylphenol, consistent with other studies that show effective removal of endocrine-disrupting chemicals during activated sludge treatment.[9−12] However, not all chemicals showed enhanced removal following the WWTF upgrade as illustrated by EDTA, which had no removal under either configuration, and NPEC which was produced during treatment under both configurations. Production of the potential endocrine-disrupting NPEC metabolites from the degradation of nonionic surfactants represents a pool of 4-nonylphenol discharged by the WWTF that is subject to further degradation in the stream.[65]

In order to evaluate implications of the WWTF upgrade on the downstream Boulder Creek ecosystem it is necessary to integrate data from different time and spatial scales including long-term water and chemical use trends, short-term chemical measurements (i.e., 1-day internal sampling), midterm chemical and biological data (28-day exposures), and the instantaneous full-scale system perturbation. The short-term internal sampling was designed to evaluate removal efficiency and minimize the effect of hourly to daily variability, whereas the mid-term fish exposure experiments were designed to incorporate dynamic hydrological and chemical conditions.

The WWTF upgrade from trickling filter/solids contact to activated sludge resulted in enhanced removal efficiency for total nitrogen and endocrine-disrupting chemicals, resulting in an "instantaneous" point-source improvement in water quality that was readily measured in the water chemistry and fish bioassays. The biological results were determined for the final WWTF effluent being discharged into Boulder Creek and the receiving water upstream from the point of discharge using on-site 28-day continuous flow exposure experiments and captures the effects of the inherent variability that occurs in the concentrations of endocrine-disrupting compounds in WWTF effluents and streams.[34−36] The integration of the full-scale WWTF and stream results provide critical information on the impact of changes in treatment technology on fish endocrine disruption and potential effects on stream ecosystems. For example, geographic information system models have been proposed as tools for predicting the effects of mixtures of endocrine-disrupting chemicals in rivers based on (1) estimated WWTF effluent concentrations of estrone, 17β-estradiol, 17α-ethinylestradiol, and 4-nonylphenol, (2) assuming that the chemicals act dose additively, and (3) using the model of concentration addition for vitellogenin induction in male fathead minnows as a biomarker.[66] These models are limited by input data which often are derived from laboratory microcosm studies or estimated from other models. This study directly links stream hydrology and chemistry, WWTF operations and effluent chemistry, and fish endocrine disruption, and how they respond to a full-scale upgrade in treatment technology, and provides integrated data that are necessary to predict potential impacts on the receiving-stream ecosystem.

Stream ecosystems respond to changes in chemistry at an inherently slower rate than engineering modifications and mesocosm experiments and are likely to occur over multiyear time intervals. The decrease in ammonium and total nitrogen loading will reduce stresses to the ecosystem caused by toxicity and eutrophication. Lower loading of endocrine-disrupting chemicals also should have a beneficial impact on the reproductive health of fish populations. Restrictions on the use of 4-nonylphenol compounds in the United Kingdom resulted in a similar reduction in WWTF effluent estrogenicity,[28] consistent with predictions based on endocrine-disrupting effects of complex chemical mixtures.[66] Although the fish exposure results following the WWTF modification indicate diminished endocrine disruption resulting from reduction in estrogenic compounds, the downstream ecosystem response will be buffered by the pool of contaminants in the stream biofilm and sediments.[59,67] Recovery of the reproductive health of free-living fish downstream from the WWTF discharge is dependent upon species specific life-history and population characteristics, as well as the reproductive response to changes in the chemical properties of the effluent.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

June 2007 and June 2008 Boulder Wastewater Treatment Facility samplings includes the following: one figure showing the pre- and postupgrade treatment processes and sampling locations, one table showing the unit process details, three tables providing analytical results and quality assurance data, and one table summarizing concentrations and removal as a function of unit process. This material is available free of charge via the Internet at http://pubs.acs.org.

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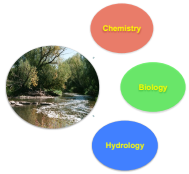
Attachment C



Infrastructure Investment Improves Ecosystem Health

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Abstract

The urban-water cycle modifies natural stream hydrology, and domestic and commercial activities increase the burden of endocrine-disrupting chemicals, such as steroidal hormones and 4-nonylphenol, that can disrupt endocrine system function in aquatic organisms. This paper presents a series of integrated chemical and biological investigations into the occurrence, fate, and effects of endocrine-disrupting chemicals in the City of Boulder Colorado's WWTF and Boulder Creek, the receiving stream.

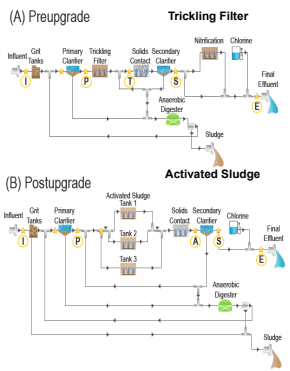
Results are presented showing the effects of a full-scale upgrade of the WWTF from a trickling filter/solids contact process to an activated sludge process on the removal of endocrine-disrupting compounds and other contaminants through each major treatment unit. Corresponding impacts of pre- and post-upgrade effluent chemistry on fish reproductive end points were evaluated using on-site, continuous-flow experiments, in which male fathead minnows (*Pimephales promelas*) were exposed for 28 days to upstream Boulder Creek water and WWTF effluent under controlled conditions.

The upgrade of the WWTF resulted in improved removal efficiency for many endocrine-disrupting chemicals, particularly 17β-estradiol and estrone, and fish exposed to the post-upgrade effluent indicated reduction in endocrine disruption relative to pre-upgrade conditions. Prior to the WWTF upgrade, wild fish downstream from the WWTF effluent showed elevated vitellogenin, female biased sex-ratios, and intersex gonads. Sampling of wild fish following the upgrade reveals recovery from endocrine disruption.

Evaluation of a Major WWTP Engineering Upgrade: Endocrine Disruption

During 2006 and 2007, the COB, implemented an approximately $50,000,000 upgrade of its WWTF from a combined trickling filter/solids contact process to activated sludge treatment. Effluent estrogenicty reflects an interaction between loading and WWTP removal efficiency.

The reduced effluent estrogen concentrations following the WWTP upgrade are attributable to improved contaminant removal efficiency emerging from increased plant capacity and retention time. The physicochemical properties of individual contaminants and the level of treatment determine their concentrations in the final WWTF effluent.



Acknowledgements

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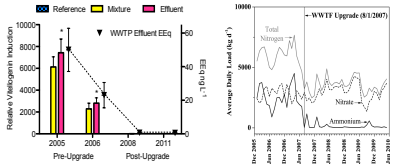
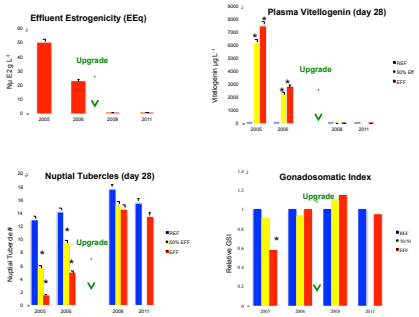
Infrastructure upgrade reduces effluent estrogenicity



A series of controlled, continuous-flow fish exposure experiments were conducted in an on-site bioassay laboratory (Barber et al., 2007, 2012; Vajda et al., 2006, 2011) to determine whether the reproductive disruption observed in wild fish could be induced by the Boulder WWTF effluent. Effluent estrogenicity declined dramatically during the study period, coincident with changes to domestic and industrial chemical usage and improved estrogen removal.

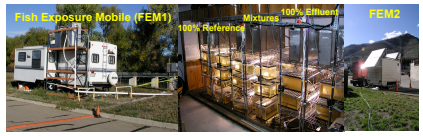
Recovery from Endocrine Disruption in Experimentally-exposed Fish



The occurrence and degree of reproductive disruption observed in adult male fathead minnows (*Pimephales promelas)* exposed to WWTP effluent paralleled reductions in WWTP effluent estrogenicity (dashed-line). The most severe reproductive disruption was observed prior to the WWTF upgrade, coincident with the highest detected effluent estrogenicity, and included rapid and severe suppression of primary and secondary sex characteristics. Significant declines in reproductive disruption paralleled measured reductions in effluent estrogen concentrations.

Exposure Conditions

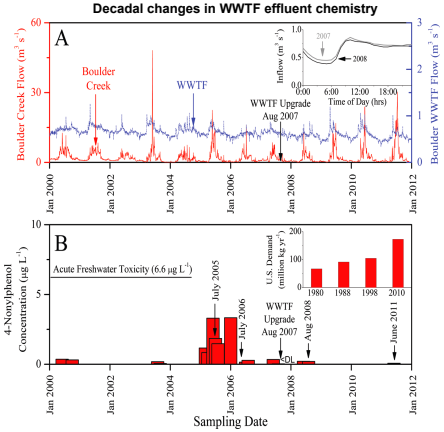
Adult fathead minnows (*Pimephales promelas*) were exposed to on-site, flow through conditions at the City of Boulder wastewater treatment plant (WWTP) to determine effects of an engineering upgrade (change from trickling filter to activated sludge) on the estrogenicity of the effluent. . Exposures were conducted on-site in a custom-built mobile flow-through laboratory. All exposure surfaces were glass, stainless steel, or Teflon. All treatments and animal care were approved by the University of Colorado IACUC.



Summary

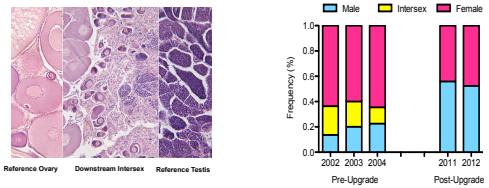
1. **Pre-Upgrade: severe reproductive disruption observed in wild and experimentally-exposed fish .**
   a. Female-biased sex ratios downstream of WWTF effluent persist between seasons.
   b. Gonadal intersex identified in multiple size-classes (20%).
   c. Elevated plasma vitellogenin among free-living downstream males.
   d. Rapid feminization and demasculinization of experimentally-exposed fish.
2. **Post-Upgrade: No evidence of estrogenic endocrine disruption in wild or experimentally-exposed fish.**
   a. No sex ratio bias downstream of WWTF.
   b. No intersex observed in downstream fish.
   c. No significant vitellogenin elevation in downstream males.
   d. No evidence of endocrine disruption in experimentally-exposed fish.

Decadal changes in WWTF effluent chemistry

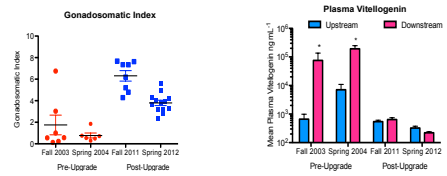


	Relative Estrogenicity	2005 Boulder Creek Reference n=6	SE	2005 COB WWTP Effluent n=7	SD	2006 Boulder Creek Reference n=6	SD	2006 COB WWTP Effluent n=6	SD
Compounds									
Steroids									
17α-Estradiol	0.06	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.0
17α-Ethynylestradiol	1.2	0.0	0.0	1.2	0.7	0.0	0.0	0.0	0.0
17β-Estradiol	1	0.0	0.0	2.1	1.0	0.0	0.0	3.2	3.2
Estriol	0.1	0.0	0.0	3.4	3.2	0.0	0.0	0.0	0.0
Estrone	0.2	0.0	0.0	75.4	28.0	0.0	0.0	59.5	74.5
Other estrogens									
Biophenol A	0.00007	32.5	46.3	41.5	44.0	17.8	27.2	10.2	5.2
1,2-Dichlorobenzene	1.00E-09	0.0	0.0	0.0	0.0	0.0	0.0	123.1	78.8
1,4-Dichlorobenzene	1.00E-07	81.2	138.0	2078.0	2914.6	0.0	0.0	1176.1	359.7
4-tert-butylphenol	3.00E-04	6.2	8.7	68.3	73.0	0.0	0.0	46.7	43.3
4-tert-pentylphenol	3.00E-04	0.0	0.0	64.8	81.8	0.0	0.0	1.9	2.2
4-Nonylphenolmonoethoxycarboxylate	2.00E-05	271.3	329.5	31630.9	9448.9	369.0	223.5	50474.8	6790.9
4-Nonylphenoldiethoxycarboxylate	2.00E-05	294.7	510.4	41633.1	7303.4	202.8	106.4	33787.6	13307.4
4-Nonylphenoltriethoxycarboxylate	2.00E-05	0.0	0.0	1452.0	687.0	0.0	0.0	509.6	242.5
4-Nonylphenoltetraethoxycarboxylate	2.00E-05	0.0	0.0	894.0	737.4	0.0	0.0	206.0	71.8
4-Nonylphenol	5.30E-05	209.5	232.4	1957.9	1307.6	73.1	33.7	238.3	104.5
4-Nonylphenolmonoethoxylate	1.00E-06	480.0	1150.0	9766.2	5814.5	67.6	71.0	1516.3	771.5
4-Nonylphenoldiethoxylate	1.00E-06	152.3	403.0	5181.4	3982.3	89.1	138.7	437.3	374.6
4-Nonylphenoltriethoxylate	1.00E-06	62.1	164.3	1910.1	1791.7	0.0	0.0	277.6	237.8
4-Nonylphenoltetraethoxylate	1.00E-06	0.0	0.0	445.1	724.6	0.0	0.0	39.3	30.7
4-n-Octylphenol	2.10E-05								
4-t-Octylphenol	3.60E-04	16.5	29.3	198.6	122.4	6.4	8.9	29.7	13.5
4-t-Octylphenolmonoethoxylate	3.60E-04	16.2	42.2	284.7	257.9	1.9	1.7	22.1	10.9
4-t-Octylphenoldiethoxylate	3.60E-04	82.4	218.1	2127.8	2687.5	8.7	10.8	53.2	66.9
4-t-Octylphenoltriethoxylate	3.60E-04	0.0	0.0	257.0	349.4	13.3	32.7	3.5	5.6
4-t-Octylphenoltetraethoxylate	3.60E-04	0.0	0.0	0.0	0.0	10.0	24.4	0.0	0.0
TEQ Average		**0.1**	**0.1**	**21.7**	**9.5**	**0.0**	**0.0**	**16.9**	**18.6**
TEQ Maximum		**0.1**	**0.2**	**102.3**	**47.0**	**0.0**	**0.0**	**52.6**	**63.3**

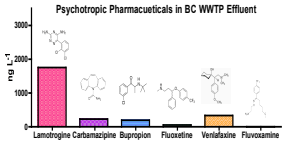
Recovery from Endocrine Disruption in Wild Fish



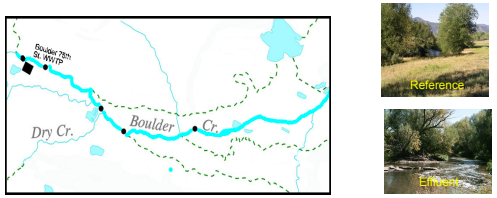
Prior to the WWTF upgrade, electrofishing surveys revealed a pattern of severe reproductive disruption in native wild fish (white sucker, *Catostomus commersoni*), downstream from the COB WWTF but not at upstream reference sites, consistent with exposure to exogenous estrogenic EACs . Reproductive effects included gonadal intersex, female-biased sex ratios, asynchronous ovarian development in females, reduced sperm abundance in males, and elevated plasma vitellogenin in males, and other evidence of reproductive disruption consistent with exposure to exogenous estrogen.



Electrofishing surveys downstream from the WWTF effluent conducted in 2011-2012, after the 2007 upgrade, failed to detect any evidence of continued reproductive disruption in wild fish. Instead, these surveys demonstrate significant recovery of gonadosomatic index, gametogenesis, and sex ratio, recovery of male-female sex ratio, and the absence of intersex fish or significant vitellogenic response among free-living, native fish downstream from the WWTF effluent. Several neuroactive pharmaceuticals are still detectable in the WWTF effluent. Ongoing studies are investigating the consequences of this persistent exposure on downstream organisms.



	Discharge (Q) or travel time (t_{travel}) from effluent outfall	Bupropion	Carbamazepine	Fluoxetine	Lamotrigine	Venlafaxine
Solubility (g/L)		312	0.017	10.7	0.17	0.72
log K_{ow}		3.35	2.61	4.33	1.46	2.99
pK_a		7.6	14	9.9	5.7	9.1
Boulder Creek above WWTF	Q = 0.45 $m^3 s^{-1}$	>5	>5	>5	5	>5
City of Boulder WWTP effluent	Q = 0.59 $m^3 s^{-1}$	237	298	44	2513	249
Boulder Creek at White Rock	t_{travel} = 2.03 h	100	144	14	1232	121
Boulder Creek above Legget Ditch	t_{travel} = 2.90 h	85	135	12	1019	111
Boulder Creek above Dry Creek	t_{travel} = 3.82 h	83	144	9	1040	130
Boulder Creek at 95th Street	t_{travel} = 5.37 h	74	148	10	1120	136

Study Sites

Boulder Creek receives effluent from the City of Boulder WWTP. During low-flow conditions, Boulder Creek is effluent-dominated below the WWTP. Boulder Creek upstream of the Boulder WWTP flows through the city of Boulder and receives all storm water runoff from the municipal area. Reference exposure water was from downstream of city of Boulder storm water runoff and upstream of all WWTP effluent.