Effects-Based Monitoring of Bioactive Contaminants Associated with Municipal Wastewater Treatment Plant Effluent Discharge to the South Platte River

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Introduction

- Over the last several years, contaminants of emerging concern (CECs), including pharmaceuticals, personal care products, and pesticides, as well as *in vitro* bioactivities have been detected in the South Platte River near Denver, Colorado, USA at concentrations and bioactivities among the highest in the nation (Bradley et al., 2017; Conley et al., 2017).
- However, given the lack of available biological effects data for most of the compounds detected, potential ecological risks associated with these contaminants are unclear.
- The present study builds upon several years of preliminary chemical monitoring research on the South Platte River as a basis for further investigation of estrogen receptor (ER), glucocorticoid receptor (GR), and peroxisome proliferator activated receptor gamma (PPARγ)-bioactive contaminants and potential responses elicited in caged fish.
- To assess the spatial and temporal distribution of CECs, surface water samples were collected and analyzed approximately bi-monthly throughout 2018 and 2019 at 11 sites along the South Platte River with varying proximities to a wastewater treatment plant (WWTP) and surrounding tributaries. Water samples were analyzed for a suite of pharmaceuticals, pesticides, steroid hormones, and wastewater indicators as well as screened for *in vitro* biological activities.
- To evaluate the potential bioavailability and significance of these biologically-active contaminants, adult fathead minnows (*Pimephales promelas*) were caged at six sites along the South Platte River at various proximities to a WWTP as well as in surrounding tributaries.

Objectives:

- Assess the spatial and temporal distribution of CECs and ER, GR, and PPARγ bioactivities associated with a historically bioactive WWTP effluent along the South Platte River.
- Utilize a caged fish system to determine the potential *in vivo* bioavailability of bioactive contaminants.

Methods

Water Samples

- Grab or 5 d composite water samples collected approximately every other month from May 2018 to October 2019 during the Spring to Fall seasons
- Solid phase extraction for *in vitro* bioassays and steroid measurements
- Analyzed for a suite of pharmaceuticals, pesticides, wastewater indicators, steroid hormones, and *in vitro* biological activities (e.g., ER, GR, and PPARγ activities)

Analytical Chemistry

- Instrumental determination of 252 analytes was conducted on the surface water samples (131 pharmaceuticals and personal care products, 69 pesticides, and 52 wastewater indicators) by the Region 8 EPA Laboratory (Denver, CO).
- Steroidal estrogen concentrations determined by the GLTED EPA Laboratory (Duluth, MN)

In vitro Bioassays

- Multiplexed *in vitro* Trans-FACTORIAL™ bioassay used to evaluate human nuclear receptors (Attagene, RTP, NC)
- Estrogen receptor agonist assay (T47D-Kbluc; Wilson et al. 2004)
- Glucocorticoid receptor agonist assay (Indigo Biosciences, State College, PA)
- Peroxisome proliferator activated receptor-gamma assay (Indigo Biosciences)

Caged Fish Study Details

Cages per site	2
Fish species	*Pimephales promelas*
Fish per cage	6M, 6F
Exposure duration	5 days
Samples collected	Mucus (metab) Plasma (steroids) Muscle (QPCR) Adipose (QPCR) Liver (QPCR & metab) Gonad (QPCR & metab)
Water samples	5 d composite at each site

Data analysis

Gene Expression Data: (Analyzed using Statistica)
- Analyzed using a one-way ANOVA, followed by Duncan's or Dunn's post-hoc test
- Differences considered significant at $p < 0.05$

***In vitro* Bioassay Data:** (Analyzed using GraphPad Prism):
- Analyzed using a non-linear regression (log-agonist vs. response)
- Significant response threshold = Mean + 3(SD) of the control media response

Caged Fish Endpoints

Gene Expression

- RNA extraction: RNeasy Mini (Qiagen)
- Quantitative polymerase chain reaction (QPCR):
  - Power SYBR Green RNA-to-Ct 1-step kit
  - Taqman RNA-to-Ct 1-step kit

Gene Targets:

ER-related:
- *vtg* = Vitellogenin

GR-related:
- *nr3c1* = Glucocorticoid receptor
- *sgk1* = Serum/glucocorticoid regulated kinase 1
- *nfkbiaa* = Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a

PPARγ-related:
- *acox1* = Acyl-coenzyme A oxidase 1 palmitoyl
- *lpl* = Lipoprotein lipase
- *fabp3* = Fatty acid binding protein 3
- *fabp1b1* = Fatty acid binding protein 1b1
- *pparg* = peroxisome proliferator activated receptor gamma

5/8/2018 Grab water → 6/27/2018 Grab water → 8/9-14/2018 Composite water; Caged fish → 11/2018 Grab water → 5-1-2019 Grab water → 6-25-2019 Grab water → 8/9-14/2019 Composite water; Caged fish → 10/7/2019 Grab water

Figure 1. Map of field sites along the South Platte River and surrounding tributaries in Denver, CO.

	Site Name	Site Abbreviation
1	Clear Creek Reference	CC-Ref
2	WWTP Upstream	WWTP-UP
3	S. Platte Cherokee	SP-Cherokee
4	WWTP Downstream Left	WWTP-DL
5	WWTP Downstream Right	WWTP-DR
6	Sand Creek Tributary of S. Platte	SC-TrbSP
7	S. Platte Downstream of Sand Creek	SP-DWN-SC
8	S. Platte Upstream of Clear Creek	SP-UP-CC
9	Clear Creek Tributary of S. Platte (Caged fish 2019 only)	CC-TrbSP
10	S. Platte Downstream of Clear Creek	SP-DWN-CC
11	S. Platte Confluence with Clear Creek- Right Bank (Caged fish 2018 only)	SPCC-R

Results

Bioactivity Surveillance

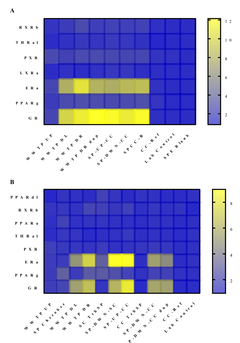

Figure 2. Results of the Attagene Trans-FACTORIAL™ assay, evaluating human nuclear receptor bioactivities in composite surface water extracts during caged fish exposures in A) August 2018; B) August 2019. Results are shown only for the endpoints that had a significant response and expressed as fold-change relative to background response. Yellow = higher activity; Blue=lower activity.

In vitro response

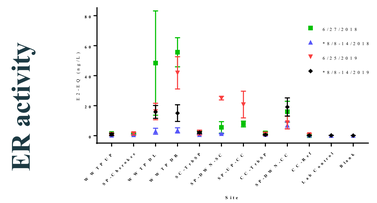

Figure 3. Estrogenic activity reported as estimated 17β-estradiol equivalents (E2-EQ) of grab and composite surface water sample extracts. Data represented by mean ± SD of sample replicates within assays. *Caged fish sample dates.

In vivo response

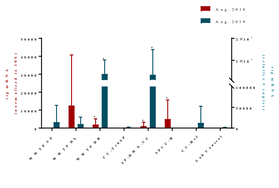

Figure 4. Relative transcript abundance of vitellogenin (*vtg*) mRNA in male livers from field-exposed fathead minnows. Data represented by mean ± SD of n = 9-14 fish per treatment.

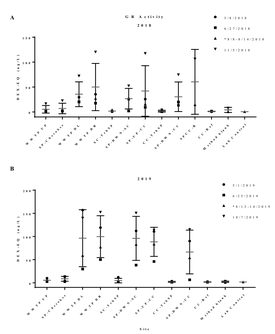

Figure 5. GR-activity of grab and composite surface water extracts reported as a dexamethasone equivalents (DEX-EQ). A) 2018 samples; B) 2019 samples. Data represented by mean ± SD of sample replicates within assays. *Caged fish sample dates.

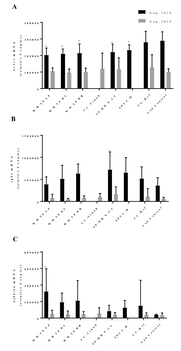

Figure 6. Relative abundance of predicted glucocorticoid receptor (GR)-related transcripts (*nr3c1*, *sgk1*, and *nfkbiaa*) in male livers from field-exposed fathead minnows. Data represented by mean ± SD of n = 9-14 fish per treatment.

PPARγ activity

No significant in vitro response.

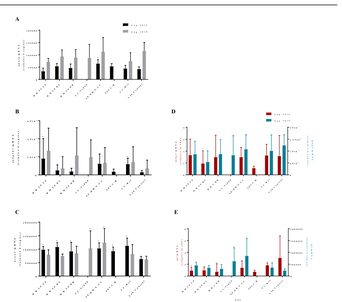

Figure 7. Relative abundance of predicted PPARγ-related transcripts (*pparg*, *fabp1b1*, *acox1*, *fabp3*, and *lpl*) in male livers from field-exposed fathead minnows. Data represented by mean ± SD of n = 9-14 fish per treatment.

Analytical Chemistry

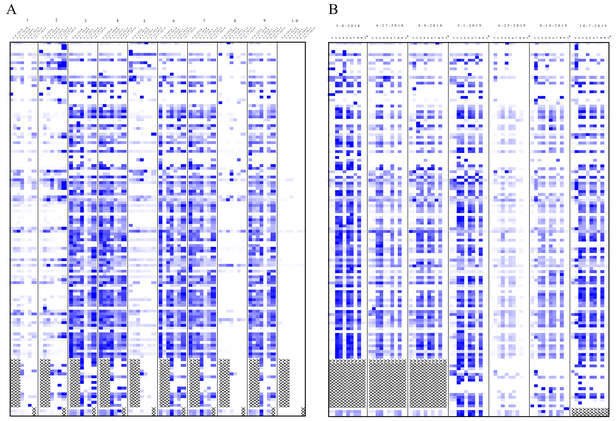

Figure 8. Ratio normalization to the maximum concentration of each respective chemical. Data arranged to highlight A) spatial and B) temporal distribution of the contaminants. Numbers correspond to the sites listed in the site key on the right. Dark blue = higher relative concentrations; white = lower relative concentrations.

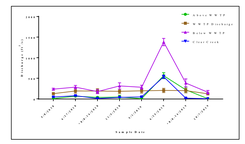

Figure 9. Mean ± SD discharge (ft^3/s) at sites along the South Platte River and Clear Creek on water sample collection dates.

Discussion and Conclusions

Temporal and Spatial Variation of CECs and Bioactivities

- Sites with the highest number and concentration of detected CECs were located within the South Platte River downstream of the WWTP. Fewer and lower concentrations of CECs were detected upstream of the WWTP and in surrounding tributaries with no direct impact of the WWTP (Fig. 8A).
- Sampling dates with the highest number and concentrations of CECs were in the spring and fall seasons (5-8-2018, 5-1-2018 and 10-7-2019). The sampling date with the overall lowest concentrations of CECs across all sites was on 6-25-2019, which also corresponded with the highest river discharge rates (Figs. 8B & 9).
- In vitro multi-factorial Attagene assays detected estrogen receptor (ER), glucocorticoid receptor (GR), and peroxisome proliferator activated receptor-gamma (PPARγ) bioactivities in surface water samples downstream of the WWTP (Fig. 2).
- Targeted *in vitro* transcriptional activation assays for ER, GR, and PPARγ corroborated bioactivities for ER (up to 55 ± 10 ng/L E2-EQ; Fig. 3) and GR (up to 156 ± 28 ng/L DEX-EQ; Fig. 5)
- There was no significant PPARγ activity detected for any of the sites during the bi-monthly sampling events using the Indigo PPARγ kits (results not shown). Relative to PPARγ activation, the Attagene assay appears to be more sensitive than the Indigo Biosciences kit.

Caged Fish Studies: In vivo Bioavailability

- Significant up-regulation of male hepatic *vtg* was observed in fish exposed at sites with corresponding *in vitro* ER activity (Fig.4).
- Despite GR-activity *in vitro*, no site-related differences in GR-related gene expression were detected in male livers, suggesting observed environmental concentrations of GR-active contaminants do not induce a detectable *in vivo* response (Fig. 6).
- Genes selected for GR-related expression were verified to be responsive to the well-known GR agonist, Dexamethasone, in a controlled laboratory study (data not shown), suggesting river concentrations associated with the 5 d caged fish exposures were insufficient to elicit a significant gene expression response.
- Consistent with the general lack of detectable *in vitro* PPARγ activity, there were no significant effects on PPARγ-related gene expression in male livers (Fig. 7).
- Although the chemicals responsible for in vitro GR- and PPARγ-mediated bioactivities are still unknown, results from the present study provide insights into the significance of these bioactivities relative to *in situ* fish exposures.

Conclusions:

- Overall, both GR- and ER-related biological activities can be detected more than 1.5 km downstream of the WWTP outflow, even after convergence with additional tributaries.
- There was no clear evidence for GR- or PPARγ-mediated responses in fish caged at various locations along the South Platte River with respect to proximity to the wastewater discharge. However, wastewater was clearly a major contributor of contaminant loading to the system and additional research to evaluate the possible significance of exposures to up to 150 ng DEX-EQ/L of GR-mediated activity under laboratory conditions is warranted.

Future directions:

- Develop a receptor pull-down method to identify unknown compounds that may be responsible for GR and PPARγ activity.

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