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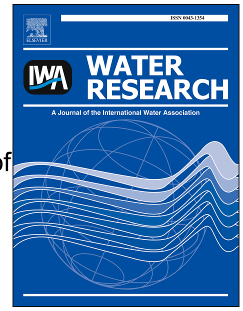


Modeling the exposure of wild fish to endocrine active chemicals: Potential linkages of total estrogenicity to field-observed intersex

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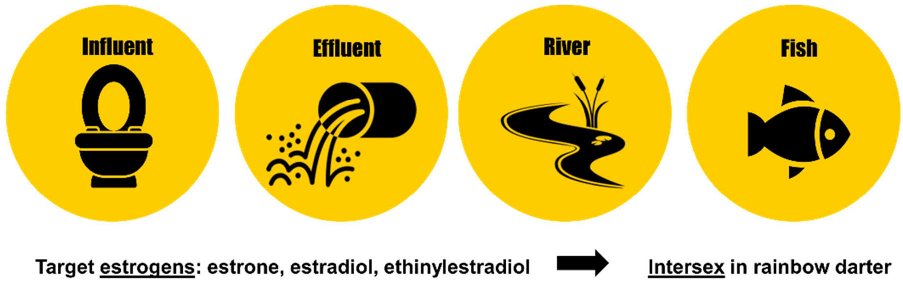
Influent
Effluent
River
Fish
Target estrogens: estrone, estradiol, ethinylestradiol
Intersex in rainbow darter

# Modeling the exposure of wild fish to endocrine active chemicals: potential linkages of total estrogenicity to field-observed intersex

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**Abstract**

Decades of studies on endocrine disruption have suggested the need to manage the release of key estrogens from municipal wastewater treatment plants (WWTP). However, the proposed thresholds are below the detection limits of most routine chemical analysis, thereby restricting the ability of watershed managers to assess the environmental exposure appropriately. In this study, we demonstrated the utility of a mechanistic model to address the data gaps on estrogen exposure. Concentrations of the prominent estrogenic contaminants in wastewaters (estrone, estradiol, and ethinylestradiol) were simulated in the Grand River in southern Ontario (Canada) for nine years, including a period when major WWTP upgrades occurred. The predicted concentrations expressed as total estrogenicity (E2 equivalent concentrations) were contrasted to a key estrogenic response (i.e., intersex) in rainbow darter (*Etheostoma caeruleum*), a wild sentinel fish species. A predicted total estrogenicity in the river of ≥10 ng/L E2 equivalents was associated with high intersex incidence and severity, whereas concentrations <0.1 ng/L E2 equivalents were associated with minimal intersex expression. Exposure to a predicted river concentration of 0.4 ng/L E2 equivalents, the environmental quality standard (EQS) proposed by the European Union for estradiol, was associated with 34% (95% CI:30-38) intersex incidence and a very low severity score of 0.6 (95% CI:0.5-0.7). This exposure is not predicted to cause adverse effects in rainbow darter. The analyses completed in this study were only based on the predicted presence of three major estrogens (E1, E2, EE2), so caution must be exercised when interpreting the results. Nevertheless, this study illustrates the use of models for exposure assessment, especially when measured data are not available.

**Keywords**

Estrogen, intersex, water quality model, exposure assessment, wastewater, rainbow darter

## 1. Introduction

The exposure of fish to endocrine active chemicals (EACs) has been shown globally to have deleterious consequences for reproductive health (Brian et al., 2005; Kime, 1999; Nash et al., 2004; Tyler and Routledge, 1998). One of the most frequent observations is the feminization of male fish with vitellogenin induction (production of estrogen-dependent protein) and intersex (ova-testis) as examples of changes reported (Jordan et al., 2016). Progress in analytical chemistry has enabled the detection of EACs at very low concentrations (Benotti et al., 2008; Carballa et al., 2004; López-Roldán et al., 2010). However, the proposed environmental quality standards (EQS) by the European Union (EU) for some EACs such as estradiol (E2) and ethinylestradiol (EE2) are only 0.4 and 0.035 ng/L respectively (European Commission, 2012). These concentrations are below the current detection limits of most routine analytical methods. As a result, some studies have utilized biological assessments (i.e., bioassays) to quantify exposures to EACs (Busch et al., 2016; Coleman et al., 2004; Escher et al., 2013; Marinho et al., 2013; Neale et al., 2017; Ohko et al., 2002). Bioassay techniques examine the combined biological activity in a mixture and can provide an indication of the potential responses in organisms exposed to complex mixtures without identifying the specific chemicals.

Despite the considerable chemical and bioanalytical monitoring of EACs in effluents and receiving environments worldwide (Agunbiade and Moodley, 2016; Escher et al., 2013; Leusch et al., 2014; Servos et al., 2005; Xu et al., 2007), there is still limited information to assess the spatial or temporal concentrations of EACs in receiving waters where technical challenges (e.g. detection limits) and cost are important considerations (Roig and D'Aco, 2016). In the absence of such data, the modeling of environmental systems can be used as an alternative approach to characterize fish exposure to EACs (Roig and D'Aco, 2016; Zhang et al., 2015). Models can be applied to evaluate current and future mitigation strategies for eliminating the target compounds through scenario testing (Kehrein et al., 2015) and assist in the design of effective monitoring programs (Roig and D'Aco, 2016). Furthermore, models can be employed to assess the potential relationship of stressor concentrations to observed effects in the wild (Jobling et al., 2009; Jobling et al., 2006). Numerous models have already been developed in recent years to predict the fate and transport of emerging contaminants such as pharmaceuticals and personal care products (Arlos et al., 2014; Balaam et al., 2010; Dale et al., 2015; Grechi et al., 2016; Kehrein et al., 2015).

Field investigations on the incidence and severity of intersex in male rainbow darter (*Etheostoma caeruleum*) in the Grand River watershed (southern Ontario) have been ongoing since 2007 (Hicks et al., 2017). The presence of severe intersex in rainbow darter has been linked to poor reproductive success (Fuzzen et al., 2015) with potential negative impacts on the fish population. However, a direct link between the exposure to specific compounds and intersex is very difficult to establish as the effluent composition and fate of EACs in the receiving environments are complex. The potential of natural estrogens (E2 and estrone [E1]) and synthetic estrogens (EE2) to cause endocrine disruption in fish has dominated many laboratory and field studies in recent years (Corcoran et al., 2010; Desbrow et al., 1998; Jobling et al., 2006; Kidd et al., 2007; Palace et al., 2009). The effects directed analysis (EDA) of the two major WWTP effluents in the Grand River suggested that the total estrogenicity was mainly contributed by E1, E2, and EE2 based on a receptor agonist screen assay (YES) (Arlos et al., 2018). However, there are many other EACs entering the receiving environment (e.g., estrogens from diffuse sources) that can interfere with the endocrine function in fish. Some responses including intersex may also be caused by androgen antagonists (Jobling et al., 2009) or chemicals such as metformin (antidiabetic) that may work through mechanisms other than receptor binding (Niemuth and Klaper, 2015). Also, the fate of other EACs may be correlated with the estrogen exposure, making it difficult to generate direct cause-and-effect relationships.

The current modeling work is focused on three major estrogens (E1, E2, and EE2) identified in the prior EDA as important contributors to the total estrogenicity in the effluents. In this study, the concentrations of E1, E2, and EE2 were simulated along the Grand River where the widespread presence of pharmaceuticals and personal care products has been documented (Arlos et al., 2015). The modeled reach also includes areas that were previously predicted (via models) to have elevated levels of estrogens (Grill et al., 2016; Hosseini et al., 2012). A major upgrade in one of the treatment plants (Kitchener WWTP) has resulted in major effluent quality changes during the study period but minimal data in effluents were available, especially during the pre-upgrade period when the environmental exposure to municipal wastewater-derived estrogens was likely at its peak. This scenario additionally provides a unique opportunity to apply models that can help assess the efficiency of WWTP upgrades. The overall goals of this study were to estimate the concentrations of select EACs (E1, E2, and EE2) in the Grand River through mechanistic water quality modeling and to determine whether the exposure to these key estrogens is consistent with the observed responses (intersex) in wild fish.

## 2. Methodology

### 2.1. Study site

The Grand River watershed in southern Ontario (~6,800 km$^2$) drains into Lake Erie and is inhabited by close to 1 million people. In addition to the non-point sources from numerous agricultural activities (~70% of total land use), the watershed also receives inputs from 30 WWTPs. The Grand River has also been extensively investigated for several biological effect endpoints on fish health since the late 2000s (Bahamonde et al., 2014; Fuzzen et al., 2015; Fuzzen et al., 2016; Tanna et al., 2013; Tetreault et al., 2011; Tetreault et al., 2013). In this study, ~80 km of the Grand River was modeled starting below a regulated water reservoir (Shand Dam) to an area that is ~2 km above the Grand and Speed River confluence (Figure 1a). This section captures both agriculture and urban gradients in the watershed and incorporates the inputs from two major (Waterloo and Kitchener) and two smaller (Elora and Fergus) WWTPs (Table S1). In 2012, Kitchener WWTP underwent major process upgrades including improved aeration, nitrification, and replacement of chlorination/de-chlorination with UV effluent disinfection.

### 2.2. Modeling strategy

The water quality modeling included three separate components: (1) source, (2) transport and fate, and (3) effects as outlined in Figure S1. The source modeling predicted the effluent concentrations from the target WWTPs and was completed as detailed in Arlos et al. (2018). The transport and fate component simulated the distribution of target EACs in the study area and was completed using a mechanistic water quality model. Finally, the effects component evaluated the potential relationship between the predicted river concentrations derived from the transport and fate model component and field-recorded intersex conditions. Due to their relatively high site fidelity (Hicks and Servos, 2017) and constant exposure to WWTP effluents throughout their life cycle, data on rainbow darter were considered suitable for quantifying the exposure impacts. The intersex data for rainbow darter at nine sites in the Grand River watershed (2007-2015) were based on the same samples compiled by Hicks et al. (2017) and were used in the concentration-response regression analysis (see section 2.5). The selection of these sites is also described in detail in Hicks et al. (2017).

A similar approach to Arlos et al. (2014) was employed to simulate estrogen concentrations in the Grand River. The Water Quality Simulation Program developed by the US Environmental Protection Agency (WASP version 7.3) was used as the model platform. This model was employed in a recent study to describe the distribution of frequently detected pharmaceuticals with varying physical-chemical properties downstream of the Kitchener WWTP (10-km reach) (Arlos et al., 2014). The model has already been calibrated for compounds that spanned the properties of those examined in the current study and was found to provide robust mechanistic predictions of pharmaceutical fate and transport (Arlos et al., 2014).

The following major steps were completed to predict the river concentrations: discretization of the river network; simulation of river transport mechanisms (i.e., advection); testing of the transport processes using a tracer compound (chloride); and integration of organic compound modeling through the addition of significant in-river fate mechanisms (e.g., biodegradation and photolysis). The first three steps were iterative in nature and were deemed as crucial in establishing a baseline model that accurately represented the mathematical structure of the system (as detailed in Arlos et al. (2014)).

The final discretized model involved 50 segments (Figure 1b) (described in the supplementary material Section A), and only the aqueous phase was considered in the discretization (i.e., no bottom segments included). Advection is the primary transport process in rivers and is driven by water flows. The internal flows in WASP under the kinematic wave flow option were propagated using Manning's Equation (Section B, supplementary material). The model was initially set up to describe water movement and its accuracy was cross-checked against the measured hydro-geometry data such as water levels and flows. Measured water level data for segments 12, 21, and 37 were used for river transport calibration. The finalized input parameters associated with the hydro-geometry and river transport are found in the supplementary material (Table S2). In addition to examining the model's accuracy in simulating water movement via measured water levels and flows, chloride was also used to determine the non-reactive constituent transport within the network. Since chloride is conservative, its assimilation in the river system is achieved via advection. Significant point sources of chloride in the river network come from urbanized creeks (Laurel and Schneider Creeks) and the WWTPs. Observed chloride values at Segments 12, 21, 23, 32, 42, and 50 were used to calibrate the transport component and were taken from Ontario's Provincial Water Quality Monitoring Network (PWQMN) database (https://www.ontario.ca/data/provincial-stream-water-quality-monitoring-network) and previous monitoring work completed in the central Grand River (Tables S3 and S4).

### 2.2.1. Modeling of target estrogens

The major inputs of target estrogens into the studied reach of the Grand River were from the four WWTPs. Although the tributaries (one river and four creeks) included in the modeled network may be receiving small amounts of estrogens from the surrounding agricultural lands (i.e., municipal biosolids/manure applications), no chemical and bioassay data for the study period are currently available to confirm this. However, data collected in the upstream reaches (above Segment 12), where the land use is predominantly agricultural, suggest low occurrence and severity of intersex (Hicks et al., 2017). Hence, it was assumed that the tributaries have negligible contributions of E1, E2, and EE2. The concentration profiles of the target estrogens in the Kitchener and Waterloo WWTP effluents were developed previously (Arlos et al., 2018) that employed population demographics, usage and excretion rates, and removal through the plant to estimate effluent data. A similar approach was completed for the effluents from the Fergus and Elora WWTPs.

The simulation of the transport and fate of the target EACs was completed by initially considering them as conservative contaminants (transport as a primary mechanism) and sequentially adding fate mechanisms responsible for their distribution in the aquatic environment. Chapra (1997) suggested that sorption is minimal when the target compounds have log octanol-water partitioning coefficients (log Kow) that are <4-5 and the suspended solids concentrations range from 1-50 mg/L. The estrogens examined in this study have log Kow's that are ~4.5 and the average suspended solids concentration in various segments ranged from 6 to 23 mg/L (PWQMN data set from 2007-2014). Hence, sorption was not simulated in this study. This decision was consistent with the results of Jurgens et al. (1999) who reported that estrogens in riverine environments are typically present in the dissolved phase. In addition, a previous modeling study by Arlos et al. (2014) in the Grand River found that inclusion of sorption had a minimal effect on the fate of modeled pharmaceuticals with log Kow of 3.2 to 4.8. Hence, only biodegradation and photolysis were deemed to be significant in the assimilation of estrogens in the aquatic environment (Balaam et al., 2010; Jürgens et al., 2002; Lin and Reinhard, 2005).

Biodegradation and photolysis processes were initialized using the approach described by Arlos et al. (2014). Briefly, biodegradation was modeled as a first-order reaction and literature-derived kinetic rate constants (Table S5) were initially corrected based on the river temperature (Table S6). Temperature profiles for each segment were taken from the nearest PWQMN site. Photolysis was also modeled as a first-order reaction and the range of rate constants was derived from the literature (Table S5).

### 2.3. Model performance measures

The Nash-Sutcliffe Efficiency (NSE), index of agreement (d), and the percent bias (PBIAS) have been recommended by Moriasi et al. (2007) (Table S7) and were used to evaluate the performance of the transport portion of the model. These criteria, however, are not applicable for simulations that have a limited number of measured data points (<50). Hence, a statistical test was completed using either the Pearson or Spearman correlation tests (measured vs. predicted) depending on the normality of the datasets (Table S8). An additional performance test using the percent difference criteria (Donigian, 2002) was also used to support the results of the correlation analysis (Table S7). These quantitative performance measures were used in addition to a subjective comparison of observed and predicted time series plots. Data were not available for all target estrogens in the modeled reach. Hence, the quality of the simulation of estrogen fate mechanisms was conducted by assessing the effects portion of the model (described in the next section).

### 2.4. Linking predicted concentrations (exposure) and intersex conditions (effects)

Although feminization of male rainbow darter has been observed at different levels of biological organization in the Grand River (Fuzzen et al., 2016; Hicks et al., 2017; Marjan et al., 2017; Tetreault et al., 2011), intersex has been found to be the most consistently observed endpoint related to reproductive changes downstream of municipal WWTPs (Fuzzen et al., 2016). Hicks et al. (2017) evaluated intersex incidence and severity from 2007 to 2015 at nine different sites (Figure 1), including periods prior to and after the Kitchener WWTP upgrades. This dataset was used as the primary biological response to which the predicted EAC concentrations were compared. It was assumed that the critical window of exposure for adult rainbow darter occurs during their gonadal recrudescence (late spring to summer) (Hicks et al., 2017). Hence, the predicted river EAC concentrations from June to August were averaged at the nine sites to provide the exposure conditions for the fish collected in the fall sampling event of that same year and the spring sampling of the following year.

The predicted concentrations were converted to total estrogenicity (EEQ) using:

$$EEQ = \sum C_i \times EEF_i \quad \text{(Equation 1)}$$

where $C_i$ is the predicted concentration. $EEF_i$ is the estrogenicity equivalency factor that describes the potency of the estrogens relative to E2. EEFs of 0.3, 1, and 1.23 were used for E1, E2, and EE2 respectively. The EEFs reflect the average potency associated with the yeast estrogen screen (YES) assay as compiled by Jarošová et al. (2014). However, there may be differences in the responses of different species and endpoints for each of the estrogens of interest and this could slightly alter the interpretation of results. EEFs for YES were presented because the measured data for selected WWTP effluents in the study (see Arlos et al. (2018)) were acquired using the YES assay. Although several estrogens in municipal wastewater effluent such as estriol (E3), BPA, and octyl/nonylphenols may contribute to the total estrogenicity, a previous study employing an effects-directed analysis (EDA) of Kitchener and Waterloo WWTPs found that the estrogens E1, E2, and EE2 dominated the total estrogenicity (Arlos et al., 2018).

One of the simplest ways to describe the relationships between exposure conditions and effects is through a dose-response model (Barnthouse, 1992). The observed intersex incidence and severity were related to the predicted EEQ values using the four-parameter Hill Equation described by Equation 2:

$$Response = \min + \frac{(max - min)}{1 + 10^{(F - EEQ) * H)}} \quad \text{(Equation 2)}$$

where the response is either intersex incidence or severity, min and max are the lowest and highest expected responses, F is the response halfway between the min and max (often described as EC50), and H is the Hillslope parameter that describes the steepness of the curve.

The term intersex incidence refers to the percentage of fish with at least one oocyte (female ovarian tissue) in the male testis. For intersex incidence, the maximum response was set to 100% (i.e., all male fish collected were intersex) whereas the minimum was set to 0% (i.e., all male fish collected identified as normal males). By comparison, the intersex severity describes the degree of feminization in each animal and is scored from 0 to 7, with 0 describing a normal male whereas 7 is used for normal female (Bahamonde et al., 2015).. Although rare, the highest recorded severity in rainbow darter was 6 (Hicks et al., 2017), so the minimum and maximum levels of severity in rainbow darter were set to 0 and 6 respectively. Prism 7 (GraphPad Software Inc.) was used to fit the regression model (Equation 2) to the data (i.e., predicted estrogenicity and intersex) and the goodness of fit ($R^2$) was employed to determine the quality of the fit between the predicted river concentrations and intersex data.

## 3. Results and discussion

### 3.1. River hydro-geometry and transport processes

The suitability of model discretization, hydro-geometry, and transport conditions was verified through the simulation of water levels and chloride concentrations at select sites. The results for three sites are shown in Figure 2 and the remainder is found in the supplementary material (Figures S2-S4). A graphical comparison of the calibrated model simulations with the measured data shows that the hydro-geometry and water movement within the network were well-characterized by the model as depicted by its ability to describe both high and low flow conditions (Figure 2a). Also, the NSE, d, PBIAS metrics for the calibration sites had ratings ranging from “satisfactory” to “very good” (Table 1) which further supported the prediction accuracy. However, there were some periods in segment 12 when the water levels were under-predicted (“unsatisfactory”, Table 1). However, visual and statistical comparisons of observed and predicted water flows were completed for segments 12, 21, and 37 and found to demonstrate an acceptable level of model performance (“satisfactory” to “very good” ratings for NSE, d, and PBIAS, Figure S2).

The good agreement between the observed and simulated chloride concentrations (Figures 2b and 2c, Table 1) further supported the conclusion that the hydro-geometry and transport conditions in the model adequately represented the conditions in the field. In particular, the model captured the different ranges of chloride concentrations measured in different segments (Figure S3). As similarly observed for water level simulation, there was no significant correlation between the measured and predicted concentrations for segment 12 when the model performance was evaluated statistically (Table 1). However, the mean percent differences for all the sites were within the calibration tolerances for water quality modeling (Donigian, 2002), suggesting that the tracer contaminant simulation was acceptable for the purposes of this study (Table 1).

### 3.2. Modeling the river concentrations of EACs

The concentrations of estrogens in the river were first simulated conservatively (assuming no degradation in the reach) and biodegradation and photolysis loss mechanisms were subsequently added. As measured EAC concentrations in the river were not available, improvements in the $R^2$ value (a goodness of fit measure) derived from the relationship between simulated concentrations and the intersex response data (Equation 2) were used as the calibration target. Under the conservative approach, an $R^2$ value of 0.755 was derived when the concentrations predicted by the model were fitted against the field-recorded intersex incidence (Figure 3a). A similar $R^2$ value (0.799) was also obtained for the intersex severity response (Figure 3b).

Although these results were deemed to be indicative of a good fit, the addition of biodegradation and photolysis mechanisms was examined to assess whether the predictions could be improved. It was found that the addition of these processes did not significantly change the results (see Table S11 for statistical analysis). The temperature-corrected biodegradation rate constants (literature-derived) in surface water were relatively low for all the target compounds (Table S6) and the model was found to be insensitive to any factors expected to impact the biodegradation (temperature correction coefficient, rate constants).

A similar trend was observed when photolysis was added to the model. Again, this was attributed to the relatively low photodegradation rate constants (literature-derived) that were employed for the target compounds. This observation was consistent with the environmental fate modeling study of pharmaceuticals in the Grand River during a low-flow condition (downstream of Kitchener WWTP) (Arlos et al., 2014). This prior study demonstrated that the first-order photolysis rate constant must be greater than 3 $d^{-1}$ before photolysis became a significant mechanism in the fate and transport of pharmaceuticals within the modeled reach (with varying physical-chemical properties). The simulations additionally revealed that considerably large values for biodegradation and photolysis rate constants would be required before substantial changes in estrogenicity were predicted (Figure S5). These exceptionally high values were unrealistic when compared to the range of values reported in the literature. Hence, the results under a conservative simulation were used to discuss the subsequent sections.

### 3.3. Temporal patterns and relationship with flows

The predicted concentrations of the target estrogens (2007-2015) at sites immediately downstream of the two major WWTPs (Waterloo and Kitchener) are presented in Figure 4. E1 was predicted to be the most abundant and contributed, on average, 51% and 65% of the total estrogenicity below the Kitchener (Segment 42) and Waterloo (Segment 23) WWTPs respectively. By contrast, EE2 (most potent of the estrogens) only contributed 17% and 18% of the total estrogenicity in the river segments immediately below Kitchener and Waterloo WWTPs respectively. Historically, less attention has been given to E1 due to its lower potency relative to E2 and EE2. However, as observed by this modeling study and many monitoring surveys worldwide (Blazer et al., 2014; Ma et al., 2016; Matthiessen et al., 2006; Sarmah et al., 2006), E1 can be present at much higher concentrations than its more potent counterparts. Ankley (2017) observed that fish (fathead minnow) can convert E1 to E2 (more potent estrogen), suggesting that an exposure to high concentrations of E1 should also be considered when assessing the risks of estrogen exposure.

Since only contaminant transport conditions were simulated in the model, the impact of river flow on predicted estrogenicity concentrations in the river is evident: low flow periods had high predicted concentrations and *vice-versa* (Figure 4). Furthermore, as the removal rates in the river via biodegradation and photolysis were determined to be minimal, the critical exposure conditions are controlled by dilution and will likely occur during low flows (Figure 4). The average predicted total estrogenicity that corresponded to flows that were less than or equal to the normal low-flow (11 $m^3/s$) was 0.45 ng/L ($s$=0.1 ng/L) E2 equivalents immediately downstream of Waterloo WWTP (Segment 23). The corresponding average predicted estrogenicity below the Kitchener WWTP (Segment 42) at similar flow conditions (<11 $m^3/s$) was 3.4 ng/L ($s$=0.9 ng/L) E2 equivalents prior to the WWTP upgrade and was reduced to 0.7

ng/L ($s$=0.1 ng/L) E2 equivalents post upgrade. This result suggests that the partial process upgrades (Figure 4b), improved the water quality downstream of Kitchener WWTP.

### 3.4. Spatial patterns of estrogen exposure

In addition to simulating time-varying conditions, the model was also employed to resolve the spatial patterns in response to the changes in the river (e.g., dilution, change in contaminant loadings after the upgrades). The spatial analysis was provided for low flow conditions as these could lead to critical exposures. A representative low flow event (<11 $m^3/s$) during the pre-upgrade period (May 20, 2012) was compared with a post-upgrade low flow condition (June 11, 2014). These dates were chosen such that the Waterloo WWTP operations and river flows were similar to avoid issues associated with river dilution (see Table S9).

Only low levels of estrogenicity were predicted in the first 50 km of the modeled section in response to the minimal release of estrogens from the two smaller upstream WWTPs (Fergus and Elora). By contrast, elevated estrogenicity concentrations were predicted below the Waterloo WWTP outfall and they persisted until Segment 50 (last modeled segment) at 1.6 ng/L E2 equivalents on May 20, 2012 (Figure 5a). The highest estrogenicity concentrations were predicted immediately below the Kitchener WWTP during low flow periods (up to 6 ng/L E2 equivalents, pre-upgrade). However, the estrogenicity concentrations at all segments downstream of this plant were substantially reduced to 0.5 ng/L E2 (Segment 50) when the process upgrades were implemented (June 11, 2014, Figure 5b).

It was observed that the 20-km distance between Waterloo and Kitchener WWTP provided limited dilution of estrogens discharged by the Waterloo WWTP during the low-flow period (evident by the unchanged spatial profile between the two plants, Figure 5). If the Kitchener WWTP source was removed from the model, an estrogenicity concentration of approximately 0.3 ng/L E2 equivalents was predicted at Segment 50 (most downstream modeled segment) because of the inputs from the upstream Waterloo outfall. This simulation suggests that although some of the estrogenicity in the river below the Kitchener outfall was due to the Kitchener effluent post-upgrade, it appears that >60% of the exposure at Segment 50 could be from the upstream Waterloo effluent (low flow only, Table S10). Hicks et al. (2017) showed low levels of incidence and severity of intersex in rainbow darter have persisted downstream of the Waterloo outfall, with similar levels below the Kitchener outfall after the upgrades. To determine the impact of future process upgrades at the Waterloo WWTP, a removal efficiency through the plant of 95% was implemented in the model and the concentrations of estrogens were simulated. The results of this simulation revealed that under a low flow scenario (June 11, 2014), the predicted estrogenicity would be reduced to ~0.2 ng/L E2 equivalents.

Overall, the modeling of the key estrogens in the Grand River is useful in evaluating the efficiency of the current WWTP operation during a critical exposure condition (low flow). These concentrations were assessed along with the intersex conditions in the Grand River to determine whether the estrogen exposures across many sites are consistent with the observed biological responses.

### 3.5. Potential linkages to biological effects observed in the field

The concentration-response curve (Figure 3) is a fitted four-parameter Hill model that employs the predicted total estrogenicity derived from the river model (Equation 1) and the intersex responses collected in the field (i.e., incidence and severity). Establishing a relationship between predicted environmental concentrations and observed biological responses contributes to a weight of evidence that helps assess the potential for wastewater contaminants (that are difficult to measure) to alter the biological endpoints in receiving environments. However, the findings presented here must be interpreted with some caution as only three estrogens (i.e., E1, E2, and EE2) were considered and their predicted river concentrations were not verified due to some limitations in the analytical method. Although a direct causal relationship is not established, the model provided predictions that fit very well with the observed intersex data for wild fish (Figure 3). The potential and limitations of water quality modeling in exposure assessments are further discussed in sections 3.7 and 3.8 below.

The concentration-response relationship (Figure 3a) suggests that a predicted river total estrogenicity of 10 ng/L would be associated with the feminization of 93% (95% CI: 89-97) of adult male rainbow darter, corresponding to a predicted mean intersex severity score of 4 (95% CI:3.6-4.4) (severely intersex fish have scores of 4-6 (Bahamonde et al., 2013)). Although an estrogenicity of 10 ng/L E2 equivalents was never predicted at any river sites in the Grand River, the high incidence of intersex (80-100%) that was mostly observed downstream of Kitchener WWTP (Segment 41, pre-upgrade) corresponded to an average predicted total estrogenicity of ~2.5 ng/L E2 equivalents (ranging from 0.8 to 6.1 ng/L E2 eq.) (Figure 3a).

The average background intersex conditions of the upstream (Segments 7, 12) and urban (Segment 19) reference sites were calculated to be 7.4% ($s$=6%) and 0.1 ($s$=0.1) for intersex incidence and severity (dataset from Hicks et al. (2017)). After the Kitchener WWTP upgrade, the predicted estrogenicity at Segment 41 (immediately below the Kitchener outfall) ranged from 0.1 to 0.6 ng/L E2 equivalents. This predicted concentration was associated with 11% (95% CI: 7-15) to 43% (95% CI: 39-48) intersex incidence with a severity of 0.1 (95% CI: 0.03-0.17) to 0.8 (95% CI: 0.7-0.9) based on the established relationship (Figure 3). The post-upgrade simulations suggest that the levels are approaching the background conditions. The predictions also represented a major improvement in exposure conditions associated with WWTP upgrades that were primarily intended for the removal of conventional contaminants (e.g., TSS, ammonia). The Waterloo WWTP will continue to contribute to the total estrogenicity, but once upgrades at both plants have been implemented (i.e., 95% removal of estrogens is anticipated), the corresponding average estrogenicity in the river below the Kitchener WWTP (Segment 41) was predicted to be ~0.2 ng/L E2 equivalents. This concentration would correspond to low intersex incidence (20% with 95% CI: 16-24) and severity scores (0.3 with 95% CI: 0.2-0.4).

The model (Equation 2) predictions are consistent with a laboratory experiment by Fuzzen (personal communication, November 13, 2017) wherein 10 ng/L EE2 (~12.3 ng/L E2 equivalents in YES) resulted in 100% female population was observed in larval fish (rainbow darter). In a separate experiment (Fuzzen, 2016), this concentration also significantly reduced the fertilization success in adult males. A similar observation (100% female population) was observed by Lange et al. (2009) when *Rutilus rutilus* (roach) were exposed to 4 ng/L EE2 (~5.0 ng/L E2 equivalents in YES) for 2 years. Furthermore, Kidd et al. (2007) observed a population collapse in fathead minnows exposed to 5 ng/L EE2 (~6.2 ng/L E2 equivalents in YES) in a whole lake experiment, whereas a life-cycle exposure of the same fish species to <1 ng/L of EE2 reduced male secondary characteristics (Parrott and Blunt, 2005).

This study provides additional support for previous work that indicated a potential relationship between estrogen exposure and intersex. Jobling et al. (2006) examined the relationship between the modeled total estrogenicity and observed intersex in wild fish population (roach) in the UK. They estimated that river sites with estrogenicities ranging from 1 to 10 ng/L E2 equivalents will cause an intersex incidence of 22% in a wild roach population. They categorized this exposure condition as medium risk whereas sites with >10 ng/L E2 equivalents were considered as having high risk. By contrast, the current study indicates that a predicted concentration of 1 to 10 ng/L of E2 equivalents was associated with an intersex incidence between 53% to ~100%, with a severity level ranging from moderate to highly severe conditions. The inconsistency between the Jobling et al. (2006) results and those of the current study may be attributed to the species difference (rainbow darter vs. roach) as well as the procedures employed in estimating the in-river estrogen concentrations (steady-state vs. time-variable model). It could also be associated with other estrogenic chemicals present in the effluent.

Fernandez et al. (2007) assessed the estrogenicity in Canadian wastewaters using *in vitro* bioassays and detected total estrogenicities ranging from 9 to 106 ng/L E2 equivalents. Early studies by Ternes et al. (1999) detected 3 ng/L, 6 ng/L, and 9 ng/L median concentrations of E1, E2, and EE2 respectively in Canadian effluents. After incorporating a 10 or even 100-fold dilution factor, it can be expected that low to moderate levels of intersex may still be associated with many effluents in Canada. However, the relatively steep concentration-response curve (Figure 3) suggests that improved treatment can dramatically reduce the intersex incidence and severity in watersheds as has been observed in the Grand River (Hicks et al., 2017).

### 3.6. Comparison with recommended thresholds of exposure

Caldwell et al. (2012) determined the predicted no effects concentration (PNECs) of 6, 2, 0.1, and 60 ng/L for E1, E2, EE2, and E3 respectively based on the species sensitivity distribution (SSD) approach. Similarly, the EU derived an EQS of 0.4 and 0.035 ng/L for E2 and EE2 respectively. Considering only the recommended thresholds for E2 from both studies, an estrogenicity level of 2 ng/L and 0.4 ng/L E2 equivalents would be associated with 72% (95% CI: 66-78) and 34% (95% CI: 29-37) intersex incidence in rainbow darter respectively and correspond to severity scores of 1.95 (95% CI: 1.8-2.1) (moderate) and 0.60 (95% CI: 0.5-0.7) (low). Despite a high incidence of intersex being associated with these predicted concentrations in the Grand River, adverse impact on rainbow darter reproduction was estimated to occur only at very severe levels (severity score of 4-6) (Fuzzen et al., 2015). Again, the results from such analyses should be treated with utmost caution, given that the relationships in the current study are based on a limited number of EACs (E1, E2, and EE2) that were predicted rather than measured. Nevertheless, this study demonstrates the potential use of the recommended thresholds from the literature when assessing potential adverse impacts resulting from estrogen exposure.

When an *in vitro* bioassay was employed in water quality monitoring, Jarošová et al. (2014) recommended a “safe” level of total estrogenicity of 0.1 to 0.4 ng/L E2 equivalents for effluent dominated streams (close to 100% effluent contribution). Another study determined a similar effect-based trigger value of 0.5 ng/L E2 equivalents when using the ER CALUX® estrogen screen assay (van der Oost et al., 2017). The exposure of rainbow darter to the trigger value reported in the literature for surface water (0.5 ng/L) would be associated with 41% (95% CI: 36.4-45.6) intersex incidence and a low severity score of 0.77 (95% CI: 0.65-0.89). Furthermore, it appears that for both intersex incidence and severity, an estrogenicity of <0.1 ng/L would be associated with relatively low occurrence (up to 10%) and severity conditions in rainbow darter (Figure 3). Whether this level (<0.1 ng/L E2 equivalents) should be set as a more stringent threshold for estrogenicity still requires additional studies employing both field and laboratory techniques that test several ecologically relevant endpoints.

### 3.7. Potential contribution of other causal agents

Previous studies in the literature have indicated that compounds other than E1, E2, and EE2 can induce intersex. For example, triclosan (anti-androgen) and a non-point source pollutant, equol (soybean isoflavone derivative), have been specifically indicated as potential agents causing endocrine disruptive effects in the wild (Jobling et al., 2009; Rostkowski et al., 2011; Wang et al., 2016). A previous study conducted by our research group (Arlos et al., 2015) on antiandrogens detected the presence of triclosan downstream of WWTPs but it rapidly degraded over a short distance (<5 km) during a low-flow sampling event. This result suggested that triclosan may not be persistent in the Grand River, but this observation requires further investigation. Also, pollution stemming from nonpoint sources is significant in the Grand River watershed (70% agricultural land use), especially in the spring (Loomer et al., 2015). However, this study assumed that the critical window of exposure to compounds causing intersex occurs when rainbow darter build their gonads in the summer months (June to August). Hence, the timing of exposure may be an important factor to consider. Obviously, the possibility of other compounds causing or contributing to intersex is not being eliminated. However, the model results are informative and can assist with future hypothesis-driven studies related to estrogen exposure. Other potential causal agents can be additionally modeled to determine whether a relationship with intersex could also be observed (potentially enhancing the current model predictions).

### 3.8. Overall implications of the model on exposure assessment

Exposure assessment quantifies the magnitude, frequency, and duration of exposure in an environmental compartment and is a key element in the risk characterization of estrogens. The assessment is often completed using advanced analytical chemistry techniques and bioassays that detect the total biological activity such as estrogenicity. These methods can be limited by sampling logistics, analytical methods, and cost. As in the case of the Grand River and other river systems, environmental samples to assess previous exposure conditions (pre-upgrade) were also not available.

Chemical measurements would have helped to validate some of the assumptions (e.g., fate mechanisms, non-point sources) and/or refine the predictions. The estrogens in the river are at extremely low concentrations which is a significant challenge in the chemical analysis (e.g. detection limits). In addition, there can be limitations with the analytical detection using LC/MS-MS methods that are prone to matrix interferences in "dirty" municipal wastewater samples. Although modeling is not a complete substitute for chemical and bioassay measurements, it is a viable option to supplement the current lack of exposure data. Further investigation and validation of the model are warranted, but the conservative estimates presented in this study suggest that the select estrogens are likely associated (or at least correlated) with the observed intersex in wild fish in the Grand River.

## 4. Conclusions

The concentrations of E1, E2, and EE2, compounds that contribute a large fraction of municipal wastewater-derived total estrogenicity in surface waters, were predicted using a mechanistic water quality model. Transport conditions appear to play a major role in the spatial and temporal distribution of the target estrogens in the Grand River, while fate mechanisms such as biodegradation and photolysis likely have minimal influence. The relationships between the predicted exposure concentrations (during the period of gonadal recrudescence for rainbow darter) and intersex incidence and severity were also developed using the four-parameter Hill concentration-response model. A predicted estrogenicity level of <0.1 ng/L E2 equivalents in river water was associated with up to 10% intersex incidence and a low severity score of <1, which is unlikely to impact the rainbow darter reproductive health. This work is consistent with the recommended thresholds for exposure, but additional work is required to validate the assumptions employed in the model. Even with the limitations identified, modeling contributes to the better understanding of risk and evaluation of potential remedial actions, and is a valuable tool that can be applied to estimate environmental exposure.

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**Table 1.** Performance measures for water level and chloride concentrations at selected calibration sites.

| Water Level | | | |
|---|---|---|---|
| **Segment No.** | **NSE (rating)** | ***d* (rating)** | **PBIAS (rating)** |
| 12 | 0.428 (US) | 0.794 (G) | 15% (S) |
| 37 | 0.848 (VG) | 0.953 (VG) | -3% (VG) |
| **Chloride** | | | |
| | **Correlation Coefficient** | **Mean PD (rating)** | |
| 12[a,c] | no significant correlation | 17 (VG) | |
| 21[b] | 0.758, $p$<0.001 | 30 (S) | |
| 23[b] | 0.531, $p$<0.001 | 25 (G) | |
| 32[a] | 0.895, $p$<0.001 | 33 (S) | |
| 42[b] | 0.223, $p$=0.0515 | 19 (VG) | |
| 50[b] | 0.836, $p$<0.001 | 32 (S) | |

[a]Datasets are normal and the Pearson correlation test was used (parametric test statistics). Normality test results are found in Table S8. [b]Datasets are non-normal so the Spearman non-parametric correlation test was used (see Table S8). [c]An explanation on potential processes that impacted the simulation in Segment 12 is presented in the supplementary material, section C. "NSE" = Nash-Suttcliffe Efficiency coefficient; "d" = index of agreement; "PBIAS" = percent bias; "VG" =very good; "G" = good; "S" = satisfactory; "US" = unsatisfactory; and "PD" = percent difference

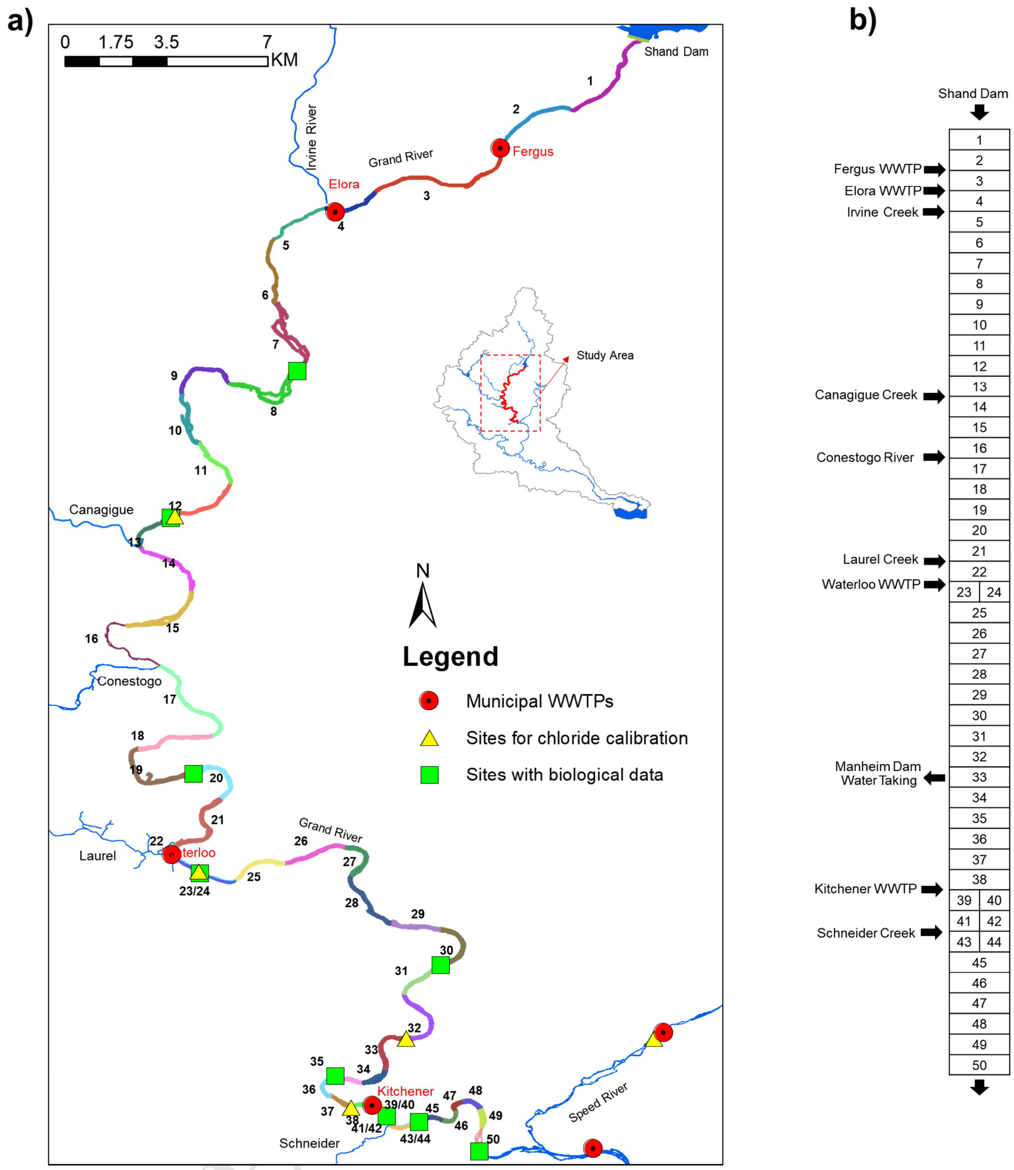



**Figure 1.** (a) Modeled area of the Grand River. Sections 23/24, 39/40, 41/42, and 43/44 represent the eastern and western divisions of the segments immediately downstream of WWTPs. (b) Segmentation profile used in the model. Black arrows indicate movement of water into and out of the network.

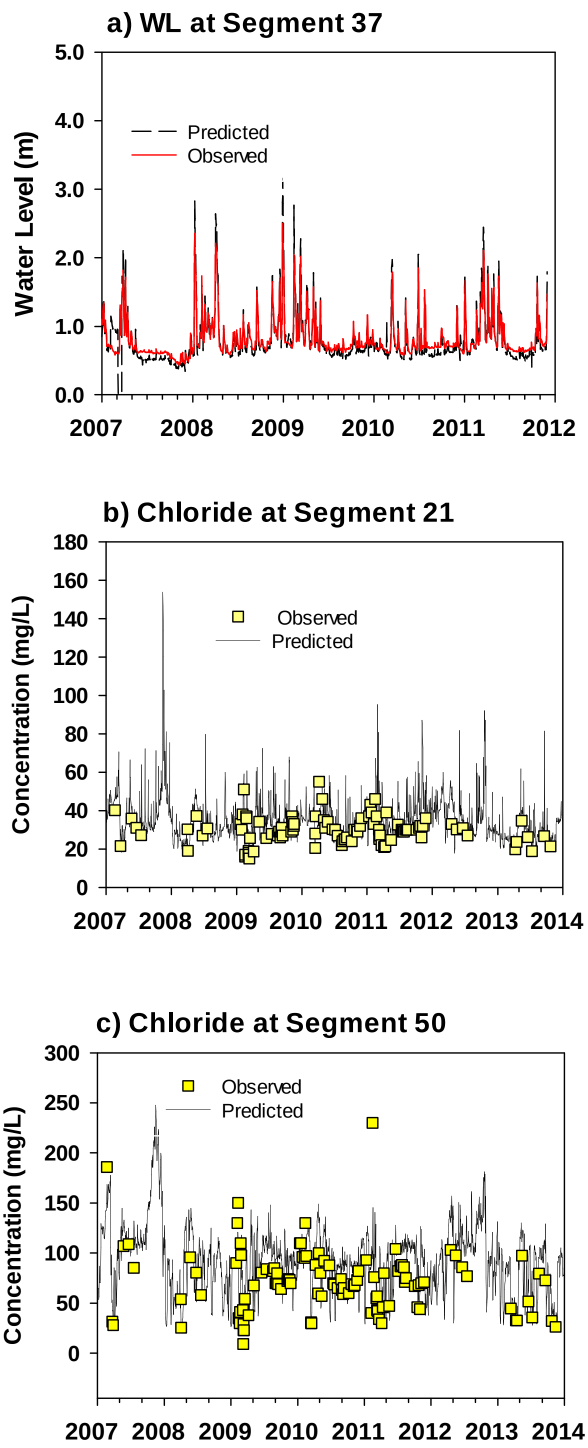



**Figure 2.** (a) Water level (WL) simulations and measured values for segment 37. Simulated and measured chloride concentrations at (b) Segment 21 and (b) Segment 50. See Figure 1 for relative locations in the modeled network.

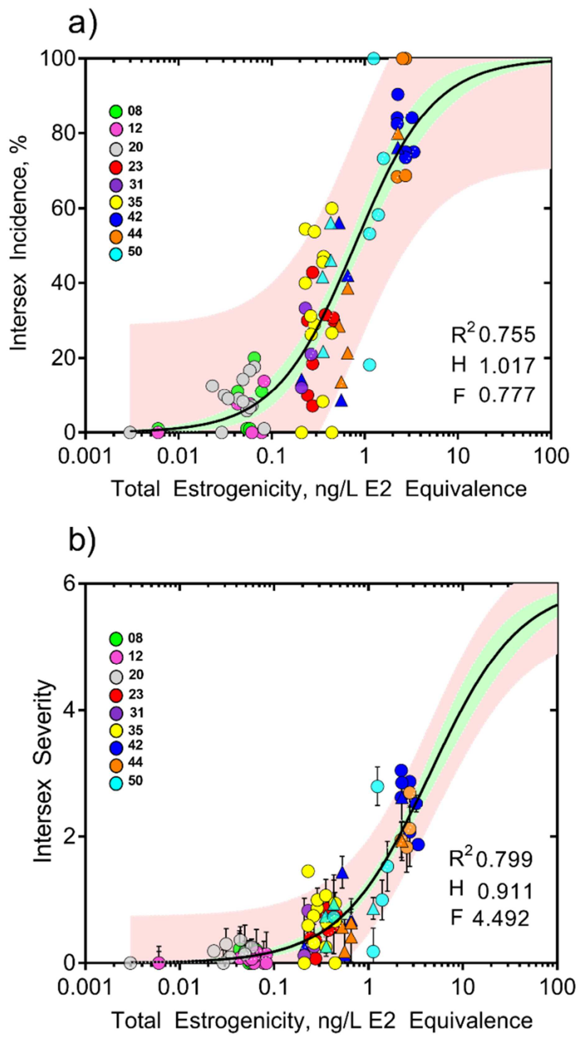
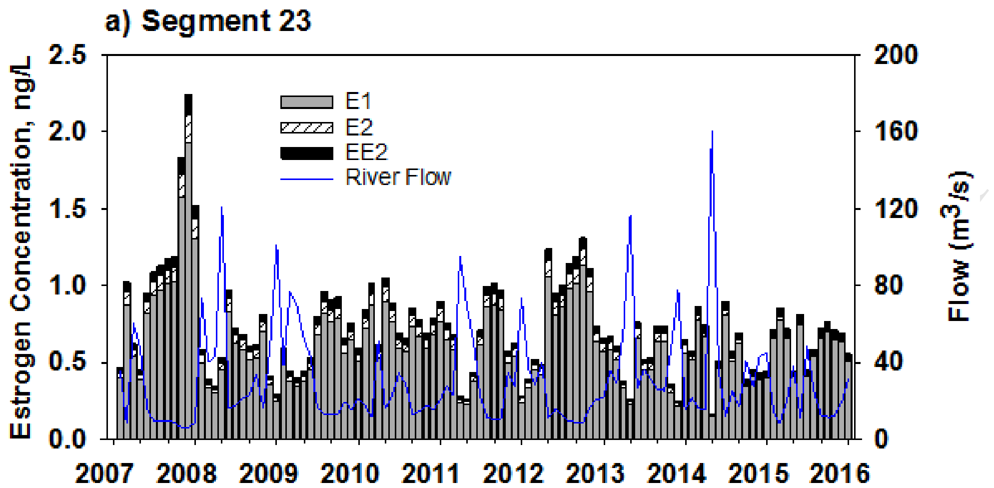



**Figure 3.** Relationship between predicted total estrogenicity and (a) intersex incidence and (b) intersex severity (error bars are standard errors). The total estrogenicity data were the averaged concentrations from June-August, the period assumed in this study as the critical window of exposure. Shaded region represents the 95% prediction (red) and confidence (green) intervals. Circles represent the sites with biological data and the triangles represent the post-upgrade period datasets for segments 42, 44, and 50. H= Hillslope parameter and F is EC50 (ng/L).



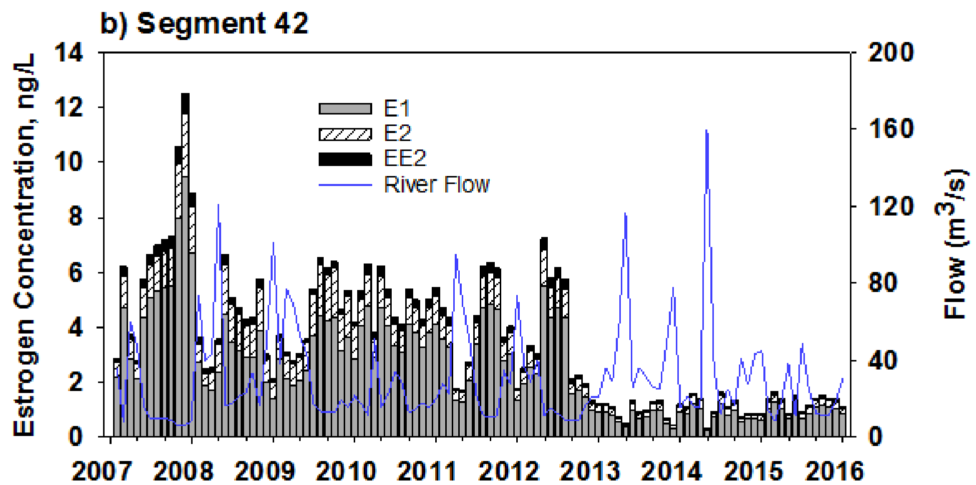




**Figure 4.** Temporal variation (monthly averaged) in EAC concentrations in segments immediately downstream of (a) Waterloo (Segment 23) and (b) Kitchener (Segment 42) WWTPs.

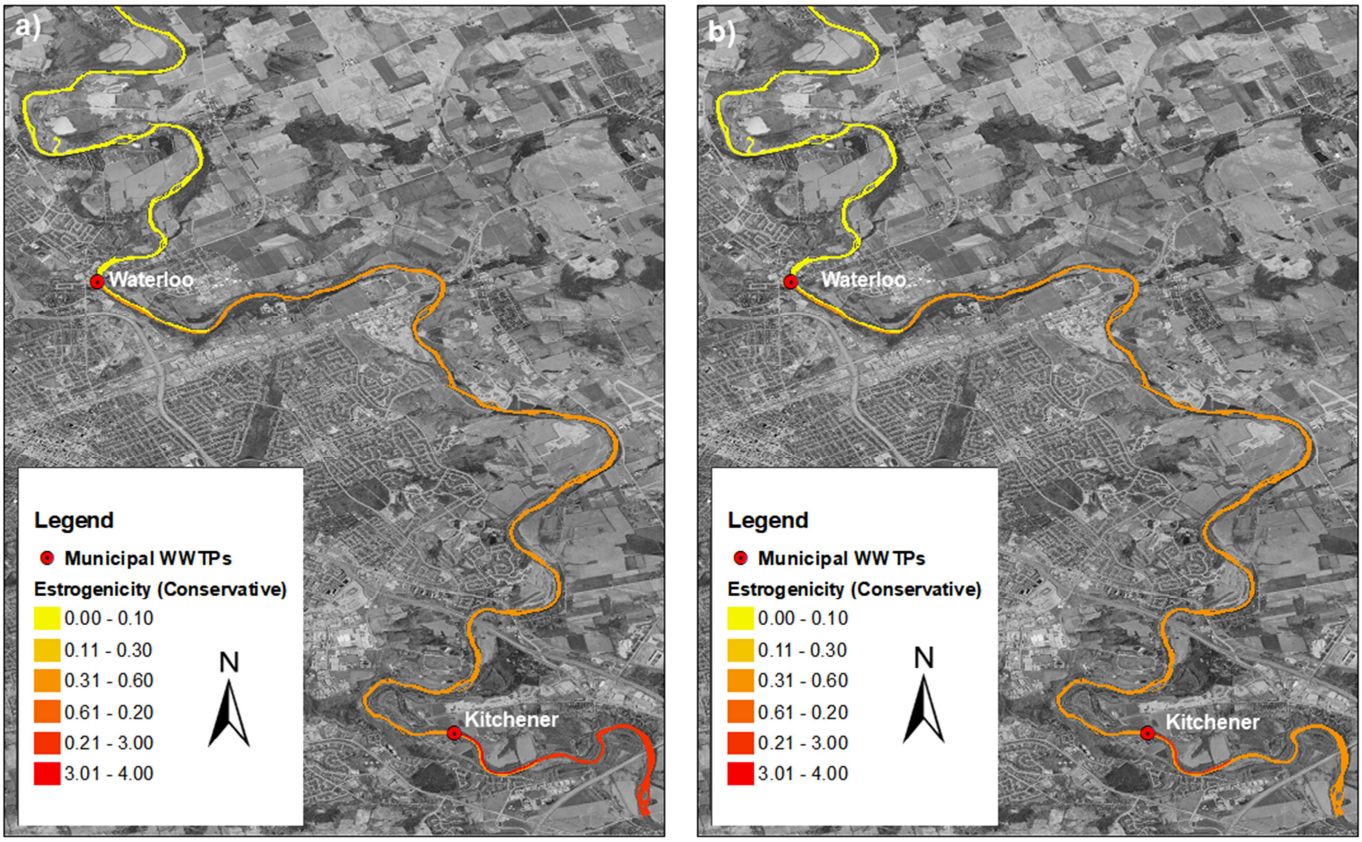



**Figure 5**. Spatial conditions of total estrogenicity: (a) low flow condition pre-upgrade (May 20, 2012) and (b) low-flow condition post-upgrade (June 11, 2014).

**Highlights**

- A water quality model was employed to predict concentrations of estrogens.
- River transport conditions played a major role in the distribution of estrogens.
- Concentrations <0.1 ng/L E2 eq. were predicted to cause minimal intersex expression.
- River estrogenicity of ≥10 ng/L E2 eq. was associated with severe intersex.