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Estrogenic Activity of Treated Municipal Effluent from Seven Sewage Treatment Plants in Victoria, Australia

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In the past few years, there has been increased interest in chemicals that have the potential to interfere with the endocrine systems of aquatic wildlife. The effluent from municipal sewerage treatment plants (STPs) is the source of much of the endocrine disrupting chemical (EDC) input into aquatic environments (Ying et al. 2002). Of the many types of compounds that show estrogenic activity, most commonly the natural and synthetic estrogens, such as 17-β-estradiol and 17-α-ethynylestradiol, are found in the highest concentrations (Ying et al. 2002). These materials are excreted daily by women during the various phase of their menstrual cycle, mainly as conjugates of sulfuric and glucuronic acids. In Australia, in most cases these chemicals are generally flushed to the sewage system. However, within the sewage treatment facility, these conjugates appear to be cleaved by microorganisms used in the treatment process, and once again become active (Johnson and Sumpter 2001). In Australia, there is very little information on overall estrogenicity, or concentrations of specific estrogenic compounds in Australian STP effluents or aquatic environments, as most effluent monitoring is concerned with nutrients, metals and organic compounds of industrial origin (Ying et al. 2004). That said, there are some reports from Australia of impacts similar to those observed in the northern hemisphere e.g. changes to the morphological characteristics of male mosquitofish (*Gambusia affinis holbrooki*) downstream of STPs in Sydney (Batty and Lim 1999).

To partly address the shortage information in Australia on estrogens in Australian STP effluents, in this reconnaisance survey we have assessed the estrogenic activity of treated municipal wastewater from seven STPs located in the southern state of Victoria Australia, using the yeast two hybrid assay reported by Shiriashi et al. (2000). The yeast two-hybrid assay is an *in vitro* reporter gene assay, which provides a simple, yet highly sensitive method to screen chemical substances for estrogenic activity by combining a 96 well plate culture method and a chemiluminescent reported gene assay. The result is a method that can then easily be applied to the screening and monitoring of estrogenicity of environmental samples. Herein we report our findings.

MATERIALS AND METHODS

Effluent samples were collected from seven STPs in the three days between November 24 and 26, 2003 from each facility at the point at which effluent enters the environment, either as recycled water or direct discharge to the receiving water. Note: samples were collected with the assistance and agreement of the STP operators on condition that they are not identified when publishing results. Hence, hereafter the

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STPs will be referred to only as STP A, B, C etc. Not all discharges could be sampled in the same manner, however, and sampling protocols were modified dependent upon on-site conditions. Grab sampling was employed where the final effluent was retained in a holding lagoon/pond prior to discharge into the environment. Grab samples were directly collected in Extran® MAO3 (Merck P/L) soaked, acid-soaked (10% nitric acid) and solvent rinsed (Methanol HPLC grade) 2 x 2.5L amber glass bottles. Where an STP utilised activated sludge treatment and the final effluent decanted after a relatively short holding time, composite samples were collected in polyethylene bottles (cleaned in the same manner as those bottles used for the grab samples) using an auto-sampler, and transferred to 2 x 2.5L amber glass bottles. All samples were stored at 4° C until extraction.

For each STP site, 500 mL samples were extracted for the measurement of estrogenicity. Prior to filtration or extraction, 5mL of an acetic acid: methanol (1:9) buffer solution was added to each 500mL sample (i.e. to each duplicate) to ensure a neutral pH. Samples were then filtered with GF/C filters (Advantec, Japan) to remove particulate matter. The sample was then passed through an Empore™ solid phase extraction (SPE) disk (47mm), which had previously been conditioned with sequential washes of 10mL methanol, 10mL dichloromethane, 10mL methanol, and 20mL deionised water (water having a resistivity of at least 18 $M\Omega\ cm^{-1}$ produced by passing singly distilled water through a Milli-Q Water Purification System), after which the SPE disks were immediately washed with 30mL of deionised water, and dried at 35°C on a hotplate for approximately 1.5 hours. Each disk was wrapped separately in aluminium foil, labelled, placed inside a labelled paper envelope, and stored at -20°C until transported to Japan for analyte elution and analysis. On the December 9, 2003, each disk was eluted with 2 x 3.5mL aliquots of dichloromethane, and the resulting solution immediately frozen (-20°C). Once all the disks had been processed, the samples were evaporated to dryness with nitrogen and the residue immediately frozen (-20° C) until analysis the following day.

Samples and standards were removed from the freezer one hour prior to analysis, thawed, and dissolved in 50μL (5μM) DMSO, effectively resulting in 10,000-fold concentration from the original effluent sample. The agonist activities of the treated municipal effluent samples were measured, both with and without possible metabolic activation by rat liver S9 preparation (Kikkoman Company, Noda, Japan), with a yeast two hybrid estrogenicity assay system using yeast cells (*Saccharomyces cervisiae* Y190) into which the human estrogen receptor (hERα) or the Japanese medaka (*Oryzias latipes*) estrogen receptor were inserted. Both were adapted to a chemiluminescent reported gene (for β - galactosidase) method employing a 96–well culture plate (Shiriashi et al. 2000). Aliquots of each sample (20μL), with or without a period of incubation with rat liver (mix 37°C, 1 h), were incubated (30° C, 4 h) with yeast cells that had been preincubated (30° C, overnight) in modified SD medium (lacking tryptophan and leucine). A mixed solution for inducing chemiluminescence and for enzymatic digestion (Zymolyase 20T) was then added followed, by a light emission accelerator solution. The chemiluminescence produced by released β-galactosidase was measured with a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan). Agonist activity was recorded as the EC x 10, which was defined as the concentration of test solution producing a chemiluminescent signal 10 times that of the blank control. Two positive controls (17β-estradiol and *trans*-stilbene) and a solvent (vehicle) control were run on each plate in both assays, and in the absence and presence of S9.

Table 1. Estrogenicity of seven Victorian STP effluents, December 2003.

STP	Estrogenic activity (ng/L EEQ)					
	hER			mER		
	-S9	+S9	RA(%)	-S9	+S9	RA(%)
A	0.3	N.D.	0	3.2	N.D.	0
B	N.D.	N.D.	0	N.D.	N.D.	0
C	2.7	N.D.	0	5.4	0.82	15
D	42	N.D.	0	28	N.D.	0
E	3.1	N.D.	0	12	3.6	30
F	13	2.1	16	8.6	5.7	66
G	12	0.93	8	55	16	29

EEQ, estradiol equivalents; hER, human estrogen receptor-based bioassy; mER, medaka estrogen receptor-based bioassay; -S9, bioassay without addition of rat liver extract; +S9, bioassay with rat liver extract; N.D., not detected; RA(%), percentage activity following incubation with rat liver extract.

RESULTS AND DISCUSSION

In this reconnaissance survey, estrogenic activity was detected in all samples by both hER and mER assays, except effluent B (Table 1). The mER assay was more sensitive, generally producing higher values of estrogenic activity than the hER assay. Conducting the assay in the presence of rat liver homogenate ((+S9) i.e. in the presence of mammalian enzymes) reduced the estrogenic response, removing the response entirely in all effluents except effluent F and G (hER), and effluents C, E, F, and G (mER). A concurrent toxicity assay (data not shown) indicated that the two samples of lowest toxicity corresponded to the samples with the highest estrogenic activity in the mER assay (-S9), suggesting that a lack of bioassay response was related to lack of estrogenic compounds, rather than the direct toxic effect of the effluent.

To the best of our knowledge, these are the first measurements of the estrogencity of Australian STP effluents to be published. The levels of estrogenicity determined are comparable to those reported in the northern hemisphere, but generally to the lower end of the range observed overseas. For instance, Matsui et al. (2000), Tilton et al. (2002), Svenson et al. (2003), and Rutishauer et al. (2004) used the YES assay to determine the estrogenicity of effluent in Japan (5-15 ng/L EEQ), the USA (44-151 ng/L EEQ), Sweden (<0.1-15 EEQ) and Switzerland (0.1-90 ng/L EEQ), respectively. Our results were also comparable with European observations, in that here was no simple correlation between estrogenicity and the size of population served by the STP, although there may be a correlation between treatment process, flow rates, and estrogenicity.

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