Canadian Journal of Pure and Applied Sciences
Vol. 1, No. 1, pp. 67-72, 2007

SENRA Academic Publishers, Burnaby, British Columbia
ISSN: 1715-9997

ASSESSMENT OF ESTROGENIC CONTAMINATION USING *IN-VITRO* YEAST ESTROGENIC SCREEN IN THE WASTEWATER OF KARACHI

M Zaheer Khan*, Jasen Nelson, Francis CP Law, Walter Leung and Hao-Feng (Howie) Lai
Department of Biological Sciences, Simon Fraser University Burnaby, BC Canada V5A 1S6

ABSTRACT

Karachi city produces discharge of wastewater of 300 mil gallons per day. There are three sewerage plants in Karachi but they are able to treat only 45 million gallon per day (15% of the total wastewater). A large number of industries discharge deadly and toxic waste into storm-drains, open nullahs or in the Lyari and Malir rivers. These include leather tanning units, pharmaceuticals, petrochemicals, refineries, chemical, textile, engineering works and thermal power plants. The Lyari River has become a putrid and toxic gutter due to discharge of effluents. Almost all chemical waste is dumped untreated into the river system from where it is taken out to sea. In this study, estrogenic activities of wastewater were determined with the samples collected from seven selected sites of Karachi city. A study of estrogenic activities was done between these seven selected areas, using the samples collected, estrogenic activity found in Site A 1.46 ng/L, site B 2.15 ng/L, site C 0.76 ng/L, site D 7.47 ng/L, site E 0.77 ng/L, site F 0.12 ng/L and site G 0.21 ng/L estradiol equivalents. While the highest estrogenic activity was found in site D 7.47 ng/L estradiol equivalents.

Keywords: Karachi, wastewater ,estrogenic, effluents, activity.

INTRODUCTION

Pakistan's cosmopolitan city Karachi, is located at the eastern coast of the Arabian Sea. It is the capital of the province of Sindh. With a population of nearly 10 million, Karachi is the largest city of Pakistan. A large number of industries discharge toxic waste into storm-drains, open nullahs or in the Lyari and Malir rivers. The Karachi coastline, which stretches over 135 km, is facing severe pollution due to a combination of industrial, port, municipal, and transportation activities in the area. The coastline is being overwhelmed with water-borne pollution being discharged in the shipping process into the marine environment (Beg, 1995). The discharge of sewage and contaminated water in rivers and water bodies not only affects on marine production, use of such water for agriculture results in the contamination of the food chain. In Pakistan, sewage water is re-channelled to irrigate crops, which contaminates them with pathogens. As a result 50% of the crops are contaminated. Groundwater may also be contaminated by untreated sewage. Water borne diseases are the killers in the city and health problems resulting from polluted water cost lot of money. Among more than 70,000 synthetic chemicals registered for commercial use, few were tested for hormonal activity. Because hormonal activity is not easily predictable, bioassays are necessary [Endocrine Disruptor Screening and Testing Advisory (EDSTAC) 1998]. *In vitro* tests were also adapted to detect estrogenic activity in wastewater and watercourses (Soto *et al*., 2004). The possible presence of estrogenic hormones in wastewater was hypothesized by Stumm-Zollinger and Fair in 1965. However, analytical techniques capable of detecting the compounds at the concentrations expected in wastewater were unavailable. Later attempts to detect estrogenic hormones in wastewater effluents and in natural waters have employed solid-phase extraction followed by analysis using gas chromatography/mass spectrometry (Desbrow *et al*., 1998; Stumpf *et al*., 1996; Schlett and Pfeifer, 1996). In recent years, endocrine disruption has been observed in wild fish and fish caged in rivers that receive significant inputs of wastewater effluents (Purdom *et al*., 1994; Harries, 1996, 1997; Jobling *et al*., 1998). As a result, concerns have been raised about the presence of endocrine disrupting chemicals in wastewater effluents. 17 β -esradiol and 17α-ethinyl estradiol most likely responsible for endocrine disruption in fish, natural and synthetic hormones (Desbrow *et al.,* 1998; Routledge *et al*., 1998). Between 0.002 and 0.10 mg of the endogenous hormone 17 β -estradiol is excreted by human per day. While pregnant women excrete as much as 30 mg per day of 17 β -estradiol (Williams and Stancel, 1996; Arcand *et al*., 1998). Water effluents are the most frequently observed steroids in surface waters and have been implicated as causative agents for widespread reproductive dysfunction in aquatic fish species in United Kingdom (Jobling *et al*., 1998). Desbrow *et al*. (1998) adopted a toxicity identification and new evaluation approach to try to identify the estrogenic compounds in wastewater effluents. They identified three sterols (17 β -estradiol, estrone, and17α-ethinyl estradiol) as the major estrogenic chemicals within the sewage effluents they investigated. The two natural estrogens (estrone and estradiol) were present at concentration up to tens of nanagrams per milliliter (1-80 ng/L), and the synthetic estrogen (ethinyl estradiol) was detected at a lower concentration (undetectable to 7 ng/L).

Some other studies in the UK (Rodgers-Gray *et al.,* 2000) and many other countries, including Netherlands (Belfroid *et al*., 1999) and Canada (Lee and Peart, 1998), have confirmed that natural synthetic estrogens are present in wastewater effluents at concentration similar to those reported by Desbrow *et al*. (1998). The objective of this study was to compare *in vitro* bioassays of seven selected sites of wastewater effluents from Karachi city.

*Corresponding author: zaheer_khan@sfu.ca

MATERIALS AND METHODS

Research sites

Karachi is biggest city of Pakistan and has a several points that discharge their effluents. For this initial study, we selected seven sites. Gulshan-e-Iqbal (site A), Shorab Goth (site B), Gabol Town North Karachi (site C), Pak Colony (site D), Lasbella (site E), Machar Colony (site F) and Liaquatabad (site G).

The wastewater samples were collected about 20~25cm deep from the surface water. The water samples were collected in the mornings between 10:00 am to 1:00 pm between Oct 15th to 17, 2005 and the weather was clear and sunny on the days of sampling, the temperature ranged from 28 to 30 Celsius.

Test sample preparation and extraction: Water from the retention pond and streams was collected by immersion of amber glass bottles 2-L bottles by U.S. Environmental Protection Agency (U.S EPA) standards from each site and added 0.02% sodium azide to avoid microbial degradation. We tried three extraction methods: dichloromethane (DCM), 6% ethyl ether-hexane, and 50% ethyl ether-hexane. Because DCM gave the best results, we used this for the field water samples.

Extraction procedure for water: The water samples were extracted within 1 day after sampling. Each water sample was 2L in volume. The 2L sample was divided to two 1L portion and each was contained to 1L separtory funnel. In each 1L portion of water sample, 60mL of DCM was added, and the sample with DCM was gently shaken for 50 times, and the sample was sat for 15 minutes. After 15 minutes, the lower organic layer was collected in a 500mL round bottom flask. The above extraction step was repeated 2 more times and the final extract was dried under rotor evaporator. After the sample was dried, it was reconstituted by 2mL of DCM and the sample was transferred to a small vial. The sample was then dried under nitrogen gas until it was totally dry. The dried extract was then reconstituted in 400µL of ethanol to obtain a 10,000X concentrated sample.

Table 1. Corresponding multiplication factors of estradiol concentration of the original water sample to the various serial dilution of the water extracts.

Serial dilution of water extract	Multiplication of estradiol concentration to the original water sample
100%	500X
10%	50X
2%	10X
1%	5X
0.2%	1X

After water extraction, the concentration of estradiol in the extract was 10,000X more concentrated than the original 2L sample (concentrated from 2L to 400µL). When YES assay was performed on the extracted samples, the concentration of estradiol was diluted 20 times (10µL of test solution in 200µL of yeast). In total, the extract was concentrated 500X. The concentration of estradiol derived from the Sigma Plot must be diluted by 500 times to obtain the original environmental concentration.

From Table 1, the concentration of estradiol in the 0.2% of water-extract correlated to the original estradiol concentration of the water sample. If other serial dilution of the samples were to be tested on the yeast assay, the correlated multiplication factor would have to be used to estimate the total estradiol concentration in the samples.

Bioassay: DNA recombinant yeast assays were used by the method developed by Gaido (1997).

Preparation of Yeast medium components

The detail medium preparation procedures for Sumpter and Jobling (1995) and Gaido (1997) yeast assays were listed in the appendix.

Sumpter Yeast Assay procedure

The Sumpter yeast strain used was *Saccharomyces cerevisiae*. Test solutions and estradiol E2 standards were serially diluted and 10µL aliquots of each concentration were then transferred to a 96 well optically flat bottom microtiter plate. Chemicals dissolved and diluted in absolute ethanol were allowed to evaporate to dryness on the assay plate. Chemicals that were insoluble in ethanol were dissolved and serially diluted in medium. Aliquots (200µL) of the seeded assay medium (medium containing recombinant yeast) and the chromogenic substrate CPRG were then dispensed to each sample well using a mutichanel pipettor. Each plate contained 12 concentrations of the 17β-estradiol to generate a standard curve (3,000 ng/L 17β-E2 to1.5 ng/L). The plates were shaken vigorously for 2 minutes and then incubated at 32°C. After 3 days of incubation, the color development of the medium was checked periodically at an absorbance of 540 nm, to obtain data with the best contrast.

After incubation, control wells appear light orange in color, due to background expression of β-galactosidase, and turbid, due to growth of the yeast. Positive wells are indicated by a deep red color accompanied by turbid yeast growth. Clear wells (containing no growth) indicate lysis of the cells, and the color may vary.

Gaido yeast assay procedure

The *Saccharomyces cerevisiae* strain BJ3505 was the strain used for the Gaido estrogen receptor assay. The yeast assay is a four day assay. On day 1, one single colony of yeast was selected from a streaked plate and

grown in the 5mL of selective medium in 50mL polypropylene tube and incubated at 30 Celsius overnight. After one night of incubation, (day 2) the overnight culture of yeast was diluted 10 fold by adding 45mL of growth medium to the tube. On day 3, the yeast was diluted by half in growth media. For each test solutions, 10µL was taken out and dispensed into flat bottom 96-well microplates and evaporate to dryness. Aliquot 20mL/plate of yeast culture into a sterile tube and 10µL/mL 10mM copper sulphate was added to the yeast culture to achieve a 100µM final concentration. 200µL of yeast culture was dispensed to each treated well. The plate was then shaken for 2 minutes and the yeast culture was incubated overnight at 30 Celsius. The following day (day 4), the yeast was re-suspended in each well. In each wells, 100µL of yeast is transferred to a new plate. The buffer solution was prepared by adding 22mg/plate of 2mg/mL ONPG and 10.9mL/plate Z-Buffer in a polypropylene tube. The mixture was dissolved in shaker at 30 Celsius. The yeast culture was resuspended in treatment plate by pipeting and aliquot 100µL of yeast from treatment plate to assay plate with multichannel pipet. When ONPG was completely dissolved, completed preparation of assay buffer by adding 29.7µL/plate of 50mM 2-Mercaptoethanol, 11µL/plate of 200 U/mL oxalyticase and 110µL 10% SDS to the Z-buffer mixture. Then, 100µL of assay buffer was added to each well. The plate was shaken for 2 minutes and staggered for 2 minutes. After 40 minutes, the plates were read at 415 nm and 595nm.

Data analysis

The E2 dose-response curve was used to quantify the unknown samples. The concentration of estradiol was generated based on the E2 dose-response curve.

For the samples that were tested, the dose-response data of each samples from the bioassays were fitted by Hill functions (sigmoid, three parameters) using the non-liner regression computer software Sigma Plot (version 8.02). The formula for the Hill function is: Response = $(V_m \times \text{Dose}^{\alpha}) / (K^{\alpha} + \text{Dose}^{\alpha})$ where V_m is the maximal response, K is the half-effective concentration (EC50, the concentration yielding half of the maximum effect), and α is the Hill coefficient (controls the slope at EC50).

RESULTS

From literature, it was found that 17-β-estradiol (E2) has the highest relative estrogenic potential in the environmental water as shown in Table 2. (Yakou *et al*., 1999 and Tanaka *et al*., 2001). Therefore, for evaluating the level of the estrogenic activities in the water samples, the dose-response curve of 17-β-estradiol was used and the concentration of estradiol was measured in each water samples.

Table 2. The relative estrogenic potential in the environmental water.

Chemicals	Relative estrogenic potential
17-β-estradiol	1.00
Estron	0.3
4-nonylphenol	0.001
4-n-octylphenol	0.000005
Di-n-butyl phthalate	------
Di-2-ethylhexyl phthalate	------
Butyl benzyl phthalate	------
Di-2-ethylhexyl adipate	------
Bisphenol A	0.00006

To summarized the data obtained, the estrogenic activities of wastewater samples at different points obtained from Karachi city in Table 3. This table shows that the average estradiol concentrations obtained from Gaido yeast assay for the water samples estrogenic activity found in Site A 1.46 ng/L, site B 2.15 ng/L, site C 0.76 ng/L, site D 7.47 ng/L, site E 0.77 ng/L, site F 0.12 ng/L and site G 0.21 ng/L estradiol equivalents. While the highest estrogenic activity was found in site D 7.47 ng/L estradiol equivalents. Comparing the results between seven sites of wastewater samples, it was found that the estradiol concentration was found to be the greatest in the site D wastewater samples (Fig. 1).

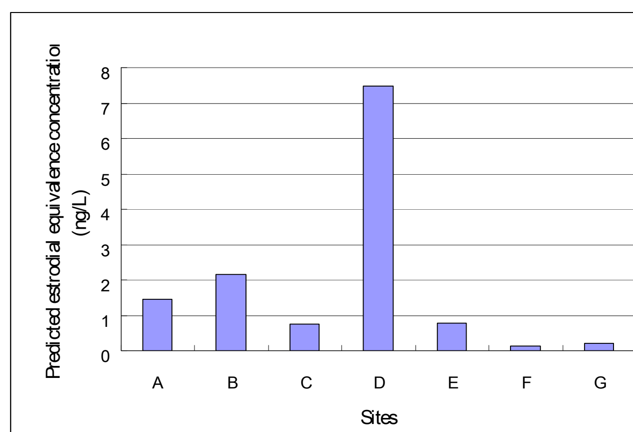

Fig. 1. Estrodial equivalence concentration for 7 Sites in the Karachi region

Note: site A: Gulshan-e-Iqbal, site B: Shorab Goth, site C: Gabol Town North Karachi, site D: Pak Colony, site E: Lasbella, site F: Machar Colony, site G: Liaquatabad

Table 3. Estrogenic activities of seven different wastewater sites in the Karachi region.

Location/ sample name	Predicted estrodial equivalence concentration (M)	Predicted estrodial equivalence concentration (ng/L)
Gulshan-e-Iqbal, site A	5.35E-12	1.46
Shorab Goth, site B	7.88E-12	2.15
Gabol Town North Karachi, site C	2.79E-12	0.76
Pak Colony, site D	2.74E-11	7.47
Lasbella, site E	2.82E-12	0.77
Machar Colony, site F	4.55E-13	0.12
Liaquatabad, site G	7.68E-13	0.21

DISCUSSION

Several chemicals theoretically can contribute to the estrogenic activity of effluents from wastewater, and hence also presumably from river water. These chemicals include natural estrogens (e.g., 17β-estradiol and estrone) and synthetic estrogens (e.g., nonylphenol and Biphenol-A) (Kirk *et al*., 2002). Which chemicals, and in what proportions, contribute to the total estrogenic activity of effluents from wastewaters is not yet completely clear, but some opinion suggested that the natural and synthetic estrogens contribute most of the overall activity in most cases (Korner *et al*., 2000; Ahel *et al*., 1994), with xenoestrogens contributing in only a minor way in most cases. Van Aerle *at al.* (2001) and Sheahan *et al*. (2002) reported that xenoestrogens are main contributors to the estrogenic level in the River Aire UK. Some Researchers have used *in vitro* methods of estrogenic activity to determine surface water or fractional effluents of wastewater for occurrence of estrogenic agents (Desbrow *et al*., 1998; Sheahan *et al*., 2002; Snyder *et al*., 2001). The environmental fates of estrogens in Wastewater are very complex and there is no universally accepted bioassay or chemical technique to quantify EDCs in the aquatic environment. Chemical analysis of EDCs is sensitive and specific but limited in that only target substances are analyzed. In vitro bioassays which are based on the interaction between EDCs and estrogenic receptors can be very useful in determining the total estrogenic activity of EDCs in a mixture (Nelson *et al*., 2007).

A comparison of estrogenic activities between seven different wastewater sites in the Karachi city was done. It was found that among the samples analyzed, estrogenic activity in Site A 1.46 ng/L, site B 2.15 ng/L, site C 0.76 ng/L, site D 7.47 ng/L, site E 0.77 ng/L, site F 0.12 ng/L and site G 0.21 ng/L estradiol equivalents. While the highest estrogenic activity was found in site D 7.47 ng/L estradiol equivalents. It has been recognized for some time that a number of chemicals in the environment possess estrogenic activity in a variety of biological systems. Among the investigated wastewater samples, it was found that the average estradiol concentrations obtained from Gaido yeast assay were Site A 1.46 ng/L, site B 2.15 ng/L, site C 0.76 ng/L, site D 7.47 ng/L, site E 0.77 ng/L, site F 0.12 ng/L and site G 0.21 ng/L estradiol equivalents. While the highest estrogenic activity was found in site D 7.47 ng/L estradiol equivalents.

The results showed that the level of EDCs in the wastewater in the environment of the Karachi region was high in site D Pak colony area. This area located between the some industries. Finally this water mixed in sea water. Currently we have no information about the estrogenic activity in marine water. We suggested that more environmental water samples should be collected and analyzed for their estrogenic activity from Layri River, Malir River, marine environment and some other selected water bodies of Karachi.

ACKNOWLEDGEMENT

We would like to thank the following individuals from University of Karachi for the collection of wastewater samples: Karim Gabol, Dr. Moazzam A Khan, Dr. Majid Mumtaz, Babar Hussain and Darkahshan Abbas.

APPENDIX

The detail medium preparation procedures for Sumpter and Jobling (1995) and Gaido (1997) yeast assays.

Sumpter yeast medium preparation

Minimal medium (pH 7.1) was prepared by adding 13.61g KH2PO4, 1.98g (NH4)2SO4, 4.2g KOH pellets, 0.2g MgSO4, 1ml Fe2(SO4)3 solution (40mg/50ml H2O), 50mg L-leucine, 50mg L-histidine, 50mg adenine, 20mg L-arginine-HCl, 20mg L-mehionine, 30mg L-tyrosine, 30mg L-isoleucine, 30mg L-lysine-HCl, 25mg L-phenylalanine, 100mg L-glutamic acid, 150mg L-valine, and 375mg L-serine to 1L of purite double-distilled water. The mixture were sterilized at 121 Celsius for 1 hour and stored at 4 Celsius.

The vitamin solution was prepared by adding 8mg thiamine, 8mg pyridoxine, 8mg pantothenic acid, 40mg inositol, and 20mL of biotin solution (2mg/100mL H2O) to 180mL double-distilled water. The solution was then filter sterilized through 0.2-μm pore size filters, and 10mL aliquots were stored at 4 Celsius in sterile glass bottles.

A 20% w/v solution of D-(+)-glucose was sterilized in 20mL aliquots at 121 Celsius for 1 hour and stored at 4 Celsius. A stock solution of 4mg/mL L-aspartic acid was sterilized in 20mL aliquots at 121 Celsius for 1hour and stored at 4 Celsius. A stock solution of 24mg/mL L-

threonine was sterilized in 5mL aliquots at 121 Celsius for 1hour and stored at 4 Celsius. A 20mM copper (II) sulfate solution was prepared and fertilized through 0.2-µm pore size filters tstock solution and stored at 4 Celsius. A 10mg/L stock solution of CPRG was made in sterile distilled water and stored at 4 Celsius in a sterile glass bottle.

Growth medium was prepared by adding 5mL of glucose solution, 1.25mL L-asparatic acid solution, 0.5mL vitamin solution, 0.4mL L-threonine solution, and 125µL copper (II) sulfate solution to 45mL single strength minimal medium in a sterile conical flask. The growth medium was then incubated at 28 Celsius for approximately 24h on an orbital shaker until an absorbance at 640nm of 1.0 was reached.

The assay medium was prepared by adding 0.5mL of the chromogenic substrate CPRG to 50mL fresh growth medium. The medium was seeded with 1mL yeast from a 24h yeast culture with an absorbance at 640nm of 1.0 prior to use.

Preparation of growth medium for Gaido

- 50mL 10X Yeast Nitrogen Base
- 50mL 20% Dextrose (20g/100mL)
- 5mL Lysine (1.8g L-lysine-Cl/500mL)
- 5mL Histidine (1.2g L-histidine-Cl/500mL)
- 1.73g Monobasic Sodium Phosphate (3.46g/L)
- 1.73g Dibasic Sodium Phosphate (3.54g/L)

REFERENCES

Ahel, M., Giger, W. and Koch, M. 1994. Behaviour of alkylphenol polyethoxylate surfactant in the aquatic environment.1. Occurrence and transformation in sewage treatment. Water Res. 28:1131-1142.

Arcand-Hoy, LD., Nimrod, AC. and Benson, WH. 1998. Endocrine modulating substances in the environment: Estrogenic effects of pharmaceutical products. Int. J. Toxicol. 17:139-158.

Beg, MAA. 1995. Environmental Degradation. Pakistan & Gulf Economist. 113-118.

Belfroid, AC., Van Der Horst, A., Vethaak, AD., Schafer, AJ., Riijs, GBJ., Wegener, J. and Cofino, WP. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Sci. Total. Environ. 225:101-108.

Desbrow, C., Rutledge, EJ., Brihty, GC., Sumpter, JP. and Waldock, M. 1998. Identification of estrogenic chemicals in STW effluent: 1. Chemical fraction and *in vitro* biological screening. Environ. Sci. Technol. 32:1549-1558.

EDSTAC. 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. Washington, DC:U.S. Environmental Protection Agency.
Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnell DP. 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143:205-212.

Harries, JE. 1996. A survey of estrogenic activity in United Kingdom inland waters. Environ. Toxicol. Chem. 15:1993-2002.

Harries, JE. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16:534-542.

Jobling, S., Nolan, M., Tyler, CR., Brighty, G. and Sumpter, JP. 1998. Widespread sexual disruption in wild fish. Environ, Sci. Tech. 35:4697-4703.

Jobling, S., Nolan, M., Tyler, CR., Brighty, G. and Sumpter, JP. 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32:2498-2506.

Korner, W., Bolz, U., Submuth, W., Hiller, G., Schuller, W., Hanf, V. and Hagenmaier, H. 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere. 40:1131-1142.

Kirk, LA., Tyler, CR., Lye, CM. and Sumpter, JP. 2002. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environ. Toxicol. Chem. 21:972-979.

Lee, HB. and Peart, TE. 1998. Determination of 17 -estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. J. AOAC. Int. 81:1209-1216.

Legler, J., Zeinstra, LM., Schuitemarker, F., Lanser, PH., Bogerd, J., Brouwer, A., Vethaak, AD., De Voogt, P., Murk, AJ. and Van Der Burg, B. 2002. Comparison of *in vivo* and *in vitro* reporter gene assay for short-term screening of estrogenic activity. Environ. Sci. Tech. 36:4410-4415.

Lorenzen, A., Hendel, JG., Conn, KL., Bittman, S., Kwabiah, AB., Lazarovitz, G., Masse, D., McAllister. TA, Topp E. 2004. Survey of hormone activities in municipal biosolids and animal manures. Environ Toxicol 19: 216-225.

Nelson, J., Bishay, F., Albert van Roodselaar., Ikonomou, M. and Law, FCP. 2007. The use of in vitro bioassays to

quantify endocrine disrupting chemicals in municipal wastewater treatment plant effluents. Science of the Total Environment 374 : 80-90.

Purdom, CE., Hardiman, PA., Bye, VJ., Eno, NC., Tyler, CR. and Sumpter, JP. 1994. Estrogenic effects from sewage treatment works. Chem. Ecol. 8:275-285.

Routledge, EJ., Sheahan, D., Desbrow, C., Brighty, GC., Waldock, M. and Sumpter, JP. 1998.

Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environ. Sci. Technol. 32:1559-1565.

Routledge EJ. and Sumpter, JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15:241-248.

Rodgers-Gray, TP., Jobling, S., Morris, S., Kelly, C., Kelly, C., Kirby, S., Janbakhsh, A., Harries, JE., Waldock, M., Sumpter, JP. and Tyler, CR. 2000. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environ. Sci. Technol. 34:1521-1528.

Stumm-Zollinger, E. and Fair, GM. 1965. Biodegradation of steroid hormones. J. Water Pollut Control Fed. 37:1506-1510.

Stumpf, M., Ternes, TA., Haberer, K. and Baumann, W. 1996. determination of natural and synthetic estrogens in sewage plants and river water. Vom Wasser. 87:251-261.

Schlett, C. and Pfeifer, B. 1996. Determination of steroid hormones in drinking and surface water samples. Vom Wasser. 87:327-333.

Soto AM. and Sonnenschein, C. 1995. The E-Screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspec. 103: 113-122.

Soto, AM., Calabro, JM., Nancy, V., Prechtl, NV., Yau, AY., Orlando, EF., Daxenberger, A., Kolok, AS., Guillette, Jr., LJ., Bruno le Bizec., Iris, G., Lange, IG. and Sonnenschein, C. 2004. Androgenic and Estrogenic Activity in Water Bodies Receiving Cattle Feedlot Effluent in Eastern Nebraska, USA. Environ Health Perspet. 112(3):346-352.

Snyder, SA., Villeneuve, DL., Snyder, EM. and Giesy, JP. 2001. Identification and quantification of estrogen receptor agonists in wasterwater effluents. Environ. Sci. Tech. 35:3620-3625.

Sheahan, DA., Brighty, GC., Daniel, M., Kirby, SJ., Hurst, MR., Kennedy, J., Morris, S., Routledge, EJ., Sumpter, JP. and Waldock, MJ. 2002. Estrogenic activity measured in a sewage treatment work treating industrial inputs containing high concentrations of alkylphenolic compounds- a case study. Environ. Toxicol. Chem. 21:507-514.

Sheahan, DA., Brighty, GC., Daniels, M., Jobling, S., Harries, JE., Hurst, MR., Kennedy, J., Kirby, SJ., Morris, S., Routledge, EJ., Sumpter, JP. and Waldock, MJ. 2002. Reduction in the estrogenic activity of a treated sewage effluent discharge to an English river as a result of a decrease in the concentration of industrially-derived surfactants. Environ. Toxicol. Chem. 21:515-519.

Sumpter JP. and Jobling, S. 1995. Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. Environ Health Perspect.103(suppl 7):173-178.

Van Aerle, R., Nolan, M., Jobling, S., Christiansen, LB., Sumpter, JP. and Tyler, CR. 2001. Sexual disruption in a second species of wild cyprinid fish (the *gudgeon, Gobio gobio*) in UK fresh waters. Environ. Toxicol. Chem. 20:2841-2847.

Williams, CL. and Stancel, GM. 1996. Estrogens and progestins. In Goodman, LS., Gilman, A. (eds.). The Pharmacological Bases of Therapeutic. Macmillan, Indianapolis, In USA. 1411-1437.