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Downstream trends of *in vitro* bioassay responses in a wastewater effluent-dominated river

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Abstract

Surface waters are becoming increasingly influenced by wastewater effluents due to drought conditions, growing populations, and urbanization. These effluents contain mixtures of trace organic compounds (TOrCs), including bioactive constituents, which are not fully attenuated by conventional wastewater treatment systems. This study investigated the occurrence of glucocorticoid receptor (GR), aryl hydrocarbon receptor (AhR), and estrogen receptor (ER) activity, as well as the overall toxicity to bacteria (BLT-Screen), in the effluent of two wastewater reclamation facilities (WRF) and downstream of the Lower Santa Cruz River, Pima County, Arizona USA, which is dominated by the WRF effluents. The GR, AhR, and ER activities and toxicity to bacteria were determined by *in vitro* bioassays during four seasons. Bioassay results showed the highest activities at the wastewater outfalls, with activities decreasing downstream of the river. Biological equivalent concentrations ranged from 9 to 170 ng/L dexamethasone-equivalents (DexEQ), 0.1 to 0.8 ng/L 2,3,7,8-tetrachlorodibenzo-p-dioxin-equivalents (TCDDEQ), and <0.005 to 0.8 ng/L estradiol equivalents (EEQ) for GR-, AhR- and ER-mediated activity, respectively. This level of biological activity at times exceeded the relevant effects-based trigger value for environmental effects, indicating a potential risk to the receiving environment. Toxicity to bacteria was low at all sites, well below the trigger value of 1.0 TU_{IC20}, which represents an undiluted water sample causing 20% toxicity in the assay. The potential inducing glucocorticoid agonists were further analysed by liquid chromatography coupled to tandem mass spectrometry. Analytical results reveal triamcinolone acetonide as the most abundant glucocorticoid with concentrations up to 38 ng/L. Similar results for DexEQ concentrations calculated from both chemical and bioassay data indicate a successful mass

balance for glucocorticoids. This mass balance illustrated lower DexEQ during summer months, which could be due to an increased attenuation from photodegradation.

Keywords: AhR, BLT-Screen, estrogenic, glucocorticoid, *in vitro* bioassay, seasonal variation

1.0 Introduction

1.1 Background

Many surface waters in the USA are influenced by wastewater effluents (Rice et al., 2013) and are becoming increasingly impacted since the volume of waste streams entering surface waters continues to rise. In 2013, the United States had nearly 15,000 municipal wastewater treatment plants processing over 32,000 million gallons per day (Pabi et al., 2013). Of those facilities, 85% of the effluents were directly discharged into surface waters. The ecosystem in surface waters receiving these effluents can experience detrimental effects as a result of exposure, such as reduced aquatic diversity (Boyle and Fraleigh, 2003; Paul and Meyer, 2001). This trend in ecosystem disruption can be linked to effluents containing mixtures of trace organic contaminants (TOrCs) such as pharmaceuticals, personal care products, natural hormones and industrial/commercial compounds, which may persist throughout treatment processes (Westerhoff et al., 2005). Treatment technologies that address TOrCs vary in cost and removal efficacy, making treatment challenging and requiring site-specific planning based on local TOrC mixtures in order to achieve desired reductions of respective compounds (Snyder et al., 2003). This is especially critical since TOrC always occur as mixtures, yet standards and guidelines applied are nearly all based on single compound exposures. Thus, the use of *in vitro* bioassays for water quality evaluation is critical and efficient, as will be demonstrated by the data presented in this manuscript.

1.2 Glucocorticoid Activity

The glucocorticoid receptor (GR) is a nuclear receptor that functions as a part of the endocrine system, responsible for subsequent regulation of glucocorticoid-related gene expression, which modulates metabolism, stress adaption, and immune system response in vertebrate animals. GR agonists include endogenous glucocorticoids (e.g. cortisone), as well as synthetic compounds manufactured to treat various health conditions including asthma, rheumatic diseases, and other inflammatory problems. As such, glucocorticoids can act as endocrine disrupting compounds (EDCs) since they interfere with hormone receptor signalling involved in maintenance of natural homeostasis.

Glucocorticoids are partially attenuated by conventional wastewater treatment when they enter municipal waste streams. Glucocorticoid concentrations in wastewater effluents have been reported to range from tens to thousands of nanograms per liter (ng/L), while receiving surface waters range from below the limits of detection (<LOD; 0.5-8 ng/L) to low hundreds of ng/L (Ammann et al., 2014; Chang et al., 2009; Li et al., 2007). Using *in vitro* bioassays, glucocorticoid activity in treated wastewater effluents has been reported in the tens to hundreds of ng/L dexamethasone equivalents (DexEQ) (van der Linden et al., 2008; Leusch et al., 2014; Jia et al., 2016). In the US, glucocorticoid activity was detected in 27% (n=115) of surface water samples collected in 14 states (Stavreva et al., 2012). According to Jia et al. (2016), reverse osmosis and monochromatic ultraviolet light sufficiently removed glucocorticoid activity from a secondary wastewater effluent, while chlorination, ozone, and microfiltration were less effective. Additionally, wastewater influence may not be the only source of GR activity in the environment. For example, surface waters that are influenced by agricultural effluents and not WWTP effluents were also observed to elicit GR response (Macikova et al., 2014).

At environmentally relevant concentrations, glucocorticoids can induce negative health impacts on aquatic organisms (Kugathas and Sumpter, 2011; Macikova et al., 2014; Guiloski et al., 2015). There is a high health risk to aquatic organisms when exposed to as little as tens of ng/L of glucocorticoids, according to fish models (Macikova et al., 2014). An effect based trigger (EBT) value of 100 ng/L DexEQ has been proposed for environmental surface waters (van der Oost et al., 2017), while an EBT value of 150 ng/L DexEQ has been proposed for drinking water (Escher et al., 2015). Only a handful of studies have measured anti-glucocorticoid activity in waste and surface waters, and no anti-glucocorticoid activity has so far been reported (<15 ng/L mifepristone equivalents MifEQ; Leusch et al., 2017). There is currently no EBT for anti-glucocorticoid activity in water.

1.3 AhR Activity

The aryl hydrocarbon receptor (AhR) is a well-known example of a xenobiotic receptor, which initiates xenobiotic metabolism in response to exposure to dioxin-like chemicals. The AhR assay has been commonly used to gauge remediation of PCBs and dioxins in environmental spill scenarios (Hilscherová et al., 2000). Some of the most potent AhR agonists are dioxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzodioxins (PCDDs), and dioxin-like chemicals including polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Dioxins originate from various sources, including industrial manufacturing and combustion of natural products (Kulkarni et al., 2008). Many AhR agonists accumulate in the environment and within animal tissues (Whyte et al., 2004), posing a significant environmental challenge because of the serious health risks associated with unnatural activation of the AhR by agonists. For instance, numerous health risk studies have linked TCDD exposure to cancer in humans (Bertazzi et al., 2001; Mandal, 2005). Dioxin-like PCB exposures may also be a factor that causes endometriosis in humans, further providing a link to endocrine disruption (Louis et al., 2005). As such, the United States Environmental Protection Agency (USEPA) set the maximum contaminant level (MCL) at 0.03 ng/L in drinking water for TCDD – one of the most potent dioxins (USEPA, 1998).

After wastewater treatment processes, dioxins are typically found at low concentrations in the dissolved aqueous phase, and they tend to concentrate in sludge due to low water solubility (Rodriguez et al., 2008). Therefore, tertiary-filtration wastewater treatment systems, which remove most suspended solids, have better removal efficiencies for dioxins and dioxin-like compounds than conventional secondary treatment systems (Dagnino et al., 2010; Rodriguez et al., 2008). Surface water samples collected downstream of a pulp mill effluent were shown to have between 0.12 to 2.67 ng/L TCDDEQ (Parrott and Tillitt, 1997), which exceeds the EBT of 0.05 ng/L TCDDEQ proposed for environmental waters (van der Oost et al., 2017). Given the high potency of dioxins, even small concentrations in the environment are of great concern.

1.4 Estrogenic Activity

Estrogenic activity is the most extensively studied class of endocrine disruption in water (Leusch et al., 2017). Natural (e.g. 17β-estradiol and estrone) and synthetic (e.g. 17α-ethinylestradiol) hormones are potent estrogenic compounds frequently detected at the low ng/L range in treated wastewater effluents, alongside less potent but often higher concentration of xeno-estrogens such as bisphenol A, nonylphenol, phthalates, metals, personal care products, and pesticides (Bolong et al., 2009). These estrogenic compounds have been linked to feminisation of wild fish in streams receiving wastewater (Tyler and Filby, 2011). Removal of estrogenic compounds during wastewater treatment varies significantly with treatment technology and chemical properties (Luo et al., 2014; Melvin and Leusch, 2016; Wang and Wang, 2016; Westerhoff et al., 2005), ranging from 40 to >99%. The estrogenic activity in treated wastewater usually ranges from below detection limit (approximately 0.01 ng/L) to 10-100 ng/L estradiol equivalents (EEQ) depending on the assay, with concentrations in surface waters usually ranging from below detection limit to 1-20 ng/L (Leusch et al., 2017). These concentrations are of concern when compared with EBTs ranging from 0.1-0.5 ng/L EEQ for chronic ecological effects (Jarošová et al., 2014; van der Oost et al., 2017) and 0.2-3.8 ng/L EEQ for drinking water (Brand *et al.*, 2013; Escher et al., 2015), depending on the assay. There is significantly less work on anti-estrogenic activity in water, and the available studies suggest anti-estrogenic activity tends to be below detection limits, <500 ng/L tamoxifen equivalents TMXEQ (Leusch et al., 2017). There is currently no EBT for anti-estrogenic activity.

1.5 Toxicity to Bacteria (Baseline Toxicity)

In addition to measuring xenobiotic metabolism (such as AhR) and specific modes of action (such as ER and GR), it is also important to understand the non-specific baseline toxicity of a water body receiving wastewater effluent. Recent non-targeted analytical methods indicate that there can be thousands of anthropogenic compounds present in wastewater effluent, with a wide range in modes of action (Singer et al., 2016; Blum et al., 2017). These highly variable chemical mixtures in wastewater effluent suggest that non-specific toxicity bioassays that can assess the overall toxicity of a water sample are an important tool for assessing the impacts of effluent in receiving environments.

Bacterial luminescence assays, such as Microtox and the Bacterial Luminescence Toxicity Screen (BLT-Screen), are commonly used for assessing the basal toxicity of water samples (Escher et al., 2014; Nguyen et al., 2016). In these assays, naturally luminescent bacteria are exposed to water samples or water extracts and non-specific toxicity is measured by quantifying inhibition of bacterial luminescence (van de Merwe & Leusch, 2015). Response in non-specific assays such as the BLT-Screen can be due to the presence of a small number of TOrCs that are highly toxic to bacteria (e.g. anti-bacterial agents) and/or complex mixtures of many compounds that are less toxic to bacteria (van de Merwe and Leusch, 2015). Therefore, these assays allow for rapid assessment of the basal toxicity of wastewater effluents and receiving waterways, providing information on relative toxicity over time and distance.

Basal toxicity can be high in wastewater samples, with toxic units (TUs) often >1 (Leusch et al., 2014; van de Merwe and Leusch, 2015), indicating that the mixture of chemicals in undiluted effluent can cause toxicity to bacteria. However, due to rapid dilution following discharge into receiving waterways, basal toxicity is generally much lower in surface water samples, with TU values values generally <1 and often <0.1 (Macova et al., 2011; Escher et al., 2014; Leusch et al., 2014).

1.6 Aim of this study

The purpose of this study was to characterize the trends of GR, AhR, and ER agonists as well as baseline toxicity to bacteria in a wastewater effluent-dependent surface flow of the Santa Cruz River (SCR) using *in vitro* bioassays. In addition, chemical analysis was performed to identify known GR agonists and provide a mass balance.

2.0 Experimental Section

2.1 Chemicals and Reagents

In vitro AhR assay media components were: alpha-MEM Cell Culture Media (Invitrogen, 12000-063), Premium Fetal Bovine Serum (Atlanta Biologicals, S11150), phenol red-free

DMEM (Gibco, 21063-045), charcoal-stripped FBS (Corning, 35-072-CV), Penicillin-Streptomycin (Gibco, 15140-122), Sodium Pyruvate (Gibco, 1136-070), and NEAA (Gibco, 11140-050). AhR assay reagents, Luciferase Assay Lysis Buffer (PR-E1531) and Promega Luciferase Assay System (PR-E1501) were purchased from Fisher Scientific, USA.

For GR assay, the LiveBLAzer™-FRET B/G Loading Kit with CCF4-AM was purchased from Life Technologies (K1095), and PBS (pH 7.4) was purchased from Gibco (10010049). HPLC grade water, methanol, acetonitrile, methyl tert-butyl ether, and acetic acid were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Twenty-eight glucocorticoid standards (Table S1) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrocortisone-d_2, prednisone-d_8 and 6α-methylprednisolone-d_2 were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Canada). Corticosterone-d_8, cortisone-d_8, fluticasone propionate-d_5, triamcinolone-$^{13}C_3$ were all purchased from Toronto Research Chemicals Inc. (Ontario, Canada). 2,3,7,8-Tetrachlorodibenzo-p-dioxin was purchased from AccuStandard (D-404N).

For the ER assay, GeneBLAzer ERα-UAS-*bla* GripTite™ cells, LiveBLAzer™-FRET B/G Loading Kit, beta-lactamase loading solutions and Dulbecco’s Modified Eagle Medium for cell culturing were purchased from Life Technologies (Carlsbad, CA). PBS, 17β-estradiol (agonist standard) and tamoxifen (antagonist standard) were purchased form Sigma-Aldrich. Poly-D-Lysine Cellware 384-well plates were purchased from Beckton Dickinson Labware (Bedford, MA).

For the BLT-Screen, *Photobacterium leiognathi* stock (ATCC® 33469™) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Ingredients for the bacterial growth and assay media (KH_2PO_4, bacto-peptone, yeast extract, NaCl, $MgSO_4.7H_2O$,

$MgCl_2.6H_2O$, $CaCl_2.2H_2O$ and KCl), the pentachlorophenol standard and white flat bottom 96-well plates were purchased from Sigma-Aldrich.

2.2 Sampling and Storage

The Lower Santa Cruz River (SCR) is a wastewater effluent-dominated stream in Pima County, Arizona. It has been ephemeral, only flowing during large rain events as groundwater withdraws have lowered the water table since the early 1900s. Pima County discharges wastewater effluent from two water reclamation facilities (WRFs) into the SCR, Agua Nueva WRF (ANWRF) outfalls and Tres Ríos WRF (TRWRF) outfalls, which flows for approximately 20 to 40 km before it infiltrates below the surface. The WRF furthest upstream of the SCR is the ANWRF (sampling site SCR-01), and the effluent from the TRWRF enters approximately 7.6 km downstream (sampling site SCR-04; Fig. 1). ANWRF has a 120 million liter per day (ML/D) limit usingflocculation/grit removal, dissolved air flotation clarification, 5-stage Bardenpho, tertiary filtration, and chloramine disinfection, while the TRWRF has a 272 ML/D capacity and is composed of flocculation/sedimentation, activated sludge, and disinfection with hypochlorite. For most the year, the water flowing in the SCR channel is almost exclusively composed of WRF effluent; therefore, it is an exceptional site to investigate how wastewater effluents and their constituents behave and interact with the environment in the absence of dilution from perennial surface water flow.

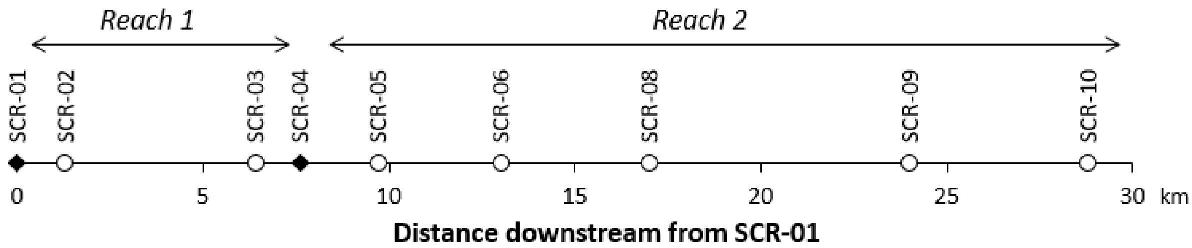

Figure 1. Schematic of the Santa Cruz River and sampling sites for this study. Wastewater discharge outlets (SCR-01 and SCR-04) are highlighted by filled diamonds. All other river sites are indicated by open circles. Samples SCR-07 and SCR-11 were field and laboratory blanks, respectively.

The SCR was sampled at nine sites on four different dates over the course of a year: May 12, 2014; September 22, 2014; December 1, 2014; and February 12, 2015. Sampling events collected 4 L grab samples from an upstream to downstream manner beginning with the ANWRF outfall (SCR-01) in the absence of precipitation-induced surface flow. Sampling sites are presented in the schematic in Fig. 1, and sampling details are provided in Supplemental Information (Table S2). SCR-01 and SCR-04 (stream km 0.0 and 7.6) are located at the outfalls of ANWRF and TRWRF, respectively. Reach 1 of this study flows from the ANWRF outfall to the downstream-most site before the TRWRF outfall, SCR-03 (stream km 6.4) or SCR-02 (stream km 1.3) if dry (Fig. 1). Reach 2 of this study flows from SCR-05 (stream km 9.7), the mixing zone of both WRF waters, to the farthest downstream site SCR-10 (stream km 28.8) or SCR08 (stream km 17.0) if dry (Fig. 1).

WRF effluent was the sole source of flow in the SCR during sampling events, thus surface flow upstream of the ANWRF was absent. In September and December, channel SCR-03 was dry, preventing samples from being collected at this site. This was also the case for the channel at SCR-09 (steam km 24.0) and SCR-10, in February. During the February event, the channel was stagnant at SCR03, but a sample was still collected.

Water samples were collected in 1 L glass amber bottles by directly filling the bottle in the river or using a polypropylene bucket at inaccessible locations to collect the sample and then pour into the 1 L glass amber glass. Before collecting the sample at each site, all equipment was rinsed three times with the respective water. During each sampling event, a field blank, using Milli-Q water, and a true triplicate sample was collected at each of the sites. Samples were transported in coolers with ice back to the lab, where they were stored in 4ºC for up to 48 h prior to extraction. Historical data of SCR water quality from quarterly river campaigns were extracted from Living River Reports (Zugmeyer, et al., 2016). The river campaign data corresponding with this study's sample collections are presented in the supplementary information (Table S3).

2.3 Sample Preparation

Samples were filtered with a 47 mm, 0.7 µm GF/F fiberglass filters (GF/F 0.7 µm, Whatman, Maidstone, UK) and solid phase extraction (SPE) was performed using previously published methods (Mehinto *et al*. 2015; Jia *et al*. 2016). Briefly, a Dionex Autotrace 280 SPE instrument was used with a Hydrophilic-Lipophilic Balance (HLB, 500 mg/6cc) cartridge (Waters Corporation, Millford, MA), which was pre-conditioned with 5 mL of methyl tert-butyl ether (MTBE), 5 mL methanol, and 5 mL of ultra-pure water. Samples were then loaded onto the cartridge, rinsed with 10 mL of ultrapure water, dried for at least an hour with nitrogen, and eluted with 5 mL of methanol followed by 5 mL of a 10/90 (v/v) methanol/MTBE mix. After elution, samples were evaporated and raised to a final volume of 1 mL, which was divided into two aliquots for bioassay and chemical analysis. The aliquot for bioassay was then further evaporated and reconstituted in DMSO to achieve a 4,000-fold enrichment. The extract aliquot for chemical analysis went through an additional clean up step (Jia et al., 2016). Briefly, a silica cartridge (500 mg/6cc, Waters), pre-conditioned with 4 mL of water-saturated ethyl acetate and 4 mL of a 90/10 (v/v) hexane/ethyl acetate was used. After the samples were loaded, they were then rinsed with 3 mL of 90/10 (v/v) hexane/ethyl acetate, dried with nitrogen, and eluted with 3 mL of 38/62 (v/v) hexane/ethyl acetate. Lastly, the samples were evaporated and reconstituted in 1 mL of methanol, resulting in a 2,000-fold enrichment. An ultrapure water sample was extracted in parallel to the samples, and no bioassay activity nor chemicals were detected in these laboratory blanks.

2.4 Glucocorticoid Activity

To quantify GR activity, the GR-GeneBLAzer assay, a commercially available stable human embryonic kidney cell line with a beta-lactamase reporter gene (GR-UAS-bla HEK 293T, Life Technologies Corporation, Grand Island NY) was used. Briefly, 6.25×10^{5} cells/mL were added to black wall, clear bottom 96-well plates (Greiner Bio One, 655090) and incubated for 16 h with SCR extracts in triplicate, in addition to a 1×10^{-7} to 1×10^{-11} M dexamethasone positive control dose curve in duplicate (Mehinto et al., 2015) to achieve an EC_{50} of 2.9×10^{-9} M. Then the LiveBLAzer™-FRET B/G substrate mixture (CCF4-AM) was added for 4 h before fluorescence was measured in a FlexStation 3 Multimode Plate Reader (Molecular Devices, Sunnyvill CA). Negative controls included cells exposed to 1% DMSO, 0% DMSO, and a cell-free blank containing 1% DMSO. The excitation wavelength was 409/20 nm, with emission at 460/40 nm and 530/30 nm for green and blue wavelengths, respectively.

To evaluate a potential laboratory bias, an interlaboratory parallel analysis for GR activity was completed on samples from two different sampling events. Aliquots from May and February extracts were analyzed by Griffith University, Southport, Queensland using GR-UAS-bla HEK 293T cells in agonist and antagonist modes (Mehinto et al., 2015). Fluorescence was measured in a FLUOstar plate reader (BMG Labtech, Germany) at 460 and 520 nm after excitation at 410 nm and the data expressed as the ratio of fluorescence at 460 divided by 520. Dexamethasone was used as the agonist reference compound ($EC_{50} = 2.0\times10^{-9}$ M), while mifepristone was used as the antagonist reference compound ($EC_{50} = 1.8\times10^{-9}$ M) in the presence of a competing dexamethasone EC_{80} concentration, as recommended for antagonist analysis in Neale and Leusch (2015). The limits of detections based on the EC_{10} and IC_{20} were 5 ng/L DexEQ and 5 ng/L mifepristone equivalents (MifEQ), respectively.

2.5 Glucocorticoid Chemical Analysis

The concentration of the 28 targeted glucocorticoids were determined using a previously published method (Jia et al., 2016). Briefly, a UHPLC-MS/MS (6490 Agilent Technologies, Santa Clara, CA) equipped with a ZORBAX Eclipse Plus C8 RRHT column (100 mm × 2.1 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA) was applied. The instrument detection limit (IDL) for each analyte is provided in the Supplemental Information (Table S4). Glucocorticoid concentrations were converted to chemical dexamethasone equivalent (cDexEQ) concentrations by multiplying the concentration by the relative potencies (REPs) listed in Table S5 (Jia et al. 2016).

2.6 AhR Activity

A chemical-activated luciferase gene expression (CALUX) AhR *in vitro* bioassay was used to quantify dioxins and other compounds that induce the activity of the CYP1A1 gene as a part of a cellular toxic response mechanism (Garrison et al., 1996). Methods described in He et al. (2014) were followed with slight modifications using a H4L1.1c2 rat hepatoma cell line. Triplicate wells at 7.5×10^{5} cells/mL were exposed to extracts and a 1×10^{-7} to 1×10^{-12} M 2,3,7,8-TCDD positive control dose curve. Negative controls included cells exposed to 1% DMSO, 0% DMSO, and a cell-free blank containing 1% DMSO. A Beckman Coulter Biomek FX liquid handling workstation performed serial dilutions on a working plate (Thomas Scientific, 1223T96). Lysed cells were shaken in a LiCONiC STX44-HRSA. A BioTek Synergy 2 plate reader auto-added 50 microliters (µL) luciferase reagent to each well and measured luminescence.

2.7 Estrogenic Activity

GeneBLAzer ERα-UAS-*bla* GripTite cells were used to quantify ER-mediated gene activation, following methods described in Escher et al. (2014) and the manufacturer's protocols, with slight modifications. The assay was run in both agonist and antagonist modes, and a serial dilution of each sample was tested on at least two separate occasions. Fluorescence was measured in a FLUOstar plate reader (BMG Labtech, Germany) at 460 and 520 nm after excitation at 410 nm, and the data expressed as the ratio of fluorescence at 460 divided by 520. 17β-estradiol ($EC_{50} = 1.4\times10^{-11}$ M) and tamoxifen ($EC_{50} = 1.5\times10^{-6}$ M, in the presence of an EC_{80} concentration of 17β-estradiol) were used as the agonist and antagonist reference compounds, respectively. The sample results were expressed as 17β-estradiol (EEQ; agonist) and tamoxifen (TMXEQ; antagonist) equivalent concentrations. The limits of detection based on the EC_{10} and IC_{20} were 0.005 ng/L EEQ and 5 μg/L TMXEQ, respectively.

2.8 Toxicity to Bacteria (Baseline Toxicity)

Baseline toxicity to bacteria was measured using the bacterial luminescence toxicity screen (BLT-Screen) assay as previously described (van de Merwe and Leusch, 2015) . Briefly, sample extracts were serially diluted in PBS in a 96-well plate. A cryopreserved aliquot of the luminescent bacteria, *Photobacterium leiognathi*, was then added to each well. Pentachlorophenol (EC50 = 0.17 μM) was used as the reference compound. After 30 min of exposure, the luminescence was measured in a FLUOstar plate reader (BMG Labtech, Germany). The inhibition of luminescence in each well was calculated relative to controls using the equation: $\% \text{ Inhibition} = \left[1 - \left(\frac{\text{lum}_{\text{sample}}}{\text{lum}_{\text{control}}}\right)\right] \times 100$, and % inhibition was plotted against the relative enrichment factor (REF) of each sample. The $IC_{20(REF)}$ is the REF that causes 20% inhibition of bacterial luminescence was calculated for each sample using the linear section of the dose-response curve (<40% inhibition). The toxicity of each sample was expressed as a Toxic Unit (TU), the reciprocal of the $IC_{20(REF)}$ value. The limit of detection was 0.03 TU (*i.e.*, REF of >33).

2.9 Bioassay Data Analysis

The average response of the blank wells from each plate were subtracted from the GR and ER raw fluorescence and AhR raw luminescence values for each well. For the GeneBLAzer data (GR and ER assays), the effect was then expressed as the ratio of 460/520 (or 530). The effect (fluorescence ratio or luminescence, depending on the assay) was then converted to a % effect using equation (1):

Equation (1)

$$\% \ effect = (signal_{sample} - signal_{negative\ control})/(signal_{max} - signal_{negative\ control}))*100$$

Where $signal_{sample}$ is the bioassay response with the sample, $signal_{negative\ control}$ is the response with a solvent negative control and $signal_{max}$ is the highest response produced with the reference compound.

The 10% effective concentration (EC_{10}) in agonist mode and the 20% inhibitory concentration (IC_{20}) in antagonist mode (Escher et al., 2014) were calculated for each sample concentration-effect curve data from equation (2):

Equation (2)

$$logEC_x = logEC_{50} + 1/s * log[x / (100-x)]$$

Where s is the slope of the concentration-effect curve of the reference compound.

Equivalent concentrations (DexEQ and MifEQ for GR, EEQ and TMXEQ for ER and TCDDEQ for AhR) were determined using equation (3):

Equation (3)

$$Equivalent\ Concentration = POD_{positive\ control} / POD_{sample} * MW$$

Where *POD* is the point of departure (*i.e.*, EC_{10} in agonist mode and IC_{20} in antagonist mode) and *MW* is the molecular weight of the reference compound in g/mol.

DexEQ and calculated DexEQ (cDexEQ) concentrations were compared in a mass balance to identify if the limited analyte set measured in chemical analysis captured the magnitude of agonist-induced GR bioactivity (Jia et al., 2016).

3.0 Results and Discussion

3.1 Glucocorticoids

3.1.1 GR activity in the Santa Cruz River

The GR agonist activity of the SCR ranged from 9 to 170 ng/L DexEQ over the four sampling events (Figure). These results are consistent with previous results for this same stream, ranging from 39 to 155 ng/L DexEQ (Jia et al., 2016). Eleven of those samples exceeded the glucocorticoid EBT of 100 ng/L for environmental waters (van der Oost et al., 2017). Across all sampling events, concentrations decreased downstream from the WRF outfalls (km 0.0 and km 7.6). Reach 1 showed a 42% to 68% reduction in concentration with the exception of the December sampling event (8% reduction). In December, the surface water had fully infiltrated upstream from km 6.4, the downstream-most site of Reach 1. The Reach 1 downstream concentration reduction ranged from 8 to 41 ng/L/km. The Reach 2 downstream concentration reduction ranged from 48% to 89% (3.9 to 8.2 ng/L/km). Antagonism (anti-GR activity) was measured in the May 2014 and February 2015 samples and was below detection limit for all samples (< 5 ng/L MifEQ), and thus not contributing to the bioassay-derived DexEQ concentrations in the mass balance.

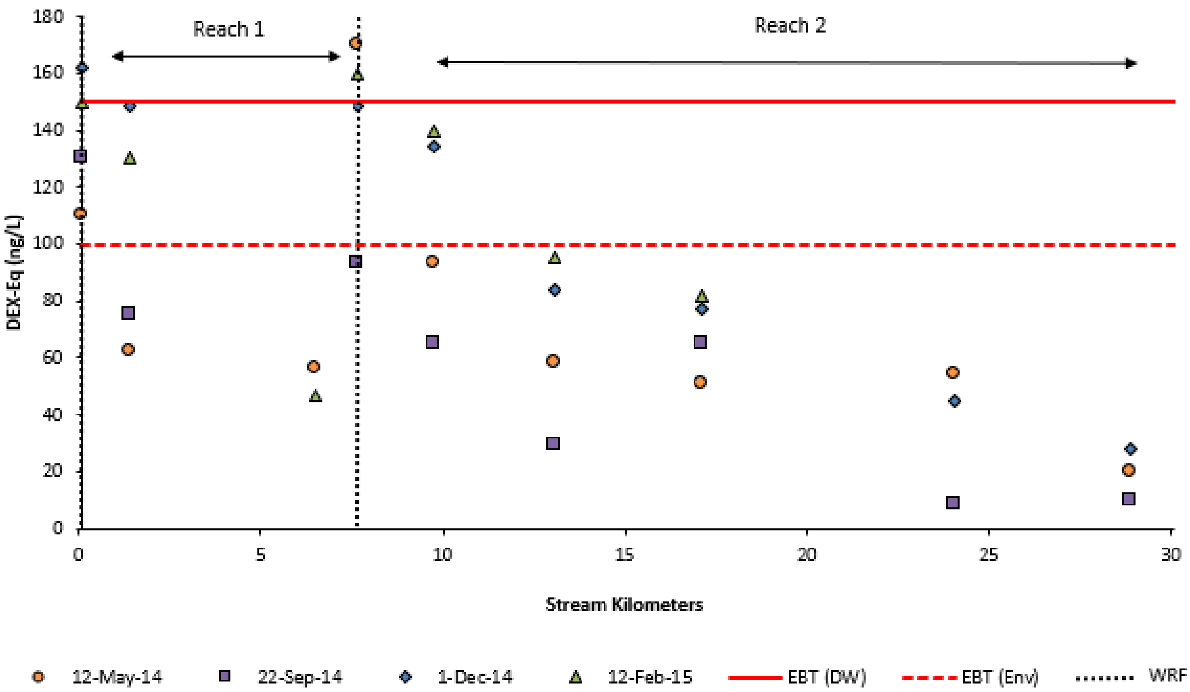

Figure 2. Glucocorticoid activity (expressed as DexEQ, ng/L) in the SCR. The dashed red line EBT(Env) at 100 ng/L DexEQ is the lowest-effect based trigger (EBT) value proposed by van der Oost et al. (2017) for environmental waters, while the full red line EBT (DW) at 150 ng/L DexEQ is the GR-GeneBLAzer EBT proposed by Escher et al. (2015) for drinking water.

The replicate inter-laboratory analysis for GR activity produced comparable results, both in terms of response of the assays to reference compounds (Table S6) and the calculated DexEQ activity in the water samples (Table S7). The mean relative standard deviation (RSD) for the DexEQ results between labs was 18.4% with the May 2014 samples and 15.6% with the February 2015 samples (Table S7), similar to a published interlaboratory parallel analysis on a GR bioassay calibration study using environmental samples (Mehinto et al., 2015). Both analyses showed the same trend of the highest bioactivity being measured at the WRF outfalls and decreased bioactivity proceeding downstream (Fig. S3).

Downstream concentration reduction may be influenced by the biodegradation or photodegradation of GR agonist compounds. Another study investigating glucocorticoid concentrations in biological wastewater treatment showed that concentrations were below or very close to reporting limits after exposure to both aerobic and anaerobic sludge (Liu et al., 2011). GR activity is associated with hydrophilic extract fractions, so decreasing downstream concentrations are not likely to be highly influenced by sorption to organic matter and sediments (Macikova et al., 2014). There is also a high possibility that GR agonists in the SCR are degraded by photolysis reactions. When exposed to 80 mJ/cm^2 ultraviolet light, (UV_{254}) complete GR agonist attenuation in a wastewater matrix was achieved in a bench scale study (Jia et al., 2016). In the SCR, effluents flow for hours to days, allowing sufficient exposure for photodegradation of some TOrCs (Quanrud et al., 2004).

3.1.2 Occurrence of GR agonists in the Santa Cruz River

Analytes were not detected in the field and laboratory blanks with the exception of the May and December field blanks containing 0.10 and 0.14 ng/L methylprednisolone (MPL), respectively. Therefore, MPL concentrations at or below these values were excluded (all December samples and two May samples: km 1.3 and km 17.0). Nine of the 28 glucocorticoid analysed were detectable in each SCR sampling event, with a total concentration ranging from 3.5 to 44.9 ng/L, with the highest concentrations at the WRF outfalls (km 0.0 and km 7.6;). Concentrations consistently decreased downstream from the WRF outfalls in all sampling events, which agrees with the GR bioassay results. The December sampling event had the greatest concentrations of glucocorticoids, where May had the lowest concentrations with about half that observed in the December sampling campaign. The GR agonist with the highest concentration was triamcinolone acetonide (TCA), which accounted for between 39% to 87% (1.4 to 38 ng/L) of total glucocorticoids. This is consistent with the findings from Jia et al. (2016) in which TCA accounted for 49% to 77% of total glucocorticoid concentration from four different WWTPs. Triamcinolone acetonide along with prednisolone (PNL) was present in all of the SCR samples. Cortisone (COR), betamethasone (BET), fluocinolone acetonide (FCA), hydrocortisone (HCT), clobetasol proprionate CBP), and fluticasone propionate (FTP) were present in >80% of samples.

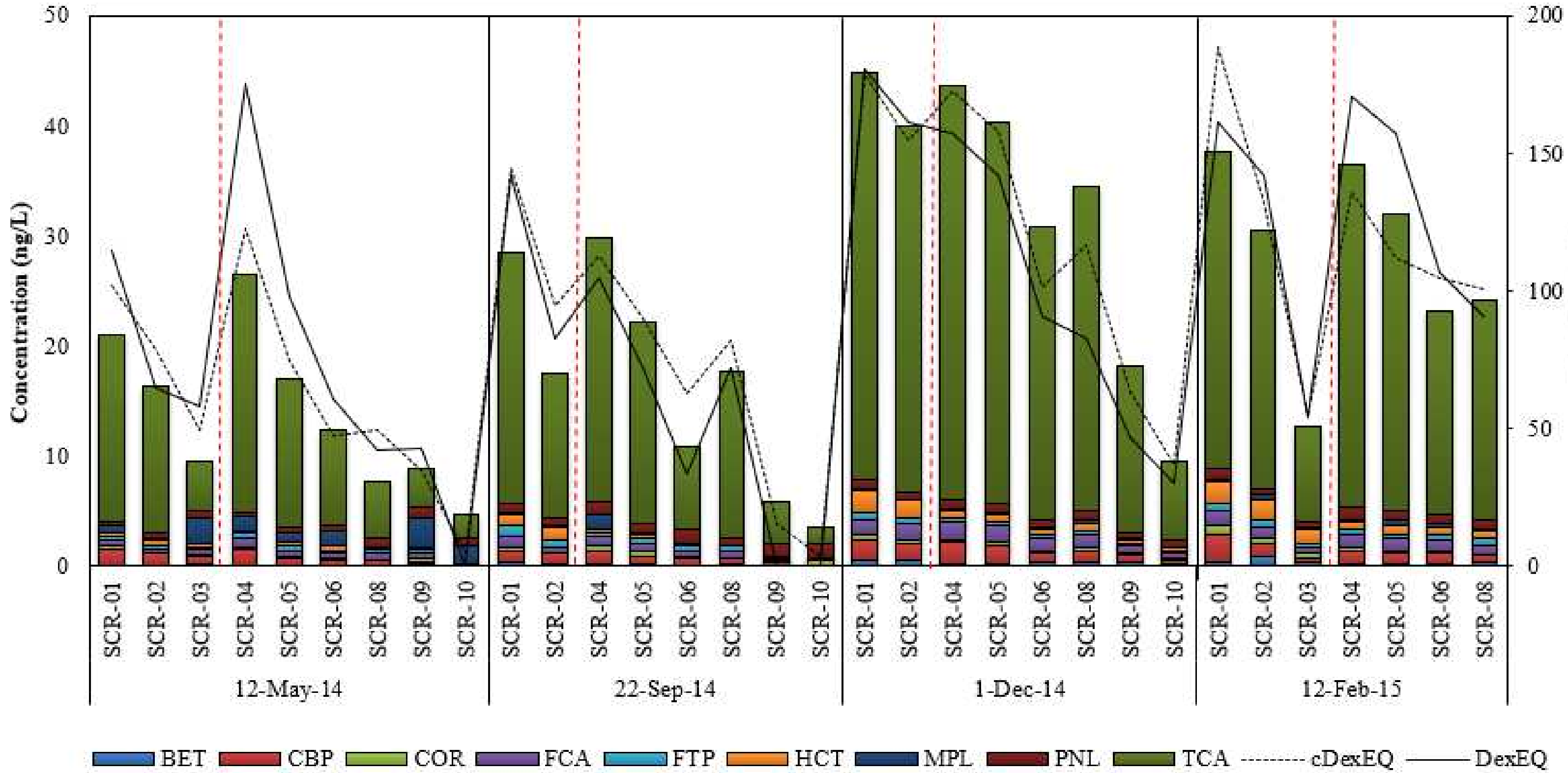

Figure 3. Concentrations of target glucocorticoids (expressed in ng/L) in the SCR. The red dashed line is the represents the second reach (ie TRWRF). The glucocorticoids detected were Triamcinolone acetonide (TCA), Prednisolone (PNL), Methylprednisolone (MPL), Hydrocortisone (HCT), Fluticasone propionate(FTP), Fluocinolone acetonide(FCA), Cortisone (COR), Clobetasol propionate (CBP), and Betamethasone (BET). These concentrations were then concerted to cDexEQ based on Jia et al. (2016) and expressed as the black dashed line. The black solid line is the DexEQ collected from the GR bioassay.

3.4.3 Glucocorticoid Mass Balance

DexEQ and cDexEQ concentrations for all samples are presented in Fig. 3 (full and dashed line, respectively), and specifically for the WRF outfalls at km 0.0 and 7.6 (SCR-01 and SCR-04, respectively) in Figure . Overall, the nine glucocorticoids identified and quantified in the WRF effluent (ANWRF and TRWRF) had a summed cDexEQ which was in good agreement with the DexEQ obtained from the bioassay data, indicating a successful mass balance. The cDexEQ concentrations were slightly higher than the DexEQ concentrations for all WRF outlet samples, except for the February and May results at km 7.6. This has also been documented and respectively attributed to the presence of antagonists (Macikova et al., 2014); however, the anti-GR bioassay results for May and February samples were below detection limit (<5 ng/L MifEQ), suggesting that this was not the case here. In addition, cDexEQ also showed the same trend of higher concentrations in the winter months compared to those collected during the summer. Analytical detection of individual glucocorticoid compounds is capable of predicting bioanalytical response in complex mixtures such as reclaimed water (Schriks et al., 2010; Jia et al., 2016). The present study provides another example of a successful mass balance of glucocorticoid receptor activity in an environmental system. The sum of TCA, FCA, CBP, and FTP contributions to cDexEQ in WRF effluents accounts for 99.5% to 99.8%, similar to those reported by Jia et al. (2016) of 97.7% to 99.8% in secondary effluents.

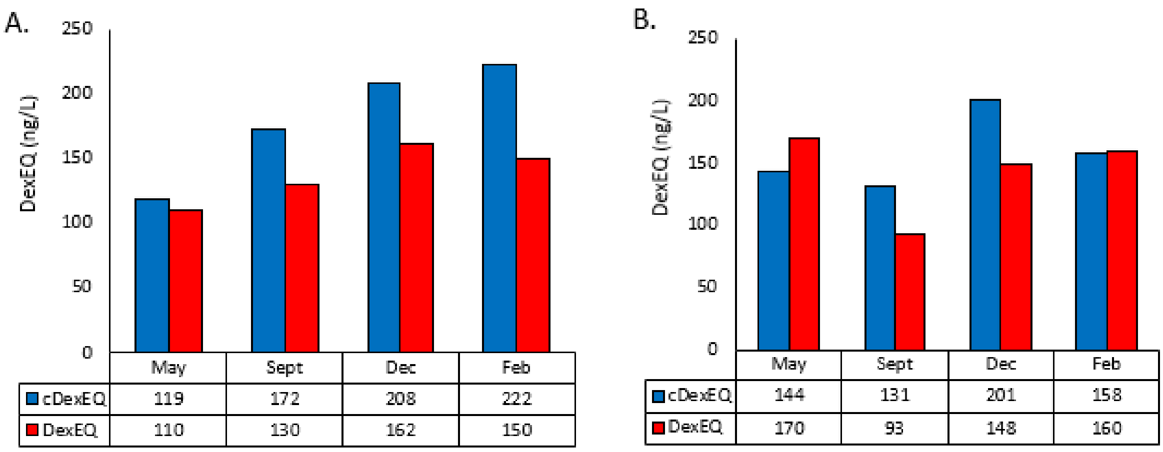

Figure 4. DexEQ vs. cDexEQ activity in ANWRF (A) and TRWRF (B) effluents (sampling sites SCR-01 and SCR-04, respectively).

3.2 AhR activity in the Santa Cruz River

The average TCDDEQ concentrations ranged between 0.1 and 1.0 ng/L (

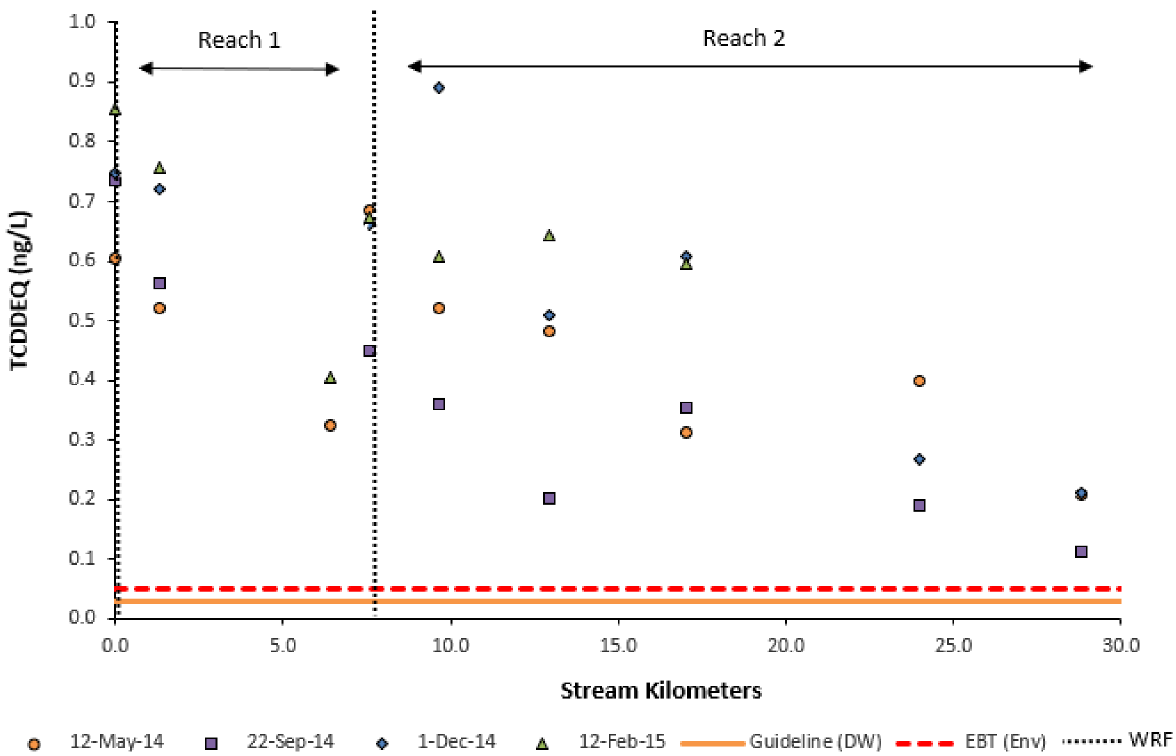

Figure 5), and every sample was above the EBT of 0.05 ng/ L for environmental waters. In addition, these values were one to two orders of magnitude above the drinking water guideline value of 0.03 ng/L set for TCDD. The amount of TCDDEQ is comparable to what was found by Dagnino et al. (2010), who reported 2.1±1.1 ng/L TCDDEQ after a lagoon-based water treatment facility (WTF). The overall trend of TCDDEQ for the SCR in this study showed the highest concentrations at the outfalls, with decreasing concentrations downstream. Reach 1 showed a 23% to 53% reduction in concentration with the exception of the December sampling event (4% reduction). However, there was no flow at km 6.4 (SCR-03) on that date. Reach 2 showed 11 to 74% downstream concentration reduction (0.003 to .02 ng/L/km). Possible causes of the downstream concentration reduction include photodegradation and sediment sorption and deposition. Kim & O'Keefe (2000) reported that dioxins and dioxin-like compounds can photodegrade in sunlight with half-lives on the order of several hours to a day. The SCR water flows down the channel from the ANWRF (SCR-01) for a few days before infiltration (Quanrud et al. 2004), which is a sufficient amount of time for photodegradation to occur. Dagnino et al. (2010) showed that AhR response was highest in wastewater suspended particles compared to the dissolved phase, making suspended particles a more-likely source of environmental contamination. Therefore, the affinity of AhR agonists to organic matter may lead to deposition in the sediments, which should be assessed as a potential sink in the SCR. The presence of AhR activity in the aqueous phase could be influenced by binding to dissolved organic carbon (DOC) or the presence of more-polar, dioxin-like compounds (Dagnino et al. 2010).

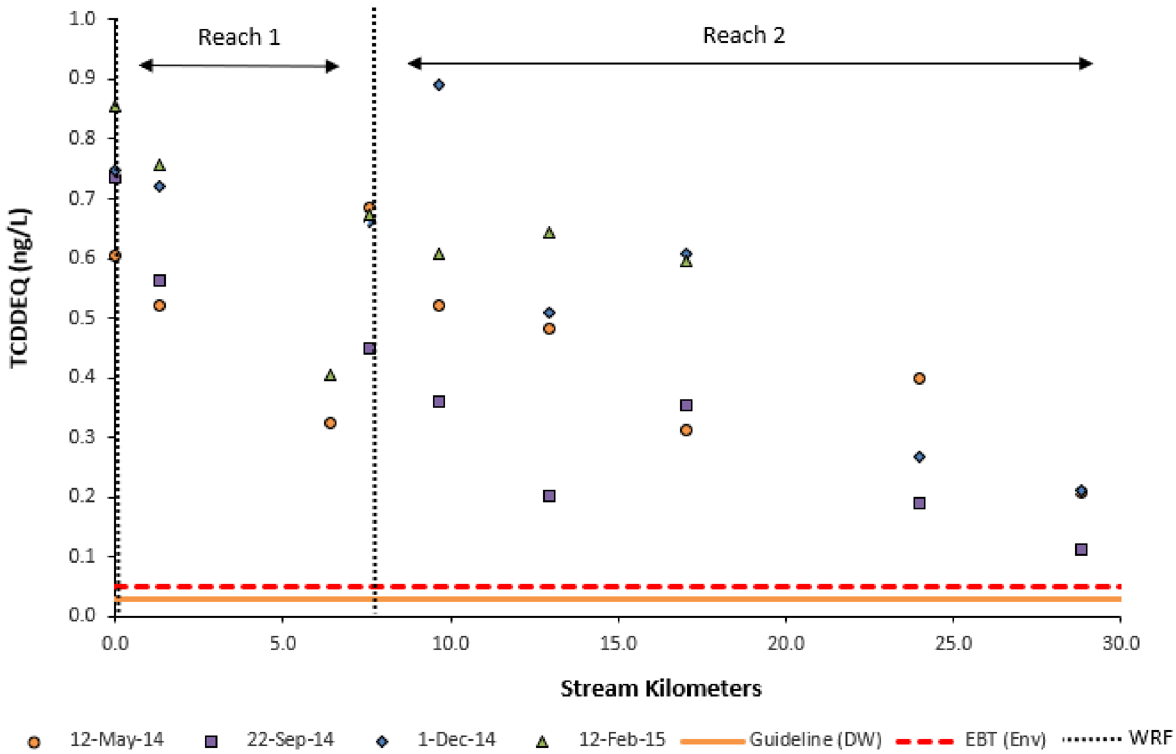

Figure 5. AhR activity (expressed as TCDDeQ, ng/L) in the SCR. The dashed red line EBT(Env) at 0.05 ng/L TCDDEQ is the lowest effect-based trigger (EBT) value proposed by van der Oost et al. (2017)for environmental waters, while the full orange line Guideline (DW) at 0.03 ng/L TCDDEQ is the guideline value proposed by the USEPA (1998) for TCDD in drinking water.

3.3 Estrogenic Activity

(Anti)-Estrogenic activity was only measured in May 2014 and Feb 2015. Estrogenic activity was generally low, with <1 ng/L EEQ in WRF effluents and the river samples. Only the two most up-stream samples in May 2014 were (slightly) above the lowest EBT of 0.1 ng/L EEQ proposed by Jarošová et al. (2014), and all other samples were below (Figure). A small decrease of 0.011 ng/L/km EEQ was apparent in Reach 1, but there was no attenuation of the low activity in Reach 2. The Feb 2015 samples were 2-25x higher than the May 2014 samples, but the activity decreased rapidly by 75-79% in both river reaches (a reduction of 0.07 to 0.13 ng/L/km EEQ) (Figure). Antagonism (anti-ER activity) was not detected in any of the samples (< 5 µg/L TMXEQ), except in SCR-01 and SCR-02 which were weakly antagonistic, just above the assay detection limit at 6 µg/L TMXEQ.

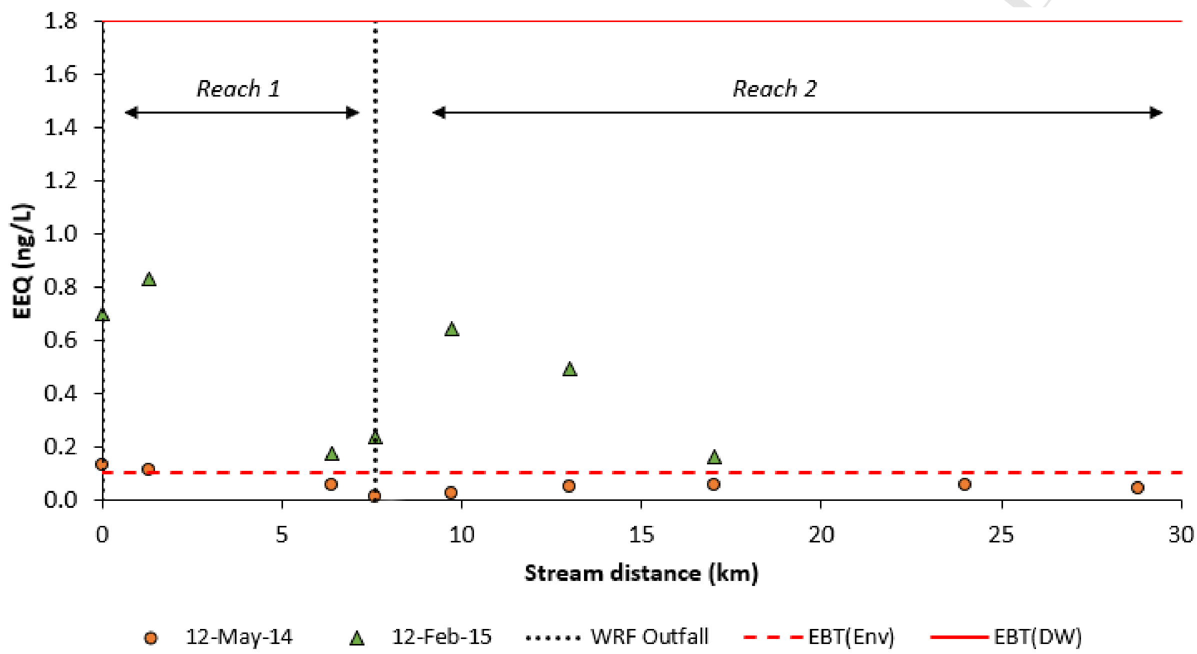

Figure 6. Estrogenic activity (expressed as EEQ, ng/L) in the SCR. The dashed red line EBT(Env) at 0.1 ng/L EEQ is the lowest effect-based trigger (EBT) value proposed by Jarošová et al. (2014) for environmental waters, while the full red line EBT(DW) at 1.8 ng/L EEQ is the ER-GeneBLAzer EBT proposed by Escher et al. (2015) for drinking water.

The estrogenic activity detected in the current study (<0.005 – 0.8 ng/L EEQ) is comparable to concentrations previously reported and predicted for wastewater-receiving surface waters elsewhere (Anderson et al., 2012; Scott et al., 2014; Van Der Linden et al., 2008). Estrogenic activity in wastewater-receiving surface waters is most likely driven by low ng/L concentrations of natural and synthetic hormones (Leusch et al., 2010), near or below their typical chemical analysis method detection limit of 1-5 ng/L. The natural hormones (such as 17β-estradiol and estrone) are usually efficiently removed during wastewater treatment with removal ranging from 74.8 to >99%, but can still frequently be detected in wastewater effluents as high as 80 ng/L (reviewed in Luo et al., 2014). Natural hormones; however, degrade rapidly in rivers due to microbial activity and photodegradation with average half-lives of 1-2 d in UK rivers (Jurgens et al., 2002). The EEQ concentrations and trends detected in the river in the Feb 2015 sampling would be typical of an input of natural estrogens such as estradiol and estrone, with a moderately high EEQ and a rapid decrease in the river. On the other hand, the synthetic hormone 17α-ethinylestradiol, the active ingredient of the birth control pill, is only moderately removed by conventional wastewater treatment (ranging from 43.8 to >99%, reviewed in Luo et al., 2014), is comparatively more persistent in surface water of 10-17d (Jurgens et al., 2002), and is one of the most potent estrogenic compounds both in *in vitro* test systems and whole animals (Leusch et al., 2009). Trace concentrations of 17α-ethinylestradiol remaining in the treated wastewater would therefore persist in the river for some time and the response in May 2014 sampling (and the baseline values in the Feb 2015) is typical of such a background contamination (Scott et al., 2014). The estrogenic activity detected here was always below the drinking water EBT of 1.8 ng/L (Escher et al., 2015) and was below the environmental EBT of 0.1-0.4 ng/L EEQ for long-term exposure (Jarošová et al., 2014) in May 2014, but above it in the Feb 2015 samples. Interestingly, while most of the Feb 2015 samples were above the long-term exposure EBT, they were at the bottom end of the window for short-term exposure (EBT ranging from 0.5-2.0 ng/L EEQ; Jarošová et al., 2014)). Considering the large difference between the two sampling events, further monitoring is necessary to determine if the higher values detected in

Feb 2015 are uncommon (in which case the risk of estrogenic endocrine disruption may be minimal) or representative of the typical concentrations in this river system.

3.4 Toxicity to Bacteria

As with estrogenic activity, basal toxicity was only measured in May 2014 and Feb 2015. The SCR samples were not particularly toxic to bacteria (Figure)., and the $TU_{IC20(REF)}$ values (0.1 - 0.3) were comparable to those reported for other surface water samples around the world (Macova et al., 2011; Escher et al., 2014; Leusch et al., 2014). All samples were well below the trigger value of 1.0 TU_{IC20}, which represents an undiluted water sample causing 20% toxicity in the assay. This indicates that although these SCR water samples may contain complex mixtures of TOrCs, the concentrations were generally low for compounds that are highly toxic to bacteria and water samples required SPE-concentration to produce a detectable toxic effect.

In the May 2014 samples, toxic unit values were relatively constant across all sites ($TU_{IC20(REF)}$ between 0.1 and 0.2). The toxic unit values of the Feb 2015 samples were generally higher than the May 2014 samples, and effluent from the second WRF (at km 7.6) increased the basal toxicity of the water slightly, to 0.25 $TU_{IC20(REF)}$, with a lag increase further downstream (at the 9.7 km site) to 0.28 toxic units. However, toxicity did decrease rapidly after that, returning to baseline values within 10 km downstream of the second WRF. The higher basal toxicity in Feb 2015 compared to May 2014 was consistent with AhR, ER and GR results, indicating higher overall toxicity in the SCR during the latter sampling period. Similarly, the lag increase in basal toxicity downstream of the second WRF was also observed for AhR (Dec 2014 and Jun 2015) and ER at both sampling times.

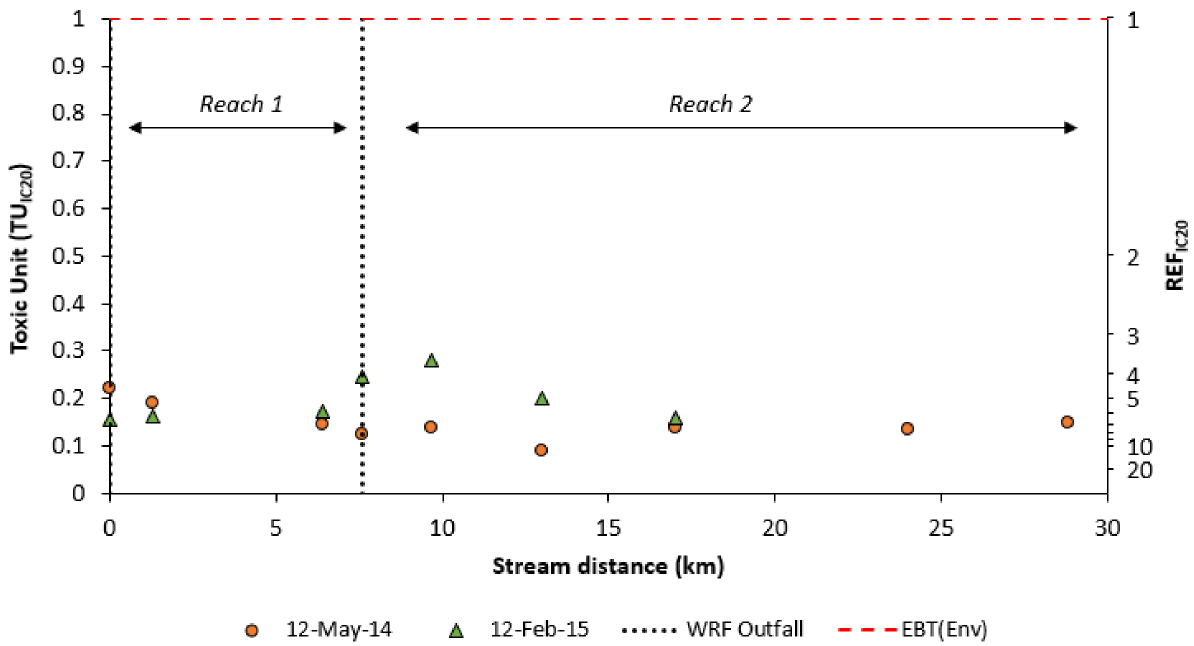

Figure 7. Toxicity to bacteria (measured by BLT-Screen) in the SCR. The red line denotes a reasonable trigger value based on reaching 20% toxicity in the assay with an undiluted sample.

4.0 Conclusions

Glucocorticoid, estrogen, and aryl hydrocarbon receptor activity (GR, ER and AhR) have been detected in the wastewater effluent-dominated flow of the SCR, often above available environmental effect-based trigger values. Overall, the activity decreased downstream from the WRFs, with up to 89%, 74%. 74%, and 43% removal seen for the GR, AhR, ER, and bacteria toxicity, respectively. These activities seem to be influenced by seasonal variations. Chemical analysis of known glucocorticoid compounds was also performed, detecting 9 out of 28 glucocorticoids analyzed, with triamcinolone acetonide (1.4 to 38 ng/L) found at the highest concentration. When converting the chemical analysis data to cDexEQ, a successful mass balance was demonstrated with the DexEQ obtained from the bioassay data, and interlaboratory comparison of bioassay result showed good agreement between laboratories, thus demonstrating the ability and reliability of bioassays to accurately monitor water quality. Further research is needed to identify whether these agonists are degrading naturally in the environment (photolysis or biodegradation) or if they are bioaccumulating and/or depositing in other environmental sinks. In addition, since some known agonists tend to be lipophilic compounds, addition research is needed that investigates the suspended solids and sediments of the SCR. As water sources are becoming increasingly influenced by anthropogenic compounds, the occurrence of these receptor agonists and other TOrCs in the environment is important to monitor; therefore, additional research is necessary to better understand the impact that these compounds pose to the aquatic environment and to humans through indirect potable reuse.

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Highlights:

- In vitro bioactivity in an effluent-dominated river measured quarterly for one year
- Bioactivity was attenuated with distance from effluent discharge without dilution
- Triamicinolone acetonide most abundant glucocorticoid pharmaceutical detected
- Biological activity exceeded effects-based trigger values for environmental effects