Canadian Journal of Pure and Applied Sciences
Vol. 11, No. 1, pp. 4067-4077, February 2017
Online ISSN: 1920-3853; Print ISSN: 1715-9997
Available online at www.cjpas.net

EXTENT OF FISH AND MUSSEL SPECIES CONTAMINATED BY ENDOCRINE DISRUPTING CHEMICALS (EDCS) IN KARACHI COSTAL AREAS: ASSESSMENT USING AN *IN-VITRO* YEAST ESTROGENIC SCREENING (YES) TEST

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ABSTRACT

The objective of the present study was to screen for Endocrine Disrupting Chemicals (EDCs) contamination in fish (*Engraulis purava*) and mussel (*Mytilus*) in 10 selected areas of Karachi coast. After the baseline survey, we selected Paradise Point, Hawks Bay, Sandspit, Manora, Baba Island, and Korangi/Phitti Creek to collect fish samples where as Buleji point 1 & point 2, Paradise point 1 & point 2, and Manora point 1 & point 2 were selected for mussel samples. The YES bioassay was used in the present study to screen marine fish and mussel for EDC. According to findings the most contaminated area is Baba Island in which the Anchovy contained 95.78ngE_2 equivalents/g wet weight of fish tissue whereas the anchovy collected from Sandspit contained 20.70ng/g of fish tissue. The second most polluted area is Korangi/ Phitti Creek where the fish samples contain an average of 77.19ng/g, ww. In the mussel samples, Buleji point 1 area contains an average of 875.23ng/g, ww, and most polluted area for mussel as compared to other study areas. Further investigation such as chemical analysis has been recommended for identification of chemicals.

Keywords: Karachi, coastal areas, YES bioassay, *Engraulis purava*, *Mytilus*.

INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) are present in hormones, pharmaceuticals, pesticides, and detergents and they are usually introduced into the aquatic ecosystem through industrial and municipal effluents along with urban and agricultural runoff. Exposures to EDCs may result in the disruption of normal functioning of hormones in vertebrates and invertebrates biodiversity. It is important to determine the levels of EDCs in different aquatic animals quantitatively using the yeast estrogen screen (YES) bioassay because the YES bioassay is an *in vitro* test that can be used to detect total EDC concentration in a sample in expensively and quickly.

In the aquatic environment some chemicals have been implicated as hormones or endocrine disruptors. The Endocrine disrupting chemicals (EDCs) are commonly used to describe environmental agents that alter the endocrine system. EDCs can be defined as an exogenous chemical that interferes with the synthesis, secretion, action transport, binding of natural hormones in the body. EDCs are responsible for behaviour, reproduction and development of animals and human (Crisp *et al*., 1998).

EDCs are a class of synthetic or natural compounds that are suspected of adversely affecting the populations of aquatic biodiversity and humans health. Estrogens are a main harmful source of EDCs that have been linked to feminization of biodiversity such as fish and reptiles (Gauillette *et al*., 1995; Sumpter and Jobling, 1995), and also the increasing incidence of testicular reduction in male fertility and breast cancer in humans (Toppari *et al*., 1996; National Research Council, 1999). Researchers Bolz *et al*. (2001), Ferguson *et al*. (2001), Peck *et al*. (2007), Stuart *et al*. (2005) and Houtman *et al*. (2006) have reported presence of EDCs in estuarine and river waters. The endocrine disrupting chemicals are thought to be especially important for development of fish, disrupting sexual development, fertility and behaviour (Rolland *et al*., 1997). Exposure to EDC's in the aquatic environment has been associated with abnormal thyroid function in fish, shellfish and birds (Moccia *et al*., 1981, 1986) and disturbed hatching in fish, turtles and birds

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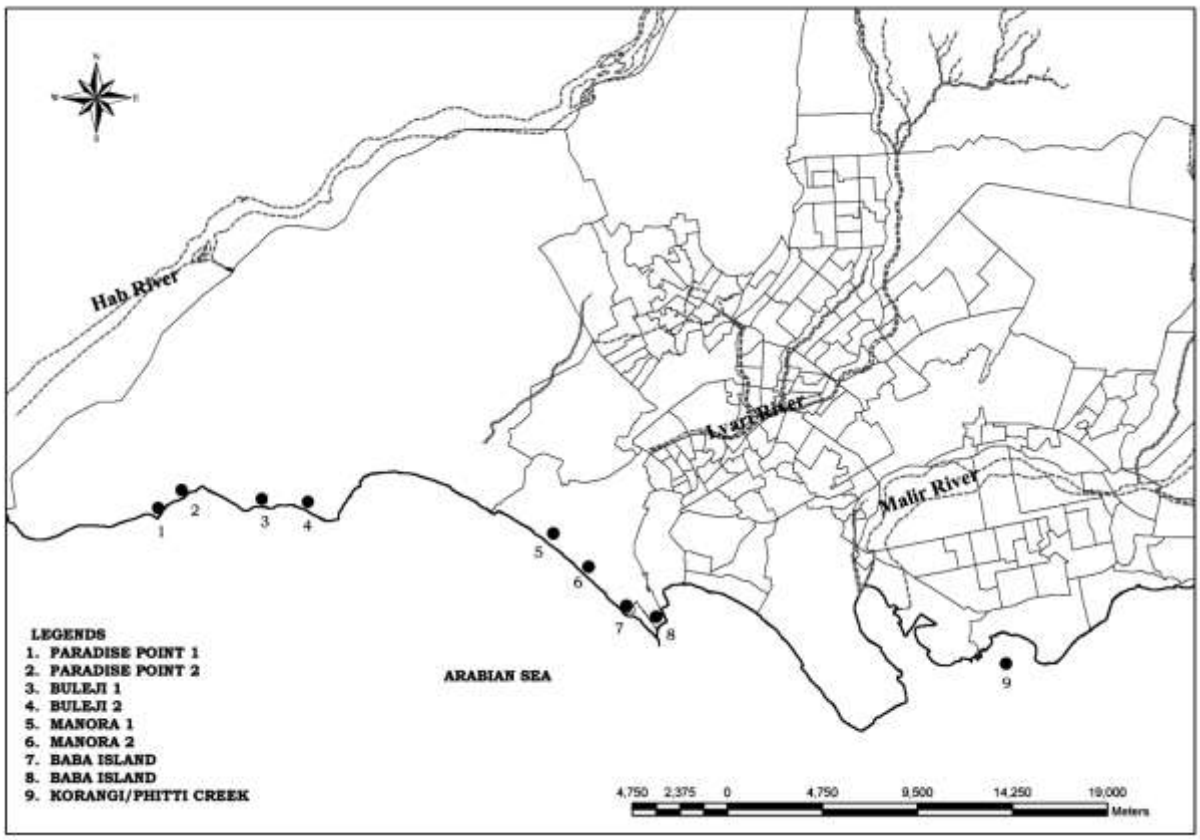

Fig. 1. Map of study areas of Karachi coast.

(Shugart, 1980; Leatherland, 1992; Mac *et al*., 1988; Bishop *et al*., 1991).

Pakistan's coastal areas have diverse rich marine biodiversity and coastal ecosystem is a complex array of creeks and delta of the Indus River. The environmental quality of coastline and marine ecosystem is continuously being degraded due to marine pollution arising from a combination of domestic and industrial activities.

Pakistan produced about 596,980 metric tons of marine fish and 25,000 metric tons of shrimp. About131,000 metric tons of fish is exported to Europe and other countries. Pollution in the coastal areas of Karachi are severely contaminating marine biodiversity (Govt. of Pakistan, 2016).

Karachi coastal areas receive 472,000m^3 domestic and industrial wastewater primarily through Lyari and Malir Rivers and from streams and drainages (KDA master plan1, 1990-2000). Presently, coastal areas of Karachi have high levels of organic pollutants and the presence of heavy metals (Ali and Jilani, 1995; Akhter *et al*., 1997; Jilani, 2014). A recent study revealed that toxic metals have been found in marine biodiversity and ecosystem in the coastal areas of Karachi. These metals include mercury, cadmium, chromium, lead, arsenic, and zinc. The mean concentration of cadmium in the fishes was found to be 0.06, 0.04, 0.06 ppm fresh weight in the fishes of Karachi harbour, Korangi creek and Gwadar East Bay, respectively. The highest concentration of cadmium was recorded both in Karachi harbour and Gwadar East Bay and obtain from Korangi creek fish. Highest concentration of metals in water and sediments was observed in Karachi Harbour area followed by Korangi creeks>Gizri creeks>Gwadar fish Harbour> Off Gwadar East Bay>Buleji (Saleem, 2002). The objective of the present study was to screen the extent of EDCs contamination in Fish (*Engraulis purava*) and Mussel (*Mytilus*) in selected areas of Karachi coast.

MATERIALS AND METHODS

Study Points

After the baseline survey and based study, we selected the following sites as study points:

For fish Anchovy sampling; Paradise Point, Hawks Bay, Sandspit, Manora, Baba Island, and Korangi/Phitti Creek, while Buleji point 1, point 2, Paradise point 1, point 2, and Manora point 1, point 2 for Sea mussel (Fig. 1).

Table 1.The dilution series for the standard curve. There are 11 dilutions and the 12th well is the methanol blank. The 100nM solution is diluted 1:3, 1:10, 1:17, 1:30, 1:50, 1:85, 1:150, 1:250, 1:1500, and 1:2500 for the assay.

Dilution Number	1	2	3	4	5	6	7	8	9	10	11	12
E2Dilution Factor	1	0.33	0.1	0.059	0.033	0.02	0.012	0.0067	0.004	0.00067	0.0004	methanol blank
Methanol volume (µL)	0	140	180	188	180	160	160	160	160	180	180	

We had hired professional fishermen for collection of live fish and mussels samples. Following collection of samples they were stored with ice and shifted to Wildlife Lab, Department of Zoology, University of Karachi for extraction.

Extraction

Three grams of Anchovy fish *Engraulis purava* / 2 gram of sea mussel *Mytilus* were cut, weighed, and placed into glass tubes separately. After 30 minutes, the fish/ mussel samples were placed in 5mL of methanol in a large glass test tube and homogenized with the Brinkmann Homogenizer (Polytron). The samples were centrifuged at 30rpm for 20 minutes. After centrifugation, the methanolic extract for each sample was removed and placed in a smaller test tube. After removing methanolic extract, the tissue sample was dissolved in 5mL of methanol and centrifuge at 30rpm for 20 minutes. After the centrifugation and the removal of the extract for the second time, the tissue pellet was extracted with 2mL of chloroform and centrifuge for 15 minutes at 30 rpm. The chloroform extract was then combined with previously removed methanolic extracts and then was centrifuged at 30rpm for 8 minutes, this allows the tissue particle to precipitate to the bottom of the extract (Peck *et al*., 2007).The final volume of the combined extract was measured before being evaporated to dryness in a rotator evaporator (Rotavapor RE 120, Büchi). The residues were reconstituted in 500µL of methanol and transferred to small vials then were sent to be dried again under a gentle stream of nitrogen. After nitrogen evaporation the residues were reconstituted in 500µL of ethanol and placed in a freezer at -40°C overnight.

Filtration Protocol

1) Samples were vacuum filtered through bond elute C18 extraction columns through the use of an extraction manifest.
2) C18 Extraction columns were pre-conditioned with 1mL methanol twice, followed by 1mL of distilled water twice.
3) Sample were then filtered through the extraction column with the filtrate being discarded.
4) 500 µL of methanol was then passed through the column to elute the sample. This portion was collected and evaporated by nitrogen gas.
5) Evaporated samples were re-constituted by adding 500µL of methanol

Sample extracts were mixed with methanol to 1, 1:5, 1:50, 1:500, 1:5000 and 1:50000 dilutions before being used in the YES assay.

Standard Dilution Series

E2 standard curves were prepared using 11 concentrations of the E2 standard solution. A pure methanol blank was also prepared. Thus, the E2 stock solution (0.01 M) was prepared by dissolving 27.24mg of estradiol in 10 mL of absolute ethanol. A 20µL aliquot of the stock solution was added to 980µL of methanol to make the substock solution. A 10µL aliquot of the substock was added to 990µL of methanol to make 100nM of working solution. A 200µL aliquot of the working solution was used as the first undiluted standard solution. From this 100 nm working solution, it was diluted with methanol to obtain 1:3, 1:10, 1:17, 1:30, 1:50, 1:85, 1: 150, 1: 250, 1:1500, 1:2500 dilutions (Table 1).

Yeast Preparation

Five days before the start of an experiment, transgenic yeast was spread on an agar plate and incubated at 30°C for 3 days. The plate was parafilmed and stored in a fridge at -10˚C. The procedure was described by Gaido *et al*. (1997) but modified for the present study according to Lorenzen *et al*. (2004). To prepare the yeast that would be used for the YES, the yeast was cultured in a 50mL polypropylene tube filled with 5mL of media on day one at 10:00 am. The culture was left in the incubation room on a shaker at 30°C overnight. On day two the yeast was diluted 1:10 by adding 45mL of media to the 5mL culture at 2:00 pm. On day 3 at 10 am the yeast culture was diluted 1:1 by re-suspending the yeast and transferring half the yeast to another 50mL propylene tube and adding 25mL of media.

Plate Preparation

10µL of the standard dilution series and the sample series were pipetted onto a 96 well plate. This is repeated twice for each standard dilution series and three times for each sample dilution. The plate was left to dry for 30-45 minutes. At 2:00 pm the yeast exposed to the estrogen on the plate by re-suspending the yeast and mixing 20mL of yeast with 200µL of copper sulphate. The copper

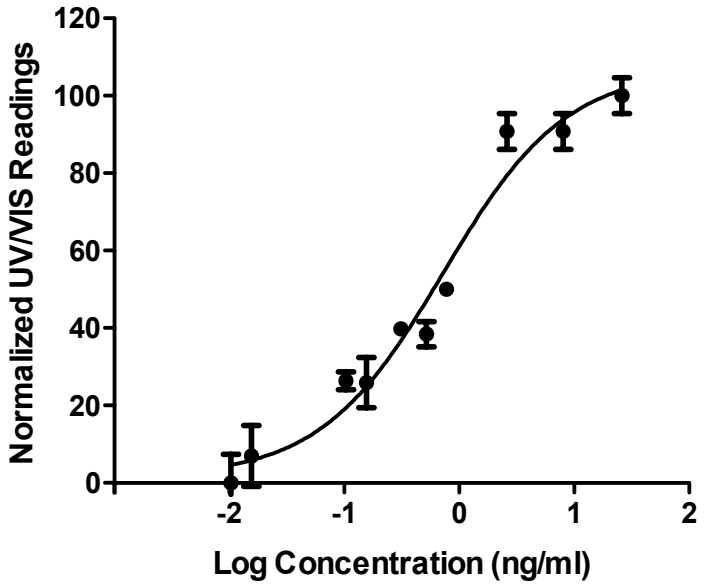

Fig. 2. This is a typical E2 standard dose-response curve. The concentration of E2 (log-transformed) is given on the x-axis; the response in percentage is given on the y-axis. Each data point is the mean ±SD of a duplicate assay. The curve shows a sigmoid shape with a maximum of 92.83%.

sulphate stimulates the production of hER in the yeast cells. 200µL of this mixture was added to each well by using a multi channel pipette. The plate is slightly shaken and parafilmed and kept in the incubation room at 30°C overnight. 2-Nitrophenyl β-D-galactopyranoside (ONPG) (0.022 grams) was dissolved in 10.9 g of Z-buffer and left in the shaker overnight. At 9:00am on the fourth day the yeast in each well was re-suspended and 100µL transferred to a new plate. Ten percent SDS (110µL) and 11 µL of oxalyticase, and 29.7µL of mercaptoethanol were added to the ONPG and then 100µL of the mixture were added to each well. Forty minutes after adding the ONPG, the plate was read by a UV/V was spectrophotometer at 415nm (over all colorimetric change) and 595 nm (for turbidity caused by yeast cells).

Data calculation and statistical analysis

Data was analyzed using Excel and Graph Pad Prism 5. The UV reading at 415 nm represents the overall colorimetric change of the sample and the reading at 595 nm represents the turbidity caused due to yeast growth. In order to obtain the colorimetric change specifically due to the reaction between ONPG and β-galactosidase, the reading at 595 nm was subtracted by 415 nm. To plot a dose response curve, the UV readings were normalized and the dilution factors were log-transformed for better data analyzing and plotting with Prism 5. The UV readings were used as the variables for y-axis and concentration factors as the variables for x-axis of the curve. The maximum of the standard curve was considered to be 100% and the sample dose-response curves were plotted compared to this maximum (Fig. 2).

The calculation of the E2 equivalence was done by using the following formula proposed by Lorenzen *et al.* (2004): E2 equivalent = [standard EC_{50} (ng/ml) / extract EC_{50}] * [volume of assay medium (ml) / (volume of extract tested (µl)] * [volume of stock extract (µl) / ww of sample (g)]. To further broaden the accuracy of our calculation EC_{20} and EC_{30} were also used in the calculation instead of just calculating using EC_{50}. To convert EC_{50} to EC_{20} and EC_{30} the following formula was used for the conversion: $EC_X = [X/(100-X)^{(1/H)}] * EC_{50}$, where the X is 20 or 30 and H is the hill slope of the dose response curve. The average of the E2 equivalence results calculated by using $EC_{20,}$ EC_{30} andEC_{50} were taken and used as the final representative of the E2 level in the samples.

RESULTS AND DISCUSSION

Exposures to endocrine-disrupting chemicals are currently regarded as the most serious anthropogenic threats to aquatic biodiversity and ecosystems.

The Malir and Lyari Rivers in the metropolitan city of Karachi are contributing about 25% and 59% of the total pollution load of Karachi City respectively, while 15% of

Table 2. The average EDC levels shown in study points (Fish samples) 2011.

Fish Sample locations	Sample I.D.	EDC (ng E2 equivalents/g fish)
Baba Island	BI 1	13.34
	BI 2	410.81
	BI 3	26.72
	BI 4	2.38
	BI 5	1.20
	BI 6	120.21
	Average	**95.78**
Hawks Bay	H 1	0.00
	H 2	0.00
	H 3	0.00
	Average	**0**
Manora	MA 1	0.00
	MA 2	23.74
	MA 3	0.00
	MA 4	326.95
	MA 5	24.53
	Average	**75.04**
Paradise point	P 1	0.00
	P 2	0.00
	P 3	0.00
	P 4	98.62
	Average	**24.66**
Sandspit	S 1	1.43
	S 2	105.61
	S 3	0.00
	S 4	0.00
	S 5	1.77
	S 6	15.39
	Average	**20.70**
Korangi/Phitti Creek	F1	63.41
	F2	134.56
	F3	0.00
	F4	0.00
	F5	399.75
	F6	0.00
	Average	**77.19**

the pollution load is directly discharged through Gharo, Gizri and Korangi Creek (Amjad and Rizvi, 2000).

Industrial toxic waste, and organic waste from the nearby cattle colony comprising of liquid dung mix with the blood from slaughterhouses are also drained into the Korangi Creek areas. Several studies Rizvi *et al*. (1988), Saifullah *et al*. (2002), Saleem and Kazi (1995) and Saleem and Kazi (1998) have already reported high levels of contaminants in the coastal areas of Karachi.

In the present work, Yeast Estrogenic Screening (YES) tests were conducted for the evaluation of Endocrine Disrupting Chemicals (EDCs) levels in the fish and mussel samples from the selected study areas of Karachi Coast. All fish samples were collected from Paradise Point, Hawks Bay, Sandspit, Manora, Baba Island, and Korangi/Phitti Creek areas, where as Sea mussel samples obtained from Buleji point 1 and point 2, Paradise point 1 and point 2, and Manora point 1 and point 2.

Study areas were surveyed and samples of fish Anchovy (*Engraulis purava*) and Sea mussel (*Mytilus*) were collected in Oct/Nov 2010 and March/April 2011. *In-Vitro* Yeast Estrogenic Screen (YES) assay techniques were used to evaluate the ECDs level in the fish and mussel samples. The most potent EDCs found in the environment are the natural and synthetic hormones such

Table 3. The average EDC levels shown in study points (Mussels samples) 2011.

Mussel Sample locations	Sample I.D.	EDC (ng E2 equivalents/g mussel)
Manora point 1	1	#
	2	876.41
	3	#
	4	364.71
	5	918.52
	6	#
	7	38.96
	8	0.00
	9	0.00
	10	0.00
	11	1306.53
	12	0.00
	13	23475.73
	14	614.56
	15	#
Manora point 2	16	0.00
	17	711.93
	18	0.00
	19	0.00
	20	0.00
	21	0.00
	22	0.00
	23	0.60
	24	0.00
	25	0.61
	26	1.00
	27	0.00
	28	47.61
	29	54.10
	30	0.00
	31	0.00
	32	0.00
	33	0.00
	34	0.00
	35	0.00
	36	33.98
Buleji Point 1	B1 A	0.00
	B1 B	27.23
	B1 C	2598.45
	Average	**875.23**
Buleji Point 2	B2 A	0.00
	B2 B	22.97
	B2 C	0.00
	Average	**7.66**
Paradise Point 1	P1 a	#
	P1 b	0.00
	P1 c	81.87
	Average	**40.94**
Paradise Point 2	P2 a	99.14
	P2 b	0.00
	P2 c	6.55
	Average	**35.23**

= No result due to sample contamination

Table 4. The average EDC levels shown in study points - 2009.

Plate and Location	Experiment	Sample	EC50+EC30+EC20/3
P1 Buleji Point 1	Sea Mussel *Mytilus*	1	60.53976
		2	4.689243
		3	14.46198
		4	7.573719
			21.81618
P2 Korangi/Phitti Creek	Anchovy *Engraulis purava*	5	213.6048
		6	12.09747
		7	1.260946
		8	0.556322
			56.87988
P3 Manora	Anchovy *Engraulis purava*	9	0.831914
		10	2.284097
		11	0.420291
		12	0.63614
			1.04311
P4 Paradise Point 1	Sea Mussel *Mytilus*	13	0.925197
		14	1.629437
		15	3.040487
		16	2.61711
			2.053058
P5 Paradise Point 2	Sea Mussel *Mytilus*	17	1.625567
		18	0.211116
		19	0.000946
		20	6.75E-05
			0.459424
P6 Buleji Point 2	Sea Mussel *Mytilus*	21	0.009465
		22	3199.153
		23	0.002393
		24	52031065
			13008566

as 17β-estradiol (E2), estrone (E1), and 17α-thynylestradiol (EE2). We used E2 as the chemical standard in our bioassay, as it is one of the most potent EDCs. E2 is produced in the ovaries, gonads, and, to a lesser extent, in the arterial walls, the brain, and adrenal cortex. E2 also acts as a growth hormone for the female reproductive organs (Hertz, 1985). The YES bioassay is an efficient and inexpensive method of detecting EDCs in aquatic samples. To find the E2 equivalents in our fish and mussel samples, the dose-response curves were compared to E2 standard curve from every experimental plate.

The average EDC levels found in the fish samples of Baba Island were found to be 95.78ng/g, 75.04ng/g in Manora, 24.66ng/g in Paradise points, 20.70ng/g in Sandspit and 77.19ng/g in Korangi/Phitti creek (Table 2).

The average EDC levels found in mussels samples were as follows, 200.91ng/g in Manora point 1, 40.46ng/g in Manora point 2, 875.23ng/g in Buleji point 1, 7.66ng/g in samples of Buleji point 2, 40.94ng/g in Paradise point 1and 35.23ng/g in samples of Paradise point 2 (Table 3).

According to present study's findings the most contaminated area is Baba Island in which the Anchovy is shown to contain 95.78ng E_2 equivalents/g wet weight of fish tissue whereas the anchovy collected from Sandspit contains 20.70ng/g of fish tissue. The second most polluted area is Korangi/ Phitti Creek where the fish samples contain an average of 77.19ng/g, ww.

In the mussel samples, Buleji point 1 area contains an average of 875.23ng/g, ww, and most polluted area for mussel as compared to other study areas. These findings are consistent with the report that the Korangi/Phitti Creek area is a polluted area of the Karachi coast because wastewaters are discharged into the sea from Korangi, Landhi, Karachi Export Processing Zone, Bin Qasim industrial areas, and Pakistan Steel Mill (Shahzad *et al*., 2009). Also, there are several tanneries, textile factories, insecticide and pesticide factories, and slaughterhouses in the area (Rizvi *et al*., 1988).

Further analysis of samples by HPLC or GC/MS have been recommended for identification of chemicals.

According to our 2009 study (Table 4), the most contaminated area was Korangi/Phitti Creek in which the anchovy is shown to contain 56.9ng E_2 equivalents/g wet weight of fish tissue whereas the anchovy collected from Manora contains only 1.04 EEQ ng/g of fish tissue. The second most polluted area was Buleji Point 1 where the mussel samples contain an average of 21.8 EEQng/g, ww. While remaining three mussel sampling sites show little or no EEQ.

Karachi city generates approximately 8,700 tons of domestic solid wastes per day (Alam, 2010). Pakistan has 17 Creeks in Sindh Coastal areas, Korangi/ Phitti Creeks being one of them. The Korangi/Phitti creek area is a polluted area of Karachi coast because several tanneries of Korangi industrial area are disposing off their untreated wastes into the creek areas.

Several studies have reported that Korangi/ Phitti Creeks has been highly polluted and are impacting marine biodiversity and human health. Three power plants located at the Karachi coastal areas use large quantities of marine water cooling; they discharge heated effluent and other pollutants to sea. Among the creeks, the Korangi and Gizri creeks are the most affected from oil discharges as well as municipal and industries activities. The present study has confirmed that Korangi/ Phittii Creeks areas have high level EDCs contamination.

Pakistan Steel Mills are located on the Gharo Creek. Thus the entire Korangi/ Phitti Creek and Gharo Creeks from an interrelated system of creeks receive industrial waste from some sources of pollution in the Karachi city. According to study conducted by National Institute of Oceanography, Steel Mills discharge cooling waste water effluent to directly adjacent area of Gharo Creek, and these effluents contain heavy metals including Cd, Fe, Zn, Cu, Pb, iron oxide particles, chlorine, oil and grease etc (Rizvi, 1997).

EDCs are introduced into the aquatic environment through industrial and municipal effluents as well as urban and agricultural runoff (Nelson *et al*., 2007; Soto *et al*., 2004, Metcalfe *et al*., 2001). Once in the environment EDCs can have harmful effects as they are transported up the food chain. The presence of endocrine disrupting compounds in the environment can cause adverse effects such as reproductive abnormalities and impaired development in aquatic biodiversity and silent killers, threatening biodiversity on a huge scale. Several adverse effects of endocrine disruption have been reported in fish, mussels, crustaceans, birds, mammals and reptiles (Canesi *et al*., 2004; Waring and Harris, 2005).

Industrial toxic waste, organic waste from the nearby cattle colony comprising liquid dung mix with the blood from slaughterhouses are also drained into the Korangi Creek areas. Earlier studies Rizvi *et al*. (1988), Saifullah *et al*. (2002), Saleem and Kazi (1995), Saleem and Kazi (1998), Saleem (2002) and Hunter *et al*. (2012) have already reported the contamination from various pollutants in the coastal areas of Karachi and they are transported up the food chain.

Many studies have proved that some industrial and agricultural chemicals have the ability to interfere with endocrine systems and hormonal functions of aquatic wild animals including fish and mussels. These EDCs are thought to be important at the larval or developmental stages of fish, disrupting sexual development, and general behaviour (Rolland *et al*., 1997). These studies and our present study results have indicated that marine biodiversity may be threatened by industrial and municipal effluents pollution especially in Korangi/ Phitti Creek area.

One new evidence at molecular level has reported that the environmental deterioration can change the genetic structure and genetic polymorphism of trout populations by cytogenetic and allozymic analysis (Was and Wenne, 2002).

In connection with screening the levels of ECD's further studies are needed at large scale screening of the levels of ECD's in the marine water, sediment, fish, mussels and prawn of all coastal areas of Sindh and Balochistan. After screening we were able to find out the hot spots, determination of level of estrogenic contamination then controlling the estrogenic contamination, protecting the aquatic biodiversity and increasing the fisheries, prawn and mussels production and preparing the aquatic biodiversity conservation plan for the coastal areas.

Further chemical analysis in the identification of chemicals is recommended for samples with high levels quantified in the yeast assay.

ACKNOWLEDGMENTS

The authors would like to thank the Higher Education Commission (HEC) Pakistan for the Sabbatical Fellowship Program grant in supporting this research study conducted at the Toxicology Program, Department of Biological Sciences, Simon Fraser University, Canada . We also thank to Prof. Jamil Kazmi, Department of Geography, University of Karachi for preparation of study areas map.

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APPENDIX

Four Hours Yeast Based Estradiol (E2) Receptor Bioassay

Day 1

1) Culture 1 colony of DSY-219 (E2 assay) into 5mL of SC-UW Media
2) Put on shaker and allow to grow overnight at 30^{o}C

Day 2

1) On the morning of day 2, dilute the yeast culture down to an optical density (OD) of 0.08 with SC-UW media
2) Allow the yeast to regrow to an OD of 0.10 on the shaker at 30^{o}C (Typically, the yeast regrows in 1.5-2 hours)
3) When the yeast is at an OD of 0.1, plate 1 µL/well of the sample to be tested on a white bottom 96 well plate
4) Add 100 µL yeast/well and incubate at 30^{o}C for two hours
5) Several minutes before the two hour incubation is complete, prepare the Tropix Gal-Screen buffer and put on ice (The buffer is prepared by diluting the substrate with B Buffer at a ratio of 1:24)
6) Add 100 µL buffer/well parafilm to prevent evaporation and incubate at room temperature for 2 hours
7) At the end of the 2 hour incubation, read the plate on the Perkin Elmer 2030 multilabel reader luminometer (The light intensity is usually the highest between 1.5 - 2.5 hours after addition of the buffer).

Received: Aug 22, 2016; Revised: Nov 24, 2016;
Accepted: Dec 16, 2016