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Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line

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ABSTRACT

There is concern regarding oil sands process water (OSPW) produced by the oil sands industry in Alberta, Canada. Little is known about the potential for OSPW, and naphthenic acids (NAs), which are the primary persistent and toxic constituents of OSPW, to affect endocrine systems. Although ozonation significantly reduces concentrations of NAs and OSPW toxicity, it was hypothesized that oxidation of OSPW might produce hydroxylated products with steroidogenic activity. Therefore, untreated and ozone treated OSPW were examined for effects on sex steroid production using the H295R Steroidogenesis Assay. Untreated OSPW significantly decreased testosterone (*T*) and increased 17 β -estradiol (*E2*) concentrations at OSPW dilutions greater or equal to 10-fold. This effect was mainly due to decreased *E2* metabolism. Analysis of *CYP19A* (aromatase) mRNA abundance and enzyme activity suggested that induction of this enzyme activity may have also contributed to these effects. Reduction of parent NA concentrations by 24% or 85% decreased the effect of OSPW on *E2* production. Although *T* production remained significantly reduced in cells exposed to ozone treated OSPW, the effect was diminished. Aromatase mRNA abundance and enzyme activity were significantly greater in cells exposed to ozone treated OSPW, however the magnitude was less than in cells exposed to untreated OSPW. No change of *E2* metabolism was observed in cells exposed to ozone treated OSPW, which may account for recovery of *E2* levels. The results indicate that OSPW exposure can decrease *E2* and *T* production, but ozonation is an effective treatment to reduce NA concentrations in OSPW without increasing effects on steroidogenesis.

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1. Introduction

The oil sands deposits in the Athabasca Basin, located in north-eastern Alberta, Canada, are one of the largest reserves of petroleum in the world (Hunt, 1979), and are increasingly being developed by a rapidly growing oil sands industry. In 2000, Alberta's oil sands industry produced approximately 604,700 barrels of marketable bitumen and crude oil per day (Alberta Energy and Utilities Board, 2004–2005), and production increased to an average of 1,184,000 barrels per day in 2008 (Alberta Energy and Utilities Board, 2007–2008). With the costs associated with bitumen recovery from oil sands decreasing due to technological advances,

and a decline in global conventional oil supplies, development of the major reserves in the Alberta Basin, which are estimated to be at least 173.2 billion barrels, is expected to continue (Williams, 2003; Alberta Energy and Utilities Board, 2006–2007).

In the surface mining oil sands industry, extraction of bitumen from oil sands involves the Clarke hot water extraction method. This process results in the production of large volumes of process-affected waters that contain sand, clay, unrecoverable bitumen, hydrocarbons, and a water soluble organic acid fraction known commonly as naphthenic acids (NAs). This wastewater is commonly referred to as oil sands process water (OSPW). OSPW is stored on-site in active settling basins, or tailing ponds, where the clarified fraction can eventually be recycled back into the extraction plant for further use. In accordance with a zero discharge policy OSPW is not intentionally released (MacKinnon, 1989; FTFC, 1995) and in 2006 it was estimated that greater than

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10^9 m^3 OSPW were currently being stored on-site in various settling basins (Del Rio et al., 2006).

NAs, or their naphthenate salts, have been identified as the primary toxic constituents of OSPW (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986; Holowenko et al., 2002). NAs are a group of naturally-occurring acyclic, monocyclic, and polycyclic carboxylic acids with the general formula of $\text{C}_n\text{H}_{2n+Z}\text{O}_2$, where n represents the number of carbon atoms and Z is zero or a negative even integer related to the number of rings (or double bond equivalents) in the molecule (Brient et al., 1995). OSPW has been found to be toxic to a range of aquatic organisms, including rainbow trout (*Oncorhynchus mykiss*), *Daphnia magna*, and *Vibrio fischeri* (reviewed in Clemente and Fedorak, 2005) as well as the green alga (*Pesudokirchneriella subcapitata*) (Warith and Yong, 1994). The toxicity of OSPW is mainly associated with lower molecular weight NAs (Holowenko et al., 2002; Frank et al., 2008a). However, a recent study suggested that increased carboxylic acid content within NA structures decreases hydrophobicity and, consequently, the toxicity of higher molecular weights NAs (Frank et al., 2008b). The mechanism of toxicity of NAs from various sources is the subject of some debate and has received little attention. However, NAs are surfactants – owing to the presence of hydrophobic alkyl groups and a hydrophilic carboxylic moiety. This had led to speculation, and some experimental evidence, that NAs do not bind to cellular receptors but rather exert their mode of acute toxicity via physical disruption of membranes, or narcosis (Roberts, 1991; Lipnick, 1993; Frank et al., 2008b).

Ultimately, there is a long-term need to reclaim the large volumes of toxic OSPW, but currently there are no proven methods to do so. Field studies have demonstrated that OSPW confined to tailings ponds for as little as 1 month was less lethal to fathead minnows (MacKinnon and Boerger, 1986). However, while removal of acute toxicity is achievable, chronic toxicity is not completely removed (Leung et al., 2001). Previous studies have also demonstrated that a certain fraction of OSPW NAs can be partially removed through aerobic microbial degradation under laboratory conditions (Herman et al., 1993; Scott et al., 2005; Han et al., 2009), but the extent of NA degradation in the field is less. NA concentrations in fresh OSPW have historically ranged between 50 and 100 mg/L, but no OSPW has been successfully remediated to concentrations less than 19 mg/L (Quagraine et al., 2005). At best, the disappearance half-lives for NAs in OSPW are 12.8–13.6 years (Han et al., 2009). Consequently, more aggressive efforts are being tested for the remediation of OSPW. One method that has already shown promise is ozonation (Scott et al., 2008). Ozonation of OSPW decreases NA concentrations and results in an effluent that is effectively non-toxic in the acute Microtox[®] microbial toxicity assay. However, little is known about the effectiveness of ozone treatment with respect to toxicity towards other organisms. Ozonation of other matrixes has been shown to change the activity of treated molecules, and in some cases results in compounds that can modulate the endocrine system (Esplugas et al., 2007; Petala et al., 2008).

Little is known about the impact of OSPW and NAs in particular on the endocrine axis, and specifically the process of sex hormone production known as steroidogenesis. Significant decreases in plasma T and $E2$ have been reported in goldfish (*Carassius auratus*; Lister et al., 2008) and yellow perch (*Perca flavescens*; van den Heuvel et al., 1999) exposed to OSPW. Reduced *in vitro* production of T and $E2$ by ovarian and testicular tissues was also reported in goldfish exposed to OSPW (Lister et al., 2008). Therefore, the objectives of this study were to: (1) assess the cellular toxicity and steroidogenic effects of untreated sediment free OSPW on sex hormone production using the H295R cell line and (2) to determine if these baseline effects are modulated by ozone treatment.

The H295R cell line expresses all key enzymes involved in steroidogenic pathways, and bioassays used to evaluate effects of

chemicals on gene expression, enzyme catalytic activities, and steroid hormone production have been established (Hilscherova et al., 2004; Zhang et al., 2005; Hecker et al., 2006, 2007; Villeneuve et al., 2007; He et al., 2008). In the present study, T and $E2$ production, $E2$ metabolism, and aromatase (*CYP19a*) gene expression and enzyme activity were also measured to investigate the effect(s) that untreated and ozonated OSPW had on steroidogenesis in the H295R cell line.

2. Methods

2.1. OSPW collection and treatment

OSPW was collected in December 2007 from the West-in-Pit, an active settling basin on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). Three 1 L batches of OSPW were filtered through a $0.45 \mu\text{m}$ cellulose membrane filter (Osmonics, Inc., Minnetonka, MN). Filtering of OSPW does not remove NAs. One batch was left untreated (control) while the two other batches were each placed in 2 L glass bottles for treatment by ozonation. Ozone was bubbled through the OSPW at a rate of 2 L/min and stopped occasionally to take samples for ultra-pressure liquid chromatography (UPLC) high-resolution mass spectrometry (HRMS) analysis of NAs (Batatineh et al., 2006). The ozonation treatment was continued until the total degradation of parent NAs in these two batches reached 24% and 85%, as determined by the remaining sum response of all UPLC–HRMS peak area corresponding to NAs. These ozonated samples will be referred to hereafter as 24% and 85% ozonated OSPW, respectively. An AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany) was used to produce ozone gas from extra dry, high purity oxygen. All glassware was cleaned and pre-treated with ozone before experimentation. Ozonation was performed at room temperature in open glass bottles on a stir plate.

It has been previously suggested that NAs have relatively low potential to partition from water to air, with Henry's Law Constants estimated to be $<10^{-6} \text{ atm} \times \text{m}^3/\text{mole}$, and bubbling of air through OSPW for 9 days resulted in only 7% reduction in naphthenic acid concentration (Han et al., 2009). Thus, physical loss of naphthenic acids during ozonation was assumed to be negligible in the current work. The pH of the initial untreated OSPW was 8.1 and this value was not affected by the ozonation.

2.2. OSPW medium preparation

Exposure media were prepared according to standard cell culture medium recommendations, except that the nano-pure water was replaced with either untreated OSPW, 24% ozonated OSPW or 85% ozonated OSPW. Dilutions of exposure medium were prepared by diluting with medium prepared with nano-pure water. All exposure media was adjusted to pH 7.4 as this is optimal for hormone production (Hecker et al., 2006).

2.3. Experimental design

H295R cells were obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA), and cultured at 37°C in a 5% CO_2 atmosphere according to previously described methods (Hecker et al., 2006). Briefly, the cell culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (SIGMA Chemical Co., St. Louis, MO, USA), supplemented with 2.5% Nu-Serum (BD Biosciences, San Jose, CA, USA), 1% ITS + Premix (BD Biosciences), and 1.2 g/l Na_2CO_3 . Prior to initiation of exposure experiments cells were plated in 24-well culture plates at a density of 3×10^5 cells/ml, and cells

were allowed to attach for 24 h. After this pre-incubation period medium was replaced with test solutions. Cells were exposed to either 10 000-, 1000-, 100-, and 10-fold diluted sediment free OSPW or full-strength untreated sediment free OSPW, 24% ozone depleted OSPW, or 85% ozone depleted OSPW.

2.4. Cytotoxicity assay

Cytotoxicity of untreated and ozonated OSPW was assessed in 96-well plates using the MTT (Mosman, 1983).

2.5. Hormone measurement

Quantification of *T* and *E2* was conducted according to Hecker et al. (2006). After a 48 h exposure period, 500 ml of medium was extracted twice with 2.5 ml diethyl ether. The solvent phase containing target hormones was evaporated under a stream of nitrogen, and then reconstituted in 250 μ l ELISA assay buffer (Cayman Chemical Company). Hormone concentrations were measured by competitive ELISA following the manufacturer's recommendations on a Dynex DSX four-plate automated ELISA processing station (Dynex Technologies, Chantilly, VA, USA). Neither the untreated nor ozone treated OSPW cross reacted with the ELISA assay.

2.6. Estradiol metabolism assay

A method was developed to measure the rate of *E2* metabolism using the H295R cell line. Briefly, 1 ml of cell suspension with a density of 1×10^6 cells/ml was plated into 24-well culture plates and cells were allowed to attach for 24 h. After this pre-incubation period medium was replaced with 250 μ l of OSPW medium spiked with 1 nM 6, 7 [3 H]-*E2* (PerkinElmer, Boston, MA, USA) was added and cells were incubated at 37 °C and 5% CO₂ for 1 h. At the end of the exposure, 200 μ l medium was collected and extracted with 500 μ l DCM, and 100 μ l supernatant was collected for liquid scintillation measurement using a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., CA, USA). The rate of *E2* metabolism was calculated by the fold change of degraded *E2* in the spiked media compared with controls.

2.7. Aromatase assay

The activity of *Cyp19a* (aromatase) was determined based on the method described by Lephart and Simpson (1991). Briefly, to measure the indirect effects on aromatase activity 1 ml of cells were plated in 24-well culture plates at a density of 1×10^6 cells/ml for 24 h, and then the medium was replaced with media prepared with either full-strength or diluted untreated OSPW, or 24% ozonated OSPW, or 85% ozonated OSPW. After 24 h exposure, the OSPW medium was removed and cells were exposed to 54 nM [3 H] androstenedione (PerkinElmer) in serum-free medium according to Sanderson et al. (2002). After 1 h exposure at 37 °C and 5% CO₂, 200 μ l of culture medium was extracted and used for measuring the relative fold change in radioactivity. The potential direct effects of OSPW on aromatase were also tested and no significant changes were observed (data not shown).

2.8. Real-time PCR

Expression of *CYP19a* and the housekeeping gene porphobilinogen deaminase (*PBGD*) was measured by quantitative real-time PCR (qPCR) using the SYBR green methods of Hilscherova et al. (2004) and Zhang et al. (2005). Briefly, cells were plated at a density of 1×10^6 cells/ml in 1 ml cell suspension per well of a 24-well culture plate. After 24 h, the medium was decanted and replaced

with fresh media prepared with full-strength untreated OSPW, 24% ozonated OSPW, or 85% Ozonated OSPW for 1, 2, 4, 8, and 24 h. Following the exposure period cells were harvested and total RNA was isolated from cells harvested from each well using the Agilent Total RNA Isolation Mini Kit (Agilent, Santa Clara, CA, USA) following the manufacturers recommendations. RNA concentrations were determined at A260 using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The first strand cDNA was synthesized from 2 μ g of total RNA using an iScript cDNA Synthesis kit (BioRad, Mississauga, ON, Canada) with an oligo dT primer according to the manufacturer's instructions. The ABI 7300 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. The primer sequences for *CYP19a* are 5'-AGGTGCTATTGGT-CATCTGCTC-3' (sense) and 5'-TGTTGGAATCGGGTCTTTATGG-3' (antisense), and the primer sequences for *PBGD* are 5'-CTGGA GGAGTCTGGAGTCTAG-3' (sense) and 5'-TGGAAATGTTACGACAGT GATG-3' (antisense). PCR reaction mixtures (20 μ l) contained 1 μ l of forward and reverse primers, 5 μ l of cDNA sample, and 10 μ l of 2 \times SYBR Green™ PCR Master Mix (Applied Biosystems). The thermal cycle profile was as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing with extension for 1 min at 60 °C; and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Melting curve analyses were performed during the 60 °C stage of the final cycle to differentiate between desired PCR products and primer-dimers or DNA contaminants. The delta-delta Ct method (Livak and Schmittgen, 2001) was used to calculate the fold change in *CYP19a* expression relative to *PBGD*.

2.9. Data analyses

All experiments were conducted in duplicate, and triplicate measurements were used for each individual exposure. Statistical analyses were conducted using SPSS 16 (SPSS Inc., Chicago, IL, USA). The normality of each data set was assessed using the Kolomogrov-Smirnov one-sample test and homogeneity of variance was determined using the Levene's test. Significant differences were evaluated by one way ANOVA with Dunnett's post hoc tests. Linear regression analysis was used to test the relationship between degree of ozonation of OSPW and either the decrease in *T* production or increase in *E2* metabolism in cells exposed to full-strength untreated and ozonated OSPW. Differences with $p < 0.05$ were considered significant. All data are presented as mean \pm standard deviations.

3. Results and discussion

3.1. Cytotoxicity of OSPW

Exposure of H295R cells to either untreated OSPW, or 24% or 85% ozonated OSPW did not produce significant cytotoxicity at any of the dilutions used (data not shown). The acute toxicity of OSPW has been demonstrated in a range of aquatic species (reviewed in Clemente and Fedorak, 2005). Using the Microtox® assay to assess acute toxicity, IC₂₀ values of approximately 10% (v/v) OSPW have been reported by Mackinnon and Boerger (1986) and Scott et al. (2008). In the current study no significant acute cytotoxicity of sediment free fresh OSPW towards H295R cells was observed using the MTT assay. This result suggests that H295R cells are less sensitive to the cytotoxic effects of OSPW than *Vibrio fischeri*. Ozonation of OSPW did not increase the cytotoxicity of OSPW. This is consistent with results reported by Scott et al. (2008) that ozonation decreased the cytotoxicity of OSPW in the Microtox® assay.

3.2. Effects of OSPW on sex hormone production

Generally, untreated OSPW decreased production of *T* and increased production of *E2* after 48 h exposure (Fig. 1). *T* production was significantly decreased by 0.45-, 0.42-, 0.32-, and 0.26-fold with exposure to full-strength untreated, 24% and 85% ozonated OSPW, and 10-fold diluted untreated OSPW, respectively (Fig. 1a). Significantly greater *E2* production was detected when cells were exposed to full-strength untreated (2.0-fold) and 24% ozonated OSPW (1.7-fold), whereas exposure to full-strength 85% ozonated OSPW did not affect *E2* production at any dose (Fig. 1b). *E2* production was also significantly increased in cells exposed to 24% ozonated OSPW diluted 10 000-fold, however, this result is likely an experimental anomaly as the effect was small and no significant effects were observed at dilutions of 1000-, 100-, and 10-fold (Fig. 1b).

Linear regression analysis of hormone concentrations released by cells exposed to full-strength untreated, 24% ozonated or 85% ozonated OSPW indicated a significant relationship between degree of ozonation and the decrease in *T* production ($r^2 = 0.578$). A significant relationship between degree of ozonation and the increase in *E2* production ($r^2 = 0.964$) was also observed.

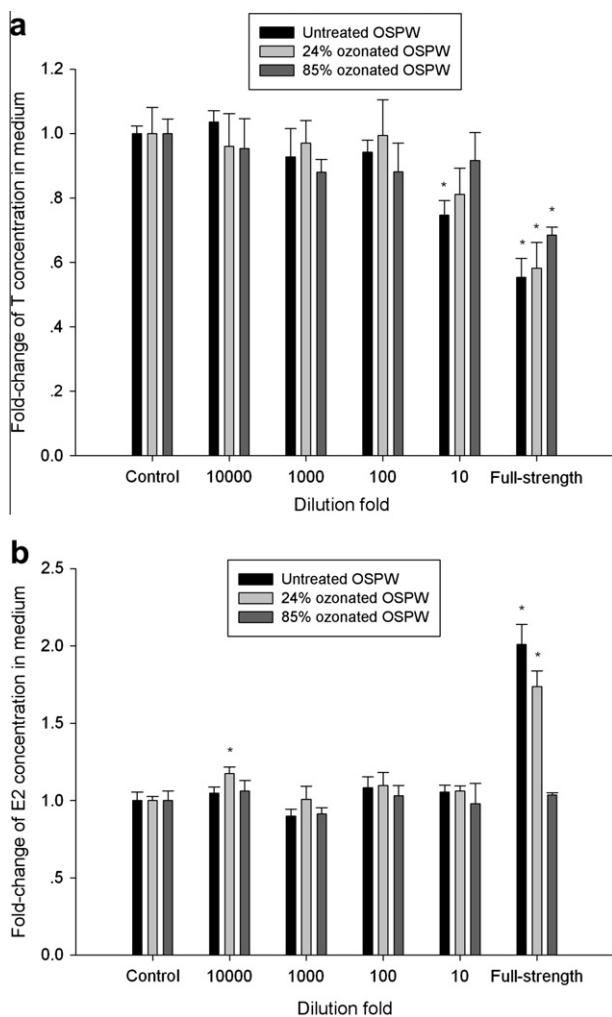


Fig. 1. Concentrations of (a) testosterone (*T*) and (b) 17β-Estradiol (*E2*) in the culture medium of H295R cells exposed to untreated, 24% ozonated, and 85% ozonated OSPW for 48 h. Values represent the Mean ± SD of the hormone concentration. Asterisks represent a significant difference compared with the control ($p < 0.05$).

3.3. Effects of OSPW on E2 metabolism

For untreated OSPW, exposure to 100- and 10-fold diluted and full-strength significantly reduced the metabolism of *E2* by 1.2, 1.4, and 2.3-fold, respectively. For 24% ozonated OSPW, exposure to 10-fold diluted and full-strength significantly reduced *E2* metabolism by 1.2 and 1.6-fold, respectively. For 85% ozonated OSPW, exposure to full-strength did not impact *E2* metabolism (Fig. 2).

3.4. Effects of OSPW on CYP19a mRNA expression and aromatase enzyme activity

Exposure to full-strength untreated OSPW significantly increased *CYP19a* mRNA expression after 2 h, 4 h, and 8 h by 1.8-, 2.0-, and 3.0-fold, respectively, post-exposure. Exposure to either full-strength 24% ozonated OSPW for 4 h or 85% ozonated OSPW for 8 h also resulted in a significant increase in *CYP19a* mRNA expression by 1.9- and 1.5-fold, respectively. In all exposure groups mRNA abundance returned to control levels by 24 h post-exposure (Fig. 3). Exposure to 10-fold diluted and full-strength untreated OSPW, full-strength 24% ozonated OSPW, and full-strength 85% ozonated OSPW increased aromatase activity 1.9-, 2.5-, 2.5-, and 2.2-fold, respectively, at 24 h post-exposure (Fig. 4).

3.5. Effect of OSPW on steroidogenesis

Although little is known about the effects of OSPW or NAs on the endocrine axis, there is information suggesting that OSPW and NAs can impact sex hormone production. Significantly less concentrations of *T* and *E2* have been reported in blood plasma of goldfish (Lister et al., 2008) and yellow perch (Van den Heuvel et al., 1999) exposed to OSPW. While the steroidogenic pathway remained intact in goldfish exposed to OSPW, reduced *in vitro* production of *T* and *E2* by ovarian and testicular tissues has been reported (Lister et al., 2008). The decrease in *T* production in H295R cells is consistent with lesser concentrations of *T* in blood plasma *in vivo* (Van den Heuvel et al., 1999; Lister et al., 2008) and lesser *in vitro* *T* production reported in fish (Tetrault et al., 2003). However, this is the first study to demonstrate an increase in *E2* production in response to OSPW exposure. Decreased *T* production was observed in cells exposed to both 10-fold diluted and full-strength OSPW. In contrast, increased *E2* production was ob-

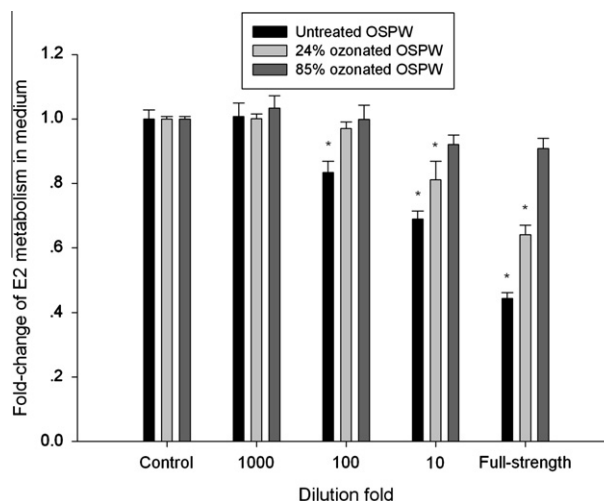


Fig. 2. Effect of OSPW on *E2* metabolism in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated OSPW. Values present the Mean ± SD of the fold-change in *E2* metabolism. Asterisks represent a significant difference compared with the control ($p < 0.05$).

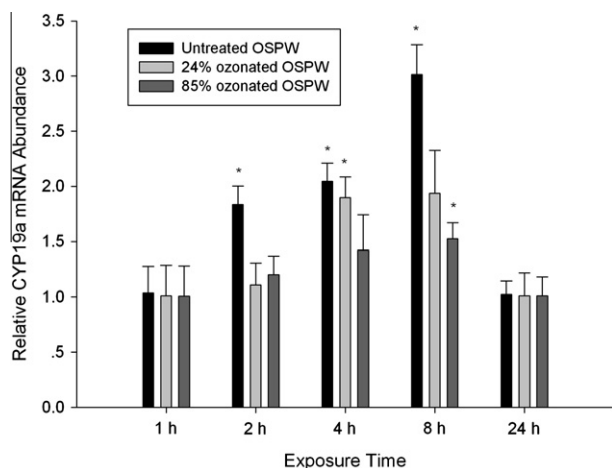


Fig. 3. Time course analysis of the effect of OSPW on *CYP19* mRNA expression in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated OSPW. Values represent the Mean \pm SD of relative *CYP19a* mRNA abundance. Asterisks represent a significant difference compared with the control ($p < 0.05$).

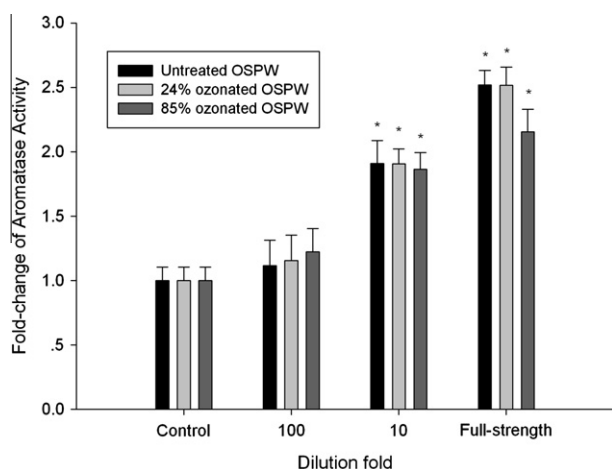


Fig. 4. Effect of OSPW on aromatase activity in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated OSPW. Values represent the Mean \pm SD of the fold change in activity. Asterisks represent a significant difference compared with the control ($p < 0.05$).

served only in cells exposed to full-strength OSPW. These results suggest that the threshold for effects on *E2* production may be greater than the threshold for effects on *T* production.

Aromatase (*CYP19a*) aromatizes *T* to convert it to *E2*. Exposure to 10-fold diluted and full-strength OSPW increased both *CYP19a* gene expression and aromatase enzyme activity and decreased *E2* metabolism. The combined effect of an increase in aromatase activity and decreased *E2* metabolism likely explains the observed increase in *E2* due to exposure to full-strength OSPW. It is unclear why no significant increase in concentrations of *E2* was observed in cells exposed to 10-fold diluted OSPW. In other studies (van den Heuvel et al., 1999; Lister et al., 2008) that have investigated the effect of OSPW on sex hormone levels no effects on either aromatase gene expression or activity, or *E2* metabolism, were investigated.

The difference in *E2* concentrations observed in this study, versus the results of *in vivo* studies, in which fish were exposed to OSPW (van den Heuvel et al., 1999; Lister et al., 2008) may be due to several factors. It is unknown whether the results from H295R cells exposed to OSPW are representative of effects on exposed fish since a complementary study was not performed. It

has been demonstrated that some chemicals exert similar effects on *E2* and *T* production in both H295R cells and in Fathead minnow (*Pimephales promelas*) ovarian explants, while others perform differently in these two systems (Villeneuve et al., 2007). Although the basis for differences in responses observed in the two systems are unclear, it has been suggested that differences in the complexity of the two systems (e.g., cells versus tissues) and species-related differences should be considered when comparing results from the H295R assay with results from fish (Villeneuve et al., 2007). The dominant steroidogenic pathways in adrenal tissue differ from those in gonad tissue (Norris, 1997). Additionally, fish and mammalian steroidogenic enzymes may differ in specificity for some chemicals (Baker, 2001). Consequently, differential regulation of sex hormone steroidogenesis in fish and mammals could result in different sensitivities and/or responses to OSPW. The results of this study also raise an interesting question regarding mammalian health. Although the effect of OSPW on hormone production in mammals is unknown, the results of this study suggest the potential for disruption of steroidogenesis. Indeed, for model chemicals with different effects on steroidogenesis, the H295R assay has been shown to be a relatively sensitive predictor of effects (Hecker et al., 2006).

One difference between this study and previous *in vivo* studies of the effects of OSPW on fish is the nature of the OSPW. The OSPW used in this study was collected from West-in-Pit (WIP), an active settling basin that was established in 1995 and receives fresh OSPW from both the Southwest Settling Basin and South East Pond (SEP). The NA concentration in the WIP OSPW used in this study was approximately 77 mg/L. In the study by Lister et al. (2008) sexually mature goldfish were caged in an experimental reclamation pond (termed P5) that was constructed from mature fine tailings (MFT) capped with uncontaminated water. The NA concentration in P5 was reported as approximately 24.1 mg/L. Although the NA concentrations in the study by van den Heuvel et al. (1999) was not reported, the effects on *T* and *E2* were observed in fish from ponds constructed in 1993; which had lesser NA concentrations than WIP OSPW (Han et al., 2009). However, van den Heuvel et al. (1999) observed no consistent relationship between exposure to OSPW or NAs and steroid hormones. In addition to the differences in NA concentration between WIP OSPW and water from these other studies the NA profile is likely to be different since aging of OSPW has been shown to decrease NA concentration, while increasing the relative importance of the hydroxylated NA fraction (Han et al., 2009). Consequently, it is possible that the differences in effects on *T* and *E2* levels between the current study and those in fish may be due to the differences in NA concentration, and NA profile including hydroxylated NAs.

3.6. Effects of ozone treatment

If the goal of reclaiming OSPW as functional lakes and wetlands, or releasing OSPW back into the environment, is to be realized then effective methods of reducing the toxicity of OSPW must be developed and validated. Ozonation of OSPW holds promise because it has been shown to rapidly decrease NA concentrations and their toxicity to bacteria as determined in the Microtox assay (Scott et al., 2008). However, different NA profiles with a greater proportion of lower molecular weight NAs was found in the treated OSPW (Scott et al., 2008). Consequently, a major concern is that low molecular weight NAs formed during ozonation, or hydroxylated NAs resembling steroids, can become more bioavailable and consequently adversely affect aquatic organisms. Indeed, ozone treatment is effective in removing both synthetic and natural estrogenic compounds and their associated activity (Alum et al., 2004; Bila et al., 2007; Maniero et al., 2008). However, questions have been raised about potential hazardous substances produced

during the ozonation process (Esplugas et al., 2007; Petala et al., 2008). As such we were interested in determining whether ozonation as a treatment method impacted the effect of OSPW on steroidogenesis in the H295R cell line.

Generally, ozonation attenuated the adverse effects of exposure to OSPW on *T* and *E2* production. The significant decrease in *T* production in cells exposed to 10-fold diluted OSPW was abolished by ozonation, since no significant decrease in *T* production was detected in either the 24% or 85% ozonated OSPW group relative to the control. In contrast, in cells exposed to full-strength OSPW *T* production remained significantly decreased in both the 24% and 85% ozonated OSPW groups; albeit the extent of the decrease appeared to be diminished with increasing ozonation. The increase in *E2* concentrations observed in cells exposed to full-strength untreated OSPW was only detected in cells exposed to full-strength 24% ozonated OSPW as exposure to 85% ozonated OSPW did not affect *E2* concentrations.

As was observed in the untreated OSPW exposure groups, aromatase mRNA abundance and enzyme activity remained significantly elevated, although to a lesser extent in cells exposed to ozone treated OSPW. Although *CYP19a* mRNA expression remained induced by each of the OSPW treatments, ozonation delayed maximal expression of *CYP19a* gene expression, and increased ozone treatment further increased the time required for maximal induction. Ozonation also impacted the effect of OSPW on the metabolism of *E2*. This attenuation was most pronounced in cells exposed to 85% ozonated OSPW, since no significant inhibition of *E2* metabolism was detected in exposure groups. In addition, when diluted sufficiently, 24% ozonated OSPW abolished the effects on *E2* metabolism. Overall, these results suggest that inhibition of *E2* breakdown is the major reason for increased *E2* in the 24% ozonated OSPW exposure groups. Furthermore, these results suggest that ozonation may abolish the attenuating effects of OSPW on *E2* production. No changes to aromatase mRNA expression or activity, or *E2* metabolism, were observed in cells, where OSPW did not affect hormone production. These results suggest that ozonation of OSPW does not impart new or enhanced steroidogenic altering properties on OSPW.

Exposure to ozone treated OSPW resulted in lesser effects on *E2* and *T* production than did exposure to full-strength OSPW. There was an inverse relationship between degree of ozonation and related decreases in NAs and effects on both *T* and *E2* production. Together, these results suggest that greater ozonation decreases the impact of OSPW on *T* and *E2* production. Again, these results support the hypothesis that ozonation of OSPW does not impart new or enhanced steroidogenic altering properties on OSPW.

4. Conclusion

In summary, exposure to untreated OSPW from an active settling basin can alter the steroidogenic pathway in the H295R cell line, and all the adverse effects are mitigated or even recovered by ozonation treatment, suggesting that ozonation may be an effective treatment method to reduce some impacts of OSPW on steroidogenesis. Further study is needed to more fully determine the *in vivo* effects of OSPW and ozonation of OSPW on steroidogenesis.

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