# Evaluating the Impact of Wastewater Effluent Discharge into the Santa Ana River

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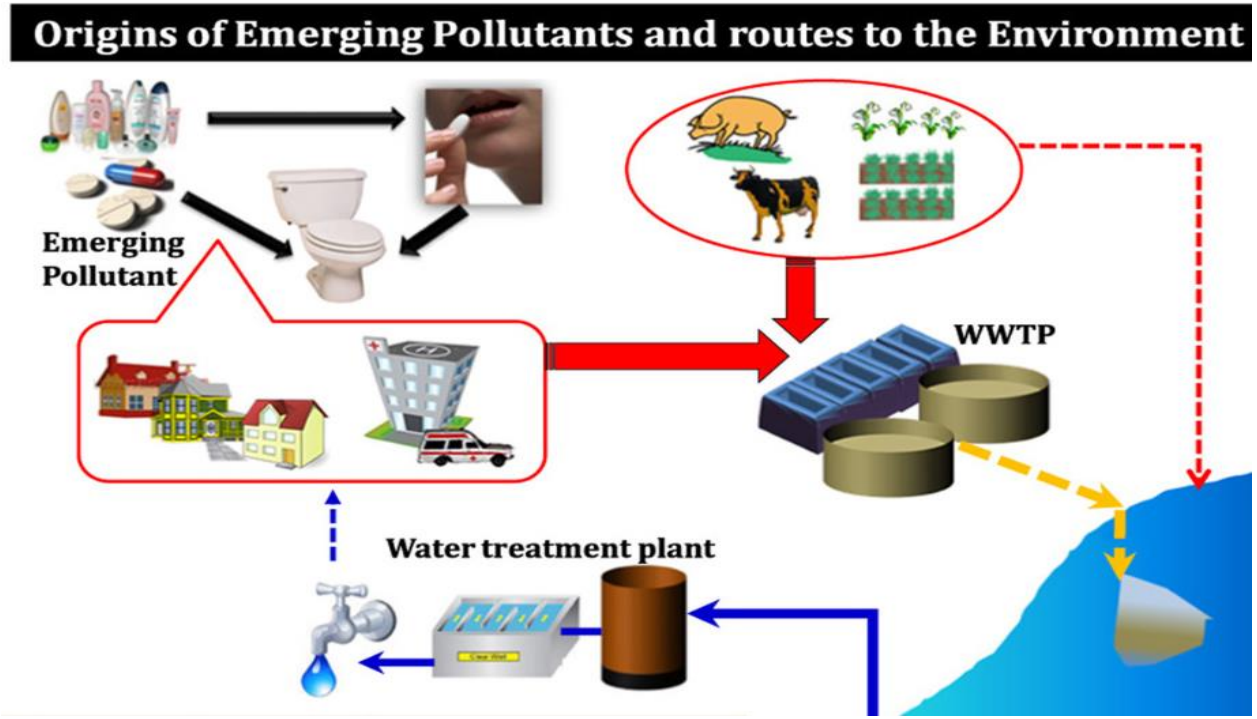
# Background



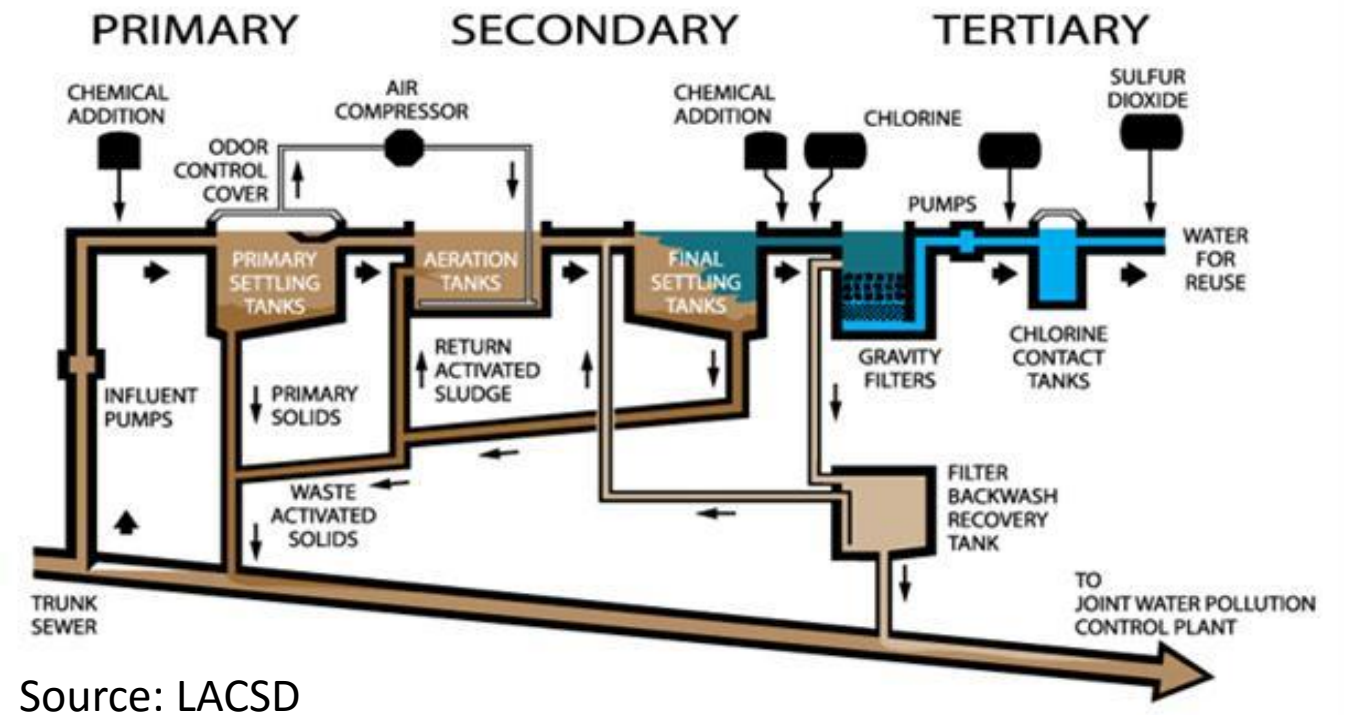

Source: Gogoi et al, 2018

- EACs in environmental surface waters is well documented
  - Effective at low concentrations (ng/L)
  - From point and non-point residential, agricultural, and industrial sources
- Treated wastewater effluent consistently found to have low concentrations of EACs
  - Inadequate contaminant removal efficiency or biotransformation of process chemicals occurs

# EACs in Wastewater Effluent

| Emerging Contaminant | Use | Log $K_{ow}$ | Effluent Concentrations (ng/L) | Surface Water Concentrations (ng/L) | Half-life (d) | Country | Reference |
|---|---|---|---|---|---|---|---|
| **Estrogens** | | | | | | | |
| Estrone | Steroid Estrogen | 3.13 | <1 - 54 | 0 - 38 | 2-3 | United States | *Ying et al, 2002; Pal et al, 2010* |
| Estradiol | Steroid Estrogen | 3.94 | <1 - 22 | 0 - 4.5 | 2-3 | United States | *Pal et al, 2010* |
| Estriol | Steroid Estrogen | 2.81 | NDA | 12 | NDA | United States | *Ying et al, 2002; Pal et al, 2010; Petrie et al, 2014* |
| Ethinylestradiol | Synthetic Estrogen | 4.15 | 0 | 2 - 4.67 | 4-6 | United States | *Ying et al, 2002; Pal et al, 2010* |
| **Androgens** | | | | | | | |
| Testosterone | Steroid Androgen | 3.32 | 0 | 4.3 - 16 | NDA | United States | *Liu et al, 2009; Pal et al, 2010* |
| Androsteneidone | Steroid Androgen | 2.75 | 0 | 44 | NDA | United States | *Liu et al, 2009; Pal et al, 2010* |
| **Alkylphenols** | | | | | | | |
| OP | Nonionic Surfactant | 4.48 | 150 | 17 | 30 | United States | *Snyder et al, 1999; Nielsen et al, 2000; Roslev et al, 2007; Vega-Morales et al, 2013; Petrie et al, 2014* |
| NP | Metabolite | 4.12 | 16,000 | 1,520 | 8.1 - 51 | United States | *Snyder et al, 1999; Nielsen et al, 2000; Roslev et al, 2007; Vega-Morales et al, 2013; Petrie et al, 2014* |
| **Phthalic acid esters** | | | | | | | |
| BBP | Plasticizer | 4.59 | 3.13 | 0.002 - 0.006 | 3.1 | Denmark, Canada | *Yuan et al, 2002; Mackintosh et al, 2006; Petrie et al, 2014; Gao and Wen, 2016* |
| DEHP | Plasticizer | 7.50 | 4.92 | 0.17 - 0.44 | 5-23 | Denmark, Canada | *Yuan et al, 2002; Mackintosh et al, 2006; Petrie et al, 2014; Gao and Wen, 2016* |
| **Bisphenol** | | | | | | | |
| BPA | Plasticizer | 3.43 | 35 - 86 | <6 - 34 | 4.5 | United Kingdom | *Cousins et al, 2006; Petrie et al, 2014* |

# Levels of Wastewater Treatment

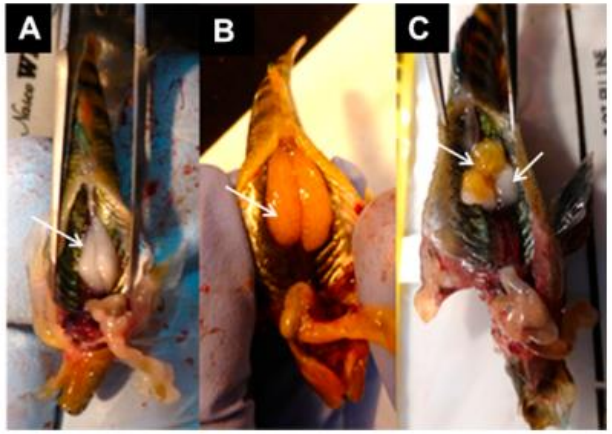


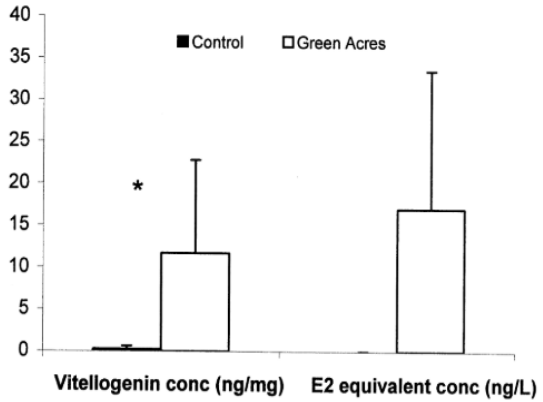

Source: LACSD

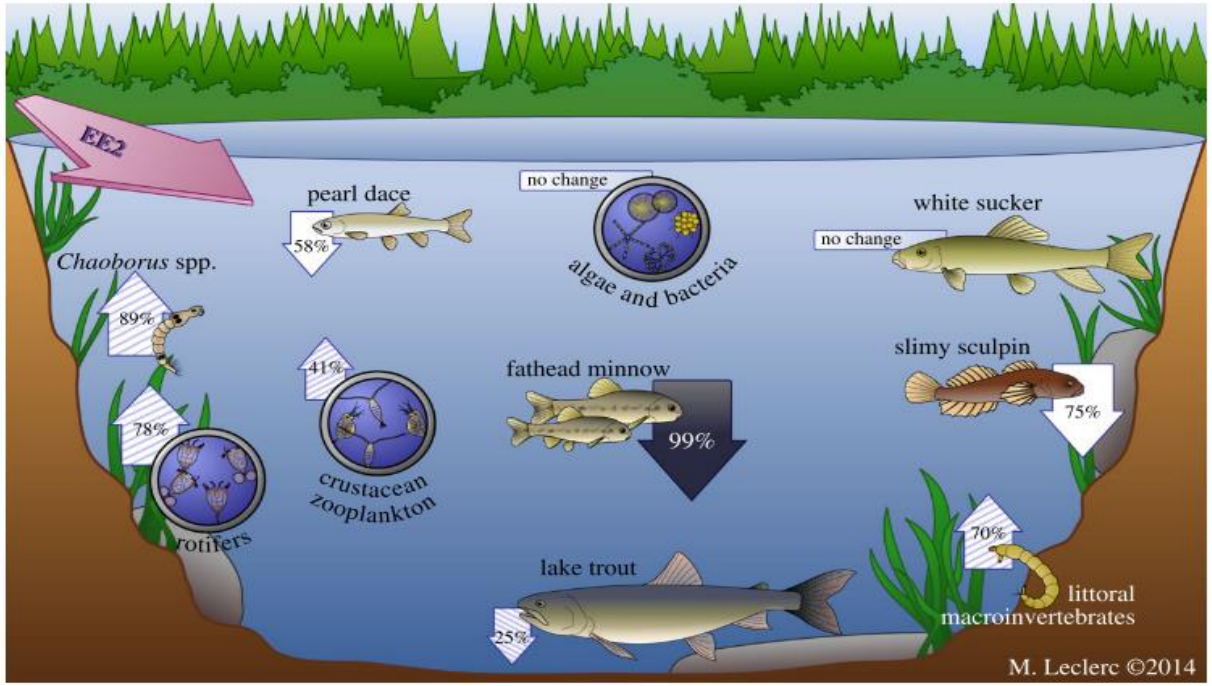
- Level of wastewater treatment can significantly effect effluent composition
- Secondary effluent EEQ – 20 – 40 ng/L EEQ
- Tertiary treatment is most efficient at removing estrogenic compounds
  - Androgens removed efficiently (>99%)
  - Estrogens more persistent due to chemical structure
    - E2 > E1 – 1-3 hours
    - EE2 degradation - ~ 5 days

# Ecosystems at Risk

- Downstream discharge sites most estrogenic due to poor effluent dispersal
  - Fish susceptibility to estrogen exposure well-documented
- Individual > Population level effects
  - Whole-Lake Estrogen Experiment
    - Indirect effects that can impact tolerant species
  - Effluent exposure studies

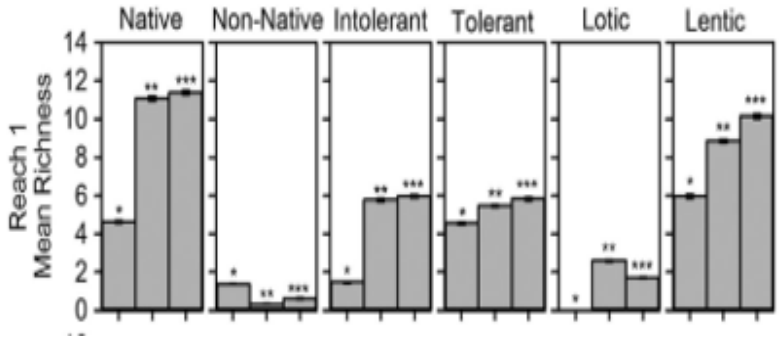


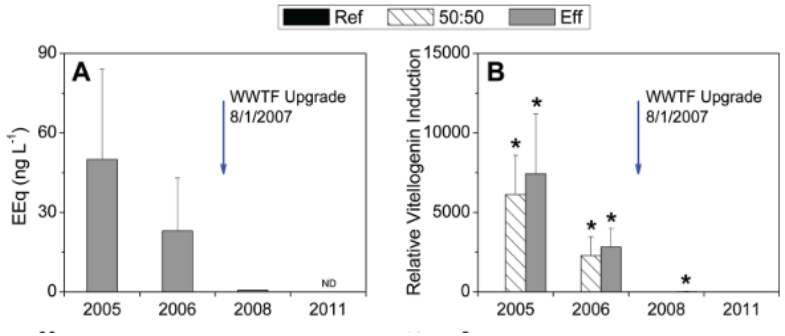




Source: Kidd et al, 2007; Kidd et al, 2012

# Effluent Dominated Rivers




Temporal changes in mean species richness for native status, tolerance level, and habitat association guilds for Trinity River of Texas fish assemblage collections taken from three reaches during three periods (Period I: 19710–1974; Period II: 1987–1988; and Period III: 1994– 2008).
Source: Perkins and Bonner, 2016



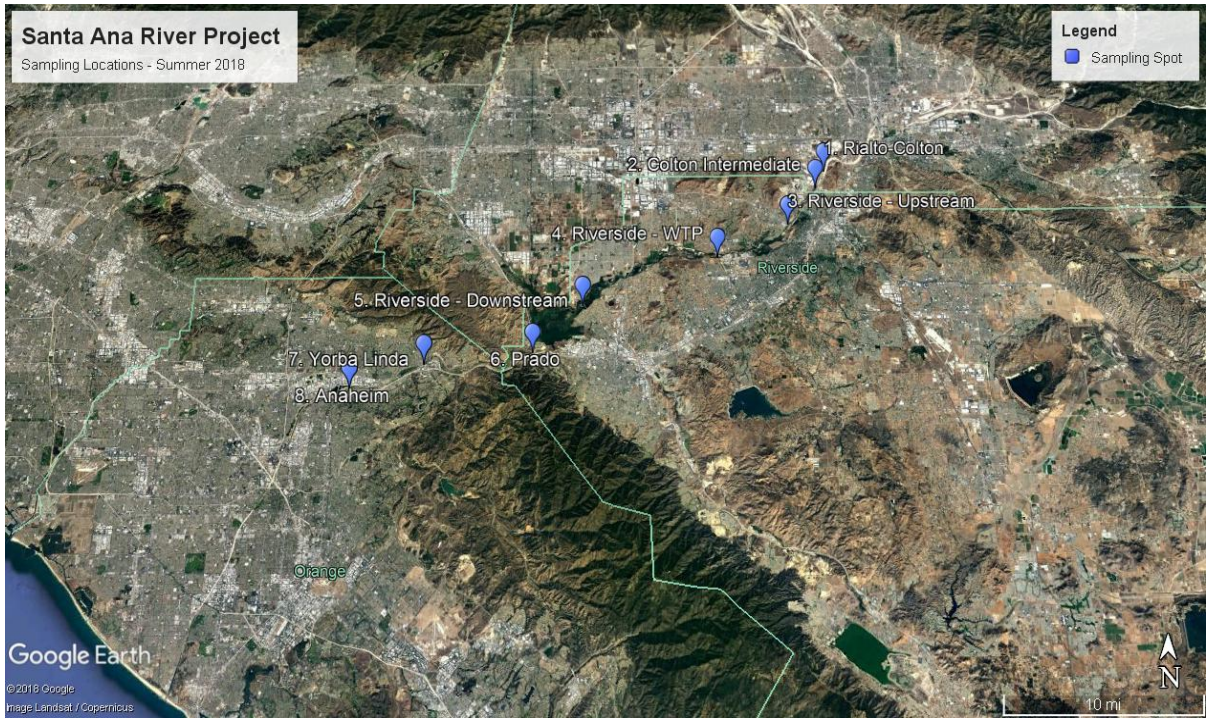

(A) Average WWTF effluent estradiol equivalency quotient (EEq) as a function of exposure experiment based on weekly measurements of multiple endocrine-disrupting chemicals;7 (B) relative plasma vitellogenin concentrations (normalized to the mean Ref concentration) in adult male fathead minnows exposed to 100% Ref, 50:50 Ref:Eff, and 100% Eff for 28 days
Source: Barber et al, 2012

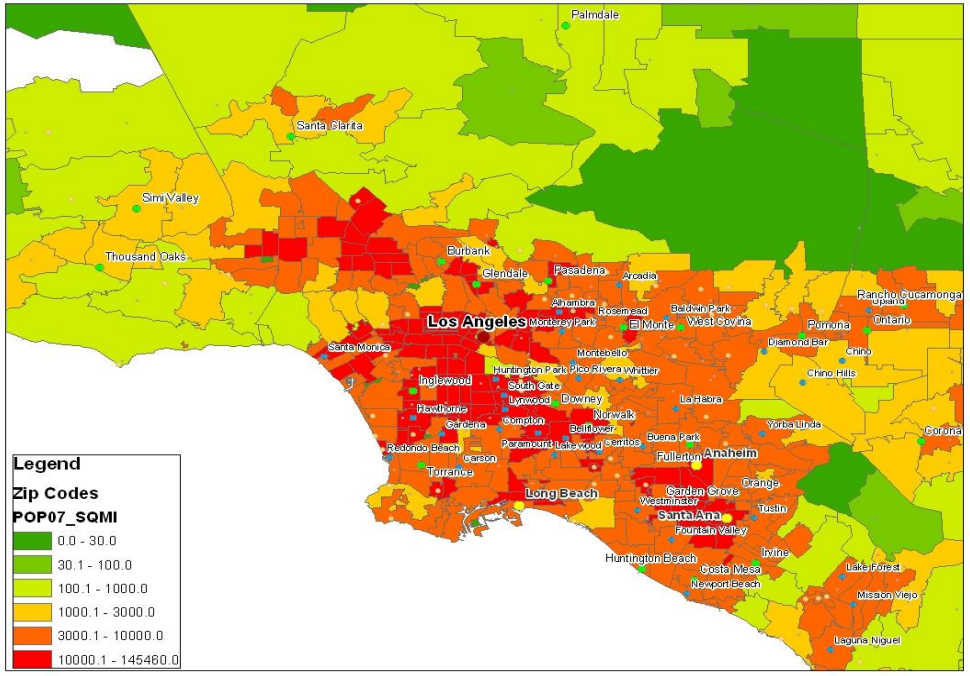
Source: Barber et al, 2012; Perkin and Bonner, 2016

- Common in highly-urbanized, semi-arid “Mediterranean” regions
- Trinity River, Texas
  - Decades of urban development > decreasing water quality and fish diversity
  - WWTP and infrastructure upgrades
    - Improvement to DO and recovery of fish assemblages
- Boulder Creek – Colorado, USA
  - Effluent 20-50 ng/L EEQ prior to upgrades
  - Trickling filter/solids contact to activated sludge treatment

# The Santa Ana River

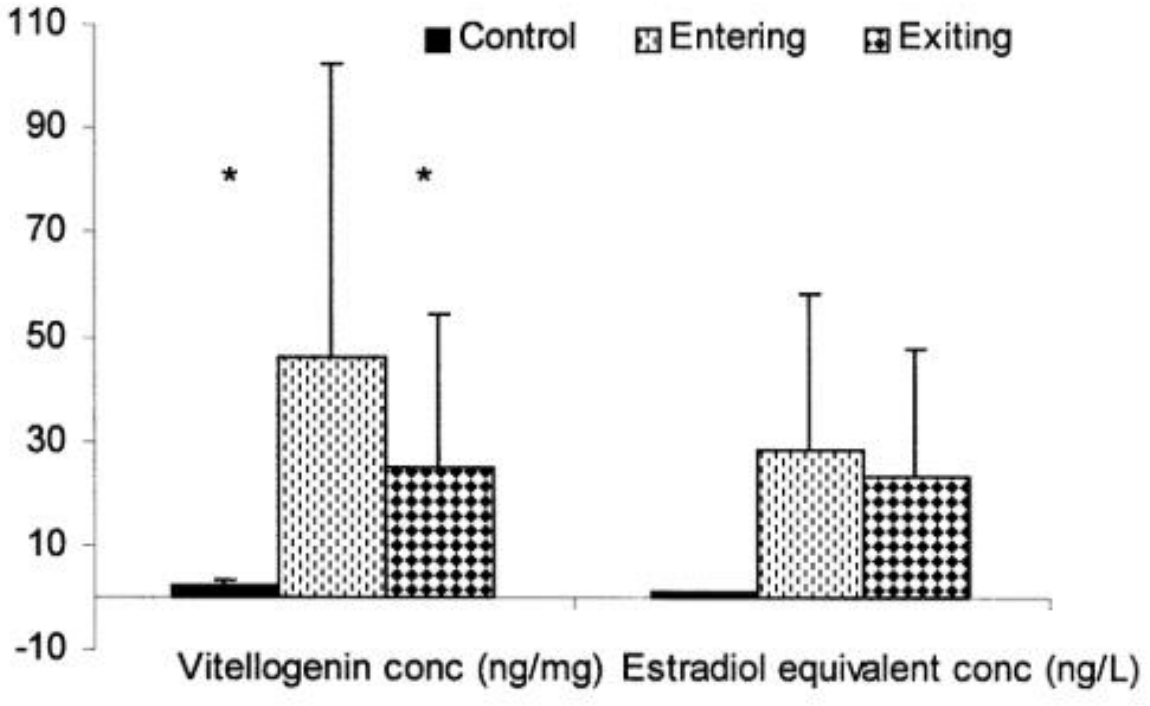
- Largest river in Southern California
  - 96 miles long – San Bernardino Mountains > Pacific Ocean
- Fed by up to 10 WWTPs; four primary plants
  - >90% effluent at base-flow conditions
- Highly urbanized watershed
  - 32% - industrial, commercial, or residential
  - 10% - agriculture; boasts one of the highest concentrations of cattle in the United States



# Evaluation of Wetland Extracts for Estrogenic Activity

- Exposed juvenile rainbow trout to Prado Dam influent and effluent
- Wetland influent vs effluent - more activity *in vitro* but similar activity *in vivo*
  - Suggests wetland treatment not as efficient at removing estrogenic compounds
- 10-fold greater *in vivo* activity compared to *in vitro*



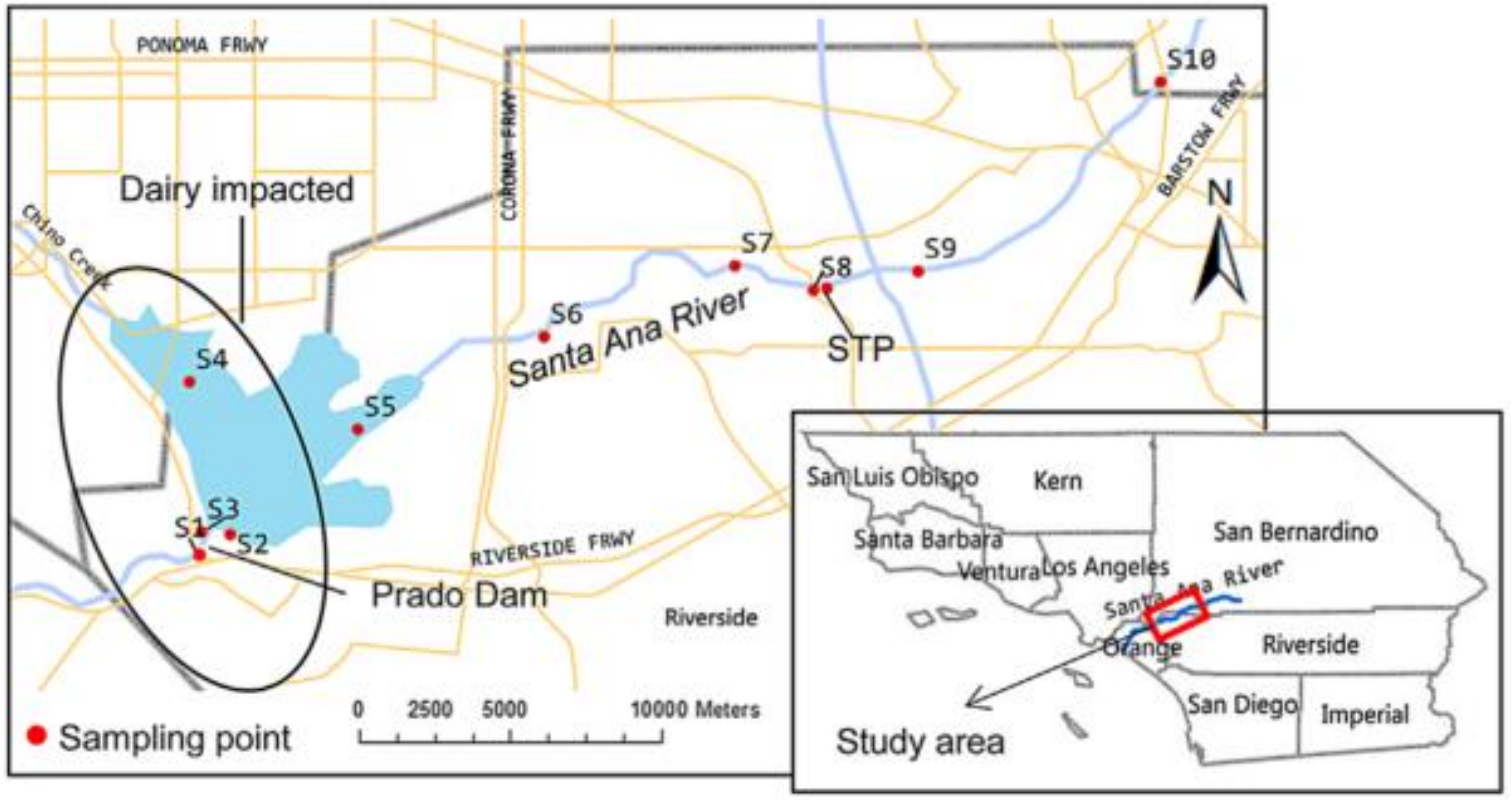

Plasma VTG levels in juvenile rainbow trout exposed to 33% of the wastewater entering and exiting Prado Wetland (left panel) and the estimated estradiol equivalent concentrations for 100% of the wastewater (right panel).
Source: Xie et al, 2002

Source: Xie et al, 2004

# Steroidal Hormones in Santa Ana River Surface Waters

(Ma et. al, 2016)

- Investigated occurrence of 13 parent and conjugated estrogens and progestogens
- Primarily focused on sites near CAFOs, WWTPs, or industrial activity
  - Assess potential wildlife risks



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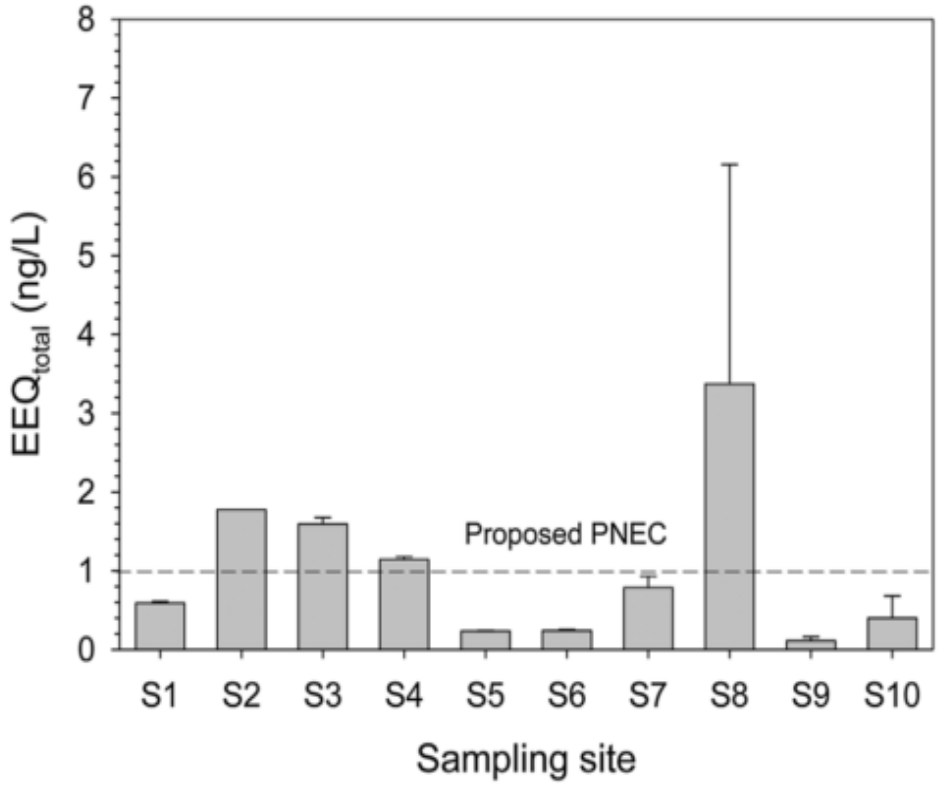



Figure 3. Average total estradiol equivalents ($EEQ_{total}$) calculated in river waters. The proposed predicted no effect concentration (PNEC) of estradiol is shown as a horizontal dash line. Error bars indicate standard deviation ($n = 2$).

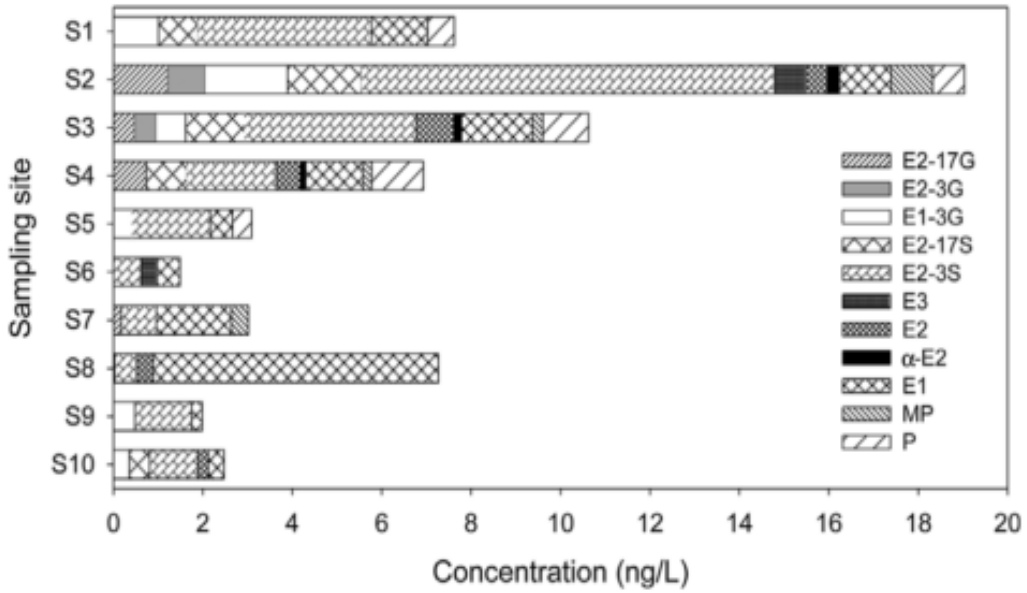
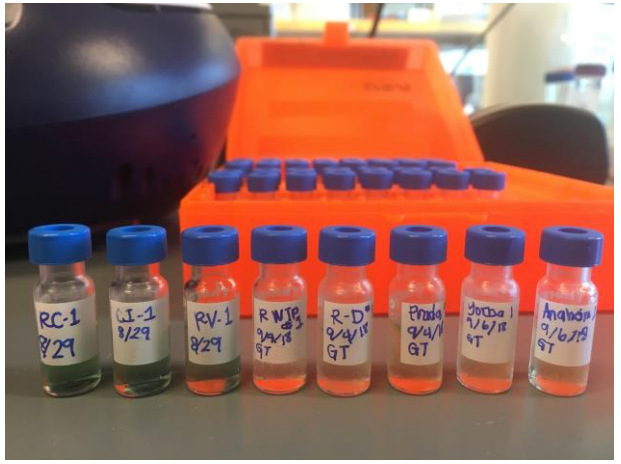



Figure 2. Concentrations (ng/L) of parent and conjugated estrogens and progestagens in surface waters of 10 sampling sites. Results were reported as averages of duplicate values. E2-17G = 17β-estradiol-17-glucuronide; E2-3G = 17β-estradiol-3-glucuronide; E1-3G = estrone-3-glucuronide; E2-17S = 17β-estradiol-17-sulfate; E2-3S = 17β-estradiol-3-sulfate; E3 = estriol; E2 = 17β-estradiol; α-E2 = 17α-estradiol; EE2 = 17α-ethynylestradiol; E1 = estrone; MP = medroxyprogesterone; P = progesterone.

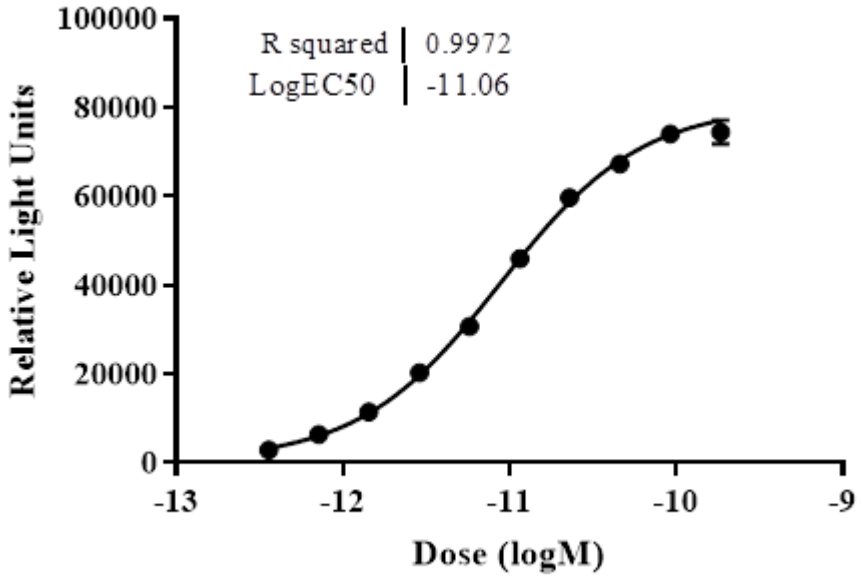
# Specific Aims

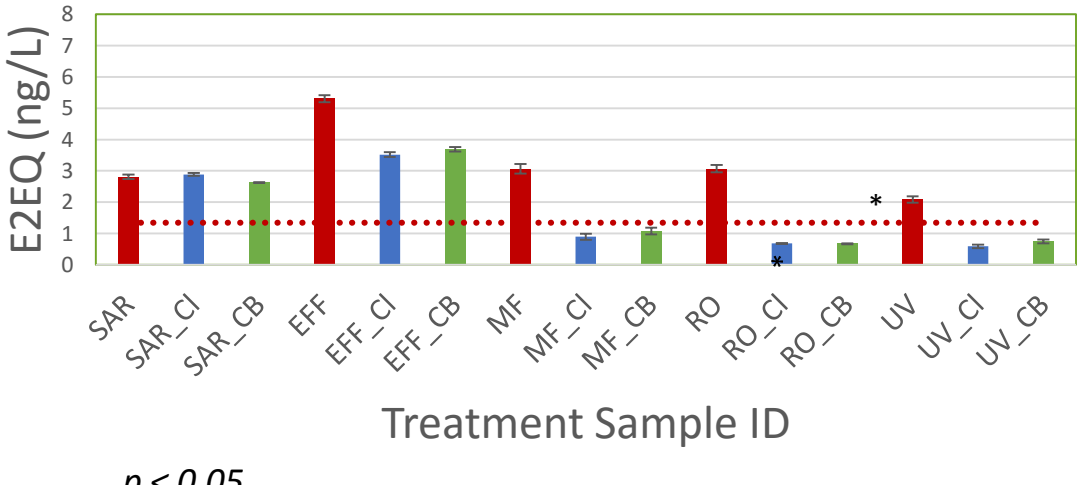
- Analyze estrogenicity of samples taken from Santa Ana River
  - Measure ER activity and quantify estrogenicity of extracts (EEQ)
  - Evaluate seasonal variation of sample activity
- Collect river water to expose Japanese Medaka (*Oryzias latipes*) to analyze extracts effects on fish feminization




# Deriving Estrogenic Equivalence from a Cell-Based Bioassay

- Use recombinant cell lines that have a reporter gene attached to the receptor of interest
  - Ex: P53 cell-line - Genotoxicity; AhR cell-line – Dioxin-like compounds
- Vm7Luc4E2 – have firefly luciferase reporter gene plasmid attached to cell estrogen receptor (ER)
  - Respond to the presence of estrogenic compounds with the induction firefly luciferase – emits light which is quantified
  - Compare samples (unknown, unknown) to known reference concentrations of 17-beta Estradiol




$p < 0.05$

# Why Vitellogenin?

- Female egg yolk protein that is produced by female fish but not by males
  - Commonly used as an endpoint for endocrine disruption
  - Easily detected in blood serum
- Gene (*vtg*) shouldn't be expressed by males unless exposed to estrogenic compounds, which leads to vitellogenesis
  - Exposure can invoke up to a 40,000 fold change in mRNA expression

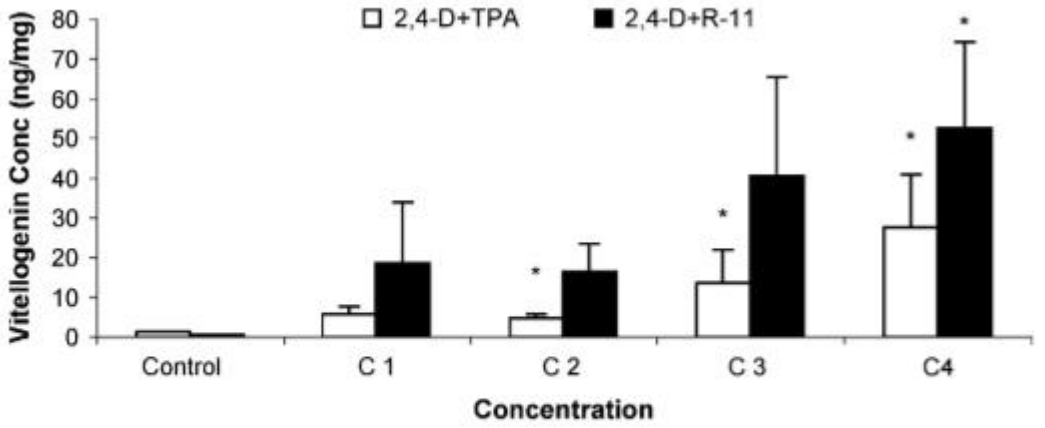



**FIG. 3.** Plasma vitellogenin levels (ng/mg) in juvenile rainbow trout exposed to the mixture of 2,4-D with TPA or R-11 for 7 days. The concentration of each chemical is provided in Table 3. * Indicates significant difference in vitellogenin levels from control at $p < 0.05$.

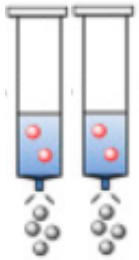
Source: Xie et al, 2004

# Methods

Santa Ana River

Collect 1L samples in triplicate

Vacuum filtration using 1.2 uM filters



Perform SPE and nitrogen evaporation to render 1 mL concentrates

Quantify estrogenicity in E2EQs via *in vivo* cell bioassays

Collect 40 liter samples

Perform 14-day exposures using Male Japanese Medaka (*Oryzias latipes*)

Collect non-native species

Collect non-native male samples from Santa Ana via minnow traps/netting or invasive removal work with CDFW

Extract livers for use in qPCR

Perform qPCR to quantify change in *vtg* expression

# *In Vitro* Cell Bioassays

- Slight decrease in estrogenicity from summer to fall
  - Small rain events in October increased SAR flow volume
- Significant increases in activity following a storm event
  - Influence of urban runoff and combined sewer overflows (CSOs)

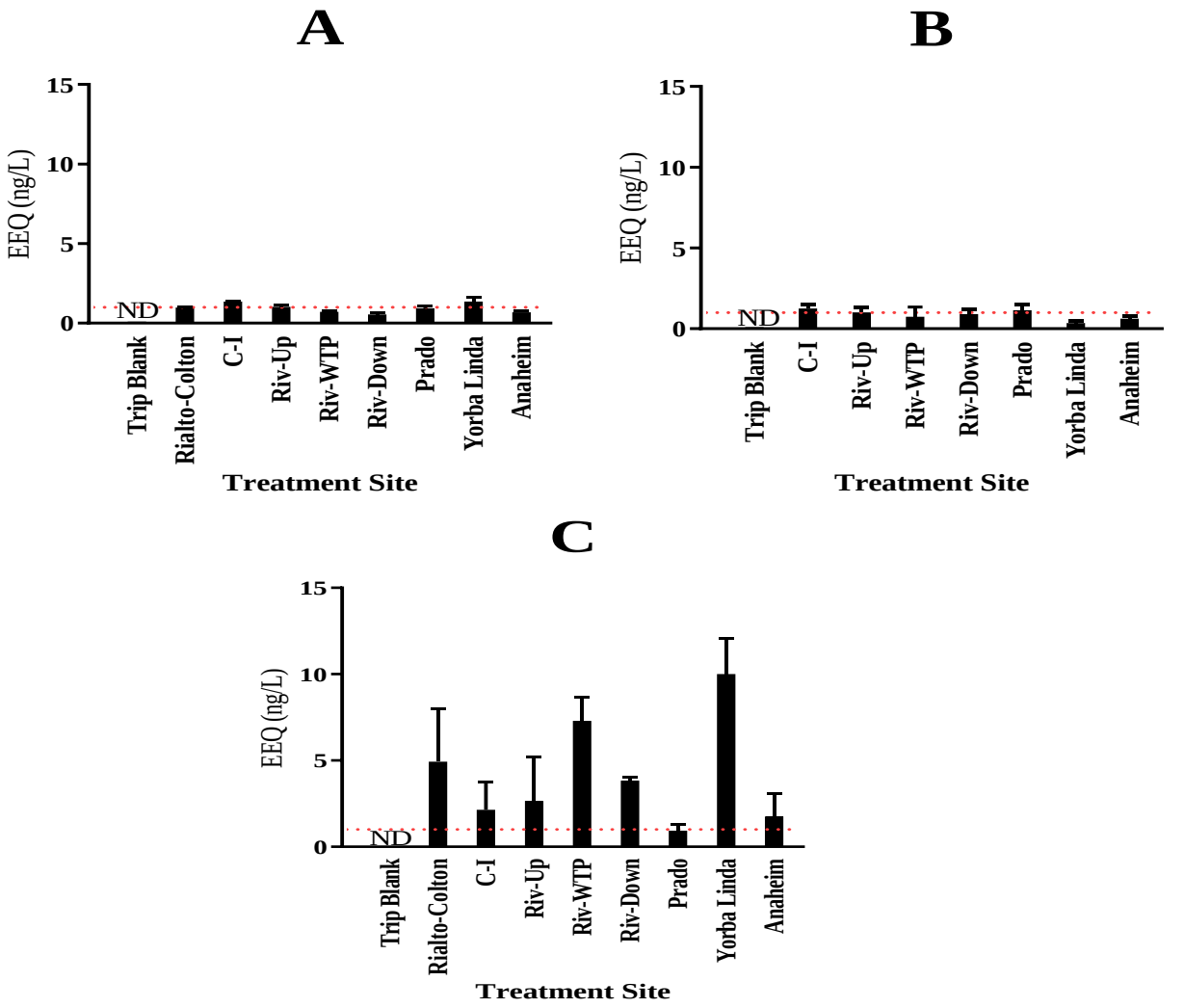



**Fig. 1.** Estradiol equivalents (EEQ) derived from treatment of Vm7Luc4E2 cells with water collected at various locations on the Santa Ana River from 2018 - 2019, A) August/ September 2018 Extracts, B) November 2018, C) January 2019. Each value represents the mean of three replicates ± SEM. Dotted line represents PNEC (1 ng/L).

# Japanese Medaka (*Oryzias latipes*) Exposure

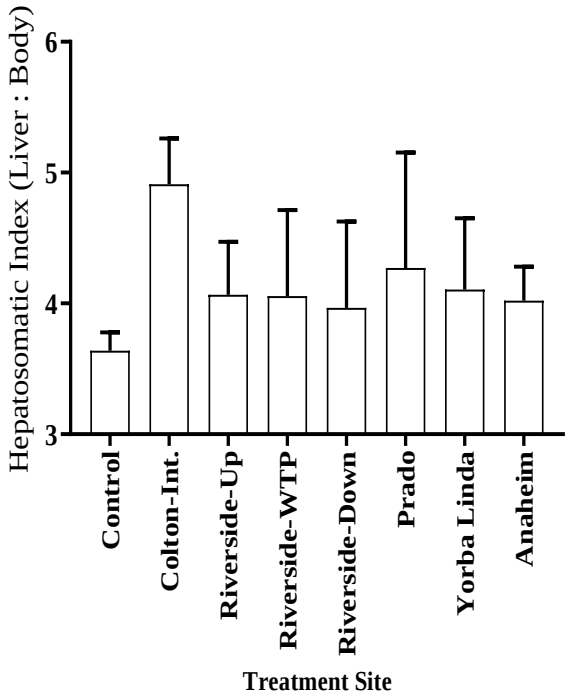



**Fig. 2.** Hepatosomatic index values in juvenile Japanese Medaka (*Oryzias latipes*) following exposure to water samples from the Santa Ana River for 14d. Each value represents the mean of three replicates ± SEM.

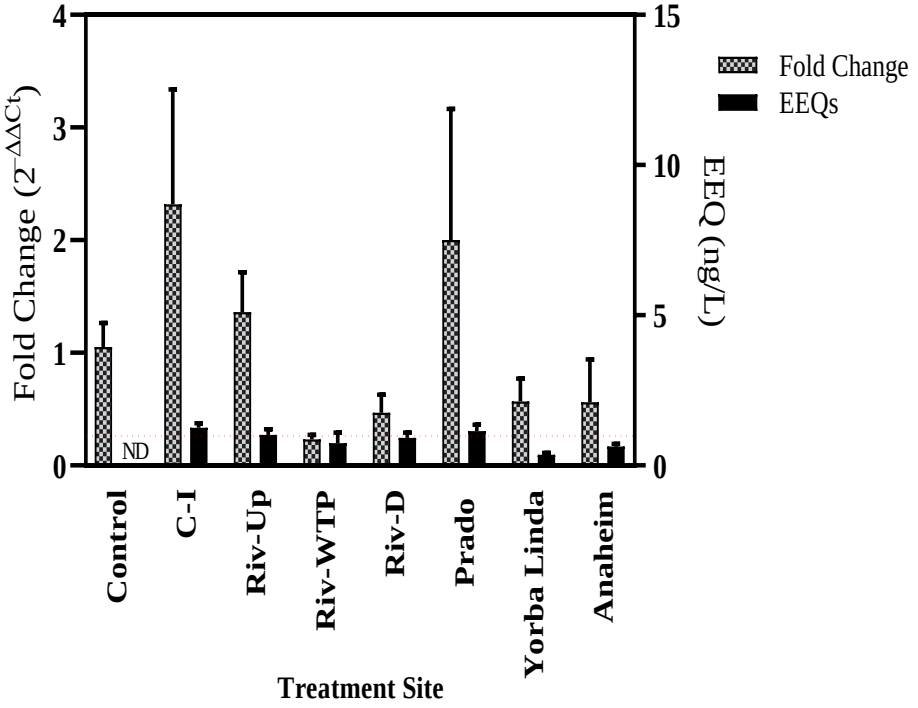



**Fig. 3.** Effects of extracts of water samples from the Santa Ana River collected in November 2018 on estrogen receptor activity from Vm7Luc4E2 cell bioassays and vitellogenin expression change in juvenile Japanese Medaka (*Oryzias latipes*) following a 14d exposure. Each value represents the mean of three replicates ± SEM. Dotted line represents PNEC (1 ng/L).

- Elevated HSI compared to control treatment
- ~ 2-fold increase in *vtg* expression relative to control at sites > 1 ng/L EEQ
  - No induction in treatments >1 ng/L EEQ
  - Not significant activity compared to previous effluent exposure studies

# Comparison of *In Vitro* and Chemical-Based EEQs

- Chemical and *in vitro* EEQs correlated significantly
  - (r = 0.756, *p* = 0.03, Pearson Correlation)
- Steroidal estrogen load greatest at RIX downstream and Yorba Linda
- Riverside WWTP downstream had minimal steroidal but significant activity *in vitro*
  - Increased influent due to storm decreases treatment efficiencies
  - Other compounds in effluent – e.g. BPA, alkylphenol ethoxylates

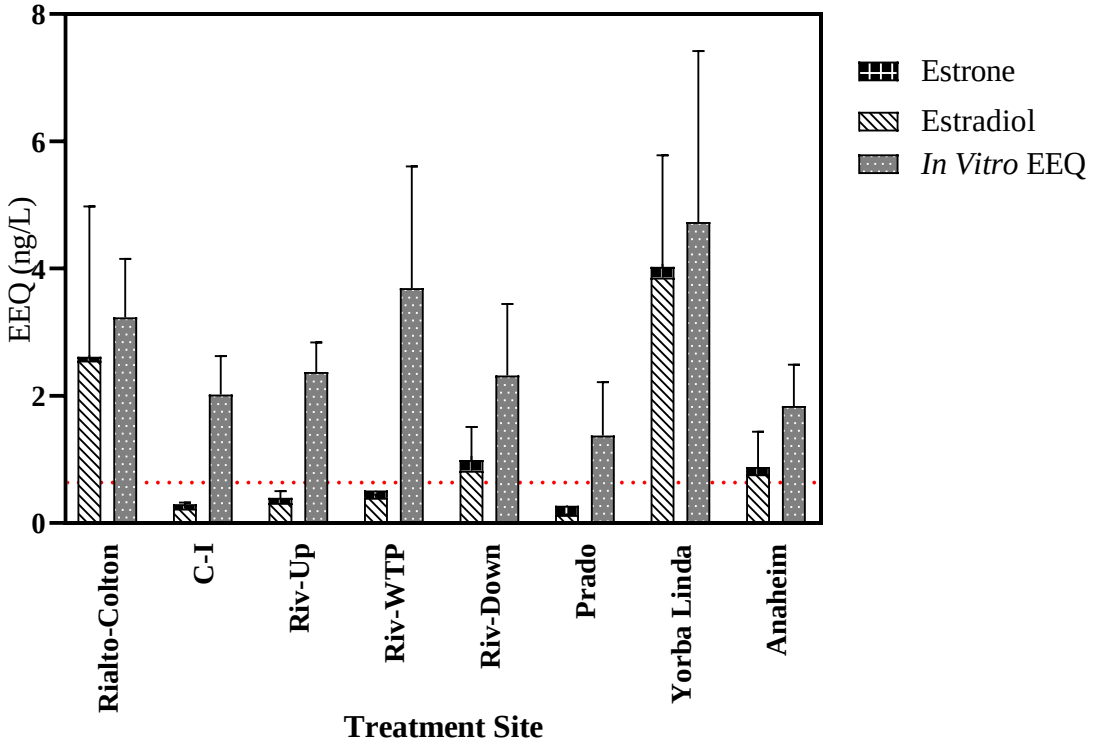



**Fig. 4.** Chemical and *in vitro* biological evaluation of water extracts from the Santa Ana River, collected in January 2019 after rain event. Each value represent the mean of three replicates ± SEM. Dotted line represents the PNEC (1 ng/L).

# Conclusions

- Tertiary treatment effective, river extracts mildly-estrogenic during base-flow conditions (1 ng/L)
  - Consistent with other tertiary effluent exposure studies
- WWTP discharge sites did not exhibit significant estrogen receptor activity compared to other sampled sites along river
- Muted *in vivo* response with small increase in *vtg* mRNA expression compared to control
  - *In Vitro* and *In Vivo* activity didn't correlate
    - Potentially due to bioavailability – adsorption to river sediments or presence of compounds that are not ER ligands but can increase endogenous E2

# Conclusions

- Chemical and *in vitro* EEQs correlated well
  - Significant increase in receptor activity following a rain event
    - Indicate non-point source EAC contributions – urban runoff or CSOs
- Estrogenic activity was variable by season
  - EEQs higher during base-flow conditions compared to after an increase in stream flow
  - Significant increase in ER activity following rain-event
    - Future studies could perform sampling events at times not influenced by active stormwater discharge in order to assess the temporal variability of estrogenic activity in the river

# Thanks!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- Heather Dyer, SBV MWD

- Department of Environmental Sciences
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Questions?