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A BASELINE STUDY TO EVALUATE THE ESTROGENIC ACTIVITIES USING *IN-VITRO* YEAST ESTROGENIC SCREEN IN THE SELECTED RAMSAR SITES AND CREEK AREAS

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ABSTRACT

Pakistan has a great variety of natural and manmade wetlands, and 19 Ramsar Sites are designated as internationally important wetlands, with a unique variety of aquatic and bird biodiversity. In the present study, a baseline study was conducted for the evaluation of estrogenic activities in the waters of three Ramsar Sites and one Creek areas. Among the selected study points of Indus Dolphin Wetland (study point1), Indus Dolphin (study point 2), Kinjhar Wetland (study point1), Kinjhar Wetland (study point 2), Haleji Wetland (study point 1), Haleji Wetland (study point 2), Creek areas (study point1) and Creek area (study point 2). During the study, selected points were surveyed two times i.e. in summer and winter, and water samples were collected in the 2nd week of November 2006 and 3rd week of May 2007. *In-Vitro* Yeast Estrogenic Screen (YES) assays techniques were used to evaluate the estrogenic activities in the water samples. Estrogenic activity recorded in the November 2006 samples of the Indus Dolphin Wetland (study point 1) was 9.31 ng/L estradiol equivalents and of the Kinjhar (study Point1) was 2.77 ng/L estradiol equivalents. In the samples collected in May 2007, the estrogenic activity found in the Indus Dolphin Wetland (study point1) was 3.14 ng/L estradiol equivalents, in the Indus Dolphin (study point 2) was 4.54 ng/L estradiol equivalents, in the Haleji Wetland (study pont1) was 1.4 ng/L estradiol equivalents, while in the Creek areas (study point1) was 7.89 ng/L estradiol equivalents and in the Creek areas (study point 2) was 2.93 ng/L estradiol equivalents. Results of this baseline study have indicated that the wetlands under study are more polluted in summer than in winter.

Keywords: Estrogenic activities, 17 β -estradiol, wetlands, Indus dolphin, pollution.

INTRODUCTION

In recent years, evidence has shown that several chemicals like estradiol, nonylphenol, bisphenol A, PCBs and some pesticides at different concentrations can cause disruption to endocrine systems and can affect hormonal control of development in aquatic biodiversity (Damstra *et al*., 2002; Hayes *et al*., 2002; Lintelmann *et al*., 2003). These chemicals are described as endocrine disrupting chemicals (EDCs). Several chemicals released into the environment can disrupt normal endocrine function in a variety of aquatic biodiversity. Environmental exposure to low and often analytically undetectable (ng/L) concentrations of endocrine disrupting compounds (EDCs) has caused physiological malfunction, such as sex reversal in fishes (Jobling *et al*., 1998). The number of anthropogenic and natural EDCs, released into the environment, is unknown since few chemicals have been tested for hormonal activity (Colborn and Clement, 1992). Globally some of the identified EDCs of potential concern have been detected in surface waters (Colborn and Clement, 1992). Generally in the aquatic environment, exposure of organisms to EDCs has been linked to endocrine effects in male fish such as vitellogenin induction and feminine reproductive organs (Aherne and Briggs, 1989; Purdom *et al*., 1994; Routledge *et al*., 1998; Tyler *et al*., 1998). It is suggested that industrial and municipal effluents as well as urban and agricultural runoff are the important sources of EDCs discharged into the aquatic environment (Desbrow *et al*., 1998; Snyder *et al*., 1999; Boyd *et al*., 2003). Pesticides and related chemicals destroy the delicate balance between species that characterizes a functioning ecosystem. Pesticides also produce many physiological and biochemical changes in fresh water organisms by influencing the activities of enzymes. Therefore, alterations in the chemical composition of the natural aquatic environment usually affect behavioral and physiological systems of the inhabitants, particularly those of the fish (Khan and Law, 2005).

The evaluation of estrogenic activities in the aquatic environment and other wildlife has not been systematically assessed in Pakistan. We have no published data about estrogenic activities in wetlands waters and their effect on aquatic biodiversity, especially economically important fish, mussels and prawn species. Pakistan has 19 Ramsar Sites designated as Internationally Important Wetlands, with a surface area of 1,343,627 hectares (Table 1). The globally endangered warbler *Prinia burnesii* and endangered Indus dolphin has been recorded in the Chashma Barrage. The Haleji Lake is a wintering site for the globally threatened pelican *Pelecanus crispus*, this wetland regularly hosts between

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Table. 1. List of Internationally Important Wetlands in Pakistan, 2005. (Source: Khan, 2005).

S. No.	Name	Location	Area (ha)
01	Astola (Haft Talar) Island	Balochistan	5,000
02	Chashma Barrage	Punjab	34,099
03	Deh Akro-II Desert Wetland Complex	Sindh	20,500
04	Drigh Lake	Sindh	164
05	Haleji Lake	Sindh	1,704
06	Hub (Hab) Dam	Balochistan	27,000
07	Indus Delta	Sindh	472,800
08	Indus Dolphin Reserve	Sindh	125,000
09	Jiwani Coastal Wetland	Balochistan	4,600
10	Jubho Lagoon	Sindh	706
11	Kinjhar (Kalri) Lake	Sindh	13,468
12	Miani Hor	Balochistan	55,000
13	Nurri Lagoon	Sindh	2,540
14	Ormara Turtle Beaches	Balochistan	2,400
15	Runn of Kutch	Sindh	566,375
16	Tanda Dam	NWFP	405
17	Taunsa Barrage	Punjab	6,576
18	Thanedar Wala	NWFP	4,047
19	Uchhali Complex	Punjab	1,243

50,000 and 100,000 water birds. Indus Dolphin Reserve is important for the survival of more than 1500 remaining individuals of the Indus dolphin *Platanista minor* (Khan, 2006). This unique species is endemic to Pakistan and listed on Appendix I of CITES and the IUCN Red List. There is abundant, albeit fragmentary, evidence that agricultural pesticides and other anthropogenic contaminants are contaminating several wetlands extensively. Such inputs can affect fundamental ecosystem properties such as primary production, which, in turn, affects habitat and resource supply of wetland fauna. Presently Haleji Lake is being polluted by the runoff from water of agricultural fields.

The coast of Sindh is characterized by the presence of several creeks, backwaters and mangrove swamps. The topographic structure of Korangi and other adjacent creek possesses special features that support different aquatic biodiversity. These creeks along the Indus Delta constitute important nursery and feeding grounds for a variety of pelagic and demersal fish and commercial prawn species. On the eastern side of Karachi, between Karachi and Keti Bunder the area extending from Korangi/ Rehi Creek at the north-eastern side is commonly known as the Korangi Creek system which further extends to Phitti, Gizri, Khuddi, Khai, Pitiani, Dobbo, Sisa, Hajamro, Turshian and Khobar Creeks.

The most important ecosystems to be found in the coastal belt are the mangrove forest in the Indus delta, which are a rich source of nutrients for a variety of marine wild species. In Pakistan, sewage water is re-chanelled 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 (Khan *et al*., 2007).

Although several natural and synthetic estrogens exist; the 17 β -estradiol (E2) has, however, been selected as the standard EDC for the development of the *in vitro* bioassays because it is the most potent natural estrogen in the endocrine system. As with many steroid hormones, 17 β -estradiol is derived from cholesterol, thus many similarities in structure exist (Fig. 1) [by sustitution of various functional groups and thus there are many similarities in structure]. E2 is primarily produced in the ovaries, and to a lesser extent in the testes; however, E2 is also produced in fat cells and brain cells (EPA, 1997; NIEHS, 2002). In testes, E2 is produced through steroidogenesis, which includes aromatization of testosterone into E2 (Fig. 2).

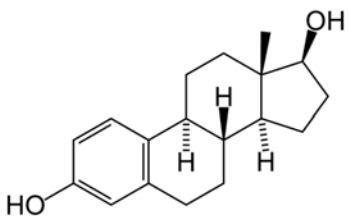

Fig. 1. Structure of 17 β -Estradiol.

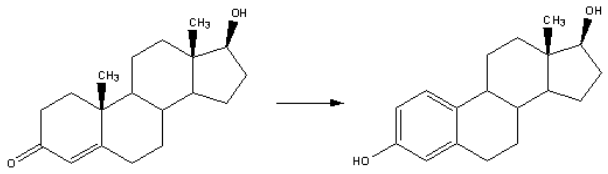

Fig. 2. Aromatization of Testosterone into 17 β -Estradiol.

17 β -estradiol is a water-soluble EDC with a log K_{ow} of 4.01; however, it may associate with organic material in water or exists in a freely dissolved form (Table 2). It is not a persistent molecule, thus exists only temporarily in aqueous media, soils, sediments and biota.

Table 2. Physical and chemical properties of 17- β - estradiol.

Property	Value
Molecular formula	$C_{18}H_{24}O_2$
Molecular weight	272.3864
Melting point	178.5°C
Vapour pressure	1.3 x10^{-8} mm Hg
Log K_{ow}	4.01
Water solubility	3.6 mg/L
Sigma # (CAS)	E4285

MATERIALS AND METHODS

For this baseline study, we selected eight study points.

Lake water Haleji and Kinjhar Watlands area were chosen for sampling (both wetlands are Ramsar sites and important for aquatic biodiversity including resident and migratory birds).

River water The Indus Dolphin Reserve area, Indus dolphin (*Platanista minor)* is an endemic species of Pakistan from River water.

Creeks areas Korangi and adjacent Creeks areas were chosen for marine water [one from nesting area of Green turtle (*Chelonia mydas)*].

The water samples were collected about one to two feet away from the coast of river, lakes and beaches and about 10~15cm deep from the surface water. Samples were collected in pre-cleaned 4L glass bottles. The water samples were collected in the 2nd week of Nov 2006 and 3rd week of May 2007 in the mornings between 10:30 to 12:00 am.

Extraction Procedure
Water samples were extracted based on a modification of the method used by Soto *et al.* (2004). Samples were extracted on the next day of sample collection. Each 4 L sample was split into 4 aliquots (1 L), and transferred into its own 2L separatory funnel containing 60 mL of dichloromethane (DCM). The separatory funnel was shaken for 5 min and the layers were allowed to separate for 15 min. The bottom (DCM) layer was removed, and 60 mL of fresh DCM was added to the remaining aqueous solution, which was mixed for another 5 min and allowed to separate for 15 min. The second extraction step was repeated and the extracts from all three extractions were combined and concentrated down to approximately 4 mL using ROTAVAP and N-EVAP evaporators (Organomation Associates, Berlin, MA) at 40°C. A 2 mL aliquot was removed and extracted by ethanol (anhydrous). For selected samples, the remaining DCM (2 mL) was dried completely and stored at -40°C and then used for YES.

Preparation of growth medium for Gaido (Gaido *et al*., 1997)

- 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)

Preparation of Standard Dilution Series
The 17β-estradiol (E2) standard dilution series ranged from 0.001 nM to 100 nM (i.e., 11 dilutions), plus a solvent (ethanol) blank. The dilutions of the aqueous samples were attained using ethanol. The concentrations used for bioassay exposures were 10, 1, 0.1, 0.01, 0.001times the initial concentration. Cytotoxicity was assessed by exposing sample dilutions to 1 nM E2; since this level of E2 produces maximal proliferation, decreased proliferation implies cytotoxicity. Cytotoxicity was confirmed visually using a microscope. Cytotoxicity affected the 10-fold aqueous samples, thus data analysis was limited to the 1-fold sample. An E2 equivalence quotient is obtained for the 1-fold sample based on the dose–response curve of E2. Trichloroacetic acid (TCA) fixation, sulforhodamine B (SRB) staining, and Tris buffer solubilization of dye, were performed according to Soto and Sonnenschein (1995). The 96-well microplates were scanned at 515 nm and 650 nm with a microplate reader.

The E2 standard dilution series was used for the YES assays. Standards and environmental extract dilutions were transferred (10 µL), in at least triplicate, into the microplate wells and allowed to dry (approximately 40 min). The YES bioassays were carried out with two different variations of recombinant yeast. These bioassays are similar in that they have been transformed to include the human estrogen receptor (hER) in their main genome, estrogen response elements (ERE) and lac-Z gene on a plasmid. Essentially, the estrogenic substance binds to an

hER which polymerizes and binds to the ERE, which controls the expression of the lac-Z gene, resulting in the transcription and subsequent release of β-galactosidase into the medium (Nelson *et al*., 2007). The two YES bioassays employ different chromogenic substances, 2-nitrophenyl-β-D-galactopyranoside (ONPG) is used in the Gaido *et al*. (1997) method. The intensity of the color is then measured by absorbance; these values are then normalized for the background activity.

The yeast cell lines, *Saccharomyces cerevisiae* for the ERTA reporter gene assays, were obtained and used with permission from their respective authors (Gaido *et al.*, 1997). The Gaido strain was obtained directly from Dr. Gaido's laboratory in the Chemical Industry Institute of Toxicology (Research Triangle Park). The bioassay was performed, respective in accordance to the procedures of Gaido *et al.* (1997).

Cell cultures were verified by running the assay with standards for one month prior to the analysis of test substances. This was to ensure the passage number was similar and the cultures were consistent (Yeast Culturing Protocol). Every week new cultures were started from plates stored at 4 ^{o}C which were never older than one month. These plates were made each month from frozen stock (Yeast Freezing Protocol). Lorenzen *et al*. (2004) provided a modified version of the Gaido *et al*. *(*1997) procedure allowing for the use of a 96-well plate with spectrophotometric measurements of endpoints. Briefly, on day 0 at 10 a.m., a yeast culture is started by adding a single colony of yeast from a streaked plate into 5 mL selective media and incubated overnight at 30^{o} C with shaking. On day 1 at 10 a.m., the culture is diluted 10 fold, by adding 45 mL of growth medium. On day 2 at 10 a.m., the culture was then diluted by 50% in growth media (Lorenzen *et al.*, 2004).

At 1 p.m., aliquots (10 µL) of standards or test solutions were transferred in triplicate to the microplate wells and allowed to dry (approximately 30 min). At 2 p.m., copper sulfate (0.1 M, final concentration in yeast culture) was added to the mid-log phase (OD_{600nm} of 0.8-1.0) yeast culture to induce hER production prior to exposure. This yeast culture was added to the 96-well plate in aliquots of 200 µL/well. After mixing for 2 min, the microplates were sealed and incubated overnight at 30^{o} C, without shaking. At the end of the 20 hours incubation, yeast cells were resuspended and 100 µL aliquots were transferred to a 96-well microplate containing 100 µL of assay buffer solution. After 2 min of shaking, the microplates were kept at room temperature for an additional 40 min, until the absorbance was read at 415 nm and 595 nm.

Results from the YES bioassays were plotted by Prism. The E2 dose–response curve was used to quantify the unknown samples. The E2 equivalence (EEQ) was generated based on the E2 dose–response curve (Fig. 3).

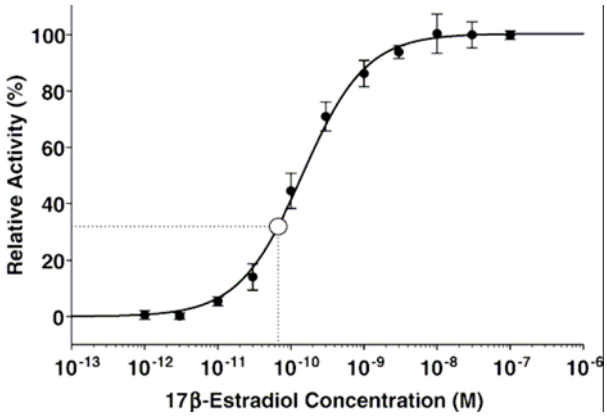

Fig. 3. A typical dose–response curve for 17β-estradiol.

RESULTS AND DISCUSSION

The environmental fates of estrogens in aquatic environment 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.

From literature, it was found that 17-β-estradiol (E2) has the highest relative estrogenic potential in the environmental water as shown in table 3 (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 3. 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

Table 4. Estrogenic activities of eight different study points of Sindh areas, water samples collected in November 2006.

Study Point	Data (average)	Predicted estrodial equivalence concentration n (M)	Predicted estrodial equivalence concentration n (ng/L)
Indus Dophin (P-1)	3.575431	11.031	9.31
Indus Dolphin (P-2)	2.948023	00	00
Kinjhar (P-1)	4.252245	10.5578	2.77
Kinjhar (P-2)	3.077898	00	00
Haleji (P-1)	2.382564	00	00
Haleji (P-2)	10.62091	00	00
Creek area (P-1)	2.773354	00	00
Creek area (P-2)	2.945462	00	00

Table 5. Estrogenic activities of eight different study points of Sindh areas, water samples collected in May 2007.

Study Point	Data (average)	Predicted estrodial equivalence concentration n (M)	Predicted estrodial equivalence concentration n (ng/L)
Indus Dophin (P-1)	10.5163	7.50327	3.14
Indus Dolphin (P-2)	10.72072	7.3425	4.54
Kinjhar (P-1)	2.52951	00	00
Kinjhar (P-2)	2.432803	00	00
Haleji (P-1)	5.879606	8.85275	1.4
Haleji (P-2)	1.865288	00	00
Creek area (P-1)	9.012585	8.10309	7.89
Creek area (P-2)	3.584123	10.5337	2.93

In the present study, a baseline study was conducted for the evaluation of estrogenic activities in the waters of three Ramsar Sites and one Creek areas among the selected study points. *In-Vitro* Yeast Estrogenic Screen (YES) assays techniques were used to evaluate the estrogenic activities in the water samples. Summarized data obtained for the estrogenic activities of water samples of Indus Dolphin Wetland (study point1), Indus Dolphin (study point 2), Kinjhar Wetland (study point 1), Kinjhar (study point 2), Haleji Wetland (study point 1), Haleji (study point 2), Creek areas (study point 1) and Creek area (study point 2) for the two seasons are shown in table 4 and 5. Estrogenic activity was recorded in the November 2006 samples as Indus Dolphin Wetland (study point 1) 9.31 ng/L estradiol equivalents and Kinjhar (study Point 1) 2.77 ng/L estradiol equivalents (Table 4), while samples collected in May 2007, estrogenic activity was found in Indus Dolphin Wetland (study point 1) 3.14 ng/L estradiol equivalents, Indus Dolphin (study point 2) 4.54 ng/L estradiol equivalents, Haleji Wetland (study point 1) 1.4 ng/L estradiol equivalents, Creek area (study point 1) 7.89 ng/L estradiol equivalents and Creek areas (study point 2) 2.93 ng/L estradiol equivalents (Table 5).

Estrogenic activities observed in Indus Dolphin samples collected from point P1 and P2, Kinjhar P1, Haleji P1, and Creek areas P1 and P2 in November 2006 and May 2007, respectively. As evident from the table 4 estrogenic activities of Nov 2006 winter samples were found varied from the summer samples May 2007 (Table 5), in the case of summer samples, estrogenic activity was found 3.14 ng/L estradiol equivalents (Indus Dolphin P1) and 4.54 ng/L estradiol equivalents (Indus Dolphin P2) in Indus Dolphin (P1 and P2) that is lower than November 2006 samples. These results indicated that the summer May 2007 samples were more polluted then the winter samples.

CONCLUSION

Results of our study have indicated that the Indus Dolphin Wetland (P1) and Kinjhar Wetland (P1) are polluted areas due to anthropogenic activities and this study also indicates the presence of Estrogenic activities in both seasons. Of the eight study points, the highest estrogenic activity has been recorded at creek P1 area. This area is busy for local fisherman activities including the cleaning of fishing boots and other related work. Normally in the summer season creek areas are very active for fishing activities and as result, the summer samples are more polluted than the winter samples. Further studies are recommended for determination of possible effects of estrogenic activities on aquatic biodiversity.

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